Cloning and characterisation of heat shock and wound-induced genes in pea (pisum sativum L.)

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Cloning and Characterisation of Heat Shock and Wound-Induced Genes in Pea (*Pisum sativum* L.)

A thesis submitted by Om Parkash Dhankher (M.Sc., M.Phil.) in accordance with the requirements for the degree of Doctor of Philosophy in the University of Durham.

Department of Biological Sciences.
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For Anshul and Akshat

Memorandum

Part of this work has been included in the following publications:

O.P. Dhankher , J.E. Drew and John A. Gatehouse (1997) Characterisation of a pea HSP70 gene which is both developmentally and stress regulated. Plant Mol. Biol. 34 : 345-352.

O.P. Dhankher and John A. Gatehouse (1997) A study of the kinetics and tissue specificity of stress-induced gene expression using promoter-reporter gene construct in transgenic tobacco plant. S.E.B. Annual Meeting, 7-11 April 1997, University of Canterbury, Canterbury.

Abstract

Plant productivity in many regions of the world is limited primarily as a result of environmental stresses. High temperature and wounding caused by pest and pathogen infection are among the main factors accounting for unpredictable and often severe yield losses worldwide. These stresses, force the plants to alter their gene expression in order to adapt to the changed environment. Attempts were made in the study to isolate and characterise the differentially expressed heat shocked and wound-induced genes to understand the underlying molecular mechanism of heat shock and wounding response. The isolation of the promoters and their use to derive the tissue-specific and high expression of the linked coding sequences will be proved practically more significant.

A cDNA clone designated LP19 was isolated from a differential screening of a cDNA library prepared from lignifying pods of pea line L59. Sequence homology analysis showed that LP19 belongs to the hsp70 gene family. Northern analysis of RNA from pods from pea lines of different genotypes, showing the presence or absence of pod lignification, showed that LP19 expression was specifically associated with lignification. Several cDNA species derived from transcripts of the LP19 gene were subsequently isolated, which showed varying positions of poly (A) addition to the 3' untranslated region. Southern blotting of genomic DNA indicated the presence of single gene corresponding to LP19.

The pea hsp70 gene corresponding to LP19 was isolated from a pea genomic library using LP19 as a probe. The pea hsp70(LP19) gene predicts an open reading frame encoding a polypeptide of 648 amino acid residues. This sequence is similar to other plant hsp70 proteins. However, unlike most other plant hsp70 genes, the pea hsp70(LP19) gene lacks an intron. 1.8 kb of 5' flanking sequence of hsp70(LP19) gene was also sequenced. The promoter region contains 6 putative consensus heat shock elements (HSEs) as well as 4 A–T rich sites upstream from TATA box.

Induction of gene expression of the pea hsp70(LP19) was observed in all organs of the plant after heat shock; the highest level of expression was observed in root, followed by stem and least in leaves. A similar expression pattern for a corresponding gene was observed in chickpea (*Cicer arietinum* L.). Other stress conditions such as salt stress and wounding failed to induce the expression of hsp70LP(19) gene both in pea and chickpea.

The pea hsp70(LP19) promoter region, including 1.8 kb 5'-flanking sequence, and the first 18 amino acids of the coding region, was fused with coding sequence for β-glucuronidase (GUS). Tobacco plants were transformed with this chimaeric gene in order to study tissue specific and developmental expression of the hsp70(LP19) promoter. Histological staining of GUS activity in transgenic tobacco plants showed that protein was present predominantly in the phloem tissue in stem, root and petioles.

In addition, developmental expression of the hsp70(LP19) gene promoter, without heat shock, was observed in petals, pollen grains, developing seeds as well as in germinating seeds and seedlings at different stages of growth. Quantitative assay of GUS activity by fluorometric assay was used to follow the time course of protein accumulation. Activity was detected within few minutes of the start of heat shock and increased to a maximum after 6 hrs. A high level of GUS activity was observed only in the heat shocked parts of the plant; no endogenous signal that spread systemically from the heat shocked areas to the rest of the plant was observed.

Pea and chickpea plants showed a transient increase of polyphenol oxidase (PPO) with maximum level at 48 hrs after wounding. No systemic induction of PPO was observed in unwounded parts in response to both wounding and MeJA treatment. In order to isolate transcripts expressed differentially in response to wounding, a pea subtractive cDNA library was made. 21 subtracted cDNA clones were partially sequenced. Most of the subtracted cDNA clones showed homology with wound or pathogen induced sequences. Northern analysis of the genes corresponding to the subtracted cDNA clones (SC3, SC7, SC12, SC33, SC57 and SC58), indicated differential expression in response to wounding. Full length or nearly full length cDNAs corresponding to 4 subtracted cDNA clones, designated SC10, SC15, SC57 and SC58, were isolated and sequenced. These cDNA clones will be further studied and efforts will be made to isolate their promoters. The tissue-specific expression will be carried out by using promoter-reporter system. These isolated cDNA clones were partially characterised and will be available for further studies to isolate their respective promoters. The tissue-specific expression will be carried out by using promoter-reporter system.

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ABBREVIATIONS

°C degree celsius

% percent

ABA abscisic acid

aa amino acids

amp ampicillin

ATP adenosine 5'-triphosphate

bp base pair

BAP 6-benzylaminopurine

BSA bovine serum albumin

CaMV cauliflower mosaic virus

cDNA copy DNA

CoA coenzyme-A

cpm counts per minute

C-terminal carboxy-terminal

DNA deoxyribonucleic acid

DNase deoxyribonuclease

DAF days after flowering

dCTP deoxycytosine-5'-triphosphate

DEPC diethylpyrocarbonate

DIECA diethyldithiocarbamate

DoDC diphenylalanine decarboxylase

ds double stranded

EC electroconductivity

EDTA ethylene diamine tetra-acetic acid

ER endoplasmic reticulum

EtBr ethidium bromide

FF Feltham First

g gram

GUS β-glucuronidase

hr(s) hour(s)

hsp heat shock proteins

IPTG isopropyl-β-thioglactopyranoside

JA jasmonic acid

kb kilo base pair

kDa kilo dalton

LB Luriani Bartini

LP lignified pod

mRNA messenger RNA

MSO Murashige

mg milligram

mm millimeter

ml millilitre

mmole milli moles

MMLV Moloney-Murine leukemia virus

M molar

mM milli molar

MeJA methyl jasmonate

MOPS 3-(N-morpholino) propane-sulphonic acid

mCi milli curie

4-MU 4-methylumbelliferone

MUG 4-methylumbeliferyl β-D-glucuronide

NAA napthalene acetic acid

NZY casein hydrolysate yeast

ng nanogram

nm nanometer

nt nucleotide

N-terminal amino-terminal

-NH2 amide

NAD nicotinamide adenine dinucleotide

NADP nicotinamide adenine dinucleotide phosphate

OD optical density

OLB oligo labelling buffer

PCR polymerase chain reaction

Pis proteinase inhibitors

POD peroxidase

PP purple podded

PPO polyphenol oxidase

pg picogram

pmole picomole

Poly(A) polyadenylic acid

RBP Rubisco binding proteins

RNA ribonucleic acid

RNase ribonuclease

rRNA ribosomal ribonucleic acid

SA salicylic acid

SDS sodium dodecyle sulphate

SSC saline sodium citrate

SSPE saline sodium phosphate

TAE tris acetate-EDTA

TE tris-EDTA

TGE tris-glycine-EDTA

T-DNA transfer DNA

TMV tobacco mosaic virus

Tris tris(hydroxymethyl) methylamine

µl microlitre

UV ultra violet light

µCi micro curie

UTR untranslated region

w/v weight for volume

X-Gal 5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside

X-Gluc 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

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Introduction

1.1 General Introduction

One of the major concerns of plant scientists today, is to work towards enhanced production and quality of food, through the application of scientific knowledge in different fields. In the developing world, agriculture dominates the economy and is the major source of employment. Population pressures will ensure that current food shortage will continue to be a problem in the future. Therefore, future demand for food will not be met without improved crop varieties, that offer higher and more stable yields, protection against abiotic and biotic factors, greater economy return to farmers and less costly quality food to consumers. The global ecosystem functions through interactions of its components. Annual losses due to drought, salinity, high temperature, weeds, pests, pathogens and other factors are enormous , both in the field and during storage. In order to enhance productivity, an understanding of abiotic and biotic components is of great significance. Plants, being sessile, have developed different mechanisms to cope with the negative effects of abiotic and biotic stresses.

In the recent past years, it has been apparent that plants respond to adverse environmental stress conditions through alteration in their physiological processes by diverting metabolic energy to cellular processes and thus may provide tolerance to stress. In general, when environmental conditions become unsuitable for optimal growth and development of the plant, new stress-related proteins are synthesized. The newly synthesized proteins appear to be more or less specific to a given environmental stress condition, for example, heat shock proteins for supraoptimal temperatures (Key et. al. 1981), water stress proteins for water deficit (Mason and Tanaka, 1988), salt stress proteins for high saline conditions (Hurkman and Tanaka, 1988), pathogenesis-related proteins (PR) and proteinase inhibitors for pest and pathogens infection (Bol et al., 1990, Farmer and Ryan, 1992).

Stress due to drought, water logging, salinity and high and low temperature are common throughout the world. The response of different abiotic stress conditions is often overlapped. For example, heat shock along with dryness caused by high temperature, also create the conditions of drought and desiccation via excessive water loss through transpiration. Similarly, high salinity causes osmotic imbalance in the cell and thus also create water deficit or drought stress. Furthermore, one particular set of genes expressed under one stress condition, are also express in response to other stresses. Heat shock proteins that expressed in response to high temperature, are also expressed in response to cold, drought and heavy metals. Osmotin and dehydrins, the proteins which are related to salt and drought stress proteins, are expressed under both salt and draught stress.

In the recent past, a number of stress-induced plant genes have been identified and characterised. This continuing effort is contributing to the understanding of the responses of plants to different stresses and may ultimately provide molecular tools for the breeding of crops better adapted to extreme environmental conditions. The development of stress tolerant crop plants through genetic engineering provides a mean to reduce the detrimental effects of different stresses on productivity. This approach has been successful to genetically engineer crops resistant to the pest and pathogens. A few genetically engineered crop lines such as cotton, potato, maize, soybean and tomato have already been released and a large number of newly evolved crop line are in pipe line. The attempts to make crops resistant to salinity, high temperature, drought and cold have not been successful yet. The major limitation in case of abiotic stresses is that tolerance to these stresses is not conferred by a single trait but is the consequences of complex gene interactions. Mayer et al., (1990) have reported that more than hundred genes are induced and probably transcripts three times that number are repressed in response to salt stress. Similar situation is observed in response to high temperature and draught stress.

1.2 Proteins Expressed as a result of thermal stress

Exposure to a high temperature stress provides the best characterised information as far as the response to different stresses at molecular level is concerned (Vierling, 1991).

All organisms respond to a rapid elevation in environmental temperature, by transcriptional activation of a set of genes that encode protective proteins called heat shock proteins (hsps). Induction of hsps as a general response to thermal stress has been demonstrated in a wide spectrum of organisms, including bacteria, fungi, animals and higher plants (Lindquist and Craig, 1989). On the basis of their approximate molecular weights in kDa, these hsps have been classified as hsp100, hsp90, hsp70, hsp60 and low molecular weight (LMW) hsps (15-30 kDa) (Neuman et al., 1989). Heat shock proteins exhibit highly ubiquitous and conserved features, and are suggested to be essential for cell survival (Lindquist and Craig, 1989).

Heat shock proteins inhibit various metabolic pathways including the synthesis of the proteins, RNA, DNA, hnRNA processing (Sadis et al., 1988). As indicated by the depletion of hs mRNAs, cessation of hsp synthesis and resumption of normal protein synthesis; the system of hsp synthesis is thought to be 'self regulated', since it "turns off" after 6-8 hrs of continuous heat shock. The level of hsp synthesis in response to a stress, is directly proportional to the severity of the stress. Return to the normal rate of hsp70 mRNA synthesis after heat shock, is much slower in cells treated with an inhibitor of protein synthesis than in untreated cells leading to the hypothesis that this response was autoregulated by one of the hsps (DiDomenico et al., 1982). This idea has been carried further in experiments that suggest self regulation of one of the hsp70 genes in yeast, at least some of which occurred at the transcriptional level (Stove and Craig, 1990).

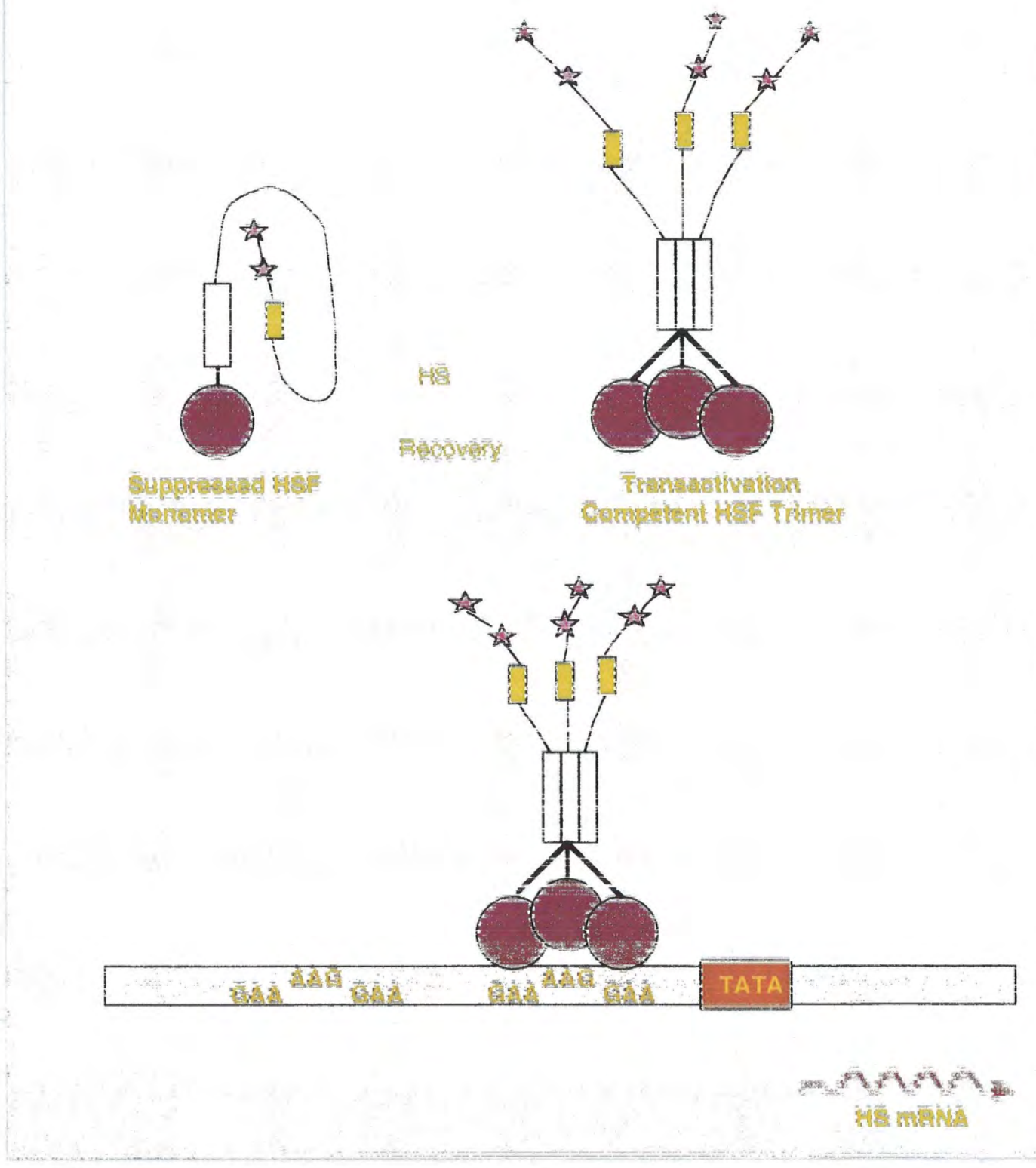
In both prokaryotes and eukaryotes, the cells respond to temperature upshift by increasing either the amount or activity of a transcription factor, specific for the heat shock genes. As a result, transcription of heat-shock genes increase, leading to rise in the concentration of hsps in the cell. In prokaryotes, the immediate trancriptional activator is the specialised σ factor called σ^{32} (Georgopolous et al., 1994). Normally only a few copies of the σ^{32} factor per cell are present, upon heat shock, a transient increase in the cellular concentration of the σ^{32} factor occurs leading to a transient increase in the rate of hsp synthesis. Interestingly, two hsps themselves, DnaK and DnaJ, act as a negative regulator of this process. They bind to σ^{32} factor, thus inactivating it and possibly contributing to its rapid degradation.

In eukaryotes, molecular regulation of the hsp response is exercised at transcriptional as well as the translational level (Hightower, 1991). Upon heat shock, the hs mRNAs are selectively transcribed, accumulated, and are also selectively translated, even though non heat shock mRNAs remain in the cells for many hours (Lindquist, 1986). The selective transcription of hs genes, involves the interaction between a *trans*-acting "heat shock transcription factor (HSF)" and the conserved *cis*-acting elements, including the heat shock consensus elements (HSE) which often occur as overlapping elements, within 100 bp of the 5' end to the transcription initiation site (Pelham, 1982). Each element consists of multiple 5 bp inverted repeats 'nGAAn' in either a head to head, or tail to tail orientation. In higher eukaryotes, the HSFs bind to HSEs only after heat-shock induction ,whereas in yeast, HSFs bind to HSEs constitutively. HSFs generally exist in the inactive state and upon heat shock (hs) they trimerise to become active and then bind to HSEs to start transcription machinery (fig 1.1). HSF activation may also involve other cellular factors, which act as intermediatory sensors to regulate the activity of HSFs, and hsps themselves may be involved. The selective translation of hs mRNAs, appears to result from a specific interaction between altered transcriptional machinery (Scott and Pardue, 1981). Baler et al. (1992) have shown that a member of the hsp70 family binds to the heat shock regulatory factors (HSF), and influences the mechanism that controls the activity of the HSF and so plays an important role in the transcriptional regulation of heat shock genes.

Heat directly leads to the denaturation of proteins. In plants in particular, it causes an increase in evaporation, and thus duplicates conditions associated with drought. Either of these forms of damage could trigger production of a second messenger for gene induction, or alternately could directly activate the heat shock regulatory proteins by forcing a change in conformation, or in protein-protein association post-transcriptionally.

A sudden shift in temperature can also lead to metabolic imbalances, it is possible that these imbalances are responsible for independent induction of heat shock factors (Kobayashi and McEntee, 1990). In most cases translation of the majority of endogenous preformed non-hs mRNAs ceases with increasing temperature (Neuman et al., 1989). This characterises the heat shock response as an emergency reaction to tolerate a sudden temperature increase. In plants as well as in other organisms, there is

Figure 1.1 Proposed mechanism of activation of a typical heat shock promoter. HSF activation, upon heat shock, occurs by unmasking the trimerisation domain (shown as long rectangles) and the transactivation domain (shown as stars). Supression of the trimerisation domain involves intramolecular coiled-coil interactions (long and short rectangles). N-terminal DNA binding domain is shown as stippled circles and C-terminal coiled-coil interaction domain as short rectangles. The transactivation competent HSF trimer binds to HSE and this interaction of HSFs with HSEs induces the transcriptional activation of HSP genes.


HS
Recovery
Suppressed HSF
Monomer
Transactivation
Competent HSF Trimer
nGAAnnnTTCnGAAnnnGAAnnnTTCnGAA
TATA
HS mRNA

considerable evidence that suggests hsp production is an essential component of the short term development of thermotolerance (Lindquist, 1986).

The importance of hsps clearly extends beyond their potential role in protection from high temperature stress. Although hsps were first characterised because of their increased expression in response to elevated temperature, some hsps are found at a significant level in normal non-stressed cells (constitutive expression), or are produced at a particular stage of cell cycles and during development in the absence of stress (developmentally regulated) (Lindquist, 1986; Morimoto et al., 1990). The constitutively expressed hsps are termed as heat shock cognate proteins (hscs). The majority of hsp70 gene family members are expressed constitutively, and abundantly in the absence of any stress. Genetic studies show that many of these proteins are essential for cell viability under normal conditions of growth (Fayet et al., 1989).

Evolutionary conservation and ubiquity of the heat shock response, indicate that hsps play some indispensable role in organisms exposed to high temperature. It has been suggested (Pelham, 1986) that hsps and hscs are involved in assembly and disassembly of proteins and protein-containing complexes, both during normal growth and after heat shock. It has also been suggested that hsps and hscs play a role in intracellular protein transport, or transmembrane targeting (Chirico et al., 1988), folding of proteins within the organelles (Miernyk and Hayman, 1996) or modulation of the activity of key proteins (Catelli et at., 1985). Therefore, hsp70s and related proteins have been included in the ATP-dependent "molecular chaperones" (Ellis, 1990). The precise mechanism by which chaperones enhance correct folding is not fully understood, but it is clear that they bind nascent polypeptides as they emerge from ribosomes (Frydmann et al., 1994), or non-native protein structures (Hartman et al., 1993) and inhibit apparent off-pathway aggregation reactions until a compact native structure is achieved. Another important role of hsps may be to provide the cells with a mechanism to handle increased amounts of abnormally folded proteins. Many of the agents and treatments that enhance the activity of hsp genes, are known to cause unfolding of pre-existing proteins or to induce the synthesis of non-folded polypeptides (Edington et al., 1989). Thus accumulation of non-folded peptides could be a signal for increased expression of hsp genes.

Classical studies *in vitro* demonstrated that protein folding is a spontaneous process dictated primarily by the linear sequence of amino acids (Alfinsen, 1973). However, new studies indicate that such events *in vivo* require the participation of accessory components i.e. "molecular chaperones" or "molecular detergents" (Hemmingson et al., 1988). While molecular chaperones themselves do not appear to directly convey information for folding or ordered assembly, they are thought to facilitate these processes by reducing incorrect folding pathways, and thereby ensure that such events occur rapidly, and with high fidelity (Ellis and Van der vies, 1991). The different proteins of hsp70, hsp60 and LMW hsp classes are present in various cellular compartments like endoplasmic reticulum (Rose et al., 1989), mitochondria and chloroplast (Amir-Sapira et al., 1990; Marshall et al., 1990), as well as being present in the cytoplasm.

1.2.1 HSP70 family

The hsp70 heat shock proteins are found in both eukaryotes and prokaryotes, and are among the highly conserved hsps. In most eukaryotes hsp70 genes occur as a multigene family, whose genes are expressed under a variety of physiological conditions. The diversity of hsp70 genes is partly accounted for by the presence of distinct hsp70 homologues in the cytoplasm (Wu et al., 1988), in the lumen of endoplasmic reticulum (Rose et al., 1989), and in the matrix of chloroplast and mitochondria (Marshall et al., 1990; Amir-sapira et al., 1990). Genes encoding hsp70 homologues have been isolated from maize (Rochester et al., 1986), petunia (Winter et al., 1988), *Arabidopsis* (Wu et al., 1986), tomato (Duck et al., 1989), soybean (Roberts and Key, 1990), pea (DeRocher and Vierling, 1995; Domoney et al., 1991) and barley (Chen et al., 1994).

The hsp70 proteins are highly homologous and show a high level of sequence conservation, with at least 50% identity at the amino acid level (Boorstein et al., 1994). Comparison of all known amino acid sequences of hsp70 protein family members, reveals that the N-terminal region (two thirds of the total sequence, about 450 amino acids) of these proteins are much more highly conserved then the C-terminal portion, suggesting a conserved domain followed by a variable region (Miernyk and Hayman,

1996). In addition, some hsp70 proteins contain N-terminal or C-terminal extensions required for targeting to, or retention in the appropriate cellular compartment. The hsp70 proteins isolated from different organisms have very similar biochemical properties. They all bind ATP with high affinity and possess a weak ATPase activity, which can be stimulated by binding to unfolded proteins and synthetic peptides (Rothman, 1989). ATP-binding activity resides in an N-terminal domain and the C-terminal domain binds to short segments of extended polypeptide (figure 1.2 A; Flaherty et al., 1990). The structure of highly conserved N-terminal ATP binding domain has been solved by X-ray crystallography (Flaherty et al., 1991), and shown that it consists of two lobes forming an ATP binding cleft, which is structurally similar to the ATP binding domain of G-actin and Hexokinase (figure 1.2 Band C) (Flaherty et al., 1990).

All hsp70s, regardless of their cellular location, bind proteins, particularly those that are unfolded. BiP (a hsp70 homologue), which is located in the endoplasmic reticulum (ER) was the first hsp70 shown to bind unfolded proteins in mammalian cells (Haas and Wabble, 1983), and KAR2p (BiP homologue), the product of the KAR2 gene in yeast (Rose et al., 1989), is induced by the accumulation of misfolded proteins inside the ER (Kozutsumi et al., 1988). The hsp70 protein of mammalian cells is capable of binding to and uncoating Clathrin-coated vesicles, in an ATP-dependent fashion *in vitro* (Chappell et al., 1986). Binding of the BiP, shows a preference for binding peptides rich in aliphatic hydrophobic amino acids, with a size of at least seven amino acids. Release of polypeptides from hsp70 is caused rather by ATP binding, and not by ATP hydrolysis as was originally proposed (Hightower et al., 1994). ATP accelerates both the rate of binding and release of peptide substrate (Schmidt et al., 1994). But the ADP form of hsp70 exhibits a higher affinity for the peptides, resulting in a more stable complex, while exchange of nucleotide facilitates release. The universal ability of hsp70s to undergo cycles of binding to and release from hydrophobic stretches of partially unfolded proteins, determines their role in a great diversity of vital intracellular functions, such as protein synthesis, protein folding, oligomerization and protein transport.

A number of mitochondrial hsps from a variety of eukaryotic cells have been isolated with a characteristic N-terminal leader sequence for targeting to the mitochondria (Watts

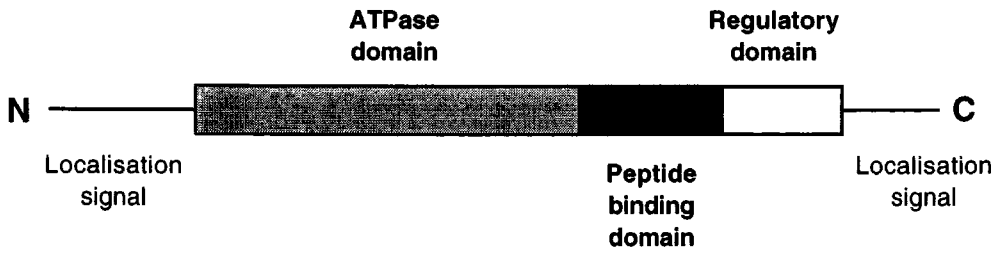
Figure 1.2 Structure of hsp70 proteins.

(a) Linear representation, emphasizing the domain structure of members of hsp70 family of chaperone proteins. The N-terminal and C-terminal localization signals are present only in the sequences of organellar forms.

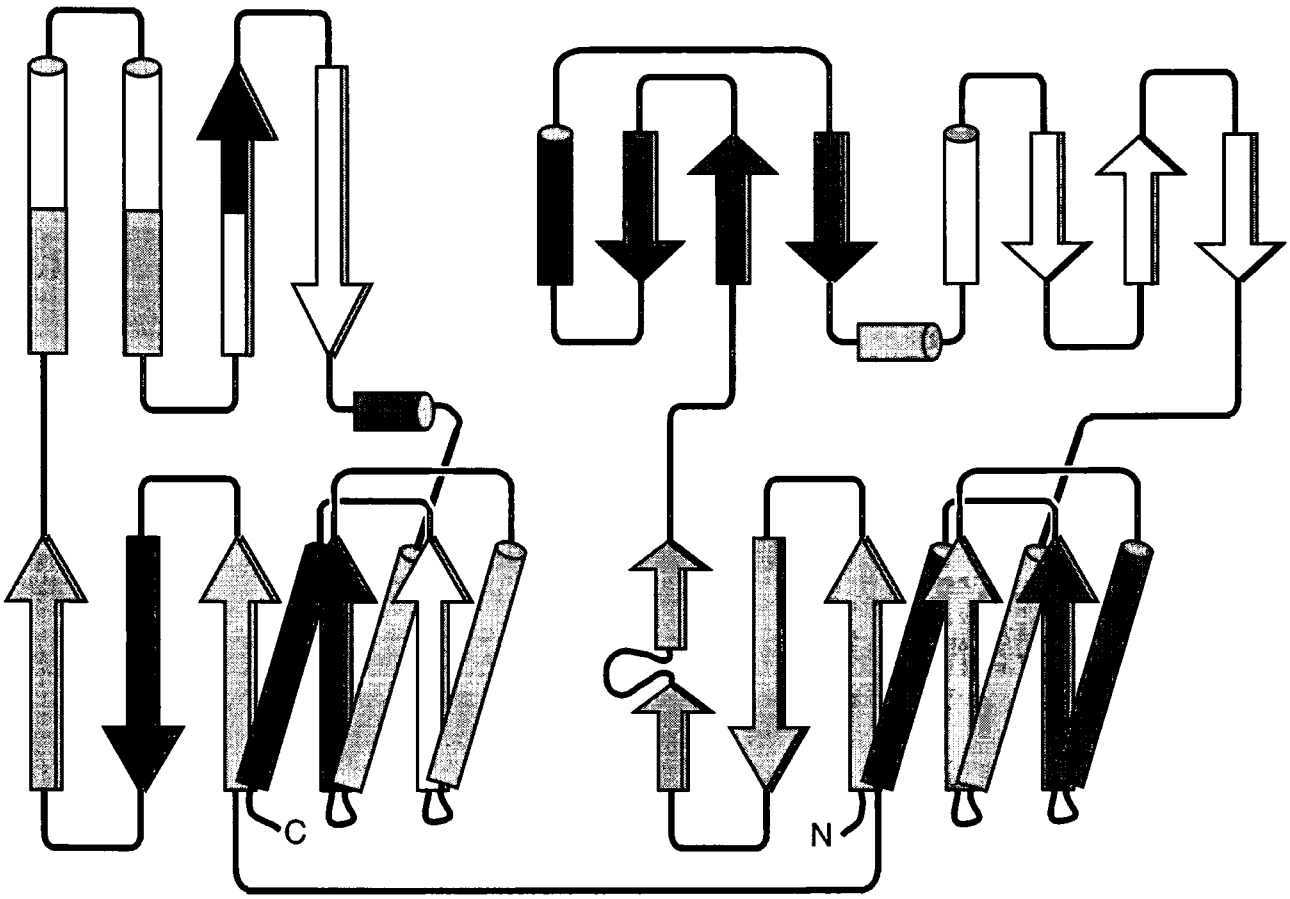
(b) Topological model of the mammalian hsc70 ATPase domain, based on X-ray analysis of the crystallized protein at 2.2 A° resolution. Cylinders represent α-helices, and arrows represent β-sheets. Shading is based on amino acid sequence conservation, with the darkest shading representing the most conservation. (Adapted and redrawn from Miernyk, 1997).

(c) Topological model of the *Escherichia coli* DnaK peptide-binding domain, also based on X-ray analysis of the crystallized polypeptide at 2.2 A° resolution. Numbers indicate the eight β-sheets, and letters indicate the five α-helices. (Adapted and redrawn from Miernyk, 1997).

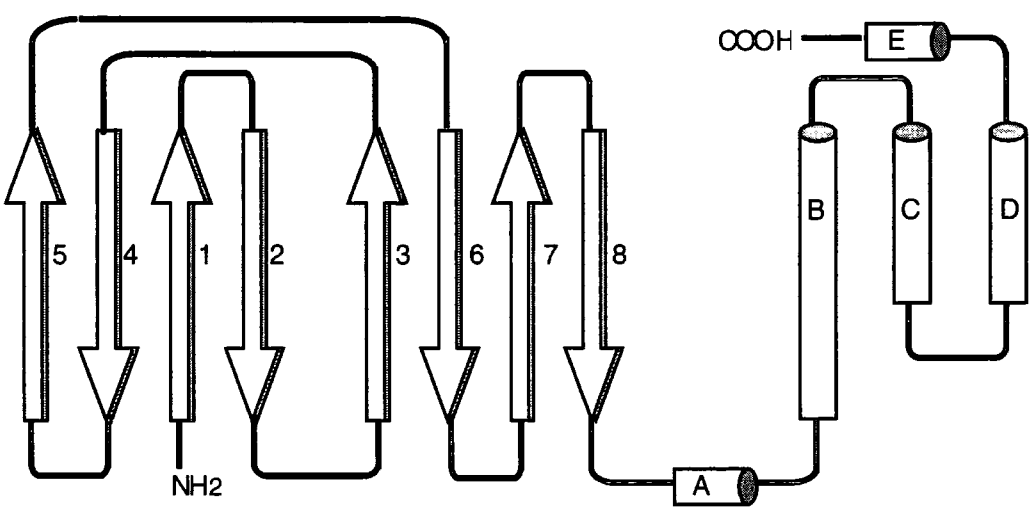
(a)


ATPase domain
Regulatory domain
N
C
Localisation signal
Peptide binding domain
Localisation signal

(b)


C
N

(c)


COOH
E
5
4
1
2
3
6
7
8
B
C
D
NH2
A

et al., 1992). The *Saccharomyces cerevisae* mitochondrial hsp70 is encoded by the nuclear encoded gene SSC1. Analysis has shown that the mitochondrial hsp70s are necessary for the import of translocated precursor proteins (Voos et al., 1993). Mitochondrial hsp70s bind precursor proteins on penetration of the mitochondrial membrane, in a manner comparable with the role of BiP in the ER. The translocated precursor is then stabilised in a partially folded state, until ATP binding permits release and further folding.

Prokaryotic bacteria *E.Coli.* have a single hsp70 called DnaK, which is typically present at approximately 1% of total cellular protein under normal conditions, and its rate of synthesis is increased in response to heat shock. *E.Coli* DnaK have about 45-50% sequence identity with the eukaryotic hsp70s, hsc70s and BiPs. A nuclear encoded protein homologous to DnaK proteins of *E.coli* has now been identified in chloroplasts of pea (Casey et al., 1994), maize and spinach (Amir-sapira et al., 1990). These proteins are located in the chloroplast stroma and are constitutively expressed. The mitochondrial and chloroplast hsp70 proteins appear to form distinct groups that are more closely related to prokaryotic DnaKs, than to other eukaryotic proteins. The presence of prokaryotic type hsp70 in chloroplast and mitochondria, is consistent with the hypothesis of an endosymbiotic origin of these organelles.

The cytosolic hsp70s are closely associated with synthesis, folding and secretion of proteins. All organisms have at least one hsp70 in the cytosol. Accumulation of unfolded proteins or secretory precursors in the cytosol has been observed to induce hsp70 expression. Mammalian cells have an abundance of constitutively expressed hsps called hsp73, and an inducible form called hsp72, without an obvious functional difference, since both can form mixed oligomers (Brown et al., 1993). Studies of yeast cytosolic hsp70s have revealed the presence of two groups, SSA and SSB, which are functionally different and transcriptionally regulated in different ways. SSA is induced by heat shock; conversely SSB genes are switched off (Werner-Washburne et al., 1989). The SSA proteins are required to maintain cell viability, and *in vitro* translation of precursor proteins into mitochondria and ER, whereas SSB proteins bind to newly synthesised polypeptides (Nelson et al., 1992).

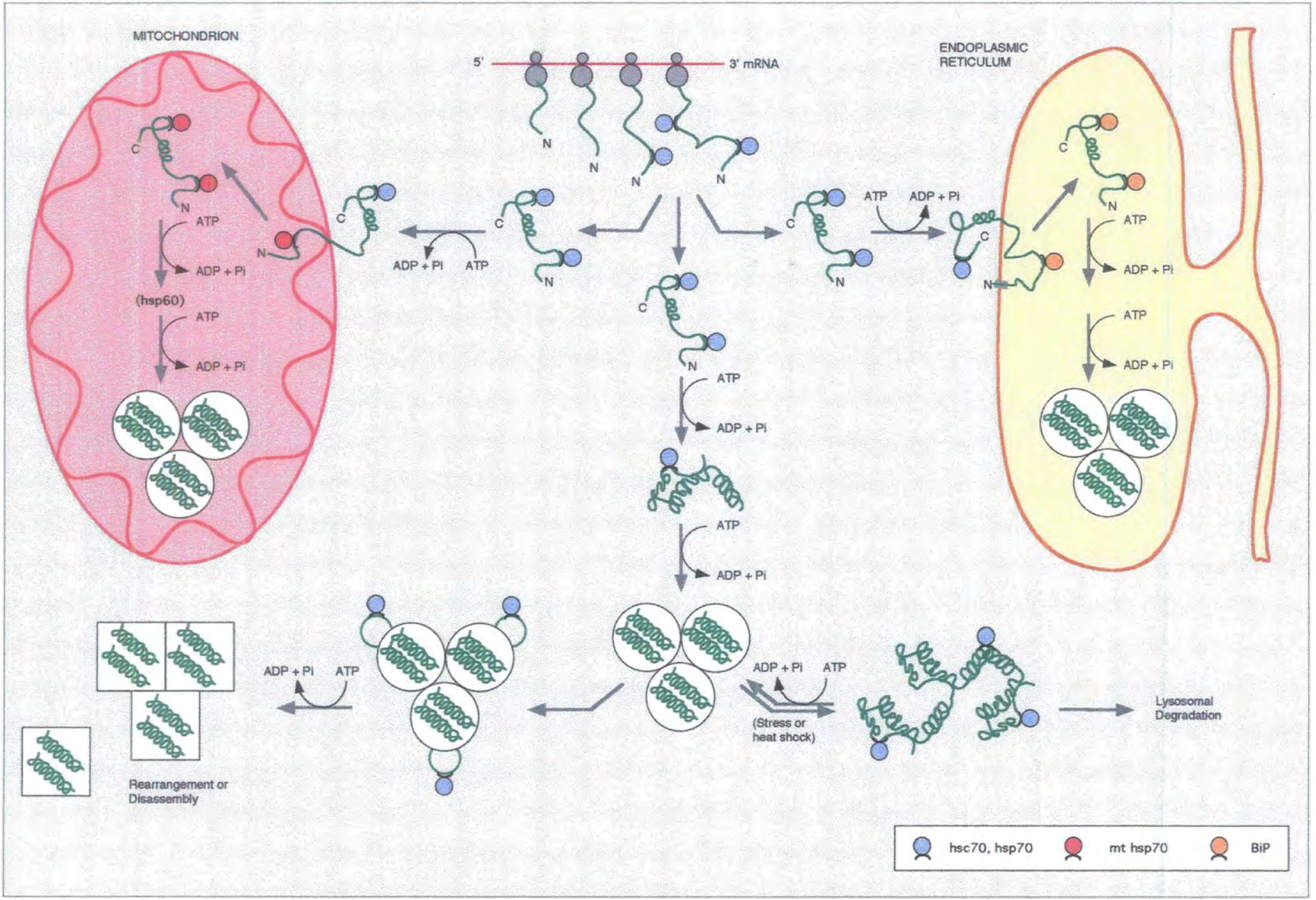
It is widely recognised that members of the hsp70 family act as molecular chaperones. Their genetic and biochemical analyses have shown a list of diverse functions, including binding of nascent chains on ribosomes, maintaining translocation-competent confirmations of ER and mitochondrial precursors in the cytosol, facilitating translocations from inside the target compartments, blocking non-productive folding interactions, and modulating the oligomeric state of macromolecular assemblies (Craig et al., 1994). These diverse actions are due to the ability of hsp70 to bind in an ATP-dependent manner to short hydrophobic stretches of 6-9 amino acids in extended confirmation. Once released, the polypeptide chain has the opportunity to complete its folding by forming intracellular interactions, or to assemble into oligomeric structures with other polypeptide chains, or to engage with the appropriate membrane translocation machinery, or with another chaperone such as hsp60 (figure 1.3).

1.2.2 HSP 60 family

HSP60 proteins are found abundantly in bacteria, mitochondria and plastids of eukaryotic cells. The members of hsp60 family in *E.coli* (GroEL), mitochondria (HSP60) and chloroplasts (Rubisco subunit binding proteins-RBP), mediate the folding of many different proteins *in vivo* and *in vitro*. Because of these activities, the members of hsp60 family have been termed "chaperonins" (Ellis and Van der vies, 1991). The chaperonins have a characteristic oligomeric structure consisting of 14 subunits of approximately 60 kDa, each arranged in two heptameric rings stacked on top of each other.

Bacterial hsp60, GroEL was identified because it is essential for the assembly of a bacteriophage, however it is also essential for the growth of *E.coli* under normal conditions (Fayet et al., 1989). GroEL binds directly to the unfolded substrate protein, stabilising it in a compact intermediate form in the absence of ATP. Upon ATP binding and hydrolysis, the polypeptide is released in a stepwise fashion from the binding site of multiple subunits of GroEL, causing a controlled folding of the protein. Another chaperonin called GroES interacts with and is required for full function of GroEL; in the absence of GroES the binding of GroEL to some of the substrates, results in release but not in productive folding of the substrate (Martin et al., 1991).

Figure 1.3 Illustration of the proposed roles of hsp70 proteins in eukaryotic cells during the folding and membrane translocation of nascent polypeptides, during molecular rearrangements or disassembly, in protection from stress and in protein turnover. (Adapted and redrawn from Gething and Sambrook, 1992).


MITOCHONDRION
5'
3' mRNA
ENDOPLASMIC RETICULUM
C
N
ATP
ADP + Pi
(hsp60)
ADP + Pi
ATP
Rearrangement or Disassembly
(Stress or heat shock)
Lysosomal Degradation
hsc70, hsp70
mt hsp70
BiP

Mitochondrial hsp60 in yeast (encoded by the essential nuclear gene MIF4), is 54% and 43% identical to GroEL and chloroplast RBP, respectively (Reading et al., 1989). Mitochodrial hsp60 has functional similarities with GroEL. They facilitate the folding and assembly of imported unfolded proteins, in an ATP-dependent manner by directly binding to the unfolded proteins in the mitochondrial matrix (Ostermann et al., 1989). Mitochondrial hsp60 has been identified in many different plant species, and maize hsp60 has been extensively characterised (Prasad et al., 1990). They have shown to be expressed at normal cell cycle and during seed germination, as well as during stress conditions. In addition to their role in folding nuclear encoded imported proteins, they may also assist in correct assembly of proteins synthesised inside the mitochondria.

The chaperonin hsp60 present in chloroplasts of higher plants is a nuclear encoded protein, first identified because of its involvement in the assembly of the hetero-oligomeric plant enzyme rubisco (Ellis, 1990). No other information is available with regard to the functional role of chloroplast hsp60 other than rubisco assembly and its expression on exposure to stress. A cytosolic hsp60 termed tail-less-complex polypeptide-1 (TCP-1; Gupta, 1990) from developing sperm cells and yeast plays a role in biogenesis of tubulin and actin.

The hsp60s have similar biochemical properties to hsp70 proteins, but they are not functionally interchangeable as each of them appears to be essential for cell viability. Even then they appear to act sequentially in a common pathway, facilitating different steps in protein folding and assembly. Evidence indicates that hsp70s bind to a peptide backbone in its extended conformation, while hsp60 binds the partially folded intermediates and mediates folding of non native proteins to the native state (Handrick and Hartl, 1993).

1.2.3 HSP90 family

Proteins of the hsp90 family range in size from approximately 80 to 94 kDa, and are the third major highly conserved group of hsp proteins, found in most of the prokaryotes and eukaryotes, and share homology to about 50% or more (Lindquist and Craig, 1988). In eukaryotes, the hsp90 proteins are abundant, essential cytosolic proteins

amounting to ~ 1-2% of total soluble proteins under non-stress conditions, and become even more abundant with stress. The hsp90 family proteins play a crucial role in cell functions, under both stress and non-stress conditions. In mammalian cells the cytosolic hsp90 homologues are often associated with actin and tubulin, several different protein kinases, and with steroid hormone receptors (Pratt et al., 1992). In yeast two hsp90s, hsp82 (stress induced) and hsc82 (constitutively expressed) have been cloned and sequenced. Disruption of both genes is lethal, and mutations in either gene causes failure of yeast to develop thermotolerance (Borkovich et al., 1989). However, in vertebrates, at least one protein similar to hsp90 is found in the cytosol and lumen of ER, this protein is induced by glucose starvation, and has been classified as GRP94 (Glucose-regulated protein; Mazzarella and Green, 1987).

Genes encoding proteins with hsp90 homologues have only recently been isolated cytoplasm of maize (Marrs et al., 1993), and ER of barley (Walther et al., 1993). The hsp90 genes in plants (like their mammalian counterparts), are developmentally regulated in addition to being controlled by temperature and other stresses (Takahashi et al., 1992). The function of hsp90s are not yet very well defined. They are known to act as molecular chaperones, bind to steroid hormone receptors in animal cells, and to interact with protein kinases (Jacob and Buchner, 1994).

1.2.4 Low molecular weight (LMW) heat shock proteins

The low molecular weight (LMW) hsps with kDa values between 15,000 and 30,000 are found predominantly in plants (Key et al., 1985). LMW hsps are abundant and heterogeneous in plant cells, and have been cloned and sequenced from a number of monocot and dicot plants such as soybean (Ming et al., 1992), maize (Jorgensen and Nguyen, 1994), wheat (Dellaqualla and Spada, 1994), sunflower (Coca et al., 1994), and pea (DeRocher and Vierling, 1994). Four gene classes i.e., class I, II, III and IV encoding LMW hsps have been distinguished in plants . These gene products consist of highly conserved hsps that are detected in different cell compartments like ER, cytosol, chloroplast and mitochondria.

Despite the considerable variations in size and complexity of LMW hsps among plant species, proteins of 17 to 23 kDa show considerable homology and often comprise the most abundant hsp class (Goping et al., 1991). Unlike hsp70 proteins, the C-terminal two thirds of the LMW hsps is highly conserved when compared to the N-terminal end. Although the functions of LMW hsps in plants remain mostly speculative, expression of hsps in absence of heat shock, suggests that in addition to defensive roles during thermal stress, they also have an important function in cell proliferation, differentiation and normal development (Bouchard, 1990). Regulation, intracellular location, and mode of peptide recognition of the numerous heat induced, and constitutively and developmentally expressed hsps of this complex gene family in plants will need further examination to determine their precise significance.

1.2.5 HSP100 protein family

The hsp100 family (also known as clp family) includes the high molecular weight hsps (>94 kDa) which are highly conserved, and have been reported in both prokaryotes and eukaryotes (Squires and Squires, 1992). A heat-inducible hsp104 from yeast has been cloned and appears to be the principle component responsible for induced thermotolerance in yeast (Sanchez and Lindquist, 1990). Sanchez et al. (1993) have shown a functional relationship between hsp104 and hsp70 genes (SSA1, SSA3, SSA4) in yeast. When hsp70 levels are reduced, hsp104 become important for growth at normal and moderately high temperatures. Conversely, when hsp104 levels are reduced, hsp70 becomes important in thermotolerance. These results strongly suggest that hsp70 and hsp104 operate on the same pathway, or on parallel pathways that partially overlap.

Members of the hsp100/clp protein family share two large blocks of sequence homology (about 200 amino acids), each containing an ATP-binding domain. The ATP-binding domains are separated by a variable spacer region, namely clpA, clpB and clpC, which are short, long and intermediate, respectively. The heat-inducible yeast hsp104, plant hsp100 and *E.coli* hsp100 belongs to the ClpB family. Additionally, several Clp genes have been characterised from higher plants, including tomato (Gottesmann et al., 1990), pea (Moore and Keegstra, 1993) and *Arabidopsis* (Kiyosue

et al., 1994), all of which are classified as clpC family members. The clpC in pea has been shown to be localised in chloroplast (Moore and Keegstra, 1993).

In contrast with hsp70 and hsp60 class chaperones, which are expressed and are functionally important at all temperatures; hsp100 proteins appear specialised to promote survival under extreme conditions (Parsell and Lindquist, 1994), but there is no well documented developmental expression of hsp100 class of proteins. The hsp100 proteins appear to functioning as chaperones to mediate protein dis-aggregation but no direct evidence has yet been shown.

1.2.6 Other roles of heat shock proteins

Despite their designation, most of the so called heat shock, or stress proteins, are in fact expressed constitutively in cells grown under normal conditions, and represent essential gene products involved in a number of important biological pathways (Morimoto et al., 1990). These proteins are designated as heat shock cognate proteins (hscs); and are expressed under normal temperatures. They seem to have similar functions during normal cellular metabolism as other hsps have under stress and during recovery from stress. These functions include unfolding, translocation and refolding of proteins (Chirico et al., 1988). Most of the hsp70 family members are expressed constitutively. Their evolutionary conservation, intracellular locations, and pattern of expression, all indicate that hsp70s play important roles, both in response to heat stress and in normal cellular function (Becker and Craig, 1994).

Certain specific hsps are produced at a particular stage of the cell cycle or during development in the absence of stress. The expression of hsp mRNAs and protein during development in the absence of heat stress has been studied in a number of eukaryotes. As already discussed in section 1.2.1, hsps are regulated developmentally in several eukaryotes, and it is evident that some hsps are regulated developmentally in higher plants. The hsp70 mRNA levels vary dramatically between organs of developing tomato fruits (Duck et al., 1989), and during the early stage of pea seed development (DeRocher and Vierling, 1995).

The genes for the heat shock proteins particularly hsp70s in plants, in addition to being under heat shock and developmental control; are activated by a variety of diverse stresses such as drought, wounding, salinity, heavy metals, arsenite or cold (Neumann

et al., 1994; Winter et al., 1988; Neven et al., 1992). Such experiments highlight the point that certain elements of the stress response cascade are common amongst different stresses (Vierling, 1991). The induction by various apparently unrelated stresses and chemical agents, suggests that hsps70 could play a general role in stress adaptation (Vierling, 1991).

The above discussion on different heat shock proteins implies that these proteins are not only involved in protein folding pathways but also play some indispensable roles under stress and during normal growth conditions. Their possible involvement in cellular aging, etiology of cancer and in immune surveillance further strengthen the view that hsps are evolutionary conserved throughout the plant and animal kingdom. Molecular and structural analysis of different hsps will help to understand their biochemical and physiological functions. The term "heat shock proteins" in the literature is misleading as it has been clear that these proteins are expressing under a variety of conditions. Therefore, a term "universal proteins" would be more appropriate to reflect their functional aspects.

1.3. Wounding stress

Plants are constantly challenged by insects, viruses, bacteria and fungi as they explore different habitats for nutrient sources. In the course of evolution, both partners of an interaction, evolve together and develop mechanisms either to benefit from the interaction, as exemplified by symbiosis, or to battle for survival, as exemplified by pathogenic interactions. When challenged by microbes or wounding by chewing insect pests, 'defense-type' reactions are triggered in the host plant (Baron and Zambryski, 1995). Wounding in plants by insect feeding can be mimicked by mechanical means such as cutting and crushing, which also shares many features of the response to pathogens. Jongsma et al. (1994), have shown that regardless of whether leaves are ingested by insects or mechanically wounded by cutting or crushing, the wound-induced accumulation of proteinase inhibitors in tomato and tobacco were at comparable levels, however, the proteinase inhibitor accumulation level after TMV infection was lower than that of insect feeding or mechanically wounding. The strong resemblance of the plant responses to wounding by herbivory, abiotic stresses and pathogen attack

have led to the speculations that wound signal transduction may proceed via similar, if not identical pathways (Baron and Zambryski, 1995).

The signalling pathways that allow plants to mount spatial and temporal defenses against the intruders are known to be complex, often resulting in superimposition of signalling processes (McConn et al., 1997). Plants respond to wounding (mechanical or insect feeding) and pathogen attack by modifying their metabolism via differential gene expression, that leads to the synthesis of several protein involved in the defence mechanism of the plants (Baldwin, 1994). The defensive genes that express in response to wounding or pathogen infection are described in section 1.4.3. Most of these genes have a role in wound healing, prevention of subsequent pathogen invasion, or deterring insects, and their expression is restricted either to the directly wounded tissue (localised response) or to the unwounded, more distal tissues (systemic response) (Bowles, 1990). A defensive signalling pathway in leaves of tomato plants is activated in response to insect attack or mechanical wounding that crushes the tissues and synthesis of several defense proteins such as proteinase inhibitors and the enzyme polyphenol oxidase are induced, in cells of both wounded and distal unwounded leaves (Ryan, 1992; Schaller and Ryan, 1996). The wound-inducibility of gene expression implies the existence of a signal transduction pathway. At the terminus of the signal transduction pathway, wound specific factor(s) bind to wound responsive promoter elements and activate gene expression.

1.3.1 Gene expression at wound site - localised response

A wound as defined by Bostock and Stermer (1989) is an external or internal injury that breaks the outer protective layer of plants and leads to the destruction of cells in a specific area of tissue. The physical rupturing of cells causes the concomitant loss of compartmentation. A wound may occur as a result from severe weather (wind, rain, hail, freezing), from herbivores or during normal growth (abrasion, growth cracks). Many pathogens gain their entry into plant body through wound sites, therefore, the study of the physiological, biochemical and molecular changes in the wounded cells and the cells in the vicinity of the wound site, may provide important clues for engineering the improved defense mechanisms into plants.

The wound will be sealed in different ways leading to the alternative wound periderm and wound barrier zone formation, depending upon the plant anatomy. It is well known that the local wound response of most monocot plants is different from that of most dicot plants. In dicots, due to the presence of cambium, the wounded tissues respond by cell proliferation, resulting in the production of wound callus. Whereas, the wounded tissues in monocots do not divide but differentiate into lignified or sclerified cells, thus producing a ring of hardened cells around the initial wound sites (Schlappi and Hohn, 1992). Initially, in both type of responses, there is an increase in transcriptional activity, associated with high metabolic activities and upregulation of defense-related genes (Bostock and Stermer, 1989). Whether this general difference in local wound response between monocot and dicot plants would lead to differences in their respective wound signal production, transmission and systemic response in unwounded plant parts, is not known.

The induction of proteinase inhibitor gene expression occurs in cells both at localised wounding sites as well as systemically. Oligosaccharide fragments released by wounding, or pathogen-derived polygalacturonases or pectolytic enzymes are immobile in plants, and are considered to play a role in signalling synthesis of localised defensive responses in cells at, or near the wound site (Koiwa et al., 1997). Genes such as tobacco proteinase inhibitor I (Linthorst et al., 1993), *mas* (mannopine synthase; Ni et al., 1995) and *nos* (nopaline synthase; An et al., 1990), are wound inducible only in wounded cells and the cells found locally around the wound site. A volatile terpenoid called volicitin, a glutamic acid-conjugated fatty acid derivative detected in the oral secretion of the beet armyworm, was shown to elicit the emission of volatile terpenoids in plants at the wound site which attracts the parasitic predators (Alborn et al., 1997). Therefore, all these studies indicate that elicitation of the response and synthesis of the signal molecules, takes place initially at the wound site and then it disperses to unwounded parts.

1.3.2 Systemic response of plant to wounding

The systemic expression of defensive genes particularly proteinase inhibitors has been extensively studied in the solanaceous plants tomato and potato. Several candidates for the systemic signal have been suggested including jasmonic acid, 18:3 (linolenic acid, a major component of plant membranes), oligogalacturonides and a 18 amino acid polypeptide called systemin (Farmer and Ryan, 1992). The activation of defensive genes by wounding, oligouronides and systemin has been proposed to occur via a lipid-derived octadecanoid pathway, in which linolenic acid is generated in receptor cells in response to signals (Farmer and Ryan, 1992), and is converted by a series of enzymatic steps to 12-oxophytodienoic acid and on to jasmonic acid (JA), leading to the transcriptional activation of defensive genes (Wasternack et al., 1996). Several plasma membrane proteins, act as potential wound induced signal molecule receptors such as a β-glucan-elicitor-binding protein (GEBP) of 70 kDa from soybean (Umemoto et al., 1997) that bind to fungal elicitor and a systemin-binding protein (SBP50) of 50 kDa from tomato leaves (Schaller and Ryan, 1994) have been isolated.

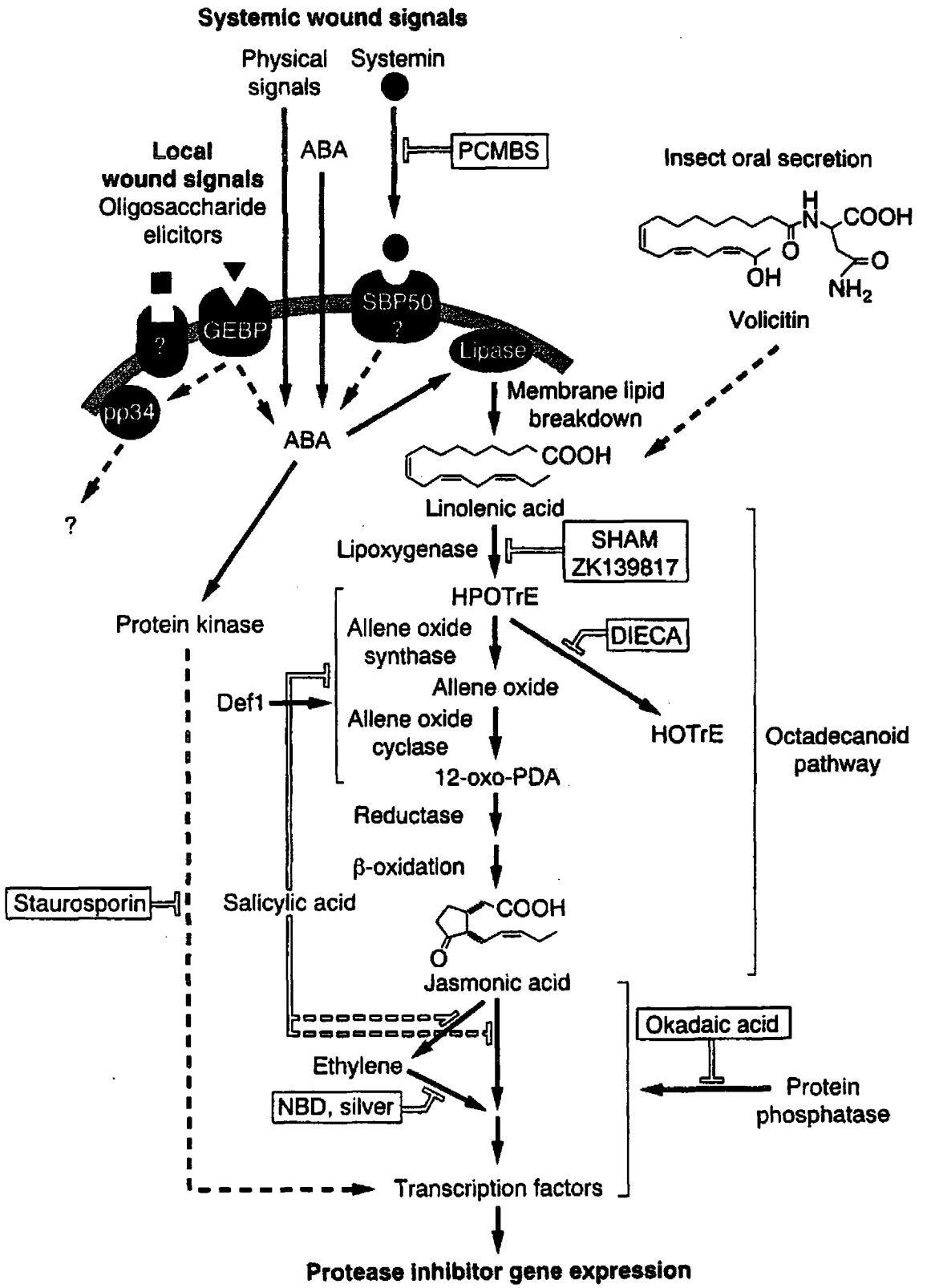
Other signals such as abscisic acid (ABA ; Pena-Cortes et al., 1995) and electrical signals (Wildon et al., 1992), have also been proposed. Phytohormones such as ABA and JA are able to trigger the proteinase inhibitor II gene expression in a manner similar to that observed upon wounding. Furthermore, ABA and JA activate proteinase inhibitor II gene expression in ABA-deficient tomato and potato mutants which fail to accumulate proteinase inhibitor II mRNA upon wounding or systemin treatment (Pena-Cortes et al., 1996). Apparently, ABA functions at a point between the systemin and linolenic acid signalling pathway, which is consistent with a role for the ABA in the transduction of systemin signal through the octadecanoid pathway. Therefore, ABA appears to be a necessary component of the systemic signalling of proteinase inhibitor synthesis, but it does not appear to act as a primary mobile systemic signal in tomato plants (Pena-Cortes et al., 1996). Evidence also indicates that the ABA signal may be transduced by a jasmonic acid-independent pathway that has a staurosporine-sensitive protein kinase as an intermediate (Dammann et al., 1997). Electrochemical signals have recently been associated with signalling proteinase inhibitor synthesis in tomato cotyledons (Wildon et al., 1992), but the velocity of electrical pulse (less than 5 min/ cm) appears to be much too rapid for the exit of the systemic wound signal out of the

wounded tomato leaves, where the systemic signal has been shown to take nearly an hour to move from one leaf to another (Nelson et al., 1983). Therefore, although electrical signals may have some role in the systemic induction, it is unlikely that they are systemic signals in tomato plants. Ethylene has also been implicated in wound or jasmonate induction of proteinase inhibitor mRNA and norbornardiene and silver thiosulfate, both inhibitors of ethylene action, could inhibit proteinase inhibitor mRNA accumulation induced by wounding or jasmonate (O'Donnel et al., 1996). These results suggest that ethylene functions downstream of, or parallel with jasmonate.

A role for the octadecanoid pathway in activating proteinase inhibitor gene by systemin (wounding) or by oligosaccharides (pathogen attacks) has been supported by several lines of evidence. Activation of proteinase inhibitor and polyphenol oxidase genes was observed by supplying systemin or oligosaccharide fragments to young tomato plants or to leaves (Constable et al., 1995). Linolenic acids or the intermediates of the pathway from linolenic acid to jasmonic acid also activate defensive genes, when applied topically to tomato leaf surfaces (Farmer and Ryan, 1992). Additionally, defense gene activation by wounding, systemin and oligosaccharide elicitors is severely depressed, at both the protein and mRNA levels by inhibitors of octadecanoid pathway such as sodium *p*-chloromercuribenzenesulfonate (PCMBS), sodium diethyldithiocarbamate (DIECA) and sodium salicylate (SA) (Farmer et al., 1994). Also a mutant tomato line (*def*1), deficient in a pathway component, was considerably more susceptible than wild type to attack by tobacco hornworm larvae (Howe et al., 1996). Therefore, it could be argued that all the enzymes necessary for converting linolenic acid to jasmonic acid are present in leaf cells and that the pathway is activated by wounding. The activation of the octadecanoid pathway by systemin (wounding) and oligouronides (pathogen attacks) and sites of action of respective inhibitors is shown in figure 1.4. The effect of these inhibitors are consistent with the hypothesis that the primary signalling molecules generated at wound or infection sites, activate defensive genes via the octadecanoid pathway.

In other plant species, wound-signalling pathways are much less completely defined. Some plants contain systemically inducible proteinase inhibitors (Cordero et al., 1994), but broader ecophysiological studies indicate that many other compounds are produced in plants to deter insect attacks (Bowles, 1990; Baldwin, 1994). It is not clear how

Figure 1.4 Proposed intracellular wound signal transduction pathway leading to the induction of proteinase inhibitor and other defensive gene expression. Filled arrows illustrate portions of the pathways proposed from direct evidence. Broken arrows illustrate inferred pathways or interactions. Open bars illustrate the effects of inhibitors. The pathway is initiated by the interaction of local or systemic signal molecules and putative plasma membrane receptors. Abbreviations: GEBP - β-Glucan-elicitor-binding protein; SBP50 - Systemin-binding protein of 50 kDa; SA - Salicylic acid; HPOTrE - 13(S)-hydroperoxylinolenic acid; HOTrE - 13-hydroxylinolenic acid; 12-oxo-PDA - 12-oxo-Phytodienoic acid; PCMB - p-chloromercuribenzene sulfonic acid; DIECA - Diethyldithiocarbamic acid; NBD - Norbornardiene; ABA - Abscisic acid. (Adapted and redrawn from Koiwa et al., 1997).


Systemic wound signals
Physical signals
Systemin
Local wound signals
Oligosaccharide elicitors
ABA
PCMBS
Insect oral secretion
COOH
OH
NH2
Volicitin
SBP50
GEBP
Lipase
pp34
Membrane lipid breakdown
ABA
COOH
Linolenic acid
Lipoxygenase
SHAM
ZK139817
HPOTrE
Protein kinase
Allene oxide synthase
DIECA
Allene oxide
Def1
Allene oxide cyclase
HOTrE
Octadecanoid pathway
12-oxo-PDA
Reductase
β-oxidation
Staurosporin
Salicylic acid
COOH
Jasmonic acid
Okadaic acid
Ethylene
Protein phosphatase
NBD, silver
Transcription factors
Protease inhibitor gene expression

many different signalling systems may be involved in higher plants, or how complex each pathway might be in its organisation. For example, studies in *Arabidopsis* have not identified a systemin homologue and specific assays have failed to demonstrate significant constitutive or wound-induced proteinase inhibitor activity (Ryan, unpublished work). *Arabidopsis*, in common with other members of the Brassicaceae, contains the constitutive glucosinolate-myrosinase system that is postulated to act as a defence against chewing insects. McConn et al. (1997), established that jasmonate is both necessary and sufficient to protect *Arabidopsis* against insect attack as an *Arabidopsis* mutant deficient in linolenic acid was unable to synthesise jasmonates, and was strikingly susceptible to devastation by a common saprophagus fungal gnat larva, *Bradysia impatiens*.

1.3.3 Defense gene products induced by wounding

Plants have developed an impressive array of defence mechanisms. Damage to plants inflicted by pathogens, predators or mechanical wounding, trigger changes in gene expression which is characterised by expression of a set of proteins. These responses includes the reinforcement of cell wall by deposition of structural proteins such as hydroxyproline-rich glycoproteins (HRGPs), glycine-rich proteins (GRPs), proline-rich proteins (PRPs), synthesis of antimicrobial compounds – phytoalexins, production of proteinase inhibitors and lytic enzymes such as chitinases and glucanases, antinutritive/toxic proteins, synthesis of chemical deterrents and an array of other responses leading to beneficial response throughout the plant body.

1.3.3.1 Proteinase inhibitors and other defensive proteins

Proteinase inhibitors (PIs)

Proteinase inhibitors are an important element of defence response in plant tissues that inhibit the activity of digestive proteinases in herbivorous insect guts and can lead to protein malnutrition, reduced growth and even death to the insects in some instances (Ryan, 1990). Induction of these inhibitors is highly regulated by a signal transduction

pathway, that is initiated by predation and transduced as a wound response both locally and systemically. Proteinase inhibitors in plants can be divided into four classes, depending upon the type of proteinase they inhibit, i.e. serine, cysteine, aspartic acid and metalloproteinases. Serine proteinase inhibitors are the most extensively studied class of plant proteinase inhibitors and are assumed to have function in defence against herbivores. Serine proteinase inhibitors are currently subdivided into 8 families based on primary sequence data (Ryan, 1990). The serine proteinase potato inhibitor I and II families whose synthesis is induced by wounding or pathogen infections are described in detail in this section. Inhibitor I inhibits chymotrypsin at its single reactive site, while inhibitor II contains two reactive sites and is able to inhibit both trypsin and chymotrypsin. Both inhibitor I and II are synthesised as precursors and after post translational modifications, the mature proteins are sequestered in the vacuole. The proteinase inhibitors form a complex upon binding to the active sites and thus repress the catalytic activity of proteolytic enzymes, by effectively blocking the active sites (Felton and Gatehouse, 1996).

Proteinase inhibitors are synthesised constitutively in specific plant organs like potato tubers and tomato fruits; they can be induced in leaves of tomato and potato plants by mechanical damage caused by chewing insects, both locally and systemically in upper non-wounded leaves (Green and Ryan, 1972), or by pathogen attack (Wingate and Ryan, 1991). The cDNA and genomic clones encoding these inhibitors have been isolated from a spectrum of plants such as tomato (Lee et al., 1986), potato (Cleavland et al., 1987), and alfalfa (Brown et al., 1985), The proteinase inhibitors initially accumulate at the site of attack, reaching maximum concentration within 12-24 hrs, and then throughout the plant over several days. A large number of compounds have been implicated in mediating the signalling leading to wound responsive expression of proteinase inhibitors (as has been described in section 1.4.2 of this chapter). A proteinase inhibitor I gene isolated from tobacco, was found to be strongly induced during the hypersensitive response to TMV infection, as well as to ethephon treatment, wounding and UV irradiation (Linthorst et al., 1993). However, contrary to the potato and tomato proteinase inhibitor I genes, the tobacco genes are not systemically inducible by wounding. This expression pattern of tobacco proteinase inhibitor, which might be located in the vacuole like potato and tomato inhibitors, resembles that of the intracellular PR proteins of Tobacco.

The promoter deletion analysis of potato and tomato proteinase inhibitor II has resulted in the identification of wound responsive *cis*-regulatory elements (Keil et al., 1990; Sanchez-Serrano et al., 1990). The transgenic tobacco plants expressing the potato and tomato proteinase inhibitor II regulated by the constitutive CaMV promoter, caused severe growth inhibition to larvae of *Manduca sexta* (Tobacco horn worm; *Coleoptera*), when fed on these transgenic plants as compared to larvae fed on wild type plants. In contrast, transgenic plants expressing inhibitor I had no effect on growth even at an expression level higher than inhibitor II (Johnson et al., 1989). More recently, the potato inhibitor II gene transferred to rice, under control of its own wound-inducible promoter (Duan et al., 1996), showed enhanced resistance to a major rice pest, *Sesamia inferens* (Pink stem borer; *Noctuidae*). Thus, evidence confirms the role of proteinase inhibitors in defense against insect pests and pathogens.

Chitinases and β-1,3-Glucanases

Chitinases and β-1,3-glucanases are fungal cell wall degrading enzymes which belong to pathogenesis-related proteins (PR proteins) that accumulate in response to pathogen and abiotic stresses. Based on sequence similarities and immunological data, PR proteins have been classified into five groups; in most of these groups acidic and basic PR proteins have been identified which are localised in the apoplast or in the vacuoles (Linthorst, 1991). Chitinases are the members of the PR-3 family and they catalyse the hydrolysis of chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine, while β-1,3-glucanases are members of the PR-2 protein family and catalyse the hydrolysis of β-glucans. Chitin and β– glucans are the main components of the cell wall of many phytopathogenic fungi (Wessels, 1993). Both chitinase and β-1,3-glucanase PR protein exists in acidic extracellular and basic vacuolar isoforms (Linthorst et al., 1990; Neuhaus et al., 1991). Genes for both proteins contain a sequence encoding an N-terminal signal peptide for transport across the membrane of the endoplasmic reticulum. By sequence alignment of these PR genes, it was found that the encoded basic isoforms carry C-terminal extensions when compared with their acidic counterparts. It has been postulated that these C-terminal extensions are the signal for targeting the basic PR proteins to the vacuoles and that they are cleaved off during the translocation event

(Neuhaus et al., 1991; Melcher et al., 1994). Acidic isoforms which do not contain a vacuolar sorting signal, are transported to the extracellular space. Apart from being differentially targeted, there are also differences in the organ specific expression pattern and in response to certain stress conditions, between the acidic and basic group of Chitinases and β-1,3-glucanases genes.

Chitinases and β-1,3-glucanases have been shown to be expressed in response to pathogens in many plants such as tobacco, potato (Kombrink et al., 1988), bean (Awade et al., 1989), and maize (Nasser et al., 1990). Plant chitinases and β-1,3-glucanases have been divided into many classes, based on their sequence similarity and inducibility. Class I consists of the basic chitinases containing a hevein domain. Class II chitinase lack the hevein domain but are otherwise similar to class I. In tobacco and many other plant species, the class I chitinases accumulate intracellularly, whereas class II chitinases are targeted to the extracellular fluid. However, the extracellular hevein domain-containing chitinase from bean disprove the generality of this rule (Margis-Pinheiro et al., 1991). Class III chitinases have both chitinase and lysozyme activity. Tobacco contains small gene families for acidic and basic class III chitinase/lysozyme, which are induced in response to TMV infection (Collinge et al., 1993).

The antifungal properties of Chitinases and β-1,3-glucanases have been confirmed *in vitro* and *in vivo* assays. Moreover, these two enzymes have been shown to be more effective when applied in combination than either protein alone (Sela-Burlage et al., 1993). Individual isoforms of the two enzymes differ in their specificity and activity, with class II acidic apoplastic chitinase in tobacco being inactive against *Fusarium solani.* In contrast, class I basic vacuolar isoforms of the two enzymes acted synergistically, while the class II chitinase also showed some action when combined with class I β-1,3-glucanase. A class V chitinase from tobacco has also been shown to act synergistically with tobacco class I β-1,3-glucanase to inhibit *Fusarium solani.* (Melchers et al., 1994). Transgenic tobacco plants, constitutively expressing bean and rice chitinases (Broglie et al., 1991; Vierheilig et al., 1993), showed enhanced resistance to *Rhizoctonia solani*, also showed that constitutive co-expression of rice chitinase and alfalfa β-1,3-glucanase in rice gave greater protection against *Cercospora nicotiana* than either protein alone. Similar synergistic interactions were observed by

Jach et al. (1995), between barley chitinase and barley β-1,3-glucanase transgenic tobacco plants expressing these proteins infected with *R.solani.*

1.3.3.2 Polyphenol oxidase (PPO) and other Oxidases

Polyphenol oxidase

Polyphenol oxidases (including monophenolase EC 1.14.18.1, diphenolase EC 1.10.3.1, and laccase EC 1.10.3.2 activities) are nuclear-encoded, copper metalloprotein enzymes located in or on the internal membrane of the plastids, in both photosynthetic or nonphotosynthetic tissues of angiosperms (Duffey and Felton, 1991). PPOs catalyse the oxidation of mono-, di-, or polyhydric phenols to quinones (figures 1.5 A&B). The quinonoid reaction products of PPO are potent electrophiles which undergo a variety of secondary reactions such as autoxidation, forming melanin, they also covalently crosslink or modify nucleophilic substituents of proteins and free amino acids. The melanin formation responsible for oxidative browning of plant tissue and extracts, represents the first detrimental effect of PPO in the post harvest physiology and processing of crop plants (Mayer and Harel, 1991). The quinones formed by these reactions also bind irreversibly to the nucleophilic side chain (-SH, $-NH_2$, -NH) of proteins, thus modifying and reducing the nutritive value of plant proteins via significant losses in lysine, histidine and thiol amino acids (Felton et al., 1992b).

Furthermore, as a result of the quinonoid reaction, reactive oxygen species (e.g. superoxide radical, hydrogen peroxide, hydroxyl radicals) may be formed, which further contribute to loss of amino acids, via carbonyl formation and to protein damage by polymerisation and/or fragmentation (Stadtman, 1993). Catecols that are attached to proteins by alkylation, or are formed by radical attack on proteins, may accumulate and then form reactive oxygen species which in turn attack other biomolecules (figure 1.6 A&B). Protein hydroperoxides may also be formed as a product of radical attack on proteins and contribute to further protein damage (Dean et al., 1993).

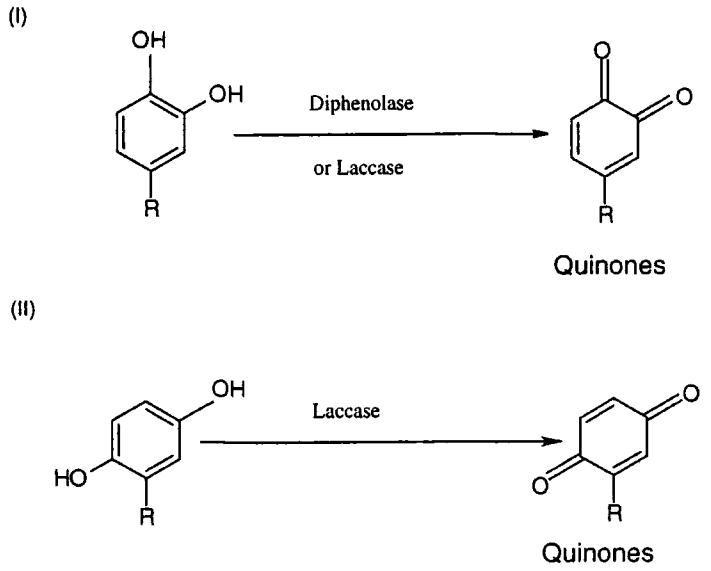


Figure 1.5 A Reaction of polyphenol oxidase and laccase with phenol. (I) Diphenolase action by polyphenol oxidase or laccase. (II) Action of laccase on p-diphenol.

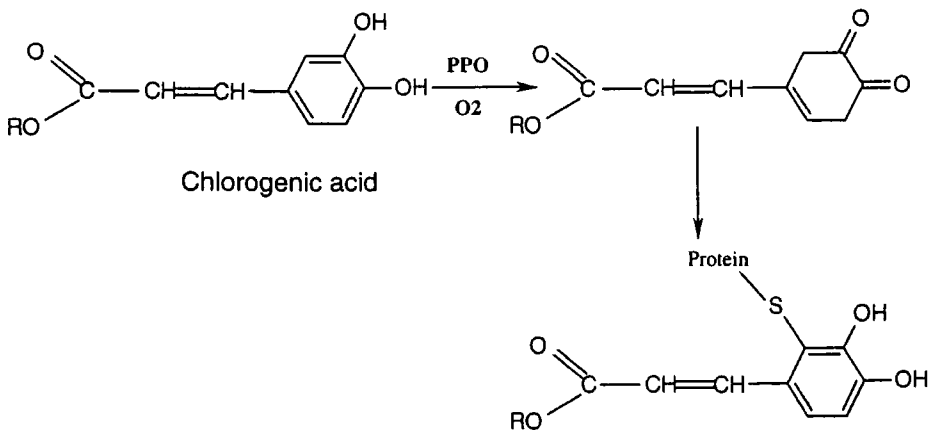


Figure 1.5 B Oxidation of chlorogenic acid and subsequent alkylation of proteins. PPO = polyphenol oxidase; S = thiol.

PPO has been purified from a number of higher plants and the resulting proteins have apparent molecular masses of 40-60 kDa (Steffens et al., 1994). Genes encoding PPO have been cloned from many plants including tomato (Sahar et al.,1992), potato (Thygesen et al., 1995), broad bean (Cary et al., 1992), grape berry (Dry and Robinson, 1994) and sugarcane (Bucheli et al., 1996). In broad bean, tomato and potato, the PPO has been shown to be encoded by multigene families (Cary et al., 1992; Newman et al., 1993; Thygesen et al., 1995), but in grape berry only a single family was identified (Dry and Robinson, 1994). PPO genes isolated from diverse groups have the strongest sequence homology in the histidine-rich domains, designated CuA and CuB. These conserved regions are thought to be involved in copper binding, have been identified by comparison with the copper binding region of the haemocyanins and tyrosinases (Steffens et al., 1994). PPO also exhibited the presence of N-terminal sequence characteristics of plastid transit peptide.

A large number of studies have shown that PPO activity increases in response to abiotic and biotic injury. Mechanical wounding of two week old tomato seedlings resulted in both localised and systemic induction of PPO (Constable et al., 1995) and a similar response was also observed in potato leaves (Thipyapong et al., 1995). An increase in PPO activity was also seen in tomato leaves upon insect feeding (Felton et al., 1992b) and inoculation with the pathogen *Pseudomans syringae* pv. tomato resulted in the systemic induction of PPO activity (Bashan et al., 1987). Differential expression of PPO in vegetative and floral organs of tomato during normal growth conditions was observed by Sahar et al. (1992).

Various physiological roles have been proposed for PPO, but now it is more widely accepted that PPO is probably involved in defence against invading pathogens or insect pests (Steffens et al., 1994; Felton, 1996). A strong correlation between PPO level in tomato leaves and reduction in growth of the tomato fruit worm *Heliothus zea* feeding on this foliage was found by Duffey et al. (1991). The proposed role of PPO is based on the ability of PPO-generated quinones to covalently modify plant proteins, thereby, decreasing the nutritive availability of dietary amino acids to herbivores or pathogens. Oxidation of the plant phenolics begins only after decompartmentation of the PPO by disruption of plant tissues by insect chewing and the quinones produced by PPO, attack those amino acids which are predicted to be most nutritionally limiting to herbivores

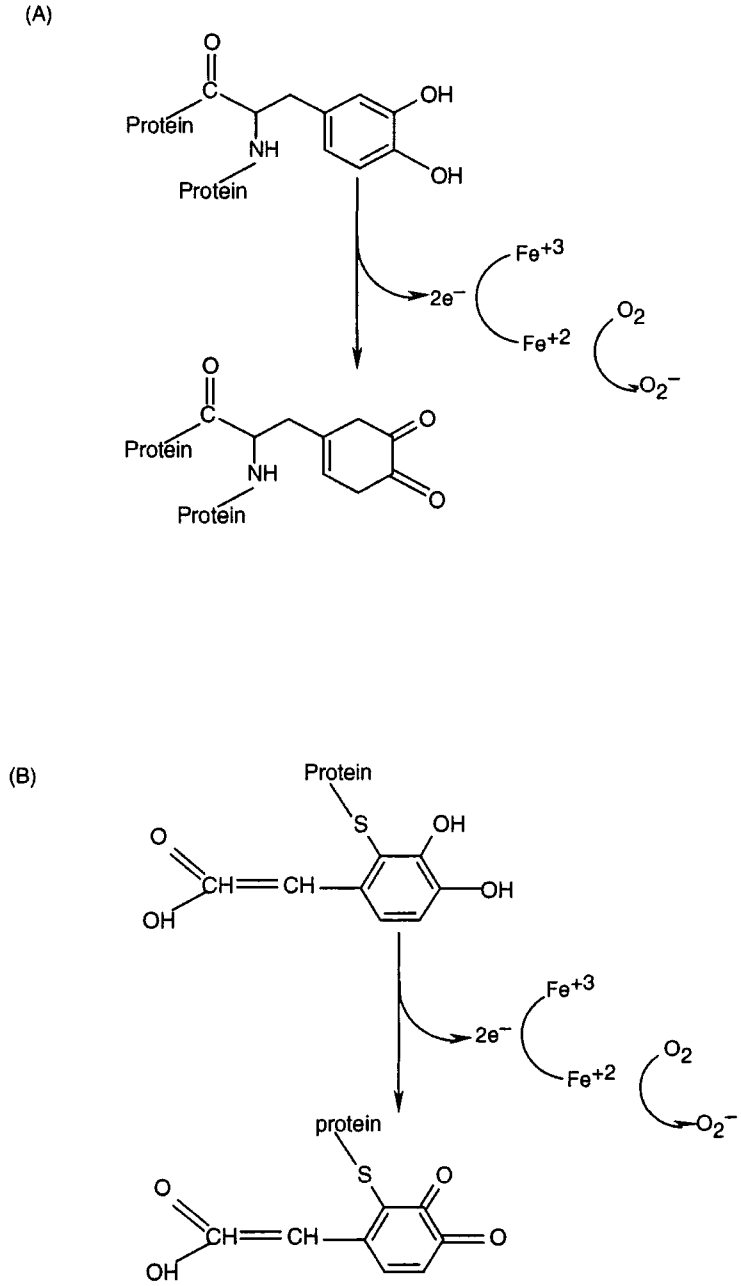


Figure 1.6 Formation of reactive oxygen species by iron-catalysed oxidations of protein catechols. (A) Formation of protein catechols by radical attack on tyrosine; (B) Formation by catechols (e.g. caffeic acid) bound to protein. (data from Dean et al, 1993)

(Felton et al., 1992b). PPO was expressed at a high level in transgenic tomato plants overexpressing prosystemin, a precursor of wound signal systemin and was induced in wild type plants by wounding, methyl jasmonate (MeJa), and systemin (Constable et al., 1995). Furthermore, foliar glandular trichome PPO in Solanaceae has been shown to confer insect resistance (Kowalski et al., 1992). Upon contact with insects, the trichome ruptures and releases an exudate and PPO which rapidly undergoes oxidative polymerisation. The polymerised hardened exudate entraps the small insects, occludes the mouth parts and causes death by starvation.

Peroxidases (POD)

Peroxidases (EC 1.11.1.7) are involved in the oxidation of many substrates such as phenolics, alcohols, aromatic acids, thiols, and ascorbic acids, by using H_2O_2 as the oxidising agent. In plants peroxidases exists as multiple isoenzymes which are coded by separate genes (Maranon et al., 1994). The peroxidases have been shown to be expressed differentially, in response to wounding and other environmental stresses.

It is well documented that POD are induced by herbivory and its oxidation products are toxic to several insects (Dowd and Norton, 1995). Transgenic tobacco and tomato plants expressing a chimaeric tobacco POD gene, have shown enhanced resistance towards *H.zea* , further supporting the evidence for a defensive role of POD (Dowd and Lagrimini, 1995). POD, like PPO, oxidises dihydroxyphenolics and gives similar oxidation products, along with some additional products. The potent alkylating agents of proteins, such as quinone methides are formed as a result of oxidation of eugenol by POD (Thompson et al., 1990). The deamination of protein lysyl α- amino groups by POD, catechols and H_2O_2, further contribute to the nutritional deficiency of amino acids to insects. The o-quinone products formed as a result of oxidation by POD and PPO, also oxidise ascorbic acids.

Lipoxygenase (LOX)

Plant lipoxygenases (EC 1.13.11.12) are non-haem iron containing dioxygenases that catalyse the oxidation of polyunsaturated fatty acids having a *cis*, *cis*-1,4-pentadiene structure, such as linoleic and linolenic acids to produce fatty acid hydroperoxides and free radical species. The hydroperoxide products which are cytotoxic, can damage membranes, proteins and DNA and may contribute to the hypersensitive response to pathogens (Shibata and Alexford, 1995). LOX, like POD also exists as multiple isozymes that may vary in pH optima, substrate specificity, product formation and tissue and cellular location. Soybean contains three LOX isozymes which are encoded by distinct genes that have been isolated and cloned. The lipid hydroperoxide products serve as intermediates in the formation of physiologically active compounds, including jasmonic acids (JA), an important signal eliciting multiple defence genes (Gardner, 1991).

LOX activity and/or gene expression has been reported to be due to the production of JA which is implicated in defence responses, during fruit ripening and senescence (Shibata and Alexford, 1995). Lox exerts multiple antinutritive effects. For example, direct removal of fatty acid substances such as linoleic and linolenic acids contributes to nutritional deficiencies. Protein quality is also adversely affected by linoleic oxidation products, and has been reported to reduce growth of *Manduca sexta* and *Heliothus zea* (Bi et al, 1994). Powell et al. (1995) reported an 80% mortality in rice brown hopper (*Niloparvata lugens*), when fed on artificial diets containing LOX.

JA and other LOX products are potent inducers of multiple defences against insects (Hildebrand et al., 1993), showing indirect resistance mediated by LOX. Application of 100μM JA to cotton and soybean plants gave rise to resistance to *Heliothus zea* (Felton et al., unpublished), whereas, fatty acid substrates of LOX applied to *Phaseolus vulgaris* foliage, reduced the fecundity of spider mites as compared to control plants (Kasu et al., 1994).

1.3.3.3 Enzymes involved in secondary product formation

Stimulation of phenylpropanoid metabolism is an important plant defence mechanism against environmental stresses, such as wounding and pathogen attacks. Plants are specialised to channel large amounts of carbon, on demand, from primary metabolism to the secondary phenylpropanoid pathway, for the biosynthesis of diverse phenylpropanoid compounds such as flavonoids, lignins, coumarins and many small phenolic molecules. These secondary plant metabolites have a multiplicity of functions, such as in pigmentation, and defence and signalling to protect plants against several stress conditions (Hahlbrock and Scheel, 1989). Flavonoid compounds can function as UV protectants and phytoalexins. Phenolic alcohols are precursors for the synthesis of lignin and suberin, which are deposited around infection and wound sites to form impenetrable barriers (Douglas, 1996).

The amino acid phenyalanine, precursor of the phenylpropanoids is provided by shikimic and aromatic amino acid pathways (Herrmann, 1995). The key steps in the phenylpropanoid pathway are shown in the figure 1.7. Phenylalanine ammonia lyase (PAL) is the first and the key enzyme in the phenylpropanoid pathway. It catalyses the synthesis of cinnamic acid by deamination of phenylalanine. Cinnnamic acid is then modified by the action of hydrolases and *O*-methyltransferases (OMT) and most phenylpropanoids are further derived from the resulting hydroxycinnamic acids. The second important enzyme is the cinnamic acid 4-hydroxylase (C_4H) which catalyses the hydroxylation of cinnamic acid to 4-hydroxycinamic acid. Cinnamate 4-hydroxylases are commonly known as cytochrome P450 monooxygenases, which are involved in a number of functions in plants, such as detoxification of herbicides, carcinogens, and insecticides along with a role in synthesis of various secondary metabolites. The third important enzyme of the pathway is the 4-coumarate : CoA ligase (4CL) that catalyses 4-hydroxycinnamic acid to hydroxycoumaryl CoA's. The latter are used in the biosynthesis of diverse compounds via specific branch pathways leading to the production of the cell wall constituents (lignins, suberins), pigments (flavonoids), ultraviolet protectants and plant defence compounds (isoflavonoids, flavonoids, coumarins and furanocoumarins).

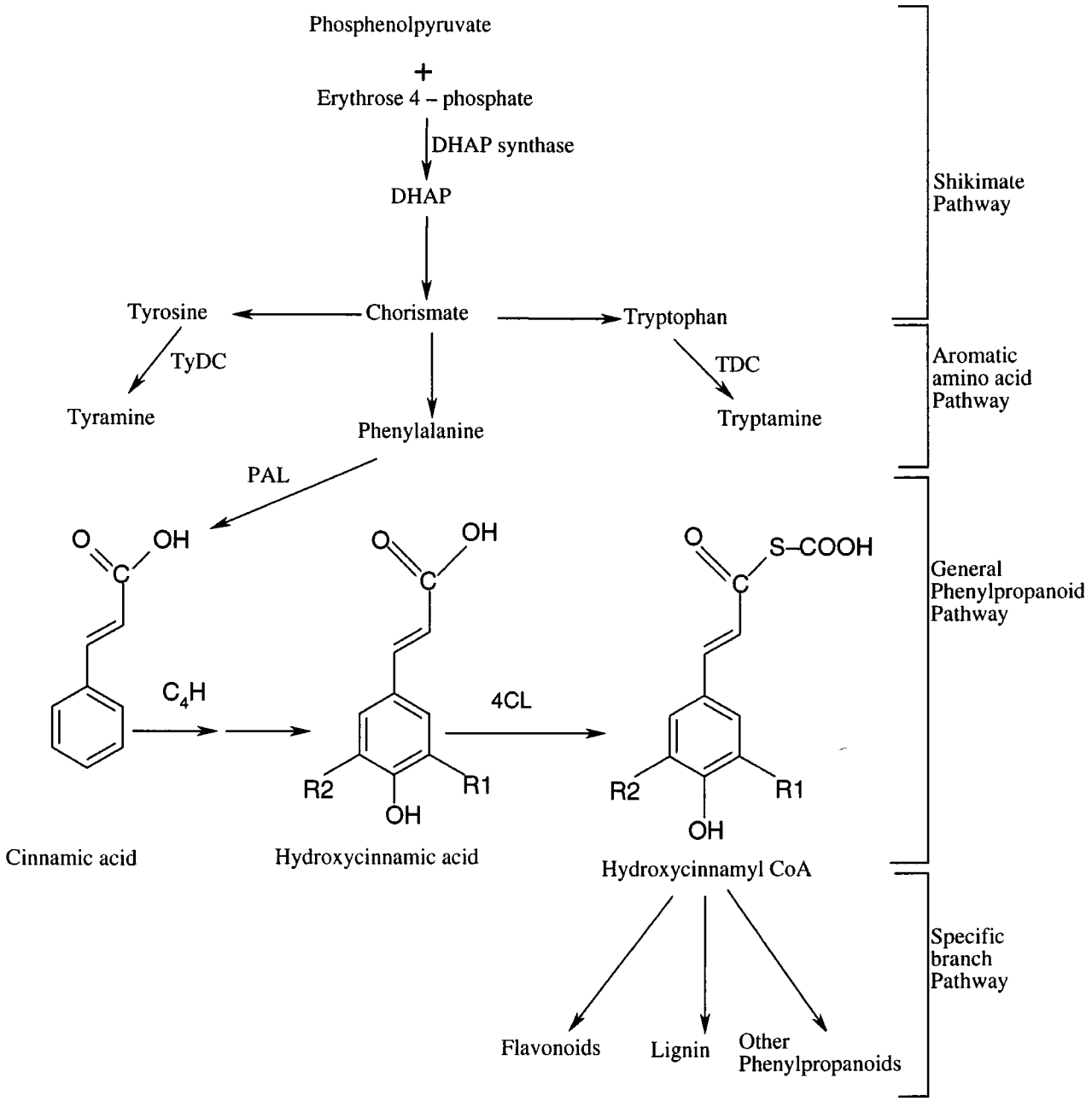


Figure 1.7 Overview of carbon flow in secondary metabolism in plants. The basic steps in the shikimate aromatic amino acids, general phenylpropanoid and specific phenylpropanoid branch pathways are indicated. The metabolic intermediates and end products are shown.
Abbreviations: R1 & R2 group on hydroxycinnamic acid may be hydroxyl and/ or methoxyl groups; TyDC, tyrosine decarboxylase; TDC. tryptophan decarboxylase; PAL, phenylalanine ammonia lyase; C_4H, cinnamyl-4-hydroxylase; 4CL, 4- coumarate: CoA ligasae. (Adapted and redrawn from Douglas, 1996).

The genes for biosynthetic enzymes of phenylpropanoid compounds are developmentally regulated in specific tissues and cell types (Liang et al., 1989; Hauffe et al., 1991), but can also be activated in response to environmental stresses such as wounding, pathogen infection, or UV irradiation (Douglas et al., 1991; Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). Genes encoding many of the enzymes involved in phenylpropanoids and lignin biosynthesis have been cloned from a number of plants, including trees (Wetten and Sederoff, 1995). In the majority of plants most of these genes are encoded by multigene families and it has been shown that individual members of a gene family are differentially expressed in response to various stresses (Koes et al., 1989; Wingender et al., 1989).

The nature of the wound-generated signal that leads to the activation of phenylpropanoid gene expression is not yet known but attention has been focused on the potential role of JA or its methyl ester as an intracellular signalling molecule (Douglas, 1996). In soybean suspension cell culture, PAL and CHS (chalcone synthase) gene expression is activated by treatment with exogenous jasmonate (Creelman et al., 1992; Gundulach et al., 1992), and also the exogenously added phytodienic acid, a precursor of jasmonate, activated PAL, 4CL and CHS mRNA accumulation in parsley (Dietrich et al., 1992). Furthermore, responsiveness of the 4CL promoter to MeJA, JA, and LA (linolenic acid) in a dose-dependent manner was observed in transgenic tobacco by Ellard-Ivey and Douglas (1996).

In addition to the activation of phenylpropanoid gene expression, the non-phenylpropanoid defense related genes such as tyrosine decarboxylase (TyDC), 3,4-dihydroxyphenylalanine decarboxylase (DODC) (Kawaleck et al., 1993), and tryptophan decarboxylase (TDC; DeLuca et al., 1989), are also transcriptionally induced in response to wounding and elicitor treatment. These represent the highly regulated branch point enzymes involved in the biosynthesis of various alkaloids and amides.

1.3.3.4 Structural cell wall proteins

The plant cell wall constitutes a preformed barrier against pathogen invasion and abrasion. Following pathogen infection or wounding, the plant cell wall is reinforced by the deposition of lignin or other phenolics, and by the enhanced incorporation and cross-linking of structural proteins (Bol and Van Kan, 1988). So far three types of structural cell wall proteins, for example extensins or related hydroxyproline-rich glycoproteins (HRGPs), Glycine-rich proteins (PRPs) and Proline-rich proteins (PRPs) have been identified in dicots and monocots. Although, the sequences of these classes of cell wall proteins can differ substantially between different species and between monocots and dicots, all of these proteins are characterised by highly repetitive amino acid motifs. The expression of these structural cell wall proteins is known to be induced in response to pathogen infection and physical damage caused by wounding, therefore, it is likely that they play an important role in plant protection (Bowels, 1990; Jose and Piugdomenech, 1993; Showalter, 1993).

Extensins

Extensins are a family of hydroxyproline-rich glycoproteins (HRGPs) and constitute the major protein in the cell wall of dicot plants (Showalter, 1993). The distinctive features of extensins are that they are highly basic in nature, with isoelectric points of ~10 due to their high lysine content and are characterised by the presence of pentapeptide 'SPPPP' motifs, which usually occur as part of longer repeating units. These pentapeptides blocks are conserved in almost all dicot extensins. The extensin amino acid composition is typically very rich in Pro, Ser, Lys, Tyr, Val, Thr and/or His. In the mature protein, the majority of the Pro residues are hydroxylated and most of both hydroxyproline and serine residues are glycosylated by post-translational modifications (Kieliszewski and Lamport, 1994). Extensins are synthesised as soluble precursors. Upon post-translational modifications, extensins are transported to the cell wall by secretory pathways and become insoluble in the cell wall matrix, presumably due to the formation of covalent cross-linkage such as isodityrosine (IDT) bridges (Liyama et al., 1994).

The term 'extensin' was originally coined by Lamport (1967) on the basis of the suggested role of extensins in cell wall extension, but recent evidence suggests that extensins strengthen the cell wall, by forming glycoprotein networks and thereby increase the resistance of the plant to pathogen invasion and mechanical wounding (Showalter, 1993). The evidence also suggests that extensins may act as an impenetrable physical barrier or may immobilise the pathogen by binding to their surfaces (Mazau et al., 1987). The extensin genes have been isolated from many plant species including bean (Corbin et al., 1987), sunflower (Adams et al., 1992), tomato (Showalter et al., 1991), potato (Bown et al., 1993), soybean (Ahn et al., 1996) and monocots (Kieliszewski et al., 1992a). Extensin genes are induced in response to mechanical wounding, pathogen attack, or ethylene treatment (Parmentier et al., 1995; Wycoff et al., 1995; Shirsat et al., 1996) and are developmentally regulated (Ertl et al., 1992). The extensins also show tissue-specific expression. Extensins are commonly associated with phloem tissues and cambium cells but can be associated with other tissues as well. Extensin gene expression and localisation can, apparently vary from plant to plant and amongest different cell and tissue types, presumably in accordance with the differing functions of a variety of cell and tissue types (Showalter, 1993).

Glycine-rich proteins (GRPs)

Glycine-rich proteins (GRPs), are characterised by the presence of glycine rich stretches, and are the second major structural protein in the cell wall of dicot plants. GRPs have been found in different tissues from many plant species like tobacco (Hirose et al., 1994), *Arabidopsis* (Carpenter et al., 1994), tomato (Parsons and Mattoo, 1994), and monocots like Sorghum (Cretin and Puigdomenech,1990), and maize (Didierjean et al., 1992). Some GRPs are thought to play a structural role in the cell walls (Showalter, 1993), others are characterised by the presence of putative RNA-binding regions (Cretin and Puigdomenech., 1990). These GRPs have been subdivided into different families such as CL-GRPs and RNA-GRPs, on the basis of their amino acid sequence. Dicot GRPs are commonly localised to vascular bundles, particularly to xylem elements and to cells that are lignified. They are usually colocalised with the PRPs (Showalter, 1993).

A distinct GRP type (CL-GRP family) has a cytokeratin-like domain, that consists of glycine stretches with interspersed tyrosine residues, followed by a cysteine-rich C-terminal domain. The second GRP type (RNA-GRP family), which is able to bind RNA is characterised by a domain with two typical RNA binding motifs. The RNA-GRPs specifically bind poly (U) and poly (G) (Ludevid et al., 1992), have a high affinity for the RNA of the same cell where they are present (Hirose et al., 1994) and are located in the nucleolar compartment (Heintzen et al., 1994). Genes encoding GRPs are known to be developmentally regulated (Heintzen et al., 1994), as well as in response to different stresses such as cold and drought (Carpenter et al., 1994), wounding (Parsons and Mattoo, 1994), by external treatment with abscisic acid (Gomez et al., 1988), and $HgCl_2$ (Didierjen et al., 1992).

Proline-rich proteins (PRPs)

The third class of cell wall proteins, the proline-rich proteins (PRPs), are composed of a number of Pro-Pro-Val-X-Lys repeats, where X is usually glutamic acid or tyrosine (Hong et al., 1990). The PRPs have been subdivided into two broad subclasses. One, those that are components of normal plant cell walls (Hong et al., 1990) and second, those that are plant nodulins (i.e., proteins produced in response to infection by nitrogen-fixing bacteria) and constitute part of the nodule cell wall (Govers et al., 1991). However, the distinction between these two classes is not clear-cut as two pea nodulin PRPs, ENOD12A and ENOD12B, are expressed in stems and flowers (Govers et al., 1991).

PRPs have been isolated from number of plants such as soybean (Lindstorm and Vodkin, 1991); potato and tomato (Ye et al., 1991), maize (Jose-Estanyol et al., 1992) and gymnosperms (Kieliszewski et al., 1992a). The developmental expression of glycine-rich proteins (GRPs) have been reported in soybean (Hong et al., 1989), also the soybean SbPRP gene family members exhibit differential expression among the various types of cells. The SbPRPs vary in size and repeat organisation/composition, therefore, it seems reasonable to assume that these variations may affect the functions of proteins within the cell wall. Regulatory studies indicate that PRPs are involved in various aspects of development, ranging from germination to the early stage of

nodulation (Jose-Estanyol et al., 1992). In addition, PRPs are also expressed in response to wounding, endogenous elicitors, fungal elicitors and ethylene (Tierney et al., 1988; Marcus et al., 1991). Members of the PRP gene family exhibit a tissue and cell-specific pattern of expression and like GRPs, the PRPs are also localised in vascular elements and some are expressed in the same cell type as extensins (Showalter, 1993). The PRPs, like the extensins and GRPs, are insolubilized in the cell wall matrix. Moreover, their insolubilization process can occur rapidly in response to stress and may be mediated by the release of hydrogen peroxide and catalysed by a wall peroxidase (Bradely et al., 1992).

Overall conclusion is that pest and pathogens cause a great loss of crop yield. The usage of pesticides and fungicides to control pest and pathogens causes health hazards and damage to our environment. An alternative approach to this problem is the development of 'in built' disease and pest resistance into crop plants via a transgenic approach. Therefore, isolation of differentially expressed, wound-induced genes, their subsequent transfer and overexpression in crop plants is a valuable strategy to manipulate the resistance against pests and pathogens.

1.4 Specific aims of the project

High temperature tolerance is one of the main components of yield stability in crop plants. Although, the response to heat shock in plants is well characterised at the level of RNA and protein synthesis, the functional role and mode of action of the heat shock proteins is only partially understood. Despite the impressive progress made in the field of molecular biology with the advent of new gene transfer techniques, manipulation of heat tolerance in crop plants has not received much specific effort. In order to understand the underlying molecular mechanism of heat shock response, the specific aim of this project was to isolate and characterise the genes differentially expressed in response to heat shock. A cDNA clone designated LP19, isolated in an ongoing project in the laboratory at Durham, was shown to encode the C-terminal region of HSP70 gene. This cDNA clone was shown to be associated with pea pod lignification. Therefore, it was decided to characterise this cDNA clone with the following main objectives:

1. Isolating and sequencing of the gene corresponding to LP19 in pea.
2. Expression analysis of LP19 mRNA under heat shock, salt stress and wounding.
3. Isolation of the promoter sequences of gene corresponding to LP19.
4. Construction of a promoter-reporter fusion and transformation of tobacco plants with a chimaeric construct in order to study the temporal and spatial regulation of LP19 gene.

A secondary aim of the project was to study the wounding response in pea plant. Crop losses as a result of wounding caused by pest and pathogen infection are also enormous. Wounding causes the induction of several genes that encode protective proteins such as proteinase inhibitors (PIs), polyphenol oxidase (PPO), pathogenesis-related proteins (PR) and cell wall proteins. Initially, it was planned to study the induction of polyphenol oxidase (PPO) activity in response to wounding and isolation of cDNA corresponding to PPO. Later on a more general approach was adopted to construct a subtractive cDNA library from wounded pea leaf tissue in order to isolate most of the mRNA transcripts which are expressed differentially in response to wounding.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Chemical reagents and equipment suppliers

All reagents were obtained from Sigma Chemical Co., St. Louis USA and BDH Chemicals Ltd. Poole, England, except those listed below. The reagents used were of analytical grade or the best available grade.

DNA and RNA size markers and restriction enzymes were obtained from Northumbria Biochemicals Ltd. (NBL), Cramlington, Northumberland; MBI Immunogen International, Sunderland; New England Biolabs (UK) Ltd, Hitchin, Hertfordshire; Promega, Southampton; Boehringer Mannheim (UK) Ltd., Lewes, E.Sussex; and Stratagene Ltd., Cambridge.

T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, *E.coli* DNA polymerase 1 large fragment (Klenow polymerase), Calf intestine alkaline phosphatase, endonuclease-free *E.coli* DNA polymerase were also obtained from Boehringer Mannheim (UK) Ltd.

Pharmacia Fine Chemicals, Uppsala, Sweden, supplied Sephadex G-50 and Ficoll 400.

pGEM-T PCR cloning kit and other plasmid vectors and PolyATract mRNA isolation kit were supplied by Promega Corporation, 2800 Woods Hollow Road Madison, WI 53711-5399, USA.

Synthetic oligo nucleotide primers T3 and T7 were purchased from Applied Biosystems Inc., Warrington, Ches.

PCR-Select™ cDNA subtraction kit and Advantage KlenTaq Polymerase mix were purchased from Clontech Laboratories Ltd. 1020 Meadow Circle, Palo Alto, CA 94303-4320, USA..

λZAP-cDNA synthesis kit and PCR-Script™ Amp SK(+) cloning kits were purchased from Stratagene Ltd. Cambridge Innovation Centre, Cambridge Science Park, Milton Road Cambridge, CB4 4GF, UK.

Genomic DNA isolation kit 'Phytopure' was purchased from Nucleon Biosciences, Coatbridge, Lanarkshire, Scotland.

Agarose was supplied by Gibco BRL Life Technologies ltd., Paisley, Scotland.

Bacto agar was obtained from Difco Laboratories, W. Molesey, Surrey.

Peptone was supplied by Becton Dickinson, Cowley, Oxon.

Yeast extract was obtained from Unipath Ltd., Basingstoke.

Radiochemicals were purchased from Amersham International Plc, Aylesbury, Bucks.

Nitro-cellulose filters, Schleicher and Schuell, grade BA-85, were supplied by Anderman and Co. Ltd., Kingston-upon Thames, Surrey.

National Diagnostics "Ecoscint" scintillation fluid was supplied by B.S & S. (Scotland Ltd.), Edinburgh.

X-ray cassettes and X-ray films were supplied by Genetic Research Instrumentation Ltd., Dunmow, Essex.

Fixer, Kodak Unifix, was supplied by Phase Separation Ltd., Deeside, Clwd.

Developer, Ilford Phenisol, was supplied by Ilford Ltd., Mobberly , Ches.

3 MM filter paper was supplied by Whatman Labsales Ltd., Maidstone, Kent.

Disposable pipette tips and Eppendorfs tubes were supplied by Greiner Labortechnik Ltd., Dursley, Glos.

Microtiter plates and petri dishes were supplied by Bibby Sterilin Ltd., Stone, Staffs.

2.1.2 Bacterial strains, plasmids and vectors

The *E.coli* strain DH5α (Gibco BRL) was used for all routine work. JM109 was used for PCR cloning by the pGEM-T method (Promega) and *Epicurian Coli* XL1-Blue MRF' supercompetent cells (Stratagene) were also used for PCR cloning by PCR-Script™ Amp SK(+) cloning method. pUC vectors were purchased from Northumbria Biologicals Ltd., Cramlington or Pharmacia Biotechs. Ltd. The *Agrobacterium tumefaciens* strain used for tobacco transformation was LBA4404. Bacterial strains, plasmid vectors and bactriophages used in genomic library and cDNA library construction and *in vivo* excision of inserts were purchased from Stratagene Ltd., Cambridge.

The bacterial strains, plasmids and bacteriophage vectors are listed below.

Bacterial Strains

DH5α F⁻, *hsd*R17 (r_K^- m_K^+), *sup*E44, Δ*lac* U169, φ80d*lacZ*Δ M15, *hsd*R17, recA1, *end*A1, *gyr*A96, *thi*-1, *rel*A1, λ⁻

HB101 *sup*E44, *hsd*S20 ($r_B^-m_B^-$), *rec*A13, *ara* –14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, *xyl* -5, *mtl* -1

KW251 F⁻, *sup*E44, *sup*F58, *gal*K2, *gal* T22, *met* B1, *hsd*R2, *mcr* B1, *mcr*A⁻, *arg* A81: *Tn*10, *rec*D1014

JM101 *sup*E, *thi* Δ(*lac* -*pro*AB), F⁻[*tra*D36, *pro*AB+, *lac* I^q, *lac* ZΔM15]

SOLR e14⁻ (*mcr* A), Δ(*mcr* CB-*hsd* SMR-*mrr*)171, *sbc* C, *rec* B, *rec* J, *umu*C::*Tn* 5(*kan* ^{R}), *uvr* C, *lac*, *gyr* A96, *rel* A1, *thi* -1, *end* A1, λ^R, [F'*pro* AB, *lac* $I^qZ\Delta M15$], Su^- (non-supressing)

SURE	e14$^-$ (*mcr* A), Δ(*mcr* CB-*hsd* SMR-*mrr*)171, *sbc* C, *rec* B, *rec* J, *umu*C::*Tn* 5(*kan* ^{R}), *uvr* C, *sup* E44, *lac*, *gyr* A96, *rel* A1, *thi* -1, *end* A1, λ^R, [F'*pro* AB, *lac* I^{q}ZΔM15], Su$^-$ (non-supressing)
XL1-Blue	recA1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup* E44, *rel* A1, *lac*, [F'*pro*AB, *lac* I^{q}ZΔM15, *Tn* 10(tet^R)]

Plasmids

pUC18	(Pharmacia): cloning vector
pUC19	(Pharmacia) : cloning vector
pBluescript	(Stratagene) : cloning vector
pGEMT	(Promega) : PCR cloning vector
pScript SK$^+$	(Stratagene) : PCR cloning vector

Bacteriophage

M13mp19	(Boehringer Mannheim) : multiple cloning site
M13mp18	(Boehringer Mannheim) : multiple cloning site as for M13mp19
Lambda ZAPII	(Stratagene) : multiple cloning site
EXAssist helper phage	(Stratagene) : *in vivo* excision

2.1.3 Growth media for bacterial and bactriophage cultures

Media routinely used for growth of bacteria and bacteriophage cultures are listed below.

Liquid Media

LB broth:	10 g bacto-tryptone, 5g NaCl, 5 g yeast extract, per litre.
LB / Maltose / $MgSO_4$:	To autoclaved medium, add filter sterilised maltose solution to 0.2% (w/v) and $MgSO_4$ to 10 mM.
YT broth:	8 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, per litre.

YEB broth:	5 g bacto-tryptone, 1 g yeast extract, 5 g beef extract (brain/heart fusion), 2 g magnesium sulphate heptahydrate and 5 g sucrose, per litre.
NZY broth:	5 g NaCl, 2 g magnesium sulphate heptahydrate, 5 g yeast extract, 10 g NZ amine, pH 7.5 with sodium hydroxide.
SOC Medium:	2% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

Solid Media

LB, YT and YEB agar :	Add 15 g agar / l of media. Autoclave and pour plates while media is still molten.
NZY agar:	NZY broth plus 15 g / l Bacto agar. Autoclave and pour plates.
NZY top agarose:	NZY plus 0.7% (w/v) agarose.
Top Agar :	10 g bacto-tryptone, 5 g NaCl, 8 g Bacto agar, per litre.
MSO medium:	4.7 g MS salt (Murashige and Skoog medium salts), 30 g sucrose, pH 5.8, 8 g agar, per litre.

Additives

Antibiotics:	Ampicillin 50 µg / ml of media, kanamycin 100 µg / ml, rifampicin 100 µg / ml , tetracycline 12.5 µg / ml, augmentin 200 µg / ml.
X- Gal:	80 mg / l of media, dissolved in N, N'-dimethylformamide.
IPTG:	238 mg / l in in N, N'-dimethylformamide (0.1 mM of the final conc. of media)

2.1.4 Commonly used buffers and other solutions

50x TAE (agarose gel running)

buffer per litre:	42 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA , pH 8.0.
10x Column Buffer: HCl	1.5 M NaCl, 0.1 M EDTA, 1% (w/v) SDS, 0.5 M Tris-pH 7.5.
5x Formaldehyde agarose gel running buffer:	0.1 M 3-[N-morpholino]propane-sulfonic acid. (MOPS) pH 7.0 with NaOH, 40 mM sodium acetate, 5 mM EDTA.
Gel loading dye:	30% (w/v) glycerol, 10 mM Tris HCl pH 8.0, 10 mM EDTA and either 0.1% (w/v) xylene cyanol and 0.1% bromophenol blue, or 0.1% (w/v) fast orange for fragments <200 bp. Mixture was prepared, sterilised by autoclaving. Aliquots stored at -20°C.
20x SSC:	per litre: 175.3 g NaCl, 88.2 g Sodium citrate, pH 7.0.
20x SSPE:	per litre: 175.3 g NaCl, 27.6 g $NaH_2PO4.2H_2O$, 7.4 g EDTA, pH 7.4.
TE buffer:	10 mM Tris / HCl pH 8.0, 1 mM EDTA pH 8.0.
Phage buffer:	20 mM Tris / HCl pH 7.4, 100 mM NaCl, 10 mM $MgSO4.7H_2O$.
SM buffer:	per litre: 5.8 g NaCl, 2 g $MgSO4.7H_2O$, 50 ml Tris / HCl pH 7.5, 5 ml 2% (w/v) gelatine solution.
50x Denhardt's solution:	per litre: 5 g Ficoll, 5 g Polyvinylpyrrolidine, 5 g BSA.
Phenol : Chloroform : isoamyl alcohol (25:24:1)	redistilled phenol was extracted several times with TE buffer and then 1 part of the lower phenol phase, mixed with 1 part of chloroform: isoamyl alcohol (24:1).
Denaturing solution:	1.5 M NaCl, 0.5 M NaOH, 1 mM EDTA, filtered and stored at room temperature.
Neutralising buffer:	3 M NaCl, 0.5 M Tris, 1 mM EDTA, pH 7.0.

2.1.5 Plant materials

Pea (*Pisum sativum* L.) seed, L59, L58 and L1390 were kindly supplied by the Weibullsholm Institute, Sweden, and Feltham First (FF) and Purple podded (PP)

cultivars were purchased from Sutton's Seeds, Torquay, Devon. L59 have a lignified parchment layer (genotype, PV), L58 has a partially lignified layer (genotype, Pv) and the lignified parchment layer is absent in L1390 (genotype, pv) (Pea Gene Bank Catalogue, 1989). Chickpea (*Cicer arietinum* L.) seeds were obtained locally. Tobacco *Nicotiana tabacum* cv. Samsun) seeds were obtained locally in the lab.

2.1.6 Growth of plant material

Pea and chickpea seeds were germinated on moistened vermiculite in the dark at 25°C. Desiccation of the seeds was prevented by intermittent spraying with water. The germinated seeds were planted at a density of 2 seedlings /5 inch pot in John Innes No. 1 compost and placed in polythene lined trays in the growth room. The compost , canes and pots were sterilised prior to use. Growing plants were maintained in a growth cabinet at 20°C temperature with 16 hrs light /8 hrs darkness.

Tobacco seeds were surface sterilised by treatment with 10% (v/v) hypochlorite solution (containing ~ 1.4% available chlorine) in a syringe for 20 min and washed with 4 changes of sterile distilled water, and then transferred onto MSO medium. The plates were sealed with Nescofilm and incubated in a tissue culture suite maintained at 25°C temperature, 65% relative humidity and 14 hrs photoperiod. After about 10 days the young seedlings were transferred to Magenta pots containing MSO medium (1 seedling/pot) and allowed to grow in a the same tissue culture room.

2.1.7 Storage of bacterial strains

The bacterial colonies were stored on agar plates upto two months kept at 4°C, sealed with Nescofilm (Nippon Shoji Kaisha Ltd. Osaka, Japan). For long term storage, the bacterial cells from a single colony on agar plates were harvested in 2 ml sterile portions of equal amounts of LB-broth and 80% glycerol, and stored at –80°C.

2.2 Methods

2.2.1 Treatment of glassware, plasticware and solutions

The sterilisation of glassware, plasticware and solutions for use with DNA was carried out by autoclaving for 20 minutes at 121°C and all the glassware for nucleic acid work was siliconised before autoclaving, using "Replicote"(Sigma). The glassware for use with RNA was siliconised and baked for at least 8 hrs at 180°C and solutions to be used with RNA were incubated overnight at room temperature with 0.1% (v/v) DEPC (Diethyl Pyrocarbonate) and then autoclaved as above. The solutions containing chemicals unstable at high temperature such as antibiotics and maltose, were sterilised by filtration through a 0.2 μ filter.

2.2.2 Stress treatments to plants

2.2.2.1 Heat-shock treatment

Two week old pea and chickpea plants growing in soil were subjected to heat shock by placing them in an oven for 4 hrs pre set at 40°C in which humidity was maintained by keeping a beaker full of water in the chamber. Transgenic tobacco (2-3 weeks after transferring into compost) were heat shocked as above. For single leaf heat shock, a single leaf was inserted into the chamber maintained at 40°C, keeping the rest of the plant outside and chamber was sealed properly to avoid any heat dissipation. For root heat shock , plants were depotted, the roots were washed, submerged in a water bath maintained at 40°C and sealed properly keeping the rest of the plant unstressed. Germinating seeds and seedlings of tobacco grown on MSO medium in petri dishes were heat-shocked only for 20-30 minutes. Tissues were collected, frozen immediately in liquid nitrogen and stored frozen until needed. Transgenic material for GUS localisation was stained as mentioned in section 2.2.26.

2.2.2.2 Salt treatment

The salt treatment solution (500 mM NaCl) was given to two week old pea and chickpea plants. The plants were maintained in salt solution for two days. Tissues were collected, frozen immediately in liquid nitrogen and stored frozen until needed.

2.2.2.3 Mechanical wounding of the leaves

Leaflets of two to three week-old pea and chickpea plants were subjected to mechanical wounding by crushing with pliers through mibrib region anf leaf blade. The tissues were collected at different time intervals, frozen immediately into liquid nitrogen and stored frozen until needed.

2.2.2.4 Methyl Jasmonate treatment

Methyl jasmonate treatment was given to 3 week-old pea plants following the method described by Constable et al., (1995). 20 µl methyl jasmonate oil (Sigma), diluted in 95% ethanol (1µl methyl jasmonate : 10µl ethanol), was applied on a 3 MM paper sheet and put into sealed glass jar along with the plants. Control plants were exposed only to 95% ethanol. The sealed jars were maintained in growth chambers under constant light until assayed. The tissues were collected at different time intervals, frozen immediately into liquid nitrogen and stored frozen until needed.

2.2.3 Minipreparations of DNA

2.2.3.1 Minipreparations of plasmid DNA for restriction analysis

Plasmid DNA was prepared by using a protocol based on the method of Birnboim and Doly (1979). Single colonies from agar plates or loopfuls from glycerol-preserved cells, were inoculated into 5 ml aliquots of YTamp medium and incubated overnight at 37°C with shaking. Bacteria were harvested by centrifugation at 3500 rpm for 10

minutes. The supernatants were poured off and bottles were inverted over absorbent paper for a few minutes. The pellet was then resuspended into 100 µl of ice cold TGE (50 mM glucose, 10 mM EDTA, 25 mM Tris / HCl, pH 8.0), mixed well by microtip, transferred to 1.5 ml Eppendorf tubes, and incubated on ice for 30 minutes. 200 µl freshly prepared solution containing 0.2 M NaOH and I% (w/v) SDS was added , mixed by inversion and kept on ice for 5-10 minutes. 150 µl of ice cold 3 M potasium acetate (600 µl 5M potassium acetate + 115 µl glacial acetic acid + 285 µl H_2O) was added and mixed well by inversion. The samples were stood on ice for 20 minutes and then centrifuged for 10 minutes at 12,000 g. To the clear supernatant 2 µl of 10 mg/ml RNase (DNase free) was added, and the tubes were incubated at 37°C for 20 minutes. The solutions were then extracted once with phenol under TE and once with chloroform/isoamyl alcohol (24 : 1), vortexing for 15 seconds, and centrifuged for 3 minutes at both stages. DNA was then ethanol precipitated for 1 hr or overnight at –20°C.

The DNA pellets were resuspended in 16 µl H_20; 8 µl 2 M NaCl and 20 µl of 13% (w/v) PEG (Polyethylene glycol 8000) were added, with mixing on addition of each solution. The mixtures were incubated on ice for 20 minutes. DNA was recovered by centrifugation for 10 minutes at 12,000g and the supernatants removed by micropipette. Pellets were washed with 1 ml of –20°C cold 70% (v/v) ethanol, tubes spun for a further 5 minutes and supernatants decanted. The pellets were vacuum dried and resuspended into 20 µl H_2O and stored at –20°C.

2.2.3.2 Minipreparation of DNA for sequencing

This was carried out following the modified procedure for pUC derived plasmid minipreps by using the Promega Wizard Miniprep DNA purification kit. Bacteria containing plasmid were grown overnight at 37°C in 10 ml YTamp aliquots and spun in a centrifuge as above. Cells were resuspended in 300 µl of cell resuspension solution (50 mM Tris HCl pH 7.5, 10 mM EDTA, 100 mg/ml RNaseA). The resuspended cells were transferred to 1.5 ml Eppendorf tubes. 300 µl cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) was added and mixed by inverting the tubes several times. Then 300 µl of neutralising buffer (1.32 M potassium acetate pH 4.8) was added and again mixed

by inverting the tubes several times. The mixtures were centrifuged in a microcentrifuge for 3 minutes. The supernatants was transferred in a fresh eppendorfs tube and re-spun for 3 minutes. The clear supernatants were transferred into 2 eppendorf tubes each (approximately 400 µl/tube). To each supernatant in each tube 500 µl of Wizard miniprep DNA purification resin was added, mixed by inverting the tubes and kept at room temperature for 5 minutes with occasional shaking.

The resin / DNA mixture from both tubes were pipetted in to a 5 ml syringe barrel and the slurry was gently pushed into a mini column with the syringe plunger. The mini column was washed with 3 ml of column wash solution (200 mM NaCl, 20 mM Tris HCl pH 7.5 and 5 mM EDTA, diluted with 95% (v/v) ethanol to a final concentration of 55% v/v). The syringe was removed, the mini column was transferred to a new eppendorf tube and spun for 1 minute at high speed to dry out the column. The DNA from the column was eluted in another eppendorf tube by applying 100 µl sterile Milli Q water heated at 70°C and centrifuging at high speed for 1 minute. 1 µl aliquots of the DNA solutions were restricted and electrophoresed with a known amount of standard DNA to estimate their concentration prior to sequencing.

2.2.3.3 Minipreparation of λ phage DNA

Lambda phage DNA was prepared by using a Qiagen>Lambda<kit, following the manufacturer's instructions. Phage were freshly titrated from stored stocks and eluted from a single plaque-plug with 100 µl phage buffer. 500 µl of host bacterial culture (KW251) grown overnight in LB media supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$, was added and then incubated at 37°C for 20 minutes with shaking. This was inoculated into prewarmed 50 ml LB media supplemented with 10 mM $MgSO_4$ and then incubated for 7 hrs at 37°C with continuous shaking for lysis of bacterial cells.

If lysis did not occur, 250 µl of chloroform was added and further incubated for 15 minutes. This mixture was centrifuged at 8,000 g for 10 minutes and the supernatant was removed and stored at 4°C. The Qiagen "midi" method was then followed using their "Tips 100" columns to purify the phage DNA, finally resuspending into 100 µl TE buffer. 5 µl aliquots were used for restriction analysis.

2.2.4 Restriction endonuclease digestion

2.2.4.1 Plasmid and phage DNA restriction

DNA samples, up to 1 μg were restricted in an eppendorf tube in a total volume of 20 μl, with an excess of the appropriate restriction enzyme (1-3 unit/μg DNA, except where higher ratios were recommended), using buffer supplied with the restriction enzyme. The restriction was performed at 37°C for 1-2 hrs. RNaseA (10 μg per reaction), which had been boiled for 20 minutes to destroy any DNAse activity, was added to the restrictions of plasmid DNA which had been prepared by the non-sequencing quality miniprep method (see section 2.2.3.1).

2.2.4.2 Genomic digests

10 - 20 μg of genomic DNA were digested with a five times excess of the appropriate restriction enzyme in the appropriate buffer supplied with the enzymes. 1 μl of RNase (10 mg/ml) was added and the volume made upto 50 μl with sterile water. The mixture was tapped and spun before incubating in a heating block at 37°C for 4 hrs.

2.2.5 Phenol extraction and ethanol precipitation of DNA

DNA solutions were extracted with Tris-saturated phenol and then with chloroform/isoamyl alcohol (24 : 1). The mixture was vortexed for 15 seconds and spun in a microcentrifuge for 3 minutes at 12,000 g at each stage. The volume of the DNA samples were adjusted to 200 μl with TE buffer, if required, and extracted with 200 μl phenol. After vortexing and centrifugation the upper aqueous phase was taken and retained. The lower organic phase (phenolic) was extracted again with 100 μl TE buffer, vortexed and spun. The aqueous phase was mixed with the previous aqueous phase, extracted with 300 μl chloroform / isoamyl alcohol, vortexing and spun as above. The aqueous phase was removed, re-extracted with chloroform / isoamyl alcohol and the final aqueous phase was ethanol precipitated using 1 μl (20 μg)

glycogen, 5M ammonium acetate (4 µl / 100 µl) and 2.5 volumes of ethanol precooled at –20°C. The mixture was kept at –20°C for at least 1 hr, followed by centrifugation at 4°C; 12,000 g for 20 minutes. The supernatant was then poured off carefully and the pellet rinsed with 1 ml of 70% (v/v) ethanol. The tube was inverted to mix, spun as above for 5 minutes and the supernatant again poured off. The pellet was vacuum dried for 5 minutes and the DNA was resuspended in sterile water and stored at –20°C.

2.2.6 Agarose gel electrophoresis

2.2.6.1 Agarose gels

Electrophoresis of DNA was carried out in a Pharmacia GNA-100 minigel apparatus or on 18.5 x 15.0 cm gels in tanks holding 2.1 litre buffer as described in Sambrook et al., (1989). 0.7% (w/v) agarose gels were prepared containing EtBr (10 µg / ml final conc.). 0.3 volumes of dye mix (10 mM Tris-HCl, 10m M EDTA pH 8.0, 1 mg / ml fast orange G and 30% (v/v) glycerol) was added to samples before loading. The gels were run at 70 volts in 1x TAE running buffer containing 1 µg / ml EtBr. *Pst*I or *Eco*47I cut λ DNA was routinely used as size marker. The gels were photographed using transmitted UV light at 300 nm.

2.2.6.2 Formaldehyde agarose RNA gel

Formaldehyde agarose gel electrophoresis was used to separate small molecular weight RNAs under denaturing conditions according to the method described in Sambrook et al. (1989). A 1.5% (w/v) agarose gel was prepared by dissolving 840 mg high quality grade agarose in 35 ml of DEPC treated water. Molten agarose was cooled to 65°C and 11 ml of 5x formaldehyde gel running buffer (0.5 M MOPS, pH 7.0, 40 mM sodium acetate and 5 mM EDTA, pH 8.0) and 10 ml of formaldehyde (37% w/v solution) were added. The gel was allowed to set for 1 hr in a fumehood prior to use. Samples of RNA in a volume of 4.5 µl (5µg) were prepared by adding the following : 2µl of 5x formaldehyde gel running buffer, 3.5µl deionised formaldehyde and 10µl deionised formamide. The samples were heated to 65°C for 15 minutes, then chilled on ice. 2µl

of sterile gel loading buffer (50% v/v glycerol, 1 mM EDTA pH 8.0, 0.25% w/v bromophenol blue and 0.25%(v/v) xylene cyanol FF) was added to the samples, mixed and loaded on the gel, which was pre-electrophoresed at 50 volts for 5 minutes. The gel was electrophoresed in 1x formaldehyde gel running buffer at 60-70 volts for 2 hrs with constant circulation of buffer. RNA size marker (Gibco-BRL) was loaded on each gel for size estimation. The formaldehyde gel was stained in 0.1% (w/v) ammonium acetate and EtBr (10 mg/ml) solution for 2-3 hrs with shaking and then destained with DEPC-treated water and photographed under UV light as above.

2.2.7 Isolation of DNA from agarose gel

The slices of agarose gel containing DNA were placed in prepared dialysis tubing with the minimum volume of TAE buffer and ends were clipped without any air inside the tube. The dialysis tube was placed horizontally, perpendicular to the flow of electric current in a Pharmacia mini gel apparatus containing electrolyte buffer, 1x TAE without EtBr. Electrophoresis was carried out for 15 minutes at 50 volts. After checking under UV light that the DNA had eluted from the gel, the polarity was reversed for 15 seconds. The buffer containing the extracted DNA was pipetted into an eppendorf tube. The gel slices and tubing were rinsed with a small (50-100 µl) volume of TAE. The DNA was then purified by phenol extraction and ethanol precipitation method as described in section 2.2.5.

2.2.8 Determination of nucleic acid concentration

2.2.8.1 Estimation of nucleic acid concentration and purity by spectrophotometery

Nucleic acid concentration and purity was determined spectrophotometerically on a Pye Unicam SP8–150 ultra-violet / visible dual beam spectrophotometer and 1 cm path length quartz cells. Absorbance reading of 1 µl of nucleic acid solution in 1 ml of sterilised water (DEPC treated water for RNA samples) were obtained at 260 nm and 280 nm. The pure DNA or RNA should have an A_{260}/A_{280} ratio of more than 1.7. A

ratio less than 1.7 indicates probable protein contamination. An O.D. of 1 at 260 nm was assumed to correspond to 50 µg/ml of double stranded DNA, 40 µg/ml for single stranded DNA and RNA, and 20 µg/ml of single stranded oligonuleotides.

2.2.8.2 Estimation of DNA concentration by the spotting method

An estimation of DNA concentration can be obtained by UV visualisation of samples spotted on EtBr agarose plates in comparison with DNA samples of known concentration. This method was taken from ZAP-cDNA Synthesis kit (Stratagene). 100 ml of a 0.8% (w/v) agarose and electrophoretic buffer media was prepared. The molten agarose was cooled to 50°C then 10 µl of 10 mg/ml EtBr stock solution was added. After mixing, the agarose was poured into 100 mm petri dishes using ~10 ml/plate. The plates were left to harden and incubated at 37°C to dry, if necessary. These plates can be stored in the dark at 4°C for up to one month.

Using a DNA sample of known concentration, seven serial dilutions in 100 mM EDTA were made to cover the range from 10 to 200 ng/µl. These standards may be stored at –20°C for up to 3 months. Using a marker, the petri dish was labelled to indicate where the samples and the standards (200, 150, 100, 75, 50, 25 and 10 ng / µl) were to be spotted. The standards were thawed and mixed thoroughly before use and 0.5 µl of each was carefully spotted onto the surface of the prepared EtBr plates. Care was taken not to dig into the surface of the plate. Capillary action was used to pull the small volume from the pipette tip to the plate surface and this avoided the formation of the bubbles. The pipette tips were changed between each samples. After all of the standards had been spotted, 0.5 µl of the DNA sample was immediately spotted onto the plate adjacent to the line of the standards. The spots were allowed to absorb into the plate for 10-15 minutes at room temperature. The plate was inverted and photographed using a UV light box. The concentration of the unknown sample was estimated by comparing it with the standards. Standards and unknowns must be spotted within 10 minutes of each other.

2.2.9 General subcloning procedure

The inserts to be subcloned and vector plasmid pUC18 or pUC19 were restricted with appropriate restriction enzyme, phenol and chloroform / isoamyl alcohol extracted and ethanol precipitated. The vector and inserts to be ligated were mixed in 1:1 Molar ratio (using 0.1 to 0.2 μg vector) with 1 μl of T_4 DNA ligase and 1 μl of 10x ligation buffer (supplied with enzyme) in a total volume of 10 μl. Ligation was performed at 15°C overnight or at room temperature for 4 hrs.

2.2.10 Cloning of genomic DNA into λ vector

The pea genomic library was kindly provided by Dr. David Bown (Durham University). The genomic DNA for use in the construction of genomic library was extracted by the method of Graham (1978) from the leaves and stipules of the purple podded pea plants. The DNA was partially restricted with *Sau*3A to yield molecules in the range of 9–23 kb suitable for cloning into the vector used. Then the size fractionated genomic DNA was cloned into Promega's λ GEM–12 *Xho*1 half site arms vector and packaged using Promega extract following the instructions from the supplied protocol.

2.2.11 Cloning PCR products

2.2.11.1 pGEM-T vector system

PCR products were cloned into Promega's pGEM-T vector system using the reagents and enzymes supplied in kit and following the manufacturer's instructions. The PCR product was first passed through IGI 'Clean Up' column to remove excess primers before cloning. Phosphorylation of 5' ends were carried out using Polynucleotide Kinase (PNK) in the presence of ATP. The ds DNA was ligated to the vector in an equimolar ratio. To calculate the appropriate amount of PCR product, the following equation was used:

$$\frac{\text{ng of vector X kb size of insert}}{\text{kb size of vector}} \text{ X insert : vector molar ratio = ng of insert}$$

The ligation was carried out at 15°C overnight. Ligated DNA was transformed into JM109 high efficiency competent cells and plated out on LBamp / IPTG / X-Gal plates. Selection for transformants was carried out by blue / white colour screening.

2.2.11.2 PCR-Script™ Amp SK(+) cloning system

Stratagene's PCR-Script™ Amp SK(+) cloning kit was used to clone PCR products in some cases, following the instructions supplied with kit. The PCR products were selectively precipitated to remove excess primers using the solutions supplied with the kit. The ends of the purified PCR products were polished using pfu DNA Polymerase. The PCR product was then cloned into PCR-Script™ Amp SK(+) cloning vector in 40:1 insert to vector ratio. The insert : vector ratio was calculated by using the following equation:

$$\text{picomoles ends / microgram of DNA} = \frac{2 \text{ X } 10^6}{\text{no. of bp X } 660}$$

The DNA was transformed into *Epicurean coli* XL1-Blue MRF' kan^R supercompetent cells by standard cell transformation method described in section 2.2.12. Transformed cells were plated out on LBamp / X-Gal / IPTG plates. The plates were incubated at 37°C overnight and selection for tranformants was carried out by blue / white colour screening.

2.2.12 Transformation of *E.coli* competent cells with plasmid vector

Competent cells commercially obtained were transformed following the suppliers instructions : 1 µl of ligated plasmid (2-10 µg) was gently mixed with 50 µl freshly thawed cells and incubated on ice for 30 minutes. The cells were then heat shocked for 90 sec at 42°C to facilitate uptake of the plasmid DNA by the cells and then chilled on

ice. 100 µl SOC buffer was added. The cells were incubated at 37°C with continuous shaking for one hour to express Ampicillin resistance and then spread over LB or YTamp X-Gal plates and incubated overnight at 37°C. The white colonies contained inserts.

2.2.13 Preparation of genomic DNA

Plant genomic DNA was isolated from 250 mg fresh tissues by using Nucleon Bioscience's "Phytopure" DNA extraction kit and according to the instructions in the manual supplied with kit. After extraction and purification, the genomic DNA was digested with RNaseA (100 µg/100 µl DNA solution) and again phenol extracted and ethanol precipitated.

2.2.14 Random primed labelling of DNA

Restricted DNA fragments used as DNA hybridisation probes were purified from agarose gel and labelled with α-^{32}P dCTP by a random priming method (Feinberg and Vogelstein, 1983). The DNA fragment was first boiled in water for 5 minutes and chilled on ice for 2 minutes before being added to the reaction mixture. The labelling reaction was set up as follows: DNA (30–50 ng) in 31 µl of H_2O; OLB (Feinberg and Vogelstein, 1984), 10 µl; BSA (10 mg / ml), 2 µl; [α-^{32}P] dCTP, 5 µl (50 µCi or 400 Ci/ mmoles); the Klenow fragment of *E.coli* DNA polymerase I–2 µl (2 units). Labelling was allowed to proceed overnight at room temperature. The reaction was stopped by adding 5 µl of 10 % (w/v) SDS. The radiolabelled DNA was separated from unincorporated label by gel filtration through a 5 ml column of Sephadex G-50 washed with 1x column buffer. The excluded peak (judged with Gieger monitor) was collected. Before use DNA probes were boiled for 5 minutes to render the DNA single stranded.

2.2.15 Scintillation counting of radiolabelled DNA

Labelled DNA in solution or precipitated onto glass fibre discs was counted by liquid scintillation in a Packard Tri-Carb liquid scintillation analyser, model 1600TR Ecoscint A was used as scintillation fluid. 5 µl of the collected labelled DNA was pippetted into 5 ml of scintillation fluid, mixed thoroughly and counted.

2.2.16 Analysis of DNA by Southern blotting

Gels containing DNA were blotted onto nitro-cellulose filters by capillary blotting as described by Sambrook et al.(1989). Prior to blotting, the gels were treated with denaturing solution for 45 minutes with one change after 30 minutes and finally with neutralising solution in same manner as denaturing solution. The gels were placed on the apparatus as described except that nappy liners were used in place of paper towels. Blotting was allowed to proceed for 16-18 hrs with 10x SSC as transfer buffer.

After blotting, the position of the wells was marked on the filter with ink and the filter was air dried for 30 minutes, then baked for 2 hrs at 80°C in vacuum oven. The blotted gel was restained with EtBr to ensure DNA transfer was complete.

2.2.17 Hybridisation of DNA probes to filters containing DNA

The nitro-cellulose filters containing DNA were processed either in sealed polythene bags in shaking water bath at 65°C or in hybridisation flasks with solution preheated to this temperature. Filters were prehybridised for at least 2 hrs in prehybridisation solution (5x SSC, 5x Denhardt's solution, 100-200 µg/ml sonicated herring sperm DNA). This solution was then replaced by hybridisation solution containing 5x SSC, 2x Denhardt's solution, 100 µg/ml sonicated herring sperm DNA, labelled probe was added and hybridisation was allowed to proceed overnight or longer at 65°C. Hybridisation solution was then removed and the filters were washed once for 30 minutes with 2x SSC, 0.1% w/v SDS; twice for 30 minutes each with 1x SSC, 0.1%

w/v SDS; once with 0.1x SSC, 0.1% (w/v) SDS until required stringency is reached. Filters were removed and blotted dry for exposure to X-ray films.

2.2.18 Autoradiography

Nitrocellulose filters probed with ^{32}P-dCTP labelled DNA were fixed on a 3 MM paper sheet and their orientation was marked with radioactive ink (writing ink with a small quantity of ^{32}P-dCTP) and placed in a film cassette. A pre-flashed X-ray film was placed between the filter and an intensifying screen within the cassette and autoradiographed at –80°C. The films were developed following the manufacturer's instructions for the developer and fixer used.

2.2.19 Library construction and screening

2.2.19.1 Construction of subtraction cDNA library

The subtraction cDNA library from wounded pea leaf tissue was made using the Clonetech PCR-Select™ cDNA subtraction kit and according to the instructions in the manual supplied with it. The overall strategy for subtractive hybridisation method is shown in figure 2.1 A&B. Subtractive hybridisation methods are used to compare two populations of mRNA and obtain clones representing mRNAs that are expressed in one population but not in the other. Poly $(A)^+$ RNA from the two types of tissues being compared are designated as "tester" from wounded tissues having differentially expressed transcripts, and "driver" from control tissues. About 2 μg of poly $(A)^+$ RNA from both categories was used and skeletal muscle poly $(A)^+$ RNA provided with the kit was used as a control reaction. First strand cDNA was synthesised using MMLV reverse transcriptase in the presence of nucleotides and buffer and a 44 base oligonucleotide including a 32 base poly (T) tail (sequences are shown in figure 2.2). ^{32}P-dCTP (10 mCi/ml, 3000 Ci/mmol) was added in the first strand synthesis reaction mixture to monitor the progress of cDNA synthesis. During second strand synthesis, RNaseH nicks the RNA bound to the first strand cDNA to produce a multitude of

Figure 2.1 A Overview of the PCR-Select Subtraction Hybridisation procedure showing the basic steps used to enrich for differentially expressed sequences.

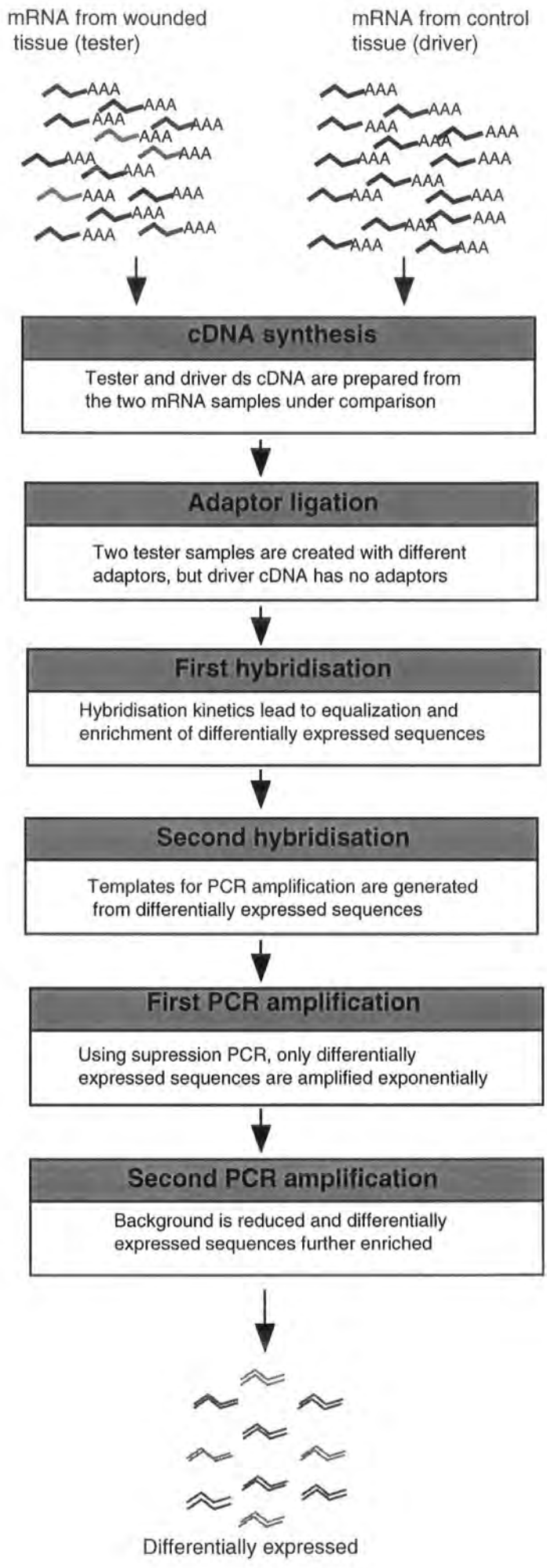
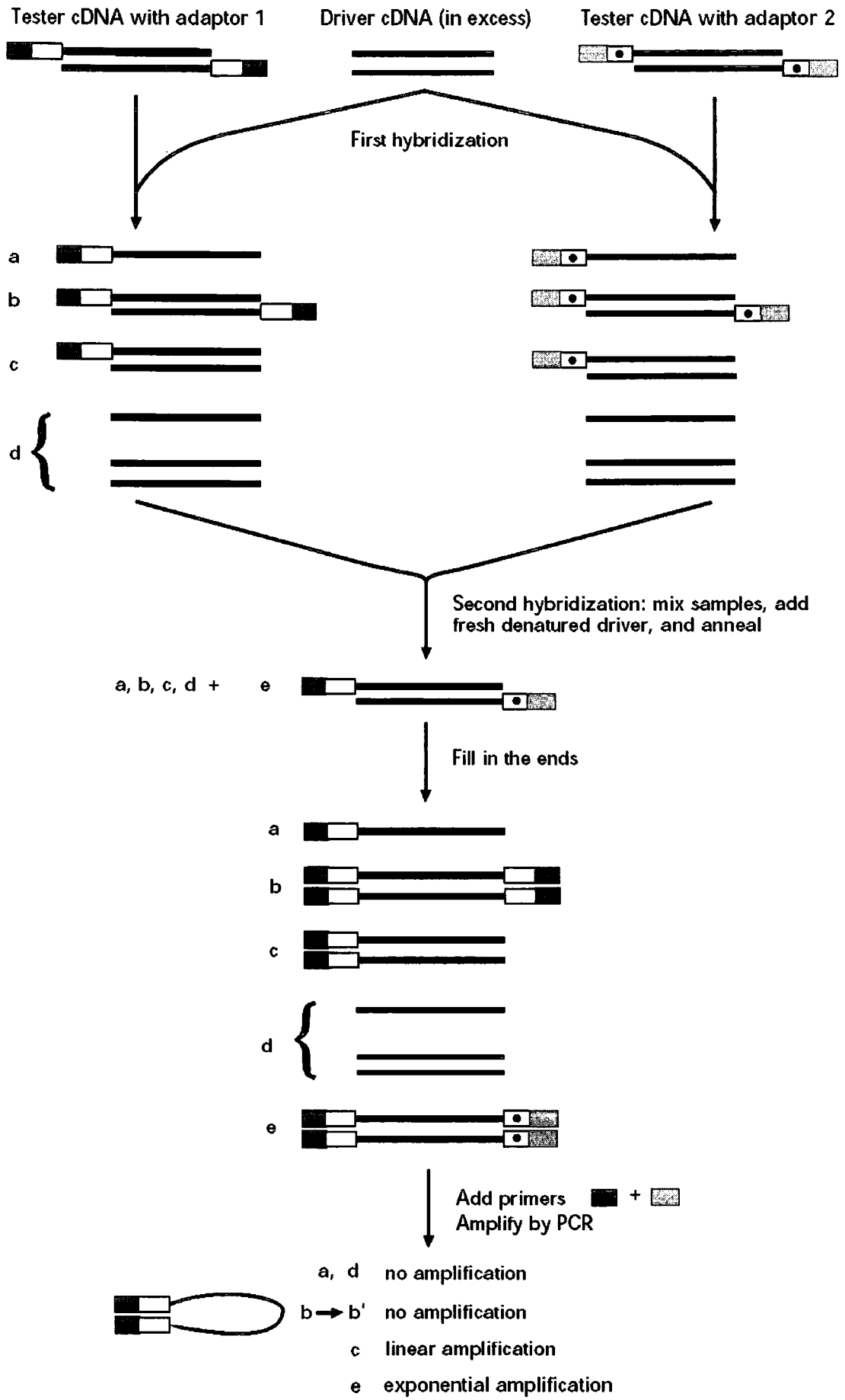

mRNA from wounded tissue (tester)
mRNA from control tissue (driver)
AAA
cDNA synthesis
Tester and driver ds cDNA are prepared from the two mRNA samples under comparison
Adaptor ligation
Two tester samples are created with different adaptors, but driver cDNA has no adaptors
First hybridisation
Hybridisation kinetics lead to equalization and enrichment of differentially expressed sequences
Second hybridisation
Templates for PCR amplification are generated from differentially expressed sequences
First PCR amplification
Using supression PCR, only differentially expressed sequences are amplified exponentially
Second PCR amplification
Background is reduced and differentially expressed sequences further enriched
Differentially expressed

Figure 2.1 B Schematic diagram of PCR-Select cDNAs Subtraction technique. Type e molecules are formed only if the sequence is absent from the driver cDNA. Solid lines represent the *Rsa* I digested tester or driver cDNA. Solid boxes represent the outer part of the adaptor 1 longer strand and corresponding PCR primer 1 sequences. Shaded boxes represent the outer part of the adaptor 2 longer strand and corresponding PCR primer 2 sequence. Clear boxes represent the inner part of the adaptors and corresponding nested primers. Tester and driver cDNAs represent the mRNA from wounded and control tissues, respectively.


Tester cDNA with adaptor 1
Driver cDNA (in excess)
Tester cDNA with adaptor 2
First hybridization
a
b
c
d
Second hybridization: mix samples, add fresh denatured driver, and anneal
a, b, c, d +
e
Fill in the ends
a
b
c
d
e
Add primers
+
Amplify by PCR
a, d no amplification
b → b' no amplification
c linear amplification
e exponential amplification

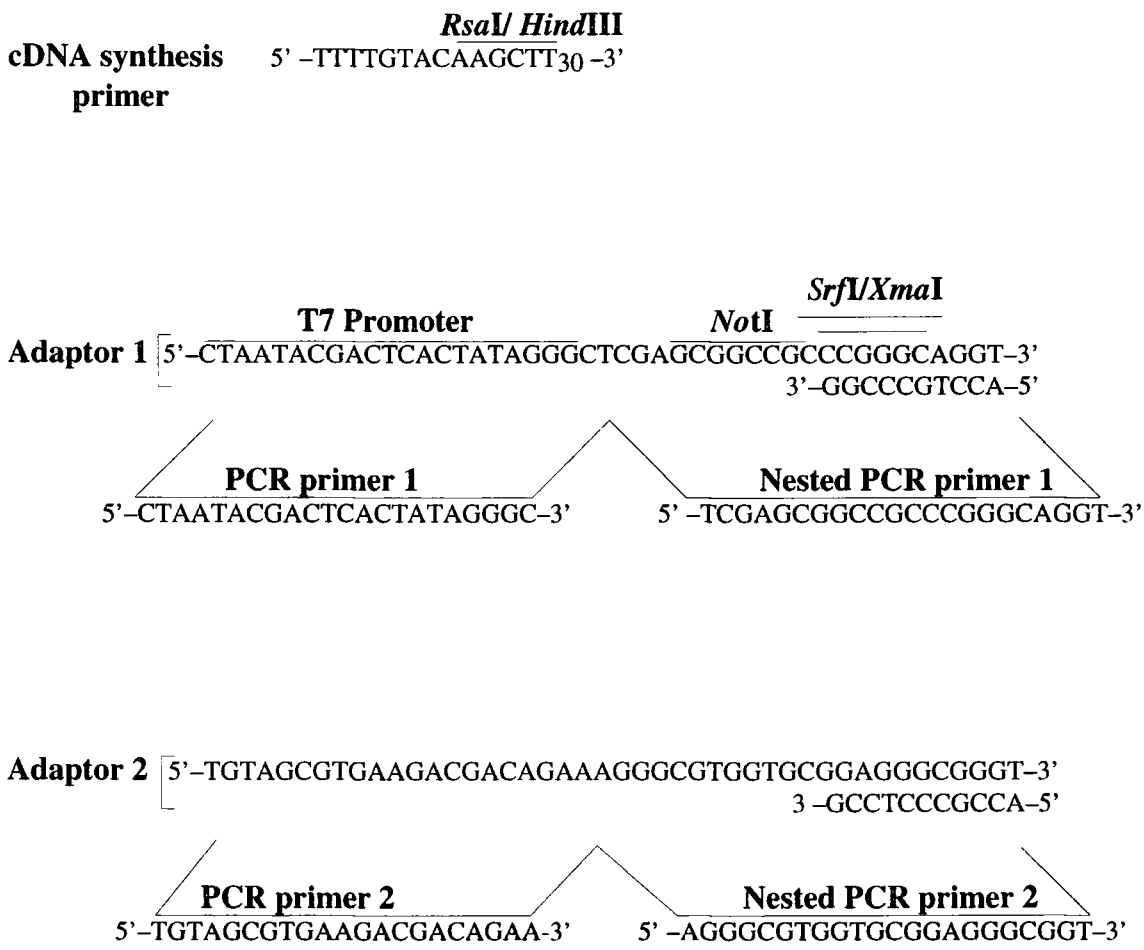


Figure 2.2 Sequences of the PCR-Select cDNA synthesis primer, adaptors, and PCR primers.

fragments, which serve as primers for DNA polymerase I. The latter nick translates these RNA fragments into second strand cDNA.

The tester, driver and control skeletal muscle double stranded cDNAs were *Rsa*I digested to generate shorter, blunt-ended fragments which are optimal for subtraction and necessary for adaptor ligation. For adaptor ligation, the tester cDNA was then subdivided into two populations and each ligated to a different cDNA adaptor. The adaptor sequences are shown in the figure 2.2. The tester cDNA ligated to both adaptors was used as ligation control which also served as positive control for ligation and later on as a negative control for subtraction. The end of the adaptors do not have a phosphate group, so that only one adaptor can attach to the 5' end of the cDNA. The two adaptors have different sequences to enable annealing of two different PCR primers once the recessed ends have been filled in.

Two steps of hybridisation were then performed. In the first, an excess of the driver cDNA (without adaptors) is added to each of the two samples of the tester (with adaptors). These were then heat denatured and allowed to anneal. High abundance sequences anneal faster than the lower abundance sequences due to second order kinetics. Low abundance sequences are enriched for differentially expressed species as non target tester cDNAs are hybridised by the excess driver cDNAs. This results in a population with low abundance tester-specific cDNAs existing as single stranded molecule with an adaptor attached.

In the second hybridisation, the two samples from the first hybridisation are mixed together and fresh denatured driver cDNA is added to further enrich for differentially expressed sequences. The complementary low abundance tester-specific ss cDNAs anneal to form the only ds molecules with different adaptors on each end. After filling in the ends with DNA Polymerase, the differentially expressed cDNAs are selectively amplified during 2 rounds of PCR amplification using Perkin-Elmer DNA Thermal Cycler 480 model. In the first amplification, only ds cDNAs with two different primer annealing sites are exponentially amplified. In the second, nested PCR is used to further reduce background and enrich for differentially expressed sequences. The PCR conditions were as follows:

Primary PCR		Secondary PCR	
30 cycles		18 cycles	
94°C	30 seconds	94°C	30 seconds
68°C	30 seconds	68°C	30 seconds
72°C	2.5 minutes	72°C	2.5 minutes

2.2.19.2 Construction of cDNA libraries

Pea cDNA libraries (L59 lignified pod cDNA library and wounded leaf tissue cDNA library) were constructed using Stratagene ZAP-cDNA Synthesis kit following the instructions from the supplier's protocol. About 5 μg poly $(A)^+$ RNA was used in each case. The first strand cDNA was synthesised with MMLV reverse trancriptase, nucleotide mixture with 5-methyl dCTP and a 50 base hybrid oligo-dT primer-linker which contains an *Xho*I site as shown below:

Poly dT

5' GAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTT 3'

"GAGA" sequence XhoI

The complete first strand will have a methyl group on each cytosine base which will protect it from digestion by restriction enzymes used in subsequent cloning steps. The restriction site *Xho*I allows the finished cDNA to be inserted into the uni-ZAP XR vector in a sense orientation (5'–3', *Eco*R1-*Xho*1) with respect to the *lac*Z promoter.

During second strand synthesis, RNase H nicks the RNA bound to the first strand to produce a multitude of fragments, which serve as primers for DNA Polymerase I. The latter nick translates these RNA fragments into second strand cDNA. The uneven termini of the double stranded cDNA were filled-in with T4 polymerase and *Eco*RI adaptors were ligated to the blunt ends. These adaptors are composed of 9 and 13 mer oligonucleotides which are complementary to each other and create an *Eco*RI cohesive end :

5' AATTCGGCACGAG 3'

3' GCCGTGCTC 5'

The 9 mer is kinased which allows it to ligate to other blunt termini available in the form of cDNA and other adaptors. The 13 mer is kept dephosphorylated to prevent it from ligating to other cohesive ends. After adaptor ligation, ligase is heat inactivated and the 13 mer is kinased to enable its ligation into the dephosphorylated vector arms. The subsequent *Xho*I digestion releases the *Eco*RI adaptor and "GAGA" sequence from the 3' of the cDNA, thus producing DNA fragments with *Eco*RI and *Xho*I cohesive ends. These DNA fragments were chromatographed on a Sephacryl spin column to remove the adaptors, the residual linker-primer and short cDNA species. The size fractionated cDNA was then ethanol precipitated and ligated to the uni-ZAP XR vector arms. The λ ZAP II library was then packaged in Gigapack II Gold packaging extract and plated in the *E.coli* cell line XL1-Blue MRF'.

2.2.19.3 Titration of DNA libraries

A host cell culture of appropriate *E.coli* strains(LE392, NM538, KW251 for genomic library, XL1-Blue MRF' for cDNA libraries) were prepared by inoculating a single bacterial colony into 10 ml of LB medium supplemented with 0.2% w/v maltose and 0.01M $MgSO_4$ and shaking at 37°C overnight. Alternatively, host cell cultures could be prepared by inoculating LB medium containing 0.2% w/v maltose and 0.01M $MgSO_4$ with 1/10 volume of an overnight grown cell culture on an appropriate antibiotic selection and shaking at 37°C for 4-5 hrs. The cell culture was first pelleted by centrifugation at 4000 g for 10 minutes at room temperature and then resuspended in 0.01M $MgSO_4$ to give a cell density of $O.D._{600}$= 0.5–1.0. Dilutions of DNA libraries (in bacteriophage lambda vectors) were made using SM phage buffer. Aliquots of 100 µl of each dilution were mixed with 100 µl of the host cell culture and incubated at 37°C for 15 to 30 minutes. For plating, the infected cells were mixed gently but quickly with 3 ml molten (48°C) LB, TB or NZY top-agar and immediately poured onto LB or NZY plates (9 cm diameter). The top agar was allowed to harden and then the plates were incubated inverted at 37°C overnight.

2.2.19.4 Amplification of DNA libraries

Host cell cultures were prepared as described above. Aliquots of the packaged mixtures of primary libraries containing about 10^5 bacteriophage were mixed in a tube with 0.6 ml of cell culture (XL1-Blue for cDNA library and KW251 for genomic library) and incubated at 37°C for 15 minutes (cDNA library) or 30 minutes (genomic library). The infected cells in each tube were plated as for titration on to a 22x22 cm square plate using 50 ml LB or NZY or TB top agar, and the plates incubated inverted at 37°C for 6-7 hrs. After phage growth 25 ml of the SM phage buffer was swirled on each plate for 4-5 hrs at 4°C. The phage buffer containing the phage was collected from each plate and chloroform was added to a final concentration of 0.5%. The amplified libraries were kept at 4°C. For long term storage, aliquots of the libraries were supplemented with dimethyl sulphoxide to 7%, frozen in dry ice-ethanol bath and kept at –80°C.

2.2.19.5 Screening of genomic library

Using the results from the titrations, primary screening was carried out. The genomic library to be screened was plated out as in the " Amplification of DNA libraries" section above. On every 22x22 cm square LB plate, about $1x10^5$ phages were plated which were then incubated at 37°C until discrete plaques were formed, then cooled at 4°C. Duplicate nitrocellulose filters were taken, overlaying the plates with filters and their position marked using a hole punch and ink. The filters were allowed to remain in contact with the plaques for 2 minutes (or 4 minutes for replicate filter). The filters were removed carefully from the plate and submerged phage side up in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. The membranes were blotted with a 3 MM paper to remove excess moisture, and then submerged in neutralisation solution (1.5 M NaCl, 0.5 M Tris/HCl pH 7.4) for 5 minutes. The filters were then soaked in 5x SSC again for 5 minutes, blotted dry with 3 MM paper and then baked in a 80°C vacuum oven for 1-2 hrs.

The filters were incubated at 65°C for 2-3 hrs in prehybridisation solution (5x SSC, 5x Denhardt's, 100 µg/ml denatured herring sperm DNA). DNA probe labelled with a-^{32}P-dCTP (see section 2.2.14) was added to the hybridisation solution (5x SSC, 2x

Denhardt's, 100 µg/ml denatured herring sperm DNA) and the membranes incubated at 65°C for 18 hrs. After hybridisation, the filters were washed to high stringency 0.1x SSC, 0.1% SDS (as described in section 2.2.17) and then exposed to X-ray films (as described in section 2.2.18). After exposure, the X-ray films were aligned with the plates, agar plugs containing areas of duplicate hybridisation removed with a cork borer and stored with phage buffer at 4°C.

Secondary and tertiary screening was performed by using 82 mm diameters filter in the same way as described above until discrete, positively hybridising plaques could be collected.

2.2.19.6 Screening of cDNA libraries

Plating of the cDNA library (10^4-10^5 plaques) on XL1-Blue MRF' cells and preparation of nitro-cellulose filters were carried out in the same way as described for screening of the genomic library. The nitro-cellulose filters containing phage DNA were incubated for 2-3 hrs in 100 ml of prehybridisation solution (5x SSC, 2x Denhardt's, 100 µg/ml denatured herring sperm DNA). The labelled probes was boiled for 5 minutes, chilled on ice, and then added to the hybridisation solution (5x SSC, 2x Denhardt's solution, 100 µg/ml denatured herring sperm DNA). Hybridisation was carried out for 18 hrs at 65°C; washed to the desired stringency at same temperature. The hybridisation was visualised by autoradiography. Plaques corresponding to strong hybridisation signals were picked up and stored in SM buffer at 4°C.

2.2.19.7 PCR amplification of primary plaques

This strategy was opted to purify only those plaques which have the largest cDNA inserts, instead of going through a labour intensive methods of processing all the primary plaques through secondary and tertiary rounds of screening. All the primary plaques were subjected to PCR amplification using T_3 and T_7 synthetic oligo primers which will amplify the cDNA inserts within the phage vector. The sequences of the oligo primers are as follows:

T3 primer 5' GCAATTAACCCTCACTAAAGGGA 3'

T7 primer 5' GTAATACGACTCACTATAGGGCG 3'

30 µl of the solution used to elute phage from the primary plaques was boiled for 5 minutes and 20 µl taken for PCR amplification. The PCR amplification mixture contained 1x *Taq* polymerase buffer (supplied with the enzyme), 2 units of *Taq* DNA polymerase, 0.2 mM 4 dNTPs, 1 µl of each primers (100 pmoles), in a total volume of 100 µl. The reaction mixture were overlaid with 100 µl mineral oil and amplified with a Perkin-Elmer DNA Thermal Cycler 2400 model. The PCR profile involved were 1 cycle (denaturation at 94°C for 3 minutes, primer annealing at 64°C for 1 minutes) and 29 cycles (94°C for 40 seconds, 60°C for 1 minutes and primer extension at 72°C for 1 minutes). The annealing temperature of the primers was calculated with the equation 4 (G+C) + 2 (A+T).

Analysis of the amplified PCR products was performed by electrophoresis of 20 µl aliquots through 0.8% w/v agarose gel which was stained with EtBr and photographed. The DNA was transferred to nitrocellulose filters by Southern blotting (see 2.2.16) and the blots processed in the same way as described in section 2.2.17. The blots were hybridised to the respective probes and the plaques which contain the biggest inserts were purified by further round of screening as described for screening of genomic library using the same hybridisation and washing conditions.

2.2.20 *In vivo* self excision of pBluescript from Uni-ZAP XR vector

Positive phage isolated from the cDNA libraries were subjected to *in vivo* self excision to form pBluescipt phagemids, which were then used in subcloning and sequencing. This was carried out using the EXAssist helper phage following the procedure in the Stratagene's ZAP-cDNA synthesis kit. 250 µl of phage stock was mixed with 250 µl XL1-Blue MRF' cells grown at 37°C for 4-5 hrs ($O.D._{600}$ = 1.0) and 1 µl of EXAssist helper phage, and incubated at 37°C for 20 minutes. A control consisting of 1 µl of helper phage and 250 µl of XL1-Blue MRF' cells was performed. The mixture was

transferred to 3 ml of 2x YT medium in a falcon tube 2059 and incubated at 37°C for 2-3 hrs with shaking. The *E.coli* cells were killed by heating at 70°C for 20 minutes and removed by centrifugation at 4000 g for 5 minutes. The rescued phagemid could be stored at 4°C for 1-2 months. One µl of saved supernatant containing phagemids was mixed with 200 µl of overnight , 30°C grown *E.coli* SOLR cells and incubated for 15 minutes at 37°C. Aliquots(100 µl and 10 µl) of the cell culture were plated on LBamp (50 µg/ml) plates and incubated overnight at 37°C. The colonies on the LBamp plates contain double stranded pBluescript containing the cloned inserts.

2.2.21 DNA Sequencing

Automated DNA sequencing was performed on an Applied Biosystems model 370A DNA Sequencer based on the dideoxy chain termination method (Sanger et al., 1977), using the PRISM Ready Reaction Dye Primer Cycle Sequencing Kit. Sequencing work was carried out by Ms. Julia Bartley, at department of Biological Sciences, University of Durham, Durham.

2.2.22 Identification of inserts in transformed bacterial cells by hybridisation

Bacterial colonies transformed with recombinant plasmid were screened by hybridisation. The individual transformed colonies were plated out using cocktail stick in a grid pattern onto two nitrocellulose filters laid on the surface of YTamp X-Gal agar plates. An untransformed colony was also transferred as a negative control. The plates and filters were marked with a sterile needle to allow the orientation of the bacterial streaks. The plates were incubated overnight at 37°C.

The master plate was sealed and stored at 4°C. The bacterial colonies on the nitrocellulose filter were lysed in the same ways described in section 2.2.19.5, by treatment with denaturing solution, neutralisation and 2x SSC solution and filters were processed. If the number of transformed colonies was less, the DNA is directly purified by minipreparation and electrophoresed on agarose gels which were then

blotted to nitro-cellulose filter. The immobilised DNA on filter was then hybridised with ^{32}P-dCTP labelled cDNA probes as described in section 2.2.17.

2.2.23 Preparation and analysis of RNA

2.2.23.1 Extraction of total RNA by guanidium thiocyanate method

Plant material for RNA extraction was harvested, frozen immediately in liquid nitrogen and stored at –80°C until required. Total RNA was extracted with guanidium thiocyanate following the instructions from the Promega protocol. 1g Frozen tissues, was ground to fine powder in a mortar and pestle and then added to 12 ml of previously chilled denaturing solution (25g guanidium thiocyanate (4M final) into 33 ml CSB buffer i.e. 42 mM sodium citrate, 0.83% N-lauryl sarcosin, stored in dark bottle and 50 mM ß-Mercaptoethanol was added fresh prior to use). The tissues were then disrupted with a high speed homogeniser such as the Brinkman Polytron set on high for 15-30 seconds. 1.2 ml of 2M sodium acetate, pH 4.0 was added and mixed thoroughly by inversion. The mixture was extracted with 12 ml of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) mixture, mixed by inversion, shaken vigorously for 10 minutes, chilled on ice for 15 minutes and then centrifuged at 10,000 g for 15 minutes at 4°C. The top aqueous phase which contains RNA was carefully removed and transferred to a fresh DEPC-treated tube. RNA was precipitated by adding an equal volume of isopropanol and precipitation was carried out overnight at –20°C. The RNA was pelleted out by centrifugation at 10,000 g for 15 minutes at 4°C and resuspended in 5 ml of denaturing solution by vortexing. The RNA was again precipitated by adding an equal volume of isopropanol as described above. After centrifugation the pellet was washed with 70% v/v ice cold ethanol and again centrifuged. Pellet was dried in a vacuum desiccator for 15-20 minutes and resuspended into 1 ml of DEPC-treated water. An 1µl aliquot, was taken for spectrophotometeric determination of concentration and an aliquot containing 5 µg of total RNA was taken for analysis by formaldehyde gel electrophoresis (see 2.2.6.2). The RNA was stored at –80°C in 3 volumes of ethanol.

2.2.23.2 Isolation of poly $(A)^+$ RNA

Isolation of poly $(A)^+$ RNA was performed from 1mg of total RNA, using a polyATract mRNA Isolation System III (Promega) according to the manufacturers instructions. This system utilises a biotinylated oligo (dT) primer to hybridise at high efficiency to the poly (A) tail of mRNA in total RNA samples. The biotin-oligo (dT) primers bind to the poly (A) tail of mRNAs in a sample of total RNA. Streptavidin coupled to paramagnetic particles (SA-PMPs) are added to the total RNA sample hybridised with biotin-oligo (dT) primers. The biotin-oligo (dT) hybridised mRNAs bind to the SM-PMPs and are then magnetised to a separation stand. The SM-PMPs are washed to high stringency to remove the aqueous phase components, and the mRNAs are eluted from the solid phase with ribonuclease-free deionised water and concentrated by ethanol precipitation.

2.2.23.3 Analysis of RNA by Northern blotting

The RNA denatured on formaldehyde gels (see section 2.2.6.2), was transferred to nitro-cellulose filters by capillary blotting as described by Sambrook et al. (1989). The gel containing formaldehyde was rinsed several times with DEPC-treated water to remove formaldehyde and then soaked into 20 x SSC for 45 minutes. Blotting was allowed to proceed for 16-18 hrs with 20 x SSC as transfer buffer. The position of the wells were marked on the filters with pencil and the filters were air dried for 30 minutes, then baked in a vacuum oven at 80°C for 1-2 hrs. The blotted gels were restained with EtBr to ensure RNA transfer was complete. The filters were prehybridised at 42°C in a solution containing 50% w/v formaldehyde, 5x Denhart's solution, 0.1% (w/v) SDS and 100 µg/ml denatured herring sperm DNA. The filters were hybridised at 42°C, with ^{32}P-dCTP labelled cDNA probes (specific activity at least $1x10^8$ cpm) and hybridisation was performed in 50% w/v formaldehyde, 2x Denhart's solution, 0.1% w/v SDS and 100 µg/ml denatured herring sperm DNA. The filters were washed to desired stringency and sensitised film was exposed for 1-2 weeks at –80°C.

2.2.24 Preparation of tissue blots

Tissue blots were prepared from different tissues to study the localisation of hsp70(LP19) gene expression. The nitrocellulose filters were made wet in deionised water for 5 minutes, then soaked into 20x SSC for at least 5 minutes and dried on 3 MM paper before tissue printing. Excess moisture from fresh cut tissues was first removed on paper towels and then sections pressed very gently on nitro-cellulose filters placed on a gel dryer under vacuum. Prints were allowed to dry and treated for 5 minutes with 10% vanadosyl complex solution for ribonuclease inactivation. The tissue blotted filters were hybridised in the same way as Northern blots described in the preceding section and probed with ^{32}P-dCTP labelled LP19 cDNA. Filters were washed to a high stringency (0.1X SSC, 0.1% w/v SDS, 50°C) and autoradiographed.

2.2.25 Plant Transformation

2.2.25.1 Construction of expression vectors

A *Hin*dIII–*Pvu*II fragment including 1.8 kb 5' flanking region and the first 18 amino acid nucleotide of coding sequences of the pea hsp70(LP19) gene was isolated and subcloned into *Hin*dIII and *Hin*cII sites of pUC18. The 1.8 kb fragment was excised from pUC18 by *Hin*dIII and *Bam*HI and was translationally fused with the coding sequence of GUS inserted into pBI101.2. The fusion site around the hsp70(LP19) promoter fragment and the GUS coding sequences was checked by nucleotide sequencing. The resulting construct was designated as pHSP70(LP19)–GUS.

2.2.25.2 Transfer of construct to *Agrobacterium* by triparental mating

Agrobacterium stain *A. tumifaciens* LBA4404 was grown for two nights at 28°C from a single colony in 5 ml of YEB rifampicin (100 µg/ml). An *E.coli* helper strain (HB101 : pRK2013) and a bacterial strain containing a recombinant plasmid (*E.coli.* DH5α : pHSP70(LP19)-GUS) was grown overnight at 37°C from a single colony in 5 ml of

LB-kanamycin (50 μg/ml) and YEB-kanamycin (50 μg/ml), respectively. Each of the cultures was spun down and resuspended in the same volume of 10 mM magnesium sulphate. This was replaced twice to remove the antibiotics. 200 μl of each culture was mixed in an eppendorf tube and spread on to YEB plates. This was repeated to give triparental mating in duplicate. The plates were incubated at 28°C for 1-2 days. Approximately 5 ml of magnesium sulphate was poured on to each plate and the cells were resuspended by gently swirling the liquid on the plate. These resuspended cells were serially diluted (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) and plated out on YEB plus 100 μg / ml rifampsin and 50 μg / ml kanamycin. The plates were incubated at 28°C for 3-4 days until colonies of triconjugant had grown to substantial size. The resulting colonies were checked by streaking on to selective plates and by Southern blotting.

2.2.25.3 *Agrobacterium* total DNA preparation

The transformed *Agrobacterium* stain strain was grown for 48 hrs at 28°C in 5 ml of YEB. A 1.5 ml of sample was removed and spun down at 1200 g for 2 minutes. The supernatant was discarded and the cells were resuspended in 400 μl of pronase buffer (380 μl of 50 mM TRIS / HCl pH 8.0, 20 mM EDTA, 0.8% sarcosyl and 20 μl of fresh 20 mg / ml pronase buffer). This was mixed thoroughly, incubated at 37°C for 1 hr and then the lysate was sheared by adding 400 μl of double distilled water and vortexing for one minute followed by passing thorough a Gilson pipette several times. The suspension was phenol and chloroform extracted. 400 μl phenol saturated under TE buffer was added to the suspension, mixed, spun and the supernatant was removed and re-extracted by phenol in the same way. The supernatant was then extracted with 400 μl chloroform saturated with TE buffer, mixed, spun and supernatant was removed which was again re-extracted by chloroform. DNA was precipitated by adding 2 volumes of 100% v/v ethanol at room temperature, the tube was swirled to mix the two layers, then shaken vigorously. The DNA was spun down and resuspended in 400 μl of Sodium acetate pH 4.8 (with acetic acid). 2 volumes of 100% ethanol was carefully added at room temperature and the mixture was incubated on ice for 10 minutes. The DNA was then spun down, washed with 70% ethanol, vacuum dried and resuspended into 100 μl of double distilled water.

2.2.25.4 Tobacco transformation using leaf discs

Leaf discs were cut from young leaves of sterile tobacco plants(*Nicotiana tabacum* cv Samsun) grown on MSO medium. The leaf discs were infected by floating them in culture of transconjugant *Agrobacterium* strain (OD_{600} =1.0) for 1-3 minutes. These were then blotted dry on sterile 3 MM paper to remove extra fluid. The inoculated leaf discs were co-cultivated on solid MSO medium plus naptheleneacetic acid (NAA) 0.1 mg/l and 6-benzylaminopurine (BAP) 1.0 g/l at 25°C and 2000-4000 lux light level, 16 hour photoperiod in sealed petri dishes until visible bacterium colonies appeared. The leaf discs were then transferred onto selection media (MSO + NAA + 200 µg/ml augmentin + 100 µg/ml kanamycin). When shoots become visible, they were cut off the leaf discs, free of callus if possible, and transferred to magenta boxes containing rooting medium (MSO + NAA + 200 µg/ml augmentin + 100 µg/ml kanamycin). Roots developed in as little as a 2-4 days. The plantlets were grown on until they were a few centimetres high and had good root system at which stage they were transferred to a peat based compost. This was done without causing any damage to roots by washing the media off carefully. The potted plantlets were first kept in sealed plastic bags to avoid wilting and they were gradually opened to air and then grown as normal tobacco plants in growth room maintained at 25°C, 65% humidity, 16 hrs light and 8 hrs dark.

2.2.26 Histochemical Localisation of GUS expression

Histochemical staining was performed according to the method described by Jefferson (1987). Fresh hand cut sections of leaf, petioles, stem, root and also the fragments of whole roots from both heat-shocked and non heat-shocked transgenic and non transgenic control tobacco plants were incubated in staining solution (1 mM 5-bromo-4-chloro-3-endolyl glucuronide (X-Gluc, Sigma), 100 mM sodium phosphate buffer pH 7.0 and 5 mM potassium ferricyanide and ferrocyanide). Staining was allowed to proceed for between 1 hr and overnight at 22°C, then cleared of chlorophyll by incubation in 70% v/v ethanol for 3-4 hrs. The sections were mounted on microscopic

slides and photographed. The material other than sections were cleared of chlorophyll by incubating in chloralhydrate solution for between 3-4 hrs and overnight.

2.2.27 Fluorometric GUS assays

β- Glucuronidase (GUS) activity was determined in protein extracts from plant tissues using the fluorometeric assay described by Jefferson (1987). Aliquots of 0.5 ml containing 50 µl extract and 1 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) were incubated at 37°C. 100 µl samples at various time points (e.g. for 0, 10, 20, 40 and 60 minutes) were taken out and added directly to 2 ml of 0.2 M Na_2CO_3. The Na_2CO_3 stops the reaction and enhances the fluorescence. The final volume was made to 2.5 ml by adding 0.4 ml of GUS extraction buffer. The reaction mixture was mixed thoroughly by inverting the cuvette. Concentration of the product i.e. 4-methylumbelliferone (4-MU), was determined from the fluorescence at 365 nm and 455 nm excitation and emission, respectively in a Perkin Elmer model LS-3B fluorimeter. A standard curve was constructed by measuring the fluorescence of serial dilutions of the reaction product, 4-MU (Sigma). GUS activity was calculated as pmoles min^{-1} μg^{-1} protein. Protein concentrations were determined according to Bradford (1976) using BSA as a standard.

2.2.28 Spectrophotometeric estimation of Polyphenol Oxidase (PPO) activity

Polyphenol oxidase activity was measured spectophotometerically following the method of Constable et al., (1995). This method depends on the decoloration of the intensely yellow 2-nitro-5-thiobenzoic acid due to the coupled reaction with the quinones formed by enzymatic oxidation (Esterbauer et al., 1977). Protein was extracted by grinding leaf tissue in 5 volumes of 100 mM sodium phosphate (pH 7.0) at 4°C and the extracts were centrifuged at maximum speed for 10 minutes in a microfuge. Clear supernatant was taken, and recentrifuged. PPO activity was assayed at 412 nm wavelength, using chlorogenic acid as substrate. The final assay mixture contained 2 mM chlorogenic acid, 50 µM 2-nitrothiobenzoic acid and 0.5–20 mg of total proteins in 1 ml of 100 mM Sodium phosphate/ 100 mM sodium citrate, pH 6.0. One unit of activity was defined

as the amount of enzyme that converts 1 mmole of chlorogenic acid to 1 mmole of chloroquinone per minutes at room temperature under the assay conditions. Protein concentrations were measured by the method of Bradford (Bradford, 1976), using BSA as the standard protein.

Chapter 3

Characterisation of a Gene Encoding an hsp70 Protein in Pea and Chickpea

3.1 Introduction

3.1.1 Isolation of LP19 cDNA clone

This project was continued from previous work in the laboratory at Durham, involving the isolation of a cDNA clone designated LP19, encoding the C-terminal region of an hsp70 protein. The LP19 cDNA clone was isolated from a cDNA library representing poly $(A)^+$ RNA prepared from developing pea pods of pea line L59, which has a lignified pod endocarp. A differential screen of the amplified L59 lignified pod cDNA library was performed following a method based on that of Olszewski et al. (1989). ^{35}S-dCTP and ^{32}P-dCTP labelled total cDNAs synthesised from 4-6 DAF pod mRNAs from L59 (phenotype, lignified endocarp, genotype, PV) and L1390 (phenotype, unlignified endocarp, genotype, pv) were used respectively, in an attempt to isolate cDNAs involved in differentiation and lignification of the pea pod endocarp. The strategy for isolating differentially expressed genes as cDNAs representing the lignified endocarp phenotype is shown in figure 3.1. Aliquots containing 100 ng of L59 and L1390 pod total cDNA were used to prepare ^{35}S dCTP and ^{32}P dCTP labelled probes respectively. The clone LP19 was selected on the basis of consistent hybridisation to a total cDNA probe from the lignifying pods of L59, but not to a total cDNA probe from pods of L1390, which do not lignify.

3.1.2 Sequence analysis of LP19 cDNA clone

The pLP19 insert was characterised by DNA sequencing. The cDNA fragment was 1072 bp in length, and contained an open reading frame of 813 nt encoding a polypeptide of 271 amino acids (truncated at the N-terminus), a 3' non-coding region of 256 nt, plus a poly (A) tail of 18 nt. A homology search for the polypeptide predicted

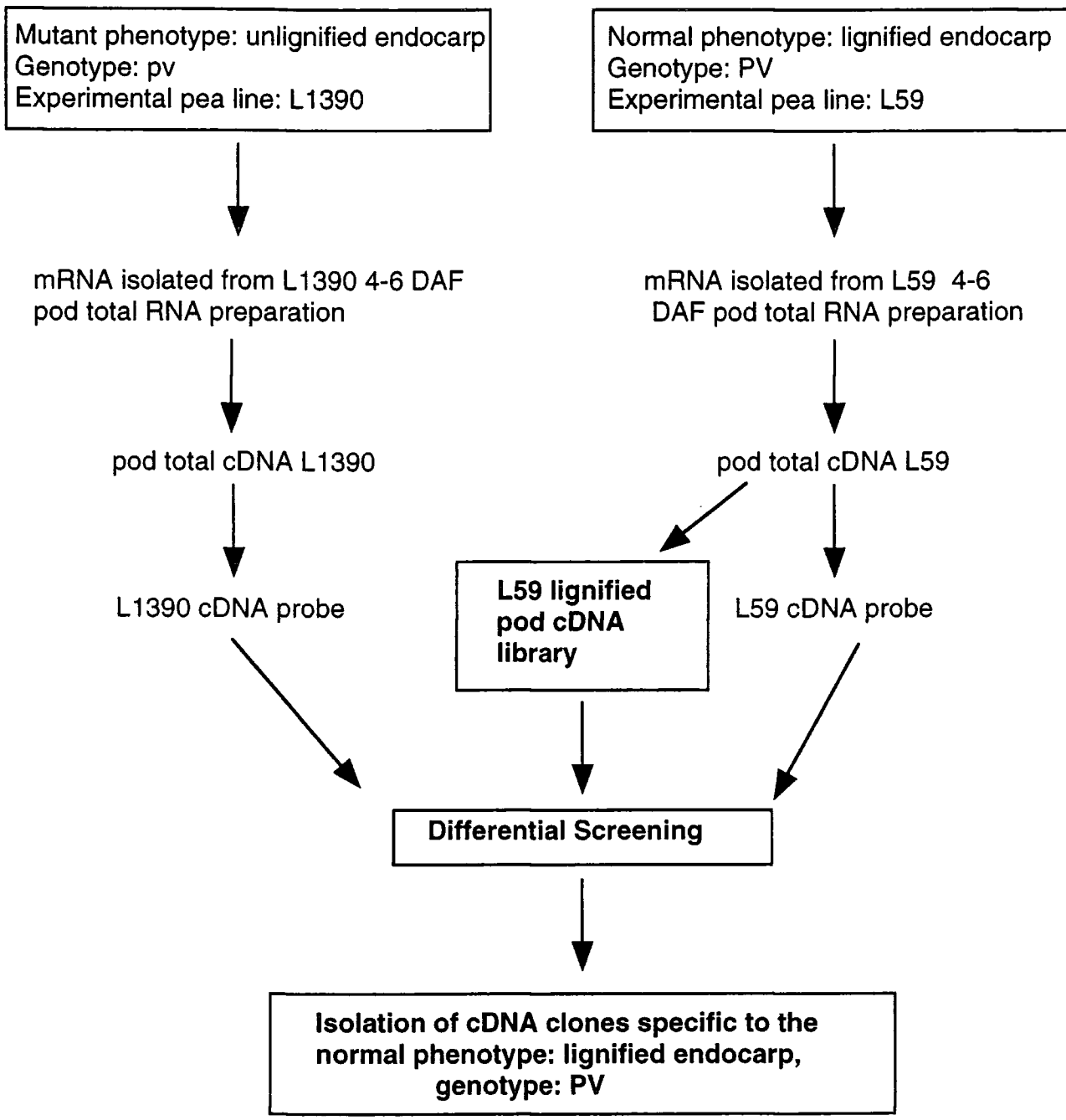


Figure 3.1 Strategy for isolating differentially expressed genes as cDNAs representing the lignified endocarp phenotype.

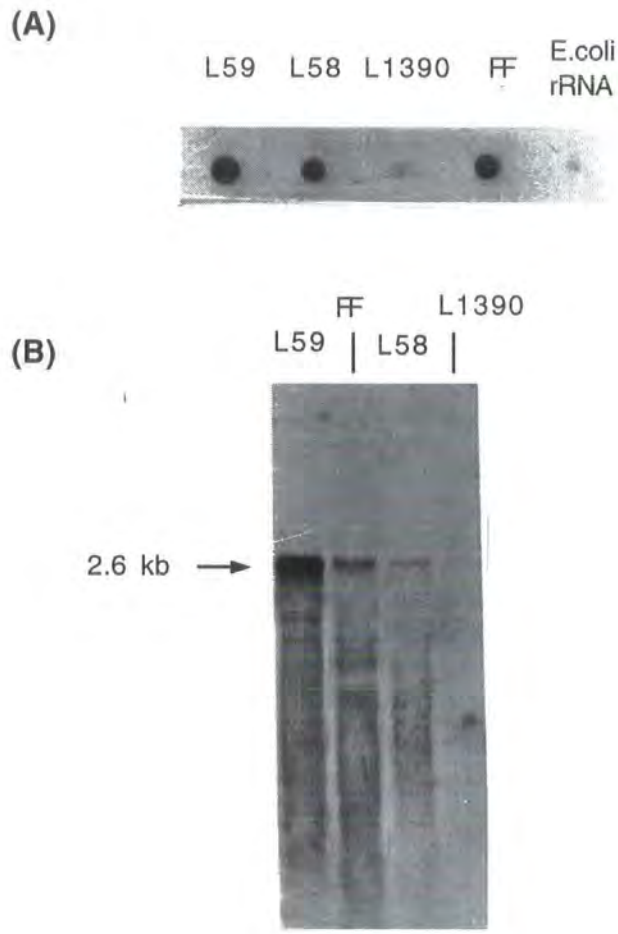


Figure 3. 2 Expression of hsp70 gene corresponding to cDNA pLP19 in developing pods from different pea lines. Total RNA was purified from pods at 4-6 days after flowering, when lignification is taking place. Pea lines L59 and FF show full lignification, L58 shows partial lignification, and L1390 has non-lignified pods.

(A) Dot blot of total RNA (5µg per dot) from pods of pea lines (and *E. coli* negative control) probed with cDNA pLP19.

(B) Separation of RNA (10µg per track) from pods of different pea lines by agarose gel electrophoresis, followed by Northern blotting, probed with cDNA pLP19.

by clone LP19 against Gene Bank data using the FASTA programme package (Lipman and Pearson, 1988), revealed similarities with the C-terminal regions of hsp70 family proteins. The highest homology was with the plant hsp70s. The high degree of homology indicates that LP19 encodes the C-terminal regions of pea hsp70 protein.

3.1.3 Screening of L59 lignified pod cDNA library for a full length LP19 cDNA

The clone pLP19 was subsequently used to rescreen the pea L59 pod cDNA library, and seven further homologous cDNA species (designated pLP19/1, pLP19/2, pLP19/3, pLP19/4, pLP19/6, pLP19/7 and pLP19/9) were isolated. DNA sequencing showed that all these cDNAs, including pLP19, could be the products of a single gene. pLP19/3 and pLP19/7 were found to be identical, as were pLP19/1 and pLP19/2. All the cDNAs showed truncation of the N-terminal sequence of the encoded polypeptide, by comparison with the homologous plant hsp70 cDNA sequences, although pLP19/1 and pLP19/2 were effectively full length (figure 3.7). None of the cDNAs showed evidence of truncation at the 3' end, since all had poly (A) tails of varying lengths. However, the sites of the poly (A) tail addition differed between the different clones. Clones pLP19/1,2 both had the longest 3' untranslated region (UTR), of 285 bp; the original clone, pLP19 had a 3' UTR of 256 bp; pLP19/4, pLP19/6 and pLP19/9 had a 3' UTR of 252 bp and pLP19/3,7 had the shortest 3' UTR, of 226 bp (figure 3.7). The position of poly (A) addition is thus highly variable in these transcripts. The 3' UTR sequence lacks a consensus poly (A) addition signal sequence (AATAAA), the best match was found to be 'AATTTA' occurring 236 bp after the stop codon.

3.1.4 Expression analysis of LP19 in 4-6 DAF pods

Dot blot analysis of total RNA from developing pods (4-6 DAF) of L59, L58, L1390 and FF, hybridised with ^{32}P-labelled pLP19 cDNA, confirmed differential expression of the mRNA corresponding to pLP19 in lignifying tissue (figure 3.2 A). Strong hybridisation was observed with pod RNA from L59, L58 and FF, where partial or

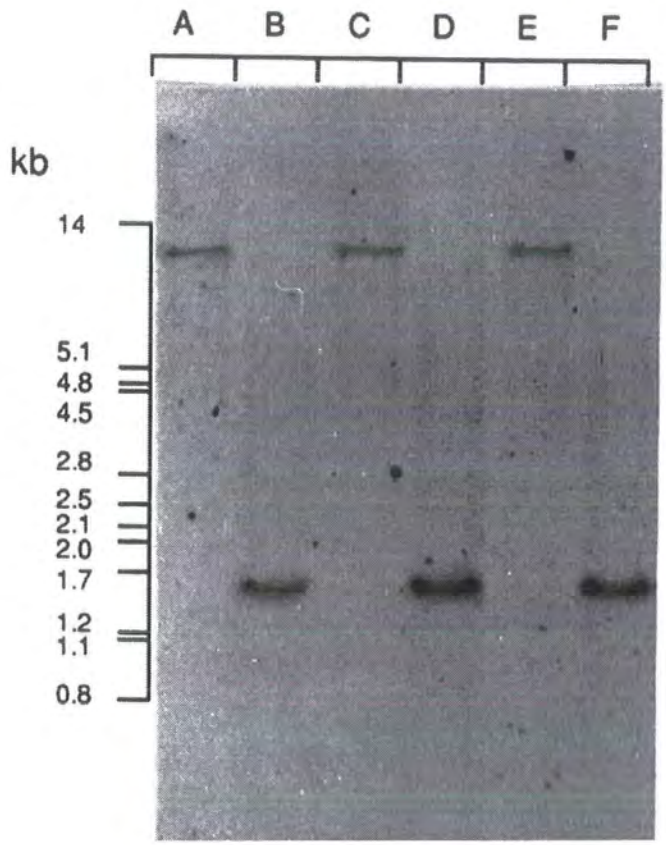


Figure 3. 3. Southern blot hybridisation of total pea leaf DNA from L59, L58 and L1390. 20µg genomic DNA from different pea lines was loaded in each lane as follows: lanes A, B: L59 (lignified pods); lanes C, D: L58 (partially lignified pods); lanes E, F: L1390 (non-lignified pods). DNA in lanes A, C and E was digested with *Bam*HI; DNA in lanes B, D and F was digested with *Hin*dIII. After separation by agarose gel electrophoresis, DNA fragments were blotted, and probed with labelled pLP19.

complete lignification of the endocarp takes place. Hybridisation to total RNA from pods of L1390, where no endocarp lignification occurs, was not above the background hybridisation to *E.coli* ribosomal RNA. This result was confirmed by Northern blot analysis of total pod RNA from the experimental pea lines. Probing with labelled pLP19 cDNA showed hybridisation to an mRNA species of estimated size 2.6 kb in L58, L59 and FF, where pod lignification takes place, but no hybridisation at all was detected in L1390, where pod lignification does not occur (figure 3.2 B). The observed hybridisation signal was significantly lower in L58, than in either L59 or FF; L58 exhibits partial rather than complete pod lignification.

3.1.5 Genomic analysis of LP19 clone

Genomic DNA from pea lines L59, L58 and L1390 was analysed using Southern blotting experiment in order to establish the presence of gene(s) corresponding to the pLP19 cDNA. Genomic DNA was restricted with *Bam*H I or *Hin*d III, separated by agarose gel electrophoresis, blotted, and probed with pLP19 cDNA. After washing to high stringency (0.1 x SSC, 0.1% SDS, 65°C), the pLP19 probe hybridised to a single band of approx. 12 kb in the *Bam*H I digests, and to a single band of approx. 1.6 kb in the Hind III digests, in all three pea lines, at an intensity which suggested that a single gene fragment was being detected (figure 3.3).

3.2 Results and discussion

3.2.1 Screening of pea genomic library and isolation of gene corresponding to pLP19

A genomic library made from purple podded (PP) pea leaf tissues (as described in section 2.2.10) was screened using ^{32}P dCTP labelled pLP19 cDNA in order to isolate the hsp70 gene corresponding to pLP19. A primary screen of $1x10^5$ plaques, yielded 8 positives on the basis of observed hybridisation to ^{32}P-labelled pLP19 cDNA. The putative positive plaques from the primary screen stock plates were subjected to secondary and tertiary rounds of screening by hybridising duplicate lifts of 50-100

Figure 3.4 Southern blot hybridisation of λ phage DNA fragments.

(A) and (B) represent *Bam*H1 cleavage products, electrophoresed on 0.7% agarose gel and transferred to a nitro-cellulose filter, respectively. The filter was hybridised with ^{32}P-labelled pLP19 cDNA, washed to a high stringency of 0.1x SSC at 65°C and exposed to x-ray film for 2 hrs at room temperature. λOPD1 (lane 1), λOPD2 (lane 2), λOPD5 (lane 3), λOPD6 (lane 4), λOPD7 (lane 5) and λOPD8 (lane 6) and size marker (M).

(C) and (D) represent *Eco*R1 cleavage products, electrophoresed on 0.7% agarose gel and transferred to a nitro-cellulose filter, respectively. The filter was hybridised with ^{32}P-labelled pLP19 cDNA, washed to a high stringency of 0.1x SSC at 65°C and exposed to x-ray film for 2 hrs at room temperature. λOPD2 (lane 1), λOPD7(lane 2), λOPD8 (lane 3), and size marker (M).

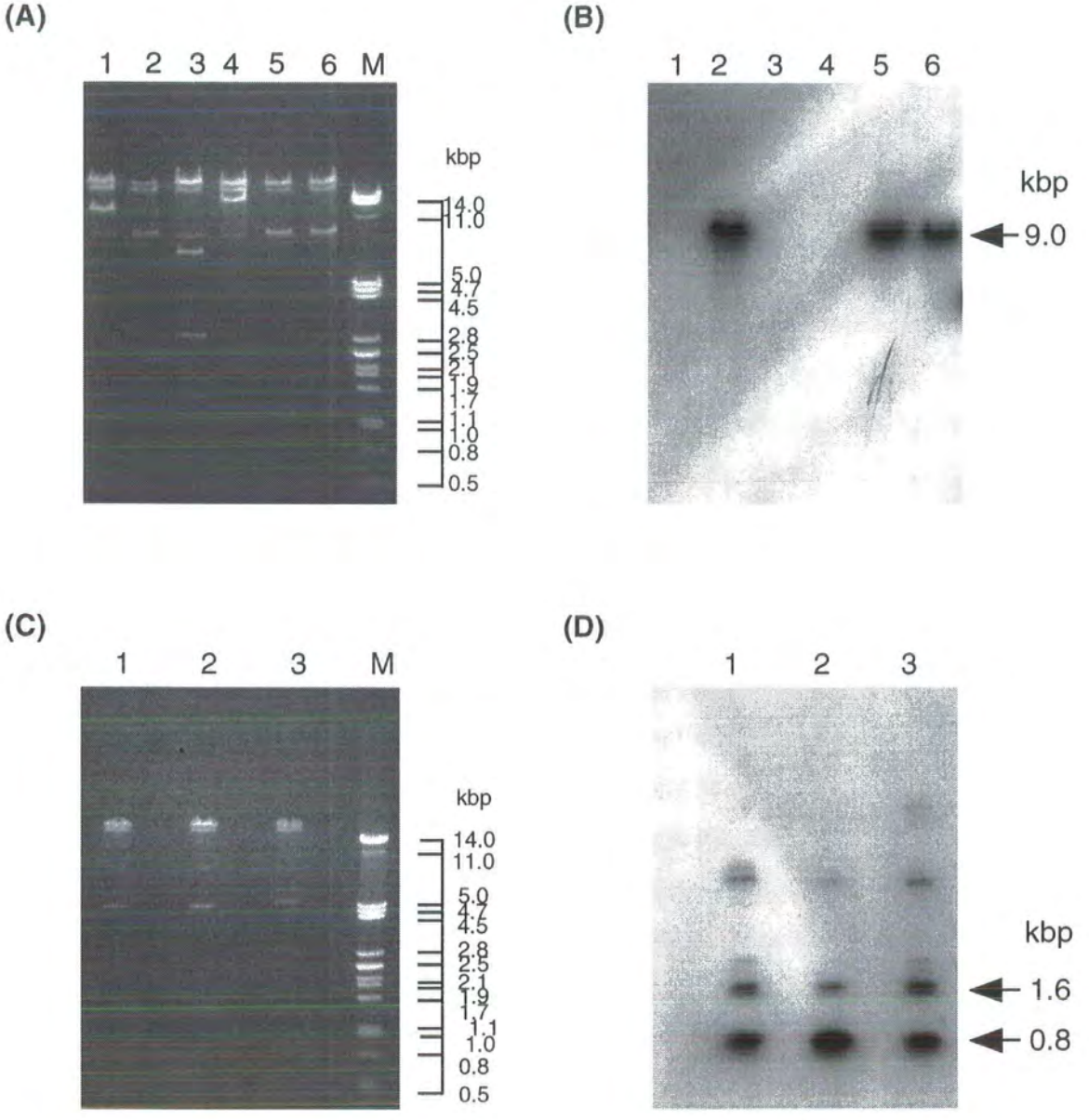
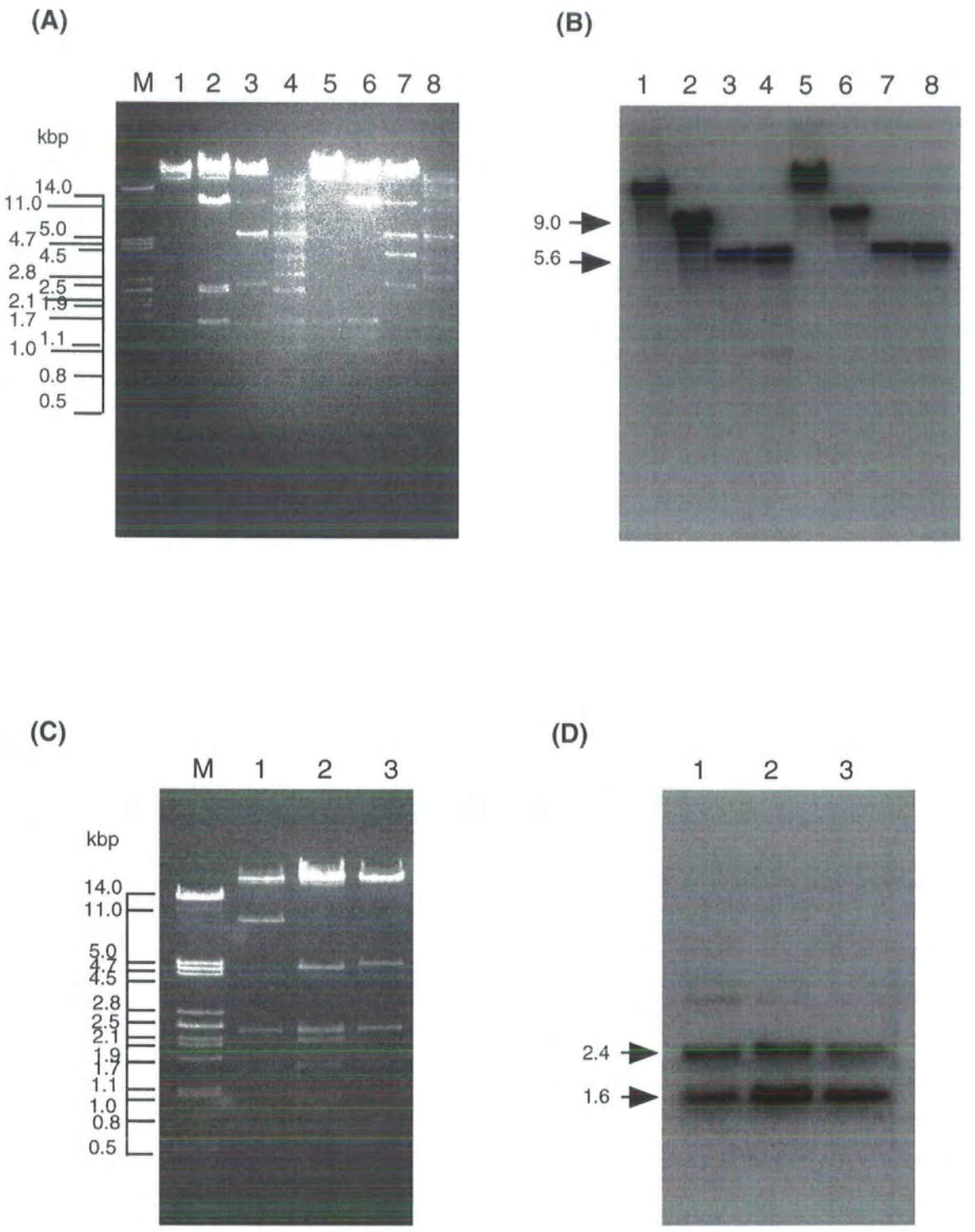

(A)
1 2 3 4 5 6 M
kbp
14.0
11.0
5.0
4.7
4.5
2.8
2.5
2.1
1.9
1.7
1.1
1.0
0.8
0.5
(B)
1 2 3 4 5 6
kbp
9.0
(C)
1 2 3 M
kbp
14.0
11.0
5.0
4.7
4.5
2.8
2.5
2.1
1.9
1.7
1.1
1.0
0.8
0.5
(D)
1 2 3
kbp
1.6
0.8

Figure 3.5 Southern blot hybridisation of genomic clones λOPD2 and λOPD7 phage DNA fragments.

(A) and (B) represent *Asp*718, *Bam*H1, *Sac*1 and *Sac*1/*Bam*H1 cleavage products, electrophoresed on 0.7% agarose gel and transferred to a nitrocellulose filter, respectively. The filter was hybridised with ^{32}P-labelled pLP19 cDNA, washed to a high stringency of 0.1x SSC at 65°C and exposed to x-ray film for 2 hrs at room temperature. Lane M (size marker), lane 1 to 4: λOPD2 restricted with *Asp*718 (lane 1), *Bam*H1 (lane 2), *Sac*1 (lane 3), and *Sac*1/*Bam*H1 (lane 4). Lane 5 to 8: λOPD7 restricted with *Asp*718 (lane 5), *Bam*H1 (lane 6), *Sac*1 (lane 7), and *Sac*1/*Bam*H1 (lane 8).

(C) and (D) represent *Hind*III cleavage products, electrophoresed on 0.7% agarose gel and transferred to a nitrocellulose filter, respectively. The filter was hybridised with ^{32}P-labelled pLP19 cDNA, washed to a high stringency of 0.1x SSC at 65°C and exposed to x-ray film for 2 hrs at room temperature. Lane M (size marker), lane 1 (*Hind*III digested λOPD2), lane 2 (*Hind*III digested λOPD7) and lane 3 (*Hind*III digested λOPD8).


(A)
M 1 2 3 4 5 6 7 8
kbp
14.0
11.0
5.0
4.7
4.5
2.8
2.5
2.1
1.9
1.7
1.1
1.0
0.8
0.5
(B)
1 2 3 4 5 6 7 8
9.0
5.6
(C)
M 1 2 3
kbp
14.0
11.0
5.0
4.7
4.5
2.8
2.5
2.1
1.9
1.7
1.1
1.0
0.8
0.5
(D)
1 2 3
2.4
1.6

plaques on nitro-cellulose filters with ^{32}P-dCTP labelled pLP19 cDNA. Tertiary screening resulted in 6 independent putative positive plaques which were designated as λOPD1, λOPD2, λOPD5, λOPD6, λOPD7 and λOPD8 after phage DNA preparation.

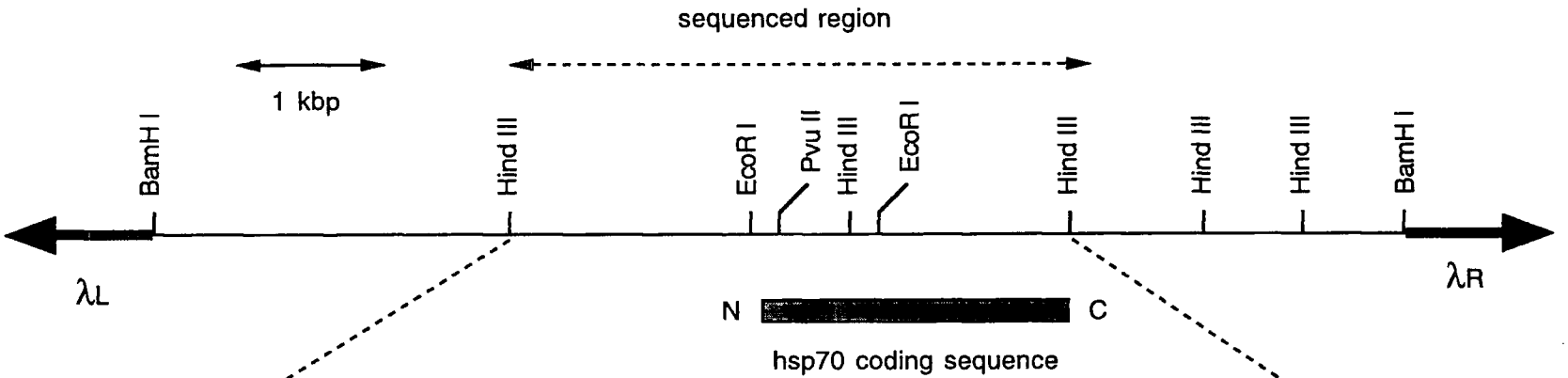
The presence of a gene corresponding to pLP19 in the above genomic clones was investigated by hybridisation of the phage DNA with ^{32}P-labelled pLP19 cDNA probe. A 9.0 kb *Bam*HI fragment of λOPD2, λOPD7 and λOPD8 hybridised strongly with the labelled probe, whereas λOPD1, λOPD5 and λOPD6 failed to show any hybridisation (figure 3.4 A&B). In *Eco*RI digests, two fragments of 1.6 and 0.8 kb size were detected. (figure 3.4 C&D). The hybridisation pattern was similar in all these three clones, indicating that all contained the same gene. The clones λOPD2 and λOPD7 were further analysed by digestion with restriction endonucleases *Asp*718, *Bam*HI, *Sac*I, and *Hind*III. In both clones, similar fragments of 9.0 kb (*Bam*HI), 5.6 kb (*Sac*I and *Sac*I/*Bam*HI) (figure 3.5 A&B), and 2.4 and 1.6 kb fragments (*Hind*III) (figure 3.5 C&D) were detected which hybridised strongly to the probe. A partial restriction map (figure 3.6) suggests that both clones represent the same hsp70 gene. The 1.6 kb *Hind*III hybridisation band of λOPD2 and λOPD7 (figure 3.5 D) corresponds to the 1.6 kb *Hind*III hybridisation band seen on genomic Southern blots (figure 3.3).

3.2.2 Subcloning and Sequencing of genomic clone λOPD7

A 4.0 kb region between two *Hind*III sites as shown in the restriction map (figure 3.6) was digested with various restriction endonucleases in order to generate small or overlapping fragments, which were then subcloned into pUC vectors and sequenced. The sequencing strategy is shown in figure 3.6 B. By using this sequencing strategy, 76% of the nucleotide sequences were determined from both strands of DNA, whereas the remaining 24% were sequenced more than once from the same DNA strand. All sequence ambiguities were removed. This gene was designated hsp70(LP19). The complete nucleotide and predicted amino acid sequence of pea hsp70(LP19) gene, including 5' and 3' nontranslated regions are shown in figure 3.7.

Figure 3.6 Partial restriction map of genomic clone λOPD7 (a), showing sequencing stategy (b), and the restriction enzyme sites for subcloning. The sequenced fragments and the direction of sequencing are indicated by arrows.

a) Partial Restriction Map of Genomic Clone λOPD7



b) Sequencing Map for Genomic Clone λOPD7

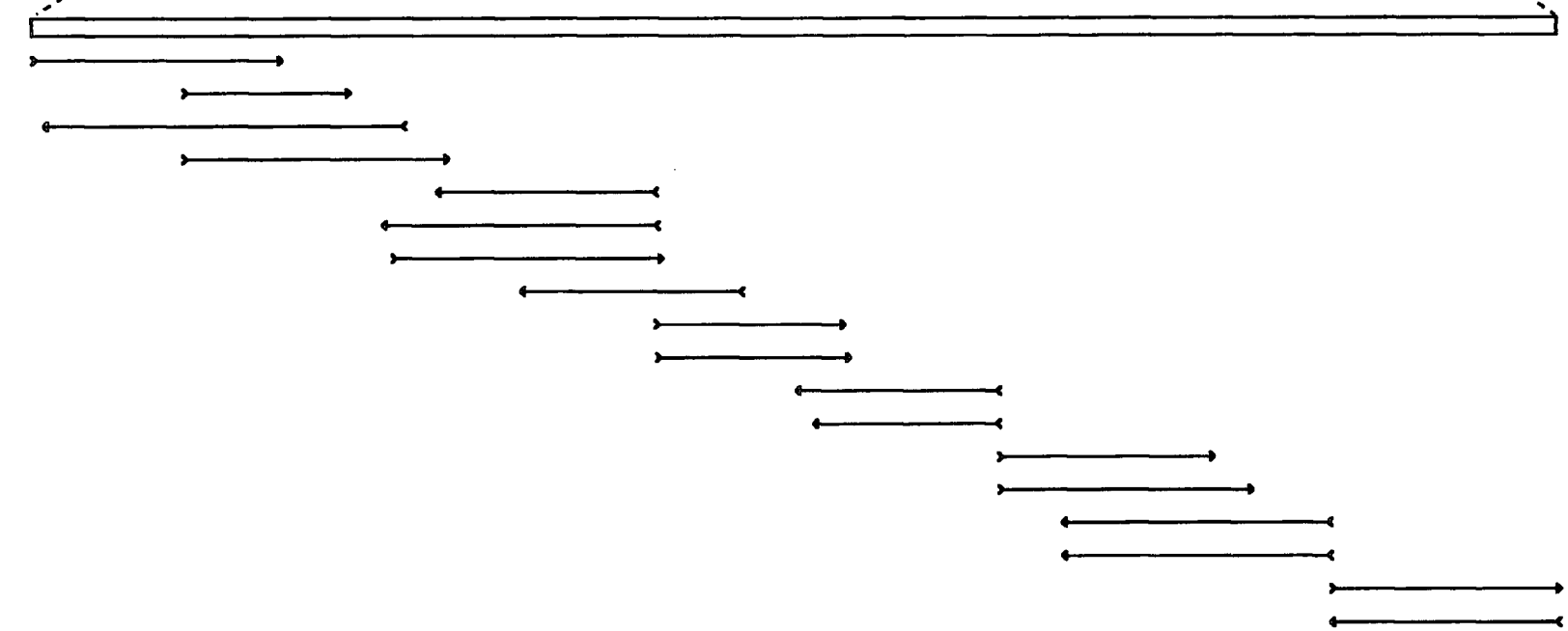
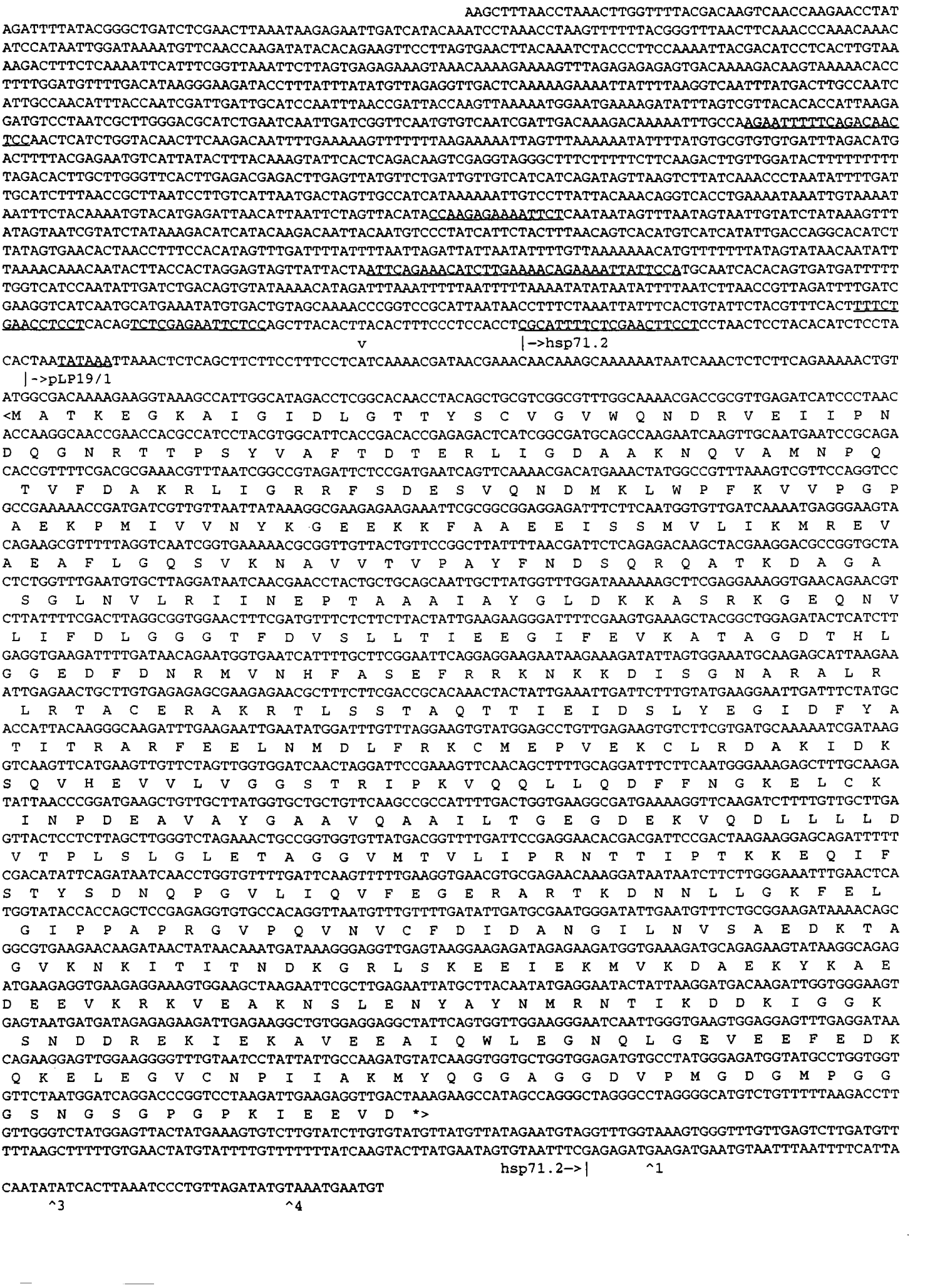


Figure 3.7 Composite sequence for cDNA species pLP19, pLP19/1,2,3,4,6,7,9, and PsHSP71.2, and the pea gene hsp70(LP19). Numbering is from the first base of coding sequence. The TATA box location (underlined), and the putative transcription start (denoted by "v" above the sequence) were identifed by using consensus sequences, and by analogy with similar hsp70 genes. Sequences in the 5' flanking region in agreement with the consensus for heat shock elements are double underlined. The start and finish of cDNA PsHSP71.2 [11] (which was not polyadenylated) are denoted by |->hsp71.2 ... hsp71.2->|. The start of cDNAs pLP19/1,2 is similarly denoted. Polyadenylation addition sites are indicated by "^" below the sequence: ^1 = pLP19/3,7; ^2 = pLP19/4,6,9; ^3 = pLP19; ^4 = pLP19/1,2.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ATCCATAATTGGATAAAATGTTCAACCAAGATATACACAGAAGTTCCTTAGTGAACTTACAAATCTACCCTTCCAAAATTACGACATCCTCACTTGTAA
AAGACTTTCTCAAAATTCATTTCGGTTAAATTCTTAGTGAGAGAAAGTAAACAAAAGAAAAGTTTAGAGAGAGAGTGACAAAAGACAAGTAAAAACACC
TTTTGGATGTTTTGACATAAGGGAAGATACCTTTATTTATATGTTAGAGGTTGACTCAAAAAGAAAATTATTTTAAGGTCAATTTATGACTTGCCAATC
ATTGCCAACATTTACCAATCGATTGATTGCATCCAATTTAACCGATTACCAAGTTAAAAATGGAATGAAAAGATATTTAGTCGTTACACACCATTAAGA
GATGTCCTAATCGCTTGGGACGCATCTGAATCAATTGATCGGTTCAATGTGTCAATCGATTGACAAAGACAAAAATTTGCCAAGAATTTTTCAGACAAC
TCCAACTCATCTGGTACAACTTCAAGACAATTTTGAAAAAGTTTTTTTAAGAAAAATTAGTTTAAAAAATATTTTATGTGCGTGTGTGATTTAGACATG
ACTTTTACGAGAATGTCATTATACTTTACAAAGTATTCACTCAGACAAGTCGAGGTAGGGCTTTCTTTTTCTTCAAGACTTGTTGGATACTTTTTTTTT
TAGACACTTGCTTGGGTTCACTTGAGACGAGACTTGAGTTATGTTCTGATTGTTGTCATCATCAGATAGTTAAGTCTTATCAAACCCTAATATTTTGAT
TGCATCTTTAACCGCTTAATCCTTGTCATTAATGACTAGTTGCCATCATAAAAAATTGTCCTTATTACAAACAGGTCACCTGAAAATAAATTGTAAAAT
AATTTCTACAAAATGTACATGAGATTAACATTAATTCTAGTTACATACCAAGAGAAAATTCTCAATAATAGTTTAATAGTAATTGTATCTATAAAGTTT
ATAGTAATCGTATCTATAAAGACATCATACAAGACAATTACAATGTCCCTATCATTCTACTTTAACAGTCACATGTCATCATATTGACCAGGCACATCT
TATAGTGAACACTAACCTTTCCACATAGTTTGATTTTATTTTAATTAGATTATTAATATTTTGTTAAAAAAACATGTTTTTTTTATAGTATAACAATATT
TAAAACAAACAATACTTACCACTAGGAGTAGTTATTACTAATTCAGAAACATCTTGAAAACAGAAAATTATTCCATGCAATCACACAGTGATGATTTTT
TGGTCATCCAATATTGATCTGACAGTGTATAAAACATAGATTTAAATTTTTAATTTTTAAAATATATAATATTTTAATCTTAACCGTTAGATTTTGATC
GAAGGTCATCAATGCATGAAATATGTGACTGTAGCAAAACCCGGTCCGCATTAATAACCTTTCTAAATTATTTCACTGTATTCTACGTTTCACTTTTCT
GAACCTCCTCACAGTCTCGAGAATTCTCCAGCTTACACTTACACTTTCCCTCCACCTCGCATTTTCTCGAACTTCCTCCTAACTCCTACACATCTCCTA
v
|->hsp71.2
CACTAATATAAAATTAAACTCTCAGCTTCTTCCTTTCCTCATCAAAACGATAACGAAACAACAAAGCAAAAAATAATCAAACTCTCTTCAGAAAAACTGT
|->pLP19/1
ATGGCGACAAAAGAAGGTAAAGCCATTGGCATAGACCTCGGCACAACCTACAGCTGCGTCGGCGTTTGGCAAAACGACCGCGTTGAGATCATCCCTAAC
<M A T K E G K A I G I D L G T T Y S C V G V W Q N D R V E I I P N
ACCAAGGCAACCGAACCACGCCATCCTACGTGGCATTCACCGACACCGAGAGACTCATCGGCGATGCAGCCAAGAATCAAGTTGCAATGAATCCGCAGA
D Q G N R T T P S Y V A F T D T E R L I G D A A K N Q V A M N P Q
CACCGTTTTCGACGCGAAACGTTTAATCGGCCGTAGATTCTCCGATGAATCAGTTCAAAACGACATGAAACTATGGCCGTTTAAAGTCGTTCCAGGTCC
T V F D A K R L I G R R F S D E S V Q N D M K L W P F K V V P G P
GCCGAAAAACCGATGATCGTTGTTAATTATAAAGGCGAAGAGAAGAAATTCGCGGCGGAGGAGATTTCTTCAATGGTGTTGATCAAAATGAGGGAAGTA
A E K P M I V V N Y K G E E K K F A A E E I S S M V L I K M R E V
CAGAAGCGTTTTTAGGTCAATCGGTGAAAAACGCGGTTGTTACTGTTCCGGCTTATTTTAACGATTCTCAGAGACAAGCTACGAAGGACGCCGGTGCTA
A E A F L G Q S V K N A V V T V P A Y F N D S Q R Q A T K D A G A
CTCTGGTTTGAATGTGCTTAGGATAATCAACGAACCTACTGCTGCAGCAATTGCTTATGGTTTGGATAAAAAAGCTTCGAGGAAAGGTGAACAGAACGT
S G L N V L R I I N E P T A A A I A Y G L D K K A S R K G E Q N V
CTTATTTTCGACTTAGGCGGTGGAACTTTCGATGTTTCTCTTCTTACTATTGAAGAAGGGATTTTCGAAGTGAAAGCTACGGCTGGAGATACTCATCTT
L I F D L G G G T F D V S L L T I E E G I F E V K A T A G D T H L
GAGGTGAAGATTTTGATAACAGAATGGTGAATCATTTTGCTTCGGAATTCAGGAGGAAGAATAAGAAAGATATTAGTGGAAATGCAAGAGCATTAAGAA
G G E D F D N R M V N H F A S E F R R K N K K D I S G N A R A L R
ATTGAGAACTGCTTGTGAGAGAGCGAAGAGAACGCTTTCTTCGACCGCACAAACTACTATTGAAATTGATTCTTTGTATGAAGGAATTGATTTCTATGC
L R T A C E R A K R T L S S T A Q T T I E I D S L Y E G I D F Y A
ACCATTACAAGGGCAAGATTTGAAGAATTGAATATGGATTTGTTTAGGAAGTGTATGGAGCCTGTTGAGAAGTGTCTTCGTGATGCAAAAATCGATAAG
T I T R A R F E E L N M D L F R K C M E P V E K C L R D A K I D K
GTCAAGTTCATGAAGTTGTTCTAGTTGGTGGATCAACTAGGATTCCGAAAGTTCAACAGCTTTTGCAGGATTTCTTCAATGGGAAAGAGCTTTGCAAGA
S Q V H E V V L V G G S T R I P K V Q Q L L Q D F F N G K E L C K
TATTAACCCGGATGAAGCTGTTGCTTATGGTGCTGCTGTTCAAGCCGCCATTTTGACTGGTGAAGGCGATGAAAAGGTTCAAGATCTTTTGTTGCTTGA
I N P D E A V A Y G A A V Q A A I L T G E G D E K V Q D L L L L D
GTTACTCCTCTTAGCTTGGGTCTAGAAACTGCCGGTGGTGTTATGACGGTTTTGATTCCGAGGAACACGACGATTCCGACTAAGAAGGAGCAGATTTTT
V T P L S L G L E T A G G V M T V L I P R N T T I P T K K E Q I F
CGACATATTCAGATAATCAACCTGGTGTTTTGATTCAAGTTTTTGAAGGTGAACGTGCGAGAACAAAGGATAATAATCTTCTTGGGAAATTTGAACTCA
S T Y S D N Q P G V L I Q V F E G E R A R T K D N N L L G K F E L
TGGTATACCACCAGCTCCGAGAGGTGTGCCACAGGTTAATGTTTGTTTTGATATTGATGCGAATGGGATATTGAATGTTTCTGCGGAAGATAAAACAGC
G I P P A P R G V P Q V N V C F D I D A N G I L N V S A E D K T A
GGCGTGAAGAACAAGATAACTATAACAAATGATAAAGGGAGGTTGAGTAAGGAAGAGATAGAGAAGATGGTGAAAGATGCAGAGAAGTATAAGGCAGAG
G V K N K I T I T N D K G R L S K E E I E K M V K D A E K Y K A E
ATGAAGAGGTGAAGAGGAAAGTGGAAGCTAAGAATTCGCTTGAGAATTATGCTTACAATATGAGGAATACTATTAAGGATGACAAGATTGGTGGGAAGT
D E E V K R K V E A K N S L E N Y A Y N M R N T I K D D K I G G K
GAGTAATGATGATAGAGAGAAGATTGAGAAGGCTGTGGAGGAGGCTATTCAGTGGTTGGAAGGGAATCAATTGGGTGAAGTGGAGGAGTTTGAGGATAA
S N D D R E K I E K A V E E A I Q W L E G N Q L G E V E E F E D K
CAGAAGGAGTTGGAAGGGGTTTGTAATCCTATTATTGCCAAGATGTATCAAGGTGGTGCTGGTGGAGATGTGCCTATGGGAGATGGTATGCCTGGTGGT
Q K E L E G V C N P I I A K M Y Q G G A G G D V P M G D G M P G G
GTTCTAATGGATCAGGACCCGGTCCTAAGATTGAAGAGGTTGACTAAAGAAGCCATAGCCAGGGCTAGGGCCTAGGGGCATGTCTGTTTTTAAGACCTT
G S N G S G P G P K I E E V D *>
GTTGGGTCTATGGAGTTACTATGAAAGTGTCTTGTATCTTGTGTATGTTATGTTATAGAATGTAGGTTTGGTAAAGTGGGTTTGTTGAGTCTTGATGTT
TTTAAGCTTTTTGTGAACTATGTATTTTGTTTTTTTATCAAGTACTTATGAATAGTGTAATTTCGAGAGATGAAGATGAATGTAATTTAATTTTCATTA
hsp71.2->|
^1
CAATATATCACTTAAATCCCTGTTAGATATGTAAATGAATGT
^3
^4

Table 3.1 Amino acid sequence homology of pea hsp70(LP19) to other HSP70s.

Sr. No.	Best Homologous HSP70s	% Homology	Accession No.
Cytoplasmic HSP70s			
1.	Soybean	93.5	P26413
2.	Tomato	87.7	P27322
3.	Petunia	87.2	P09189
4.	Spinach	87.1	L26243
5.	*Arabidopsis*	85.9	S46302
6.	Pea PsHSP71	85.7	S44168
7.	Rice	85.6	X67711
8.	Maize	84.0	P11143
9.	Carrot	83.1	P26791
10.	Chlamydonas	81.2	P25840
11.	Yeast SSA1	75.2	P10591
12.	Human	74.5	P48791
ER HSP70s			
13.	Tobacco	65.2	Q03684
14.	Human	64.2	P11028
Plastid and Mitochondrial HSP70s			
15.	Pea plastid	48.0	Q02028
16.	Pea mito.	49.8	P48791

Amino acid sequence identity was calculated with the CLUSTAL W (1.7) multiple sequence alignment program, using the default paramenters. All hsp70 sequences compared with pea hsp70(LP19) were retrieved from Gene Bank database following the accession numbers shown in the table.

3.2.3 Sequence analysis of a hsp70(LP19) Gene

The sequenced region containing the hsp70(LP19) gene is 4091 bp long, comprising 1849 nt of 5' untranslated DNA, an open reading frame of 1947 nucleotides and 295 nt of 3' untranslated region. The open reading frame encodes a polypeptide of 648 amino acid residues with a calculated mass of 74.5 kDa. Comparison of nucleotide sequences of the cDNA and genomic clones shows that there is no intron present in the gene. The coding and 3' flanking regions of this gene were identical to the pLP19 cDNAs, establishing that hsp70(LP19) is the gene from which the cDNAs are derived.

As shown in table 3.1, the polypeptide predicted by pea hsp70(LP19) has high amino acid sequence homology with other hsp70s from diverse sources. The hsp70(LP19) protein is more homologous to cytoplasmic hsp70s of soybean (93.5%), petunia (87.2%), maize (84.5%), tomato (87.7%), carrot (83.1%), rice (85.6%), pea PsHSP71 (85.7%), spinach (87.1%), *Chlamydomonas* (81.2%), yeast SSA1 (75.2%), human (74.5%), followed by ER localised hsp70 from tobacco (65.2%) and human (64.2%) but is less homologous to pea plastid (48%) and mitochondria (49.8%) localised hsp70s. The amino acid sequence of hsp70(LP19) is strongly conserved, when compared to other hsp70s, throughout the ATP binding domain and peptide binding domain, but diverges in the carboxy-terminal regulatory domain. However, the carboxy terminal regulatory domain of hsp70(LP19) has the eight amino acid sequence "GPKIEEVD", which is similar to the consensus for other hsp70s; the last four amino acids form a motif "EEVD", which is conserved throughout all plant and animal hsp70s. A hydropathy plot analysed by Kyte-Doolittle hydropathy program (Kyte and Doolittle, 1982), indicated a high frequency of hydrophilic amino acids. The amino acid sequences of the C-terminal region, especially between 490 and 610, contains by far the greatest concentration of hydrophilic residues within the entire protein as shown in figure 3.8. The amino acid sequence predicted by pea hsp70(LP19) does not have an amino terminal leader sequence characteristic for import into ER, mitochondria or chloroplasts.

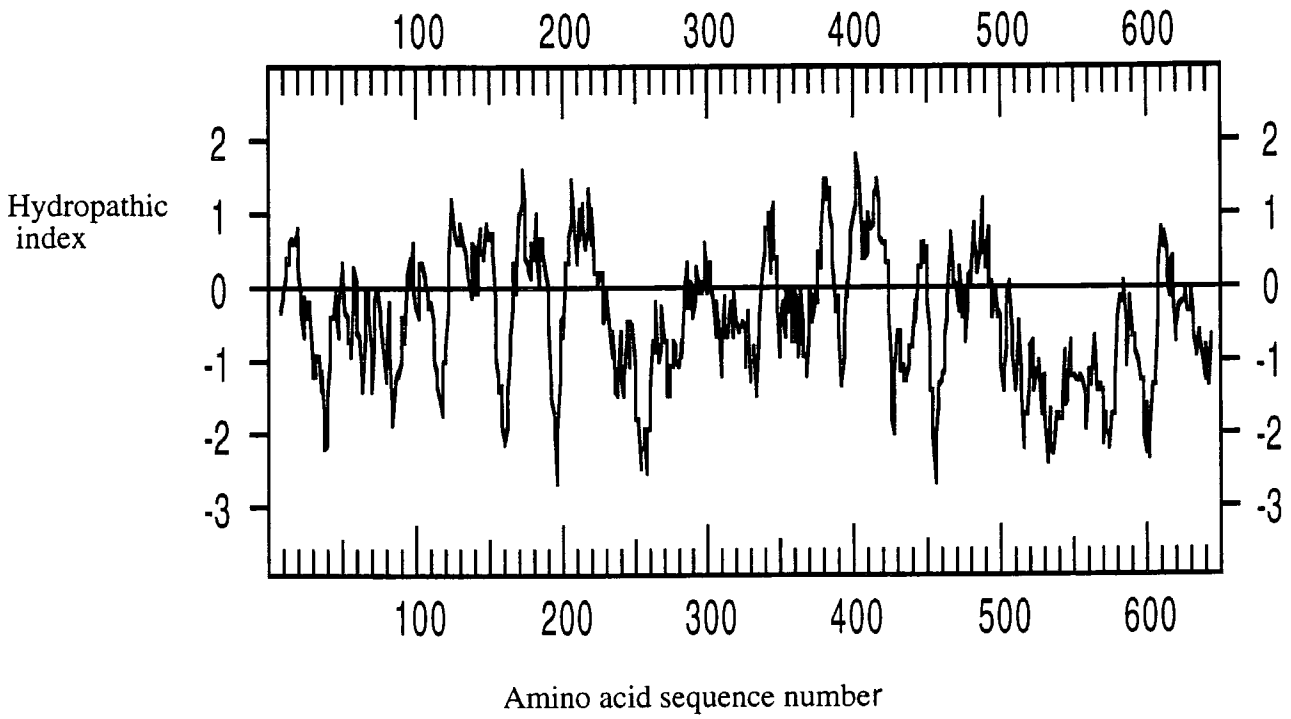


Figure 3.8 A Kyte and Doolittle (1982) hydropathy plot of the hsp70(LP19) amino acid sequence determined using a DNA strider programme package (Marck, 1988).

3.2.4 Sequence analysis of 5'-flanking region of hsp70(LP19)

1849 bp of 5' flanking sequence upstream of the translation start codon of the gene hsp70(LP19) was sequenced. The putative TATA box is located at position –28 upstream of the predicted transcription start site (figure 3.7), a common distance in eukaryotic genes. Analysis of the 5'-flanking end of this genomic clone revealed the presence of several perfect and imperfect matches to consensus heat shock promoter elements (HSEs), represented by 5 bp "nGAAn" inverted repeats, either head to head or tail to tail in orientation (Amin et al., 1988), present upstream of the presumptive TATA-box. The heat shock regulatory elements (HSEs) are the binding sites for heat shock transcription factors (HSFs). The original heat shock element module [CTnGAAnnTTCnAG] was defined by Pelham (1982) as a sequence required for the heat inducibility of the *Drosophila* hsp70 gene. Further analysis by Amin et al.(1988) led to the more precise definition of an HSE as a repeating array of the 5 bp 5'-nGAAn-3', where each repeat is inverted relative to the immediately adjacent repeat. HSEs can start with either a 'nGAAn' repeat or with its complement, 'nTTCn'.

The first consensus heat shock element, HSE I, is 30 bases upstream of the TATA-box. The other elements HSE II, HSE III, HSE IV, HSE V and HSE VI are positioned at -73 to –92, –98 to –112, –348 to –367, –640 to –654 and –1099 to –1123, relative to the TATA box respectively, as shown in figure 3.7. All the HSEs have three to six 5 bp units except HSE V which has only two 5 bp repeats. The variable number of perfect and imperfect 'nGAAn' inverted repeats has also been described in other heat shock gene promoters from different plants and animals such as soybean (Roberts and Key, 1991), *Arabidopsis* (Wu et al., 1988), maize (Rochester et al., 1986), petunia (Winter et al., 1988), *Drosophila* (Amin et al., 1988), and yeast (Slater and Craig, 1989). Figure 3.9 shows the different regions of hsp70(LP19) containing all perfect and imperfect HSEs aligned with the HSE containing region of the other heat shock genes from plants and animals. HSE VI of hsp70(LP19) appeared to consist of interrupted arrangements of GAA/TTC blocks by the insertion or deletion of extra nucleotides between repeating units. Between GAA/GAC and TTC blocks there is an insertion of one extra nucleotide and between the second TTC and GAC blocks there is a deletion of one nucleotide base. Insertions of extra nucleotides are also present in HSEs from other hsp70s such as human hsp70-A. In some heat shock regulatory

TCCCTCCACCTCGCATTTTCTCGAACTTCCTCCTAAC -24 hsp70(LP19)

----------TCTCGAGAATTCTCCAGCTTA -72

TATTCTACGTTTCACTTTTCTGGAACCTCCTCACAG- -93

AGGAGTAGTTATTACTAATTCAGAAACATCTTGAAAA -347

ATTTTCTAGTTACATACCAAGAGAAAATTCTCAATAA -639

AAATTTGCCAAGAATTTTTCAGACAACTTCCAACTC -1098

TTCCTAATCTCGTATATTCTCGAACTCACTCTAATT -45 Soy HSP70

------TGATCAAACACTCGAGAACTCTCTAGCCAG -94

TTCGATTTCTCTCTTATTCTGTTTTCTTTCTGGAACA -124

TTTTAGTATTATAAATTTTATGAAAATTTAATTAAA -275

CCAAACATCAACACAAGTGATTGAAAATTGAAAACAT -596

ACCAATTTTTGACCGTCCGATGGAAACTCTAGCCTCA -150 Arabi.HSP70-1

TCACAACCATCGAACATTCTCGAAACATTTTAACAA -227

CAACGGTTATAAATAATTACAGAACATTCTGGAAAC -125 Arabi.HSP70-2

GGCCAAGGTCGCCCCGTGCCCGAATCTTCTGGACGC -44 Maize HSP70

CCAACACCCTCCACTCCTCCAGAGCCTTCCAGAACC -91

CTACGGCTATAAACTCCACTAGAACGTTCATGGAAA -18 Petunia HSP70

AGCGCGCCTCGAATGTTCGCGAAAAGAGCGCCGGAG -34 Droso. HSP70

TACTGCTCTCGTTGGTTCGAGAGAGCGCGCCTCGAA -57

TTGGCAGAAAGAAAACTCGAGAAATTTCTCTGGCCG -175

AAAATAAAGCGAATATTCTAGAATCCCAAAACAAAC -235

CCTTTTTCTGTCACTTTCCGGACTCTTCTAGAAAA -227 Droso.HSP26

GTTCTTTTGCGCTCTTTCTAGAAACTTCGGCTCTC -515

AAATTATTCTTCTTTTTCCAGAACGTTCCATCGGC -182 Yeast YG100

TTGCAAACACGATTTTTCTGGAACGTACACGATTA -45 Soy. HSP17

GAGAAGTCCTGAAGTTTATCGAATCATCTAAAACT -104

GACGGGAGGCCAAACCCCTGGAATATTCCCGACCT -89 Human hsp70A

GTGAATCCCAGAAGACTCTGGAGAGTTCTGAGCAG -170

Figure 3.9 Nucleotide sequence containing the heat shock consensus elements from different HSPs genes. GAA/TTC blocks (or matching nucleotides) are in boldface. The position of the last nucleotide is indicated to the right of each sequence. The nucleotide numbering systems used are from the original publications except hsp70(LP19) where the numbering started from 'TATA' box. The sequences are from genes encoding soybean HSP70 (Roberts and Key, 1991); soybean HSP17 (Nagao et al., 1985); *Arabidopsis* HSP70-1 and HSP70-2 (Wu et al., 1988); maize HSP70 (Rochester et al., 1986); petunia HSP70 (Winter et al., 1988); *Drosophila* HSP70 (Amin et al., 1988); *Drosophila* HSP26 (Cohen and meselson, 1985); and yeast SSA1 hsp70 (Slater and Craig, 1989).

elements, substitution has occurred in some of the GAA/TTC blocks. The substituted blocks GAG, GAC/TCC, TCT or CTC are frequently found in many hsps, as shown in fig 3.9.

In addition to HSEs, there are four A–T rich regions, ranging from 20 to 50 A–T bases, positioned at –260 to –300, –468 to –507, –734 to –757 and –1063 to –1111, from the TATA–box, and two sites having simple GAGA repeats at positions, –1430 to –1439 and –1458 to –1466.

3.2.5 Expression analysis of hsp70(LP19) gene in pea and chickpea tissues under stress conditions

Heat Shock

Total RNA preparations from heat shocked leaves, roots and stem tissues were analysed by Northern blot, and probed with α^{32}P dCTP labelled cDNA clone pLP19. In each case, hybridisation to a single band of 2.6 kb was observed, with no detectable hybridisation to RNA from control tissues. As a proportion of total RNA, the level of pLP19 mRNA was greatest in root tissues, with lower accumulation in stem and very low accumulation in leaves (figure 3.10 A). Similar results were observed when the pLP19 probe was used to detect the presence of the corresponding mRNA species in heat shocked and control chickpea plants (fig 3.10 B); again mRNA levels were highest in root tissue, lower in stem and least in leaves.

Salt stress and Wounding

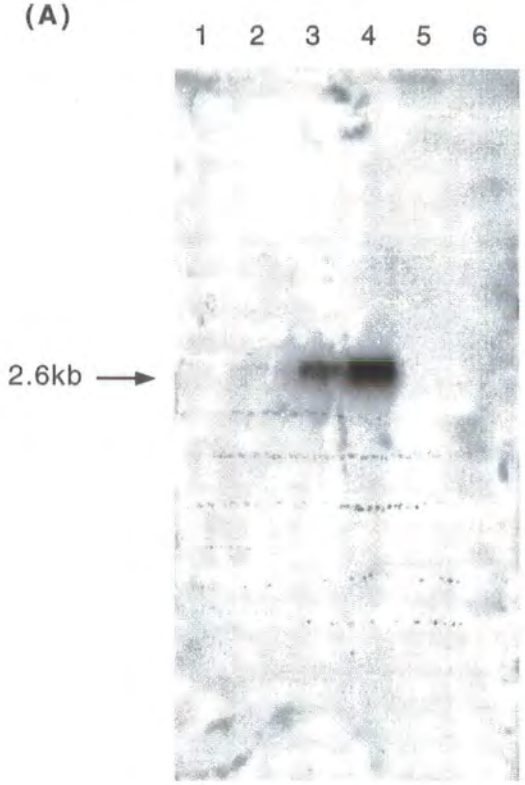
In order to study the expression of the hsp70(LP19) gene under stresses other than heat shock, pea and chickpea plants were subjected to salt stress and mechanical wounding. Total RNA extracted from leaf, stem and root of pea and chickpea plants under salt stress, or after wounding, was hybridised with α^{32}P dCTP labelled pLP19 cDNA. No detectable expression of sequences hybridising to pLP19 was observed after either salt stress (figure 3.10 A&B) or wounding (figure 3.11 A&B)

Fig. 3.10. Expression of hsp70 genes corresponding to cDNA pLP19 in heat shocked tissues of pea and chickpea. Total RNA was purified from tissues as designated, separated by agarose gel electrophoresis, subjected to Northern blotting, and probed with cDNA pLP19.

(A) Expression in pea (line FF). Lanes 1,3,5 contain RNA isolated from leaf tissue; lanes 2,4,6 contain RNA isolated from root tissue. RNA in lanes 1 and 2 was isolated from control plants (normal growing conditions), in lanes 3 and 4 from heat-shocked plants, and in lanes 5 and 6 from salt-stressed plants.

(B) Expression in chickpea. Lanes 1,3,5 contain RNA isolated from leaf tissue, lanes 2,4,6 contain RNA isolated from root tissue, and lanes 7,8,9 contain RNA isolated from stem tissues. RNA in lanes 1,2 and 7 was isolated from control plants (normal growing conditions), in lanes 3,4 and 8 from heat-shocked plants, and in lanes 5,6 and 9 from salt-stressed plants.

(A)



(B)

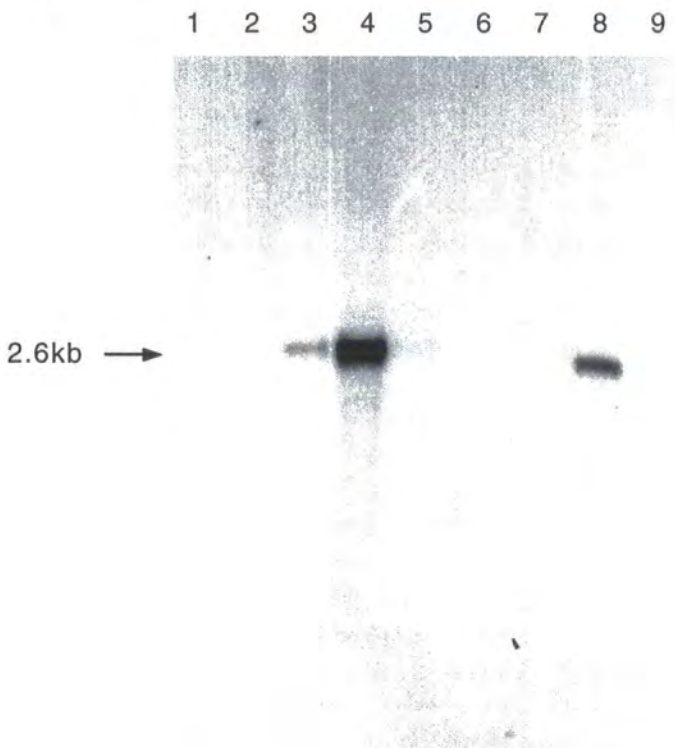
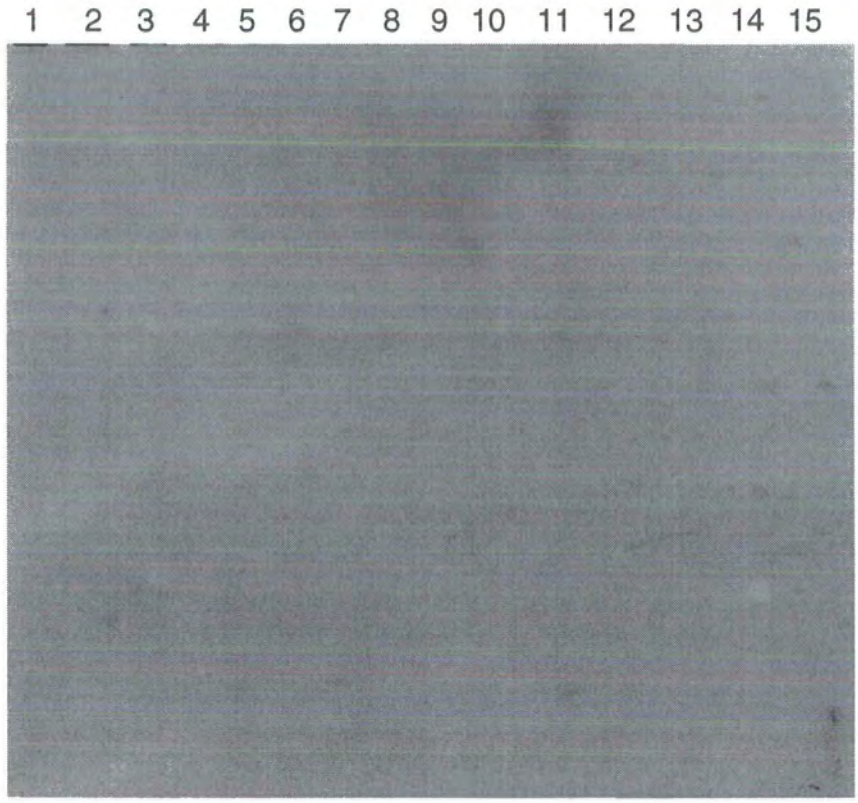


Figure 3.11 Expression of hsp70(LP19) genes in pea and chickpea tissues in response to wounding. Total RNA was purified from tissues as designated, separated by agarose gel electrophoresis, subjected to Northern blotting, and probed with cDNA pLP19.

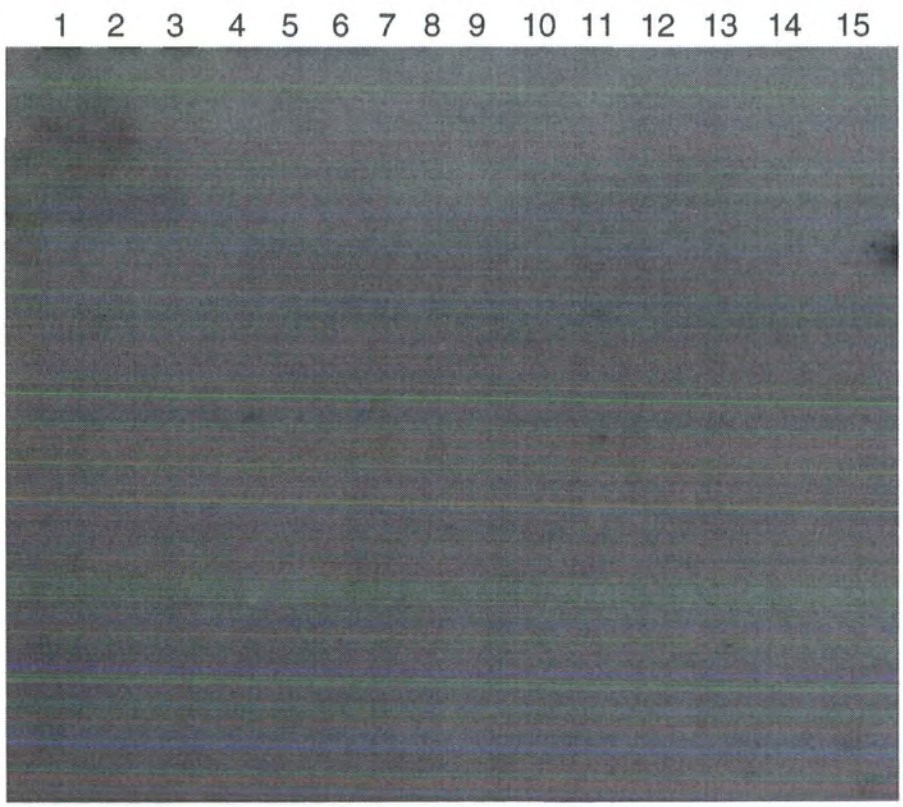
(A) Northern blot of pea total RNA. Lanes 1, 2, 3, 4, 5 contain RNA isolated from leaf tissue; lanes 6, 7, 8, 9, 10 contain RNA isolated from stem tissue and lane 11, 12, 13. 14, 15 contain RNA isolated from root tissue. Lanes 1 (Control leaf), lane 2, 3, 4, 5 (RNA from leaves of 6,12, 24, 48 hrs after wounding, respectively). Lane 6 (stem control), lane 7, 8, 9, 10 (RNA from stem tissues of 6, 12, 24, 48 hrs after wounding, respectively.). Lane 11 (root control) and lane 12, 13, 14, 15 (RNA from root tissues of 6,12,24,48 hrs after wounding, respectively).

(B) Northern blot of chickpea total RNA. The pattern of RNA samples loading is in the same order as mentioned in pea blot.

(A)



(B)



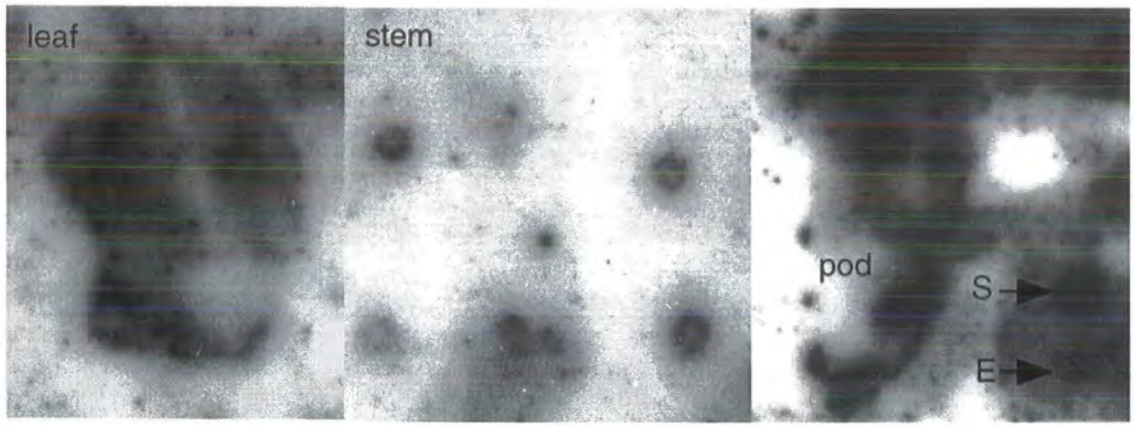
3.2.6 Localisation of hsp70(LP19) gene expression by tissue prints in heat shocked pea stem, leaves, young pods and embryos

Tissue printing is a crude way to show the localisation of gene expression in different tissues, instead of using *in situ* hybridisation which is a tedious and time consuming process. Tissue blots were prepared from different tissues of heat-shocked pea plants, such as leaves, stem, pods and seeds, to study the localisation of hsp70(LP19) gene expression. The plants were 10-11 weeks old with pods having mid-mature seeds. The pea variety used (Feltham First) has lignified pods. The tissue printed nitrocellulose filters were probed with ^{32}P-labelled pLP19 cDNA. A considerable amount of hybridisation was observed in all heat-shocked tissues, while no hybridisation was detected in tissues from control plants (figure 3.12 A&B). Hybridisation was detected throughout stem and leaf tissues whereas in pods strong hybridisation was present in the wall on both ends of the pods and in the placental region along the ventral suture. In some seeds a hybridisation signal could be detected in the cotyledons as well as the in the embryonic axis, whereas, in others only the embryonic axis showed the hybridisation. As previously mentioned (section 3.1) LP19 transcripts are differentially expressed in lignified pod tissues in pea lines which have a differentiated lignified endocarp and thus the localisation of the hybridisation signal in the pod wall showed that the tissue printing is giving valid results.

3.3 Discussion

In plants, the effect of thermal stress (hs) on gene expression has been studied in detail. The complexity and abundance of hsps in plants, in response to heat stress, have stimulated the development of several studies aimed at understanding their biological role, which is probably of a wider physiological and environmental significance than a simple response to a temperature increase (Vierling and Sun, 1987). Recent results have shown that heat shock proteins play multiple roles in an organism, not only under stress conditions, but also in normal growth and development. These roles can be catered for by the presence of multiple genes encoding hsps with different functions, comprising both different hsp gene families (hsp70, hsp90, etc.), and diversity within

(A)



(B)

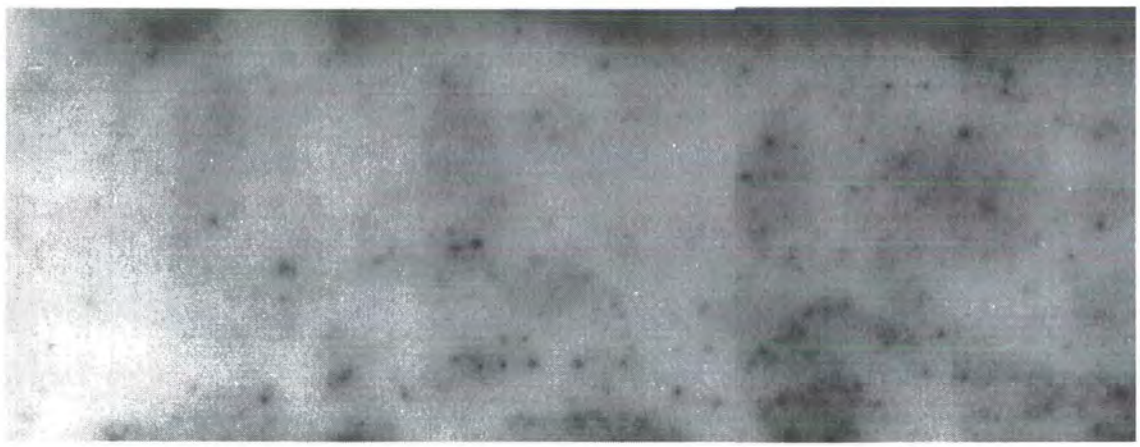


Figure 3.12 Localisation of hsp70(LP19) genes in heat shocked (**A**) and non heat shocked control (**B**) tissues of pea by tissue printing. The tissue printed nitrocellulose filters were probed with ^{32}P-labelled pLP19 cDNA. The hybridisation was performed overnight at 40°C in 50 ml of a solution containing: 5x SSC, 2x Denhardt's reagent, 50% formamide and 100µg/ ml herring sperm DNA. The filters were washed to a high stringency of 0.1x SSC, 0.1% SDS at 50°C for 20 minutes. S- seed, E- embryo.

gene families. The hsp70 gene family in plants has been shown to contain genes encoding proteins present in different cellular compartments; even within the cytoplasmic hsp70s, differential expression patterns of different hsp70 genes has led to suggestions that these proteins play subtly different roles in plant development. The hsp70 gene family also includes genes encoding so called 'heat shock cognate (hsc)' proteins which are expressed during normal development.

The primary sequences of stress-induced hsp70s and constitutively expressed hsc70s are highly conserved across phylogenetic boundaries (Boorstein et al., 1994), and also in their overall tertiary structure (Flaherty et al., 1991). There might be subtle differences in function between hsp70 and hsc70 proteins. A recent paper (DeRocher and Vierling, 1995) described three cDNAs encoding cytoplasmic hsp70 homologues in pea, which are induced to different extents in leaves by heat shock, and are expressed in zygotic, or zygotic and maternal tissues in developing seeds. The nucleotide and the predicted amino acid sequence of one of these cDNAs, designated PsHSP71.2, is contained within the pea hsp70(LP19) gene sequence; the 5' and 3' termini of this cDNA are marked on the gene sequence in figure 3.7. In agreement with results presented here, expression of PsHSP71.2 was only detected in leaves in response to heat shock, and was not present under normal growth conditions. Roots and stems were not examined in the earlier paper. PsHSP71.2 was also expressed in the cotyledons and axes of developing pea seeds, but only during the later stages of development (mid-maturation onwards); it was not present in seed coats. Differential expression of mRNA species corresponding to the three cDNAs isolated by DeRocher and Vierling (1995) was considered to be "consistent with the hypothesis that there are functional distinctions between cytoplasmic hsp70s".

The amino acid sequence comparison of cytoplasmic hsp70(LP19) with hsp70 from other sources, indicated that plant cytoplasmic hsp70 proteins are more closely related to mammalian or yeast counterparts than they are to the organellar forms from the same plant (table 3.1). The lower homology of cytoplasmic hsp70 amino acid sequences with organellar hsp70s indicates their evolutionary divergence. The striking homology among a wide range of plant species (table 3.1) illustrates that plant cytoplasmic hsp70s are strongly conserved. Comparison of amino acid sequences between hsp70(LP19)

and other plant hsp70s, and the absence of a leader sequence in the pea hsp70(LP19) gene, further supports the assertion that pea hsp70(LP19) is a cytoplasmic protein.

The sequence comparison with other plant hsp70s has also revealed that the C-terminal domain is more divergent than the N-terminal conserved domain. The less conserved C-terminal domain is found among various hsp70 proteins. These findings support the contention of Bienz (1984) that there is much less selection pressure on the function of the C-terminal domain or the C-terminal domain is species specific. There is a high proportion of glycine and proline in the last 40 amino acid residues of the C-terminal domain.. The presence of the conserved C-terminal tetrapeptide motif 'EEVD' is expected, since deletion or mutation of this motif in hsp70s affects the ATPase activity and the ability to interact with substrate. C-terminal conserved tetrapeptide motif has been found in all cytoplasmic 70 kDa stress proteins from all sources investigated so far. This motif has been shown to interfere with the ability of human hsp70 to interact with HDJ-1 (human DnaJ-like protein) and in the refolding of denatured firefly luciferase (Freeman et al, 1995). However, an unusual feature of the hsp70(LP19) sequence is that this gene lacks an intron, whereas other plant hsp70s (with the exception of soybean ; Roberts and Key, 1991) have one or more introns. The soybean gene is the most similar to pea hsp70(LP19) in encoded amino acid sequence, and probably represents the soybean homologue of hsp70(LP19). Despite this, no significant homologies were observed between the two genes in non-coding sequence, as compared by dot-plot and aligning software, although both genes contain multiple HSEs.

The presence of introns in hsp genes has been related to the role of their encoded protein products. Heat shock cognate (hsc) genes were defined by Lindquist (1986) as genes that express at normal growth temperature and contain one or more introns, whereas the genes encoding stress-induced heat shock proteins (hsp) contain no introns. The absence of introns from these hsp70 genes could have arisen from the need to express heat shock (hs) genes quickly, when intron processing would be too time consuming (Lindquist, 1986). Intron splicing is inhibited by severe hs both *in vitro* (Bond, 1989) and *in vivo* (Labhart and Reeder, 1987). The absence of introns in the pea hsp70(LP19) gene, which is heat induced, supports the above definition. However, this definition of hsp and hsc genes has been opposed by reports showing

that the heat induced hsp70 genes of maize (Rochester et al., 1986) and petunia (Winter et al., 1988) contain introns. These heat induced hsp70 genes from petunia and maize encode proteins that are more homologous to the protein products of hsc genes than those of other hsp70 genes. Other hsp70 genes also contain introns, for example a nuclear encoded chloroplast hsp70 gene from pea has been shown to contain seven introns (Casey et al., 1994). The sequence data obtained for hsp70 genes in plants do not therefore support Lindquist's definition, but suggest that the main distinguishing feature is the location of the encoded protein.

In all examined instances, cytoplasmic hsp70 proteins in plant cells are the product of a small multigene family. *Arabidopsis*, for example, has 4-6 genes encoding these proteins. At least three cytoplasmic hsp70s in pea, including PsHSP71.2, which is a homologue of hsp70(LP19) have been reported by DeRocher and Vierling (1995). The reason for the presence of multiple cytoplasmic hsp70 is probably to ensure sufficient proteins because functional cytoplasmic hsp/hsc70 proteins are absolutely essential for cell viability. It is known that the newly synthesised polypeptide chains begin to fold immediately upon release from the ribosome, if undeterred. This would be disastrous for the cell because cytoplasmic proteins only fold into the correct conformations after reaching a certain minimum size. In microbial, plant and animal cells, it has been shown that more than half of all nascent polypeptides are associated with hsp70 proteins, very shortly after emerging from the ribosomes (Beckmann et al., 1990). Therefore, it is likely that cytoplasmic hsp70 chaperone proteins act to forestall folding, misfolding and aggregation. Recently, in an intriguing report, Lee and Schoffl (1996), have shown that antisense downregulation of one cytoplasmic hsp70 gene led to the simultaneous downregulation of another cytoplasmic hsp70 protein gene.

The 5' flanking end of the pea hsp70(LP19) gene contains 6 site having nGAAn, repeats corresponding to heat shock elements (HSEs), with one or more elements present immediately upstream of the TATA box. All heat shock promoters from different genes in a diverse array of organisms have the same 5 bp building blocks of HSE, i.e. inverted 5 bp repeats "nGAAn". Periodically arranged GAA segments, repeated at 2-nucleotide intervals and in alternating orientations, is a key feature of heat shock regulatory elements. The transcriptional activation of heat shock genes requires the activity of two or more binding sites for heat shock factor (HSF) trimers. These

sites act synergistically to activate transcription which is consistent with the finding that hs promoters often contain multiple HSEs (Fernandez et al., 1994). Shuey and Parker (1986), suggested that HSF binding to a single regulatory element induces local DNA bending and perhaps productive HSF-DNA interactions involve bending of the binding sites. Therefore, the presence of multiple contact points in a binding site may be a prerequisite for HSF-induced bending. The HSEs in pea hsp70(LP19) have three to six 5 bp units, except HSE V which only has two 5 bp repeats. However, an exact match of the 5 bp repeats is not necessary for functionality, as the binding of the HSF can occur if only two of the 5 bp units are present (Fernandez et al., 1994). In addition, a HSE can tolerate up to a 5 bp insertion between repeating units, provided the phase of the repeats is maintained (Amin et al., 1988). The number of HSEs and the distance between them in promoter regions of different heat shock genes can also vary. For example, the upstream region of the Drosophila hsp70 gene contains 4 HSEs, each composed of three or four 5 bp units, while the upstream region of *Drosophila* hsc83 contains a single HSE with eight 5 bp units (Xio and Lis, 1989). Another important variable is the degree of homology of the bases in each 5 bp unit to the standard 'nGAAn' motif.

Variability in sequences of HSEs influences the affinity with which HSFs bind to HSEs of a particular hs gene. The GAA blocks probably represent the contact points for HSFs and are therefore important for the function of heat shock regulatory elements. Mutation in the GAA region reduces the relative binding affinity with HSFs. Amin et al. (1988), studied the effect of the substitutions in the GAA block on the functionality of the regulatory elements and found that first base G in the block is absolutely important for the functionality followed by second base A and leaving the last base A as flexible. The HSF-HSE interaction appears to be strikingly similar in all organisms.

In addition, further potential regulatory elements are present, in the form of A-T rich sites, which act as enhancers for the thermo-inducibility of gene expression (Gurley et al, 1993). Proximal A-T rich sites contribute more to enhancement when compared to distal ones, and the pea hsp70(LP19) gene has two sites, of approximately 40 bases each, within 500 bases of the TATA box. There are also two distal sites with simple GAGA repeats, which are close to each other; these could represent the binding site of the "GAGA" factor which acts to "open" the promoter enabling access of HSFs via

nucleosomes and histone displacement. Lee, (1992), reported that HSFs are unable to dislodge nucleosomes and act only on nucleosome free promoters potentiated by bound 'GAGA' factor, TFIID and a paused polymerase. The GAGA elements are found in the regulatory regions of a variety of heat shock and developmentally expressed genes and have been shown to act positively on the expression of the hsp genes (Gasser and Lis, 1990). Therefore, it is speculated that the "GAGA" elements are necessary for the maintenance of the heat shock promoter regions in an accessible configuration. Taylor et al. (1991), has shown that in the case of humans, heat shock transcription factors (HSFs) failed to bind HSEs if the promoter was associated with the nucleosomes. *Drosophila* transformants carrying mutations in GAGA repeats showed that these elements are required for creating the nuclease-hypersensitive region in the promoter (Lu et al., 1993).

The pea hsp70(LP19) clearly has a complex pattern of expression. Not only does its heat shock response show partial tissue specificity, in that expression decreases in the order roots>stems>leaves, but it is also expressed as part of normal plant development, in developing seeds (DeRocher and Vierling, 1995), and in pods as described in section 3.1.4. The expression pattern of LP19 mRNA in 4-6 DAF pods by Northern blot analysis indicates correlation with pod lignification. Expression is highest in pods of L59 and FF which both have a differentiated endocarp with lignification commencing at around 4-6 DAF. The expression level of LP19 mRNA was lower in L58 which has partially lignified endocarp and undetectable in L1390 which does not have a differentiated lignified endocarp. Histological staining with phloroglucinol (Drew, 1994) established the onset of lignification in the endocarp of L59 and FF at around 6 DAF, whereas in L58, partial lignification of the endocarp was observed in 12 DAF pods and no lignification at all was observed in pea line L1390 even after 12 DAF.

The analysis of genomic DNA by Southern blotting shows hybridisation to a single band in experimental pea lines L59, L58, and L1390 indicating that the hsp70(LP19) gene is present in all the lines. Thus the lack of hybridisation of the LP19 cDNA to L1390 4-6 DAF pod total RNA cannot be due to the gene being absent in this line. It is unlikely that the change in hsp70 gene expression is a causative factor in the absence of pod lignification in L1390 (lignification in other tissues in this line is not affected), but it may play a role in the change in developmental pattern caused by the genes

corresponding to the classical loci *p* and *v*. The expression of hsp70 genes during development and differentiation have also been shown in many other plant species (Kurtz et al, 1986; Lindquist; 1986). Expression of tomato hsc70 has been detected in secretary tissues and organs with rapidly dividing and differentiating cells (Duck et al., 1989). It has also been suggested that some conserved developmental functions require the aid of hsc/hsp70 in ovaries (Winter and Sinabaldi, 1991). Cordewener *et al.* (1995) have shown a strong correlation between the phase of the cell cycle, nuclear localisation of hsp70 and induction of embryogenesis in *Brassica napus* . Therefore, developmental expression of a specific hsp70 which is heat regulated, in vegetative tissues, suggests that the unique hsp70 functions required during stress are also required during development.

The hsp70(LP19) mRNA did not show any expression under salt stress and wounding. This is contrary to the reports which have claimed the expression of some hsp70 homologues is induced under a variety of stresses such as cold (Neven et al., 1992), heavy metals (Winter et al., 1988; Neumann et al., 1994) and wounding (Kalinski et al., 1995). Possibly different hsp70 genes are responsive to different stresses, and they do not share a common signal cascade mechanism. Alternatively, the heat shock elements of hsp70(LP19) are not responsive to other stresses.

It is interesting to note that hsp70(LP19) seems to be expressed in pods, but not seed coats, and that it is expressed in both pods and seeds during phases of development when tissues are under osmotic stress; the pod desiccates on lignification, and developing seeds undergo desiccation during mid- and late-developmental stages, when PsHSP71.2 mRNA, the product of hsp70(LP19), is present (DeRocher and Vierling, 1995). On the other hand, a plastid localised hsp70 is detected during early stages of pea seed development (Domoney et al, 1991). An accurate specification of the role of the polypeptide encoded by hsp70(LP19) is not possible with the data available at present. However, it could be explained that it might be advantageous for a population of hsp70s in seeds and pods to remain bound to their targets for an extended period of time to protect them in the low water environment of the desiccating seeds and during pod lignification.

The molecular regulation of heat shock response is imposed at both transcriptional and translational levels. Upon heat shock, hs mRNAs are selectively transcribed and accumulated, and are selectively translated even though the non-heat shock mRNAs remain in the cell for many hours (Lindquist, 1986). The complexity of transcriptional regulation of the hsp70 gene family is further indicated here with the isolation of hsp70(LP19) mRNA transcripts with heterogeneous 3' untranslated regions (UTRs). All the LP19 transcripts have an identical 3' untranslated region up to the differing polyadenylation sites, suggesting that they are all transcribed from one gene. This assertion was further supported by genomic blot analysis, which indicated that hsp70(LP19) was present as a single copy gene. Multiple polyadenylation sites have also been observed in other eukaryotic genes (Parnes et al., 1983; Hernandez-Lucas et al., 1986). Hence, the presence of variable poly (A) 'tails' on these cDNAs shows that they cannot be artefacts. The 3' UTR of hsp83 in *Leishmania* is involved in temperature dependent regulated decay (Aly et al, 1994). The 3' UTRs of several other genes are implicated in gene regulation and RNA processing (An et al, 1989). Therefore, it is assumed that hsp70(LP19) expression may be regulated in the differentiating cells by some mechanism involving the generation of heterogeneous 3' UTRs and subsequent RNA processing.

The association of hsp70(LP19) gene with pod lignification is an important and new finding. No other report has been published to establish a link between hsp70 expression and lignification. Therefore, the results of the present study opens the door for further interesting research to elucidate the mechanism of how the hsp70 gene or its product may be associated with the lignification process in plants.

Chapter 4

Tissue-specific and Developmental expression of pea hsp70(LP19) gene using Promoter-Reporter Construct

4.1 Results

4.1.1 Construction of promoter-reporter chimaeric gene

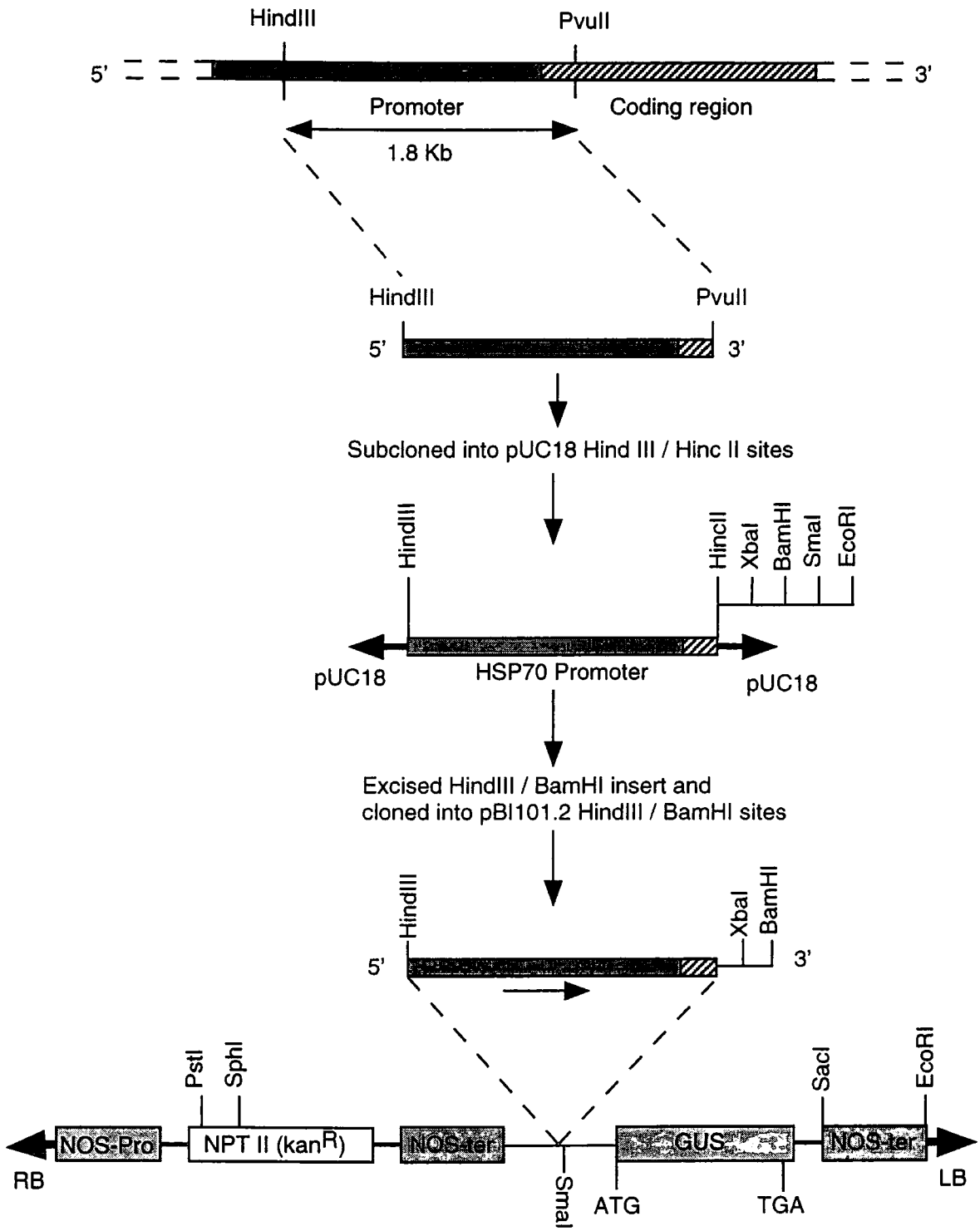
A 1.8 kb 5' fragment of pea hsp70(LP19) gene promoter containing the transcription start site, the translation start codon and the first 18 amino acids of the hsp70(LP19) polypeptide, was translationally fused with the coding sequence of *uid*A (gusA) reporter gene encoding the β-glucuronidase (GUS), and assembled into pBI101.2. As shown in figure 4.1A, the 1.8 kb *Hin*dIII/*Pvu*II fragment was first inserted into *Hin*dIII and *Hin*cII sites of pUC18. By using *Bam*HI site of pUC18, the 5' fragment was excised as a *Hin*dIII+*Bam*HI fragment, and finally assembled into pBI101.2 at *Hin*dIII+*Bam*HI site (figure 4.1). The resulting construct was designated as pHSP70(LP19)-GUS. The construct was verified by sequencing showing that the reading frame had been preserved. The sequences around the fusion site are shown in figure 4.1.

4.1.2 Transformation of *Agrobacterium tumefaciens* with pHSP70(LP19)-GUS

The pHSP70(LP19)-GUS construct was introduced into the *Agrobacterium tumefaciens* strain LBA4404 by a triparental mating between *E.coli* strain DH5α containing the construct pHSP70(LP19)-GUS, *E.coli* strain HB101 containing the helper plasmid pRK2013 and the *A. tumefaciens* strain. Transformation was confirmed by Southern analysis of bacterial DNA isolated from kanamycin resistant *A. tumefaciens* colonies. The DNA was restricted with *Hin*dIII and *Bam*HI, in order to

Figure 4.1 Structure of chimaeric gene construct pHSP70(LP19)-GUS used for tobacco transformation; restriction sites relevant for cloning are included. RB, T-DNA right border; LB, T-DNA left border; Kan^R, kanamycin resistance cassette consisting of the neomycin phosphotransferases II (NPTII) gene fused to nopaline synthase promoter (NOS-Pro) and terminator (NOS-ter); GUS, β-glucuronidase coding sequence.

Pea HSP70(LP19)Promoter - GUS Chimaeric Construct



Sequence around promoter/GUS fusion:

BamH I

```
... CA ATG NAG AGG ACT CTA GAG GAT CCC CGG GTA GGT CAG TCC CTT ATG ...
    V  X  R   T   L   E   D   P   R   V   G   Q   S   L   M
```

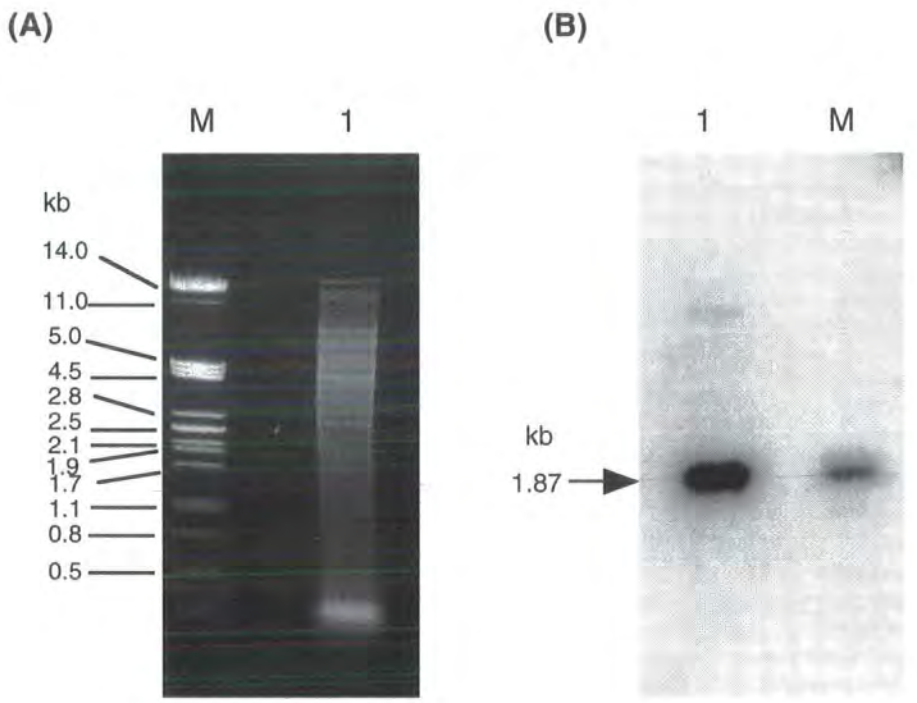


Figure 4.2 Southern blot hybridisation of genomic DNA from *Agrobacterium tumefaceans* transformed with the chimaeric construct pHSP70(LP19)-GUS. (A) and (B) represent HindIII/BamHI digested DNA electrophoresed on 0.7% w/v agarose gel and transferred to nitrocellulose filter, respectively. Filter was probed with ^{32}P-labelled 1.8 kb promoter region of hsp70(LP19) gene and was washed to a high stringency of 0.1x SSC, 0.1% SDS at 65°C for 30 minutes. Lane M (size marker λ PstI) and lane 1(HindIII/BamHI digested *A.tumefaceans* DNA)

release the 1.8 kb hsp70(LP19) gene promoter fragment. Following transfer to nitro-cellulose filter by Southern blotting, the presence of the promoter fragment was confirmed by hybridising the filter with ^{32}P-dCTP labelled 1.8 kb hsp70(LP19) gene promoter fragment (figure 4.2).

4.1.3 Expression analysis of pHSP70(LP19)-GUS in transgenic tobacco and identification of functional transgenics

The pHSP70(LP19)-GUS construct was introduced into tobacco (*N. tabacum* cv. Samsun) by a standard *Agrobacterium* mediated transformation method employing leaf discs. A total number of 23 transformed plants were regenerated from kanamycin resistant calli, and were successfully grown to maturity in growth rooms. All 23 primary transformants were analysed using histochemical staining with X-Gluc and fluorimetric GUS assays (Jefferson et al., 1987). Three weeks after potting, plants were subjected to heat shock at 40°C for 4 hours as described in section 2.1. Hand cut transverse sections of stem, petiole, leaf and root were stained in X-Gluc and examined. The control experiments included sections from transformants not subjected to heat shock and the wild type tobacco plants subjected to high temperature. A detectable amount of GUS expression was observed in 19 out of 23 primary transformants after heat shock. No staining was detected in the controls, or transformants without heat shock.

Extracts from the leaves of all the 19 heat shocked functional transgenics were fluorimetrically assayed for GUS activity. On the basis of level of GUS expression (table 4.1), the plants were grouped into 3 categories, high, medium and low expressers according to their levels of GUS expression. Among the high expressers, primary transformant number 14 was found to be the highest, with GUS activity 65.7x 10^{-3} nmoles MU/μg protein/min.

Table 4.1 Estimation of GUS activity in primary transformants.

Primary Transformant numbers	**GUS activities (4-MU nmoles min^{-1} μg^{-1} protein ($x10^3$)**
1	10.1
2	11.54
3	0.3
4	0.71
5	25.25
6	30.12
7	0.011
8	39.34
9	1.25
10	0.14
11	2.2
12	1.9
13	3.2
14	65.51
15	26.02
16	0.1
17	1.1
18	35.40
19	0.8
Control	0.013

4.1.4 Determination of the number of transgene loci by segregation of seeds on kanamycin selection

Transformed tobacco plants were allowed to self and set seeds, which were collected at maturity. Segregation of the germinating T_1 seeds on media containing kanamycin was used to determine the number of the transgene loci present in the transgenic tobacco plants. If the nptII transgene (selectable marker) has integrated into a single locus

within the tobacco genome, the expected ratio of kanamycin resistant : sensitive seedlings, is 3 : 1. If T-DNA, containing the nptII gene, has integrated into 2 loci, then the expected ratio of the seedlings should be 15 : 1 (resistant : sensitive), unless the loci are linked. If linkage occurs, the segregation ratio would be lower than the expected and depends upon the degree of linkage. For the plant lines in which T-DNA copies have been integrated into 3 loci in the plant genome, the expected ratio is 63 : 1. A ratio lower than this will indicate the presence of linkage whereas a higher ratio indicate the presence of more than 3 transgene loci.

Three primary transformants were tested i.e. numbers 2, 14, and 19. The kanamycin resistant : sensitive ratio observed in transformants 2 and 14 was approximately 3 : 1, indicating the presence of the transgene at a single locus (table 4.2). In transformant number 19, the ratio was 59.2: 1, indicating that the transgene had integrated at 3 sites in the genome.

Table 4.2 Segregation of germinating seeds, the product of self fertilisation, on medium containing kanamycin (100 mg/l medium). The ratio of resistant : sensitive seedlings were tested for fit to the expected ratios for 1, 2, and 3 transgene loci using the chi-squared test (rejection at $p > 0.05$).

Plant number	Resistant Seedlings	Sensitive Seedlings	Ratio R : S	Transgene Loci Number
2	226	78	2.89 : 1	1
14	204	64	3.19 : 1	1
15	296	5	59.2 : 1	3

4.1.5 Southern analysis to confirm the transgene copy number

The number of transgene copies integrated into the transformed tobacco plants was estimated by Southern blot hybridisation using a ^{32}P-labelled probe corresponding to the gusA coding region (isolated from plasmid pBI221 commercially obtained from Clontech). Genomic DNA isolated from primary transformant number 14 was digested separately with *Hin*dIII and *Bam*HI, restricting the DNA at an internal site within the

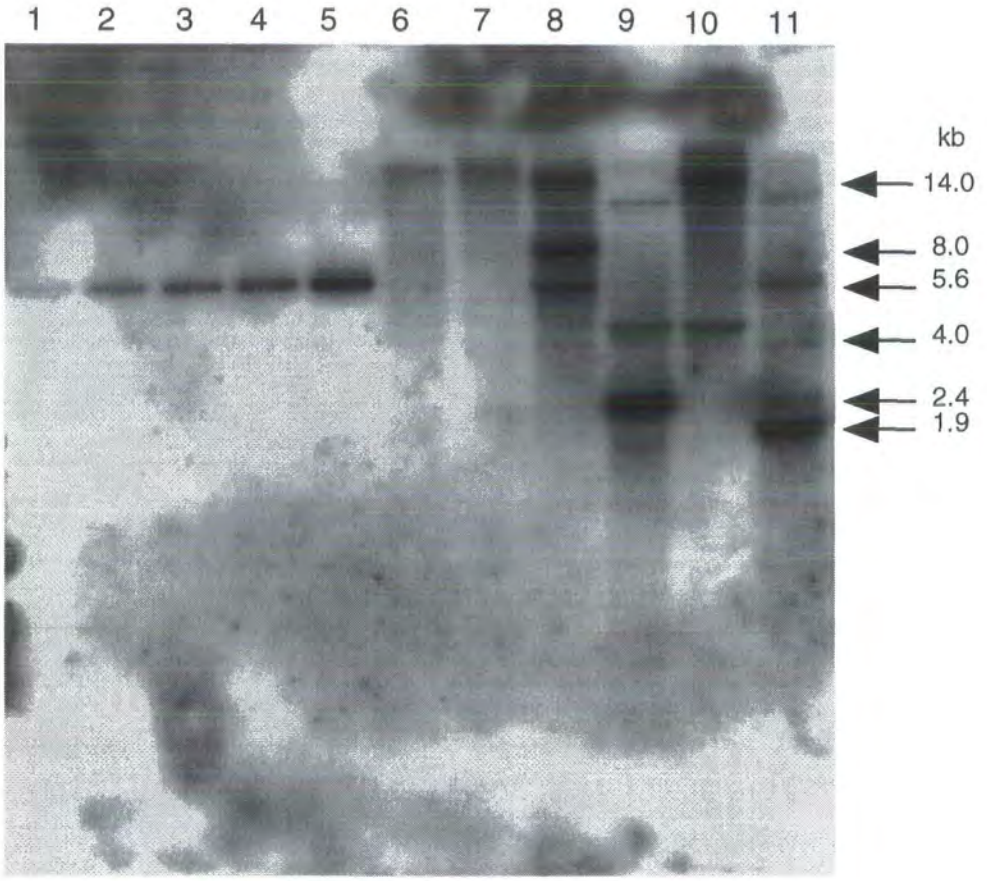


Figure 4.3 Southern blot analysis of genomic DNA from tobacco plants transformed with pHSP70(LP19)-GUS transgene. Total genomic DNA from wild type control plants and primary transformant number 14, after restriction with appropriate enzymes, was fractionated on 0.7% w/vagarose gel and blotted to nitrocellulose filter. 10µg of DNA was loaded in each lane. The filter was hybridised with ^{32}P-labelled GUS probe. Lane 1-5, *Bam*HI linearised pBI221 DNA used for reconstruction; lane 1 (2pg), lane 2 (4pg), lane 3 (6pg), lane 4 (8pg), lane 5 (16pg); lane 6–7, wild type DNA restricted with *Hin*dIII (lane 6) and *Bam*HI (lane 7); lane 8 to 11, DNA from transformant 14 restricted with *Hind*III (lane 8), *Hind*III+*Eco*RI (lane 9), *Bam*HI (lane 10) and *Bam*HI+*Sac*I (lane 11).

T-DNA, and also with *Hin*dIII+*Eco*RI (to confirm the construct) and with *Bam*HI+*Sac*I (to confirm the gusA gene). Hybridisation of the restricted DNA with the gusA probe was expected to detect the gene within the T-DNA border fragment. The T-DNA copy number was determined by reconstruction of BamHI linearised pBI221 vector DNA and hybridisation with the gusA probe (figure 4.3).

The digest of genomic DNA with *Hin*dIII (lane 8), showed strong hybridisation of the GUS probe to a band of approximately 8 kb. Hybridisation to DNA digested with *Bam*HI (lane 10) also detected a single band of approximately 14 kb. For DNA digested with *Hin*dIII+*Eco*RI (lane 9), a band of 2.4 kb size was detected, representing the gusA coding region and a fragment of the promoter region, as an *Eco*RI site exists in the promoter 300 bp upstream of the fusion site. In the case of DNA restricted with *Bam*HI+*Sac*I (lane 11), hybridisation of a band of 1.9 kb, corresponding to the gusA coding sequence was observed. The DNA from wild type non-transformed tobacco digested with *Hin*dIII (lane 6) and *Bam*HI (lane 7) did not show any hybridisation. In all the digestions of transgenic plant DNA, 2 to 3 extra bands hybridising at low intensity were detected. This was considered as due to partial digestion; for example, the observation of a band of 4 kb in DNA digested with *Hin*dIII+*Eco*RI (lane 10) corresponds to a partial restriction product of the construct. Due to the presence of the *Eco*RI site in the construct, the construct was restricted into 2 bands of 2.4 kb and 1.6 kb representing the gusA coding region and promoter fragment respectively (figure 3.6). Therefore, the 4 kb construct fragment represents a case of partial digestion. Comparison of the hybridisation intensity of genomic DNA bands hybridised to the gusA probe and the reconstruction for T-DNA copy number, indicate the presence of a single copy of the transgene in the tobacco genome.

4.1.6 Expression analysis of the pHSP70(LP19)-GUS gene

In order to study the pattern of transgene expression in transgenic tobacco plants under heat shock and during plant development without heat shock, it was considered important to characterise tobacco transformants which have the transgene integrated at a single locus. This would simplify the interpretation of expression pattern, which might be more complex should the transgene be integrated at more than one locus. Therefore,

transformant number 14 with a single transgene locus and the highest GUS activity, was selected for further analysis. The GUS expression was also studied for transformants numbered 2 and 19. Similar profiles of GUS expression were observed for all these lines in subsequent experiments.

4.1.6.1 Tissue-specific expression of the pHSP70(LP19)-GUS gene

Histochemical analysis of GUS activity conferred by the hsp70(LP19) gene promoter was carried out in different tissues of the heat shocked primary tobacco transformants. In leaf petiole tissue, whereas the internal and external phloem were intensely stained, cells constituting epidermis, cortex, xylem and pith remained unstained (figure 4.4 a and b). At higher magnification a little staining was observed in a few parenchymatous cells adjacent to the phloem tissue as well. Similar phloem-specific staining was observed in the stem (figure 4.4 c and d) and roots (figure 4.4 e and f). In whole root tissue (figure 4.4 e) staining due to GUS expression was observed in the phloem as two strands along the central xylem core. Similarly transverse section of the root also (figure 4.4 f) show the phloem-specific staining. The vascular-specific expression has been reported for barley Hvhsp17 gene promoter (Raho et al., 1996), although this was not specific to the phloem. The staining of leaf, on the other hand, showed variations and did not show such tissue-specific expression. As seen in figure 4.4 g, the whole leaf surface including trichomes, vein reticulum and midrib showed positive staining. Similarly, in cross section (figure 4.4 h), GUS staining was detected in all cells types such as epidermis, mesophyll and in the vascular elements of the leaf. In some cases staining was only detected in the trichomes and vascular region. A similar pattern of expression in leaf has also been reported by Prandl et al. (1995), for the soybean GmHSP17.3-B promoter. No GUS staining was observed in any tissue of the heat shocked untransformed control plants and non heat shocked transformed plants.

Seeds of the selfed primary / T_0 transformants representing one each of the above three expresser categories were selected for further studies. The T_1 seedlings from these seeds, germinated on kanamycin (100µg/ml) selection media exhibited segregation into 3 resistant : 1 sensitive in case of transformants 2 and 14 whereas in transformants number 19, this ratio was 63 : 1. The resistant seedlings were transferred to soil and

Figure 4.4 Histochemical localisation of GUS expression in HSP70(LP19)-GUS transgenic tobaccos. Co, cortex; E, epidermis; EP, external phloem; IP, internal phloem; M, midrib; Pi, pith; T, trichome; X, xylem.

(a) Transverse section of stem (x40)

(b) Transverse section of stem (magnified) (x100)

(c) Transverse section of petiole (x32)

(d) Transverse section of petiole (magnified) (x80)

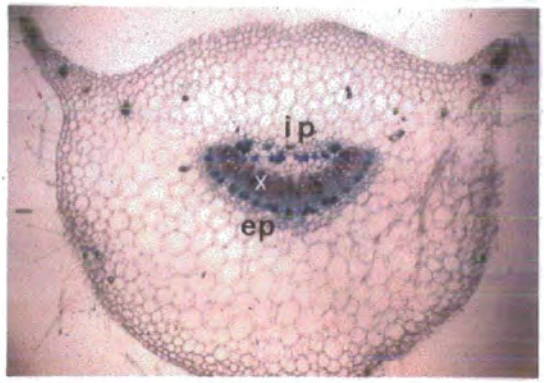
(e) Whole root portion stained (x32)

(f) Transverse section of root (x100)

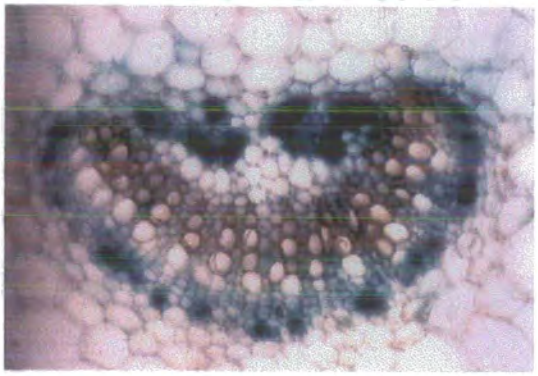
(g) Whole leaf portion stained (x32)

(h) Cross section of leaf (x100)

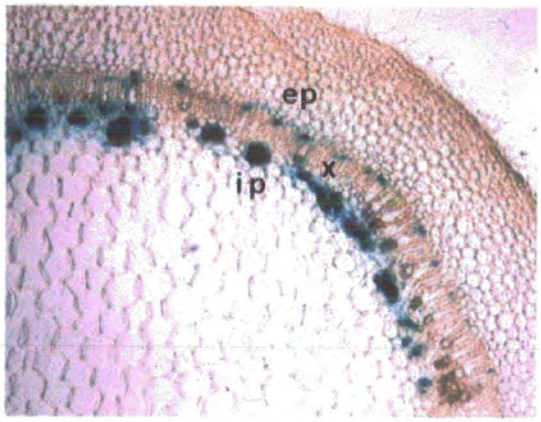
(a)



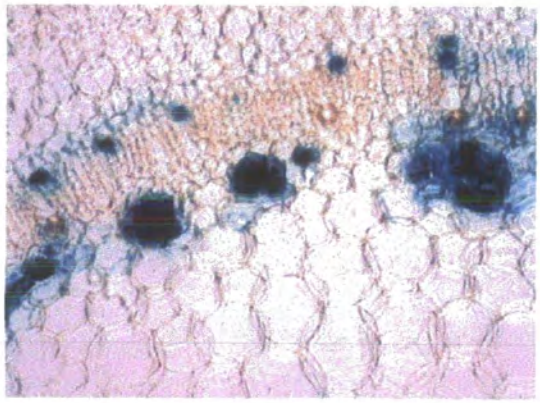
(b)



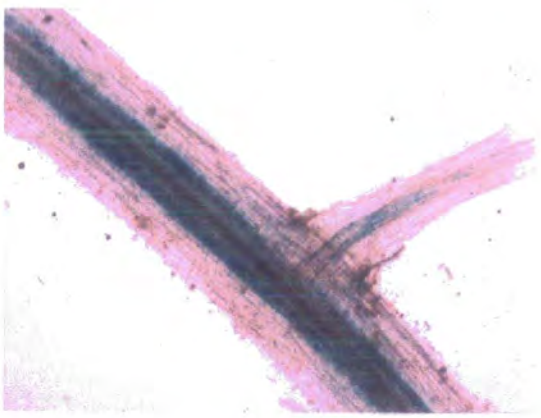
(c)



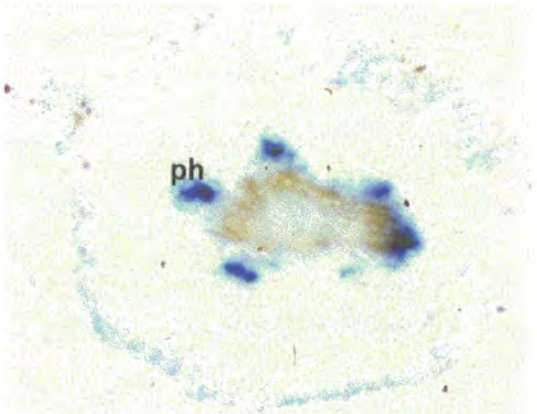
(d)



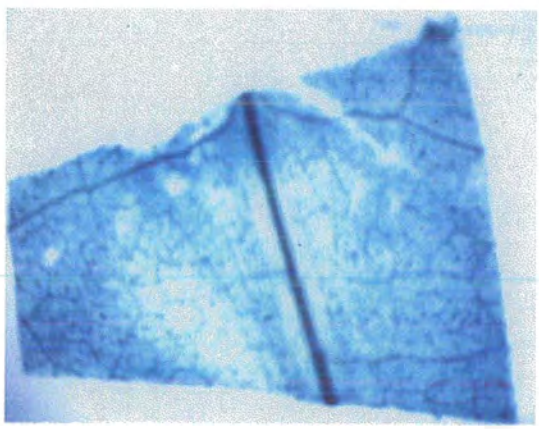
(e)



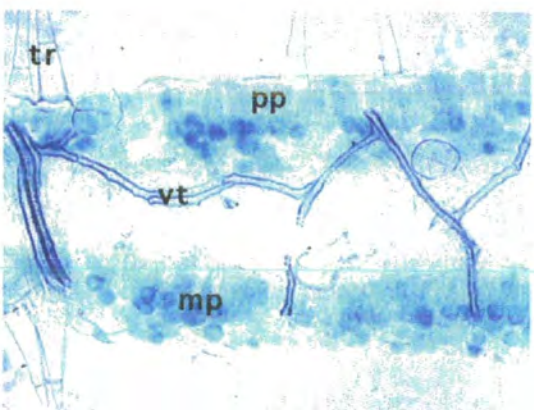
(f)



(g)



(h)



the T_1 plants were heat shocked when 6 weeks old. The treatments were as for the primary transformants and the GUS activity assayed in the section of the root, stem, petiole and leaf also showed staining as observed for primary transformants. These studies of the transgenics representing two generations showed that the chimaeric construct pHSP70(LP19)-GUS is stably inherited.

4.1.6.2 Developmental expression of the pHSP70(LP19)-GUS gene

The developmental expression of the hsp70(LP19) gene promoter was studied in floral tissues, germinating seeds and seedlings at different stages of growth. No heat shock treatment except where mentioned was given during this study. All the samples were stained at 22°C to avoid synthesis of GUS during the staining reaction. As shown in figures 4.5 and 4.6, the histochemical localisation of GUS activity in the reproductive organs and during seed germination stages, indicated organ specific and differential regulation of the transgene.

In the flowers, petals did not exhibit any GUS activity at the young non pigmented stage. The presence of GUS staining coincided with the appearance of the pigmentation at the tip of the elongated corolla tube at anthesis stage and was detectable in the pigmented petals of the fully opened flower (figure 4.5 a). In the stamens, only the anthers showed GUS expression (figure 4.5 b and c). With all the pollen grains pushed out, no staining was observed in the anther wall, showing that the expression of the transgene was in the pollen grains only. As is clear from figure 4.5 d, GUS staining was intense in the central mass of the pollen grains, and no blue staining was visible in the exine and intine layer of the pollen wall. The pollen grains were counted under the microscopic field by making a grid on the slide. Approximately 50% of pollen grains in an anther were found to be unstained and thus without any GUS expression (table 4.3), indicating gametophytic expression of a single gusA gene and that the plants used were hemizygous. This expression pattern was found to be stably transmitted through meiosis to the progeny as a single dominant Mendelian trait.

Table 4.3 The segregation of the pollen grains during microsporogenesis by counting the GUS stained and non-stained pollen grains.

Plant Number	Pollen Grains Stained (S)	Pollen Grains Non-stained (NS)	Ratio S : NS
14	4368	4032	1.16 : 1

In the ovary GUS expression was studied at three developmental stages. Stage I represented young ovary from the unopened flower, stage II represented ovary from the just opened but unpollinated flower and stage III represented the mature ovary from the fully opened flower. At stage I, GUS activity was detectable in the whole of the ovary. No GUS activity was detected in the style and stigma. At stage II, only the placenta and the developing ovule in the loculi were stained blue and in stage III, GUS activity could be seen only in the developing seeds (figure 4.5 e, f and g). These ovaries were found to have seeds which were fully stained, half stained or not stained at all (figure 4.5 h). The GUS staining pattern in seeds, again indicates the segregation of the transgene. Sections of mature dry seeds showed GUS staining only in the embryo.

In order to determine the status of hsp70(LP19) gene promoter activity in germinating seeds and seedlings, seeds from selfed T_0 primary transformants were sown on selective MS media, in the absence of heat shock. After 48 hrs of imbibition, GUS staining was observed in the micropylar end of the seeds near the point of emergence of the radicle (figure 4.6 a). After 4 days, on emergence, the embryo showed GUS expression only in the cotyledons and epicotyl of the axis. On the other hand, no staining was observed in the radicle and the hypocotyl. There was a clear delineation between epicotyl and hypocotyl (figure 4.6 b). When the seedlings were 6-7 days old, GUS expression in the cotyledons decreased and started appearing in the roots (figure 4.6 c); in the older seedlings at 14 days of age, GUS activity in the leaves and cotyledonary leaves declined considerably, becoming more pronounced in the root tissue (figure 4.6 d). At later stages no constitutive expression of GUS was observed anywhere in the young plants.

Figure 4.5 Histochemical localisation of GUS expression in HSP70(LP19)-GUS transgenic tobaccos in floral organs without hs treatment.

(a) Tobacco petals before staining (left) and after staining (right) (bar- 1cm)
(b) Stamens in young flower before anthesis (bar- 1 mm)
(c) Stamens expressing HSP70(LP19)-GUS transgene (Left) and wild type (right) (bar- 1 mm)
(d) Pollen grains from dehisced anthers (x160)
(e) Ovary stage I (very young in bud condition) (bar 1 mm)
(f) Ovary stage II (nearly opened but unpollinated flower) (bar 1 mm)
(g) Ovary stage III (mid-mature from fully opened flower) (bar 1 mm)
(h) Seeds from half mature capsule (x80)

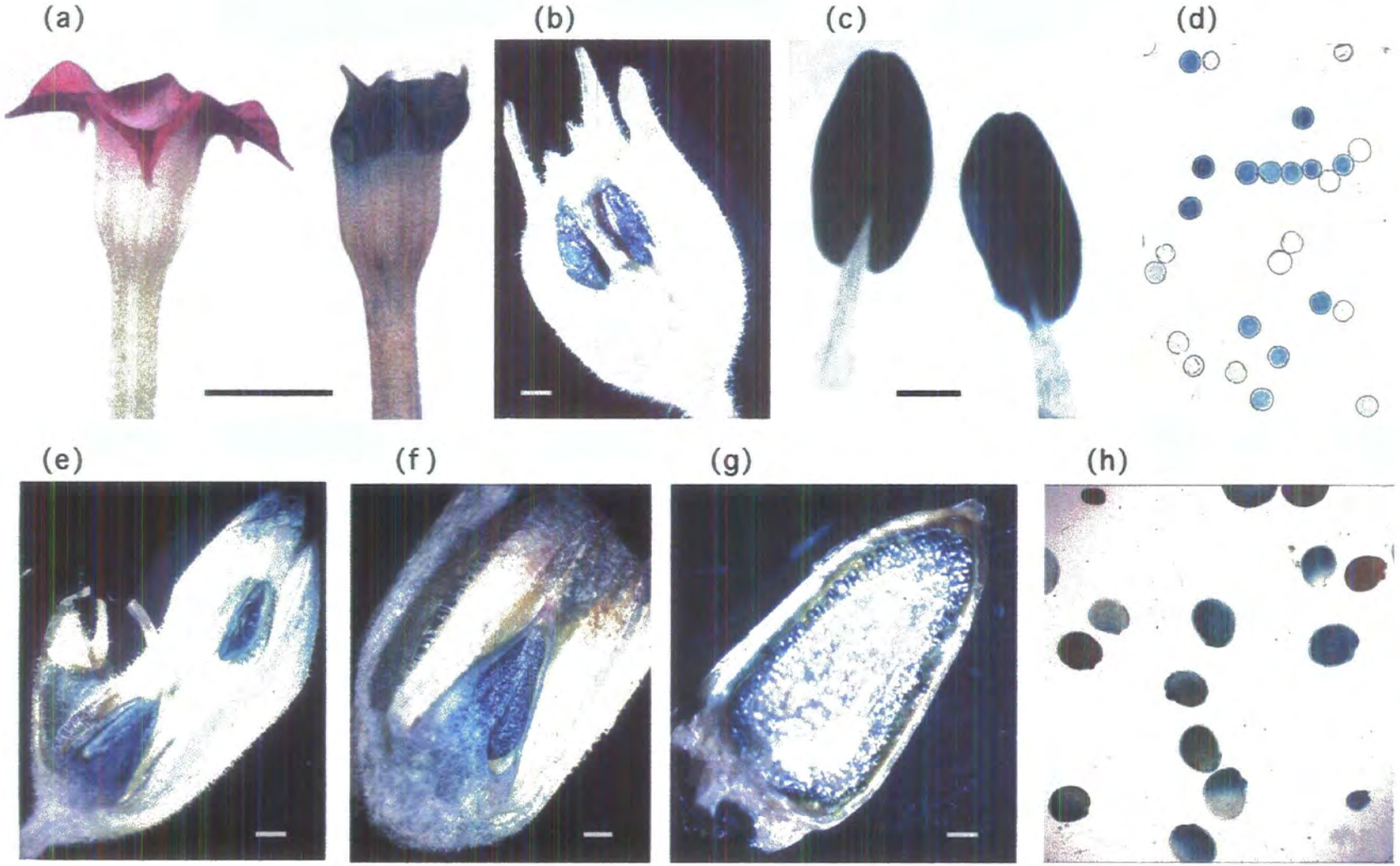

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Figure 4.6 Histochemical localisation of GUS expression in germinating seeds and seedlings.

From (a) to (d) non heat shocked.
(a) Seeds after 48 hrs of imbibition (x32)
(b) 4 days old embryo (x40)
(c) 6-7 days old seedling (x25)
(d) 14 days old seedling (x10)

From (e) to (h) after heat shock
(e) Seeds after 48 hrs of imbibition (x32)
(f) 4 days old embryo (x40)
(g) 6-7 days old seedling (x25)
(h) 14 days old seedling (x15)

From (i) to (l) wild type heat shocked.
(I) Seeds after 48 hrs of imbibition (x32)
(j) 4 days old embryo (x40)
(k) 6-7 days old seedling (x25)
(l) 14 days old seedling (x15)

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)

The germinating seeds and seedlings at different developmental stages as described in the preceding paragraph were heat shocked at 40°C for 20 minutes to study how these transgenic plants responded to high temperature stress. As compared to the non-heat shocked seeds (figure 4.6 a) where GUS staining was seen only in the micropylar end, the heat shock led to expression of GUS activity in all parts of the seeds except the seed coat (figure 4.6 e). At the 4 day stage of seed germination, the heat shock led to expression of GUS activity in the radicle and hypocotyl, in addition to activity found in the cotyledons and epicotyl of the non heat shocked seeds (figure 4.6 f). Similar observations were made for heat shocks at later stages of seedling growth (figure 4.6 g and h). As a negative control, when non-transformed seeds and seedlings were GUS stained before and after heat shock, no GUS staining was detected in any tissue at any stage (figure 4.6 i, j, k and l).

4.1.7 Time course induction of the hsp70(LP19) gene in transgenic tobacco

To study the rate at which the plants respond to high temperature stress, the time course induction of the hsp70(LP19) gene in transgenic tobacco clone 14 was analysed in terms of GUS expression levels by fluorimetric assays. Time course induction of the transgene in terms of GUS levels was studied in seedlings as well as in mature plants.

The seeds were sown on kanamycin (100µg/ml) selection media in petri plates and 14 day old seedlings were subjected to heat shock at 40°C over a time course ranging from 0 to 40 minutes. After heat shock treatment, seedlings were immediately frozen in liquid air and then extracted in GUS extraction buffer. Non-transformed tobacco seedlings subjected to heat shock were used as controls. A basal level of GUS expression was observed in the transformed tobacco seedlings prior to heat shock. The basal expression level of GUS declined in the early time stages and then appeared to increase to its maximum at 20 minutes of heat shock. After 20 minutes, GUS levels started to decline returning to the basal level after 40 minutes (figure 4.7).

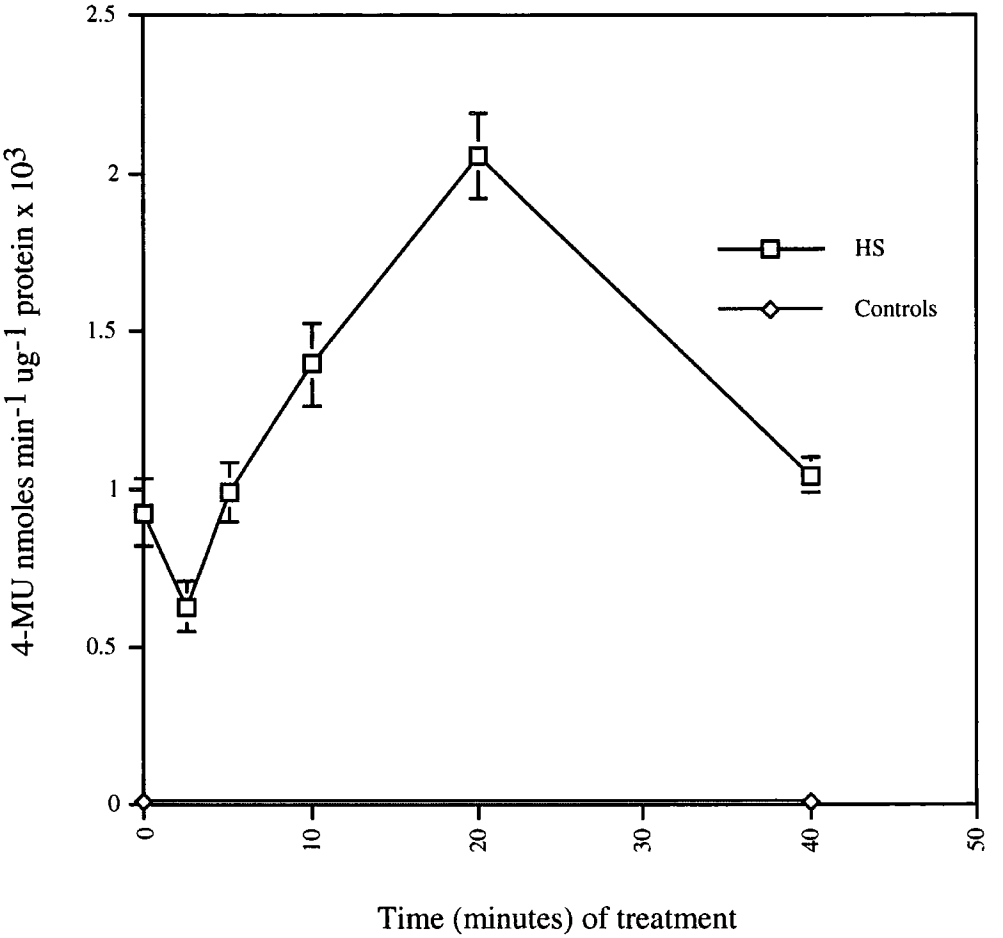


Figure 4.7 GUS activity in seedlings of transgenic line number 14 exposed to elevated temperature for different times. Seedlings were grown on MS selective media, exposed to heat at 40°C for different periods of time and assayed for GUS activity.

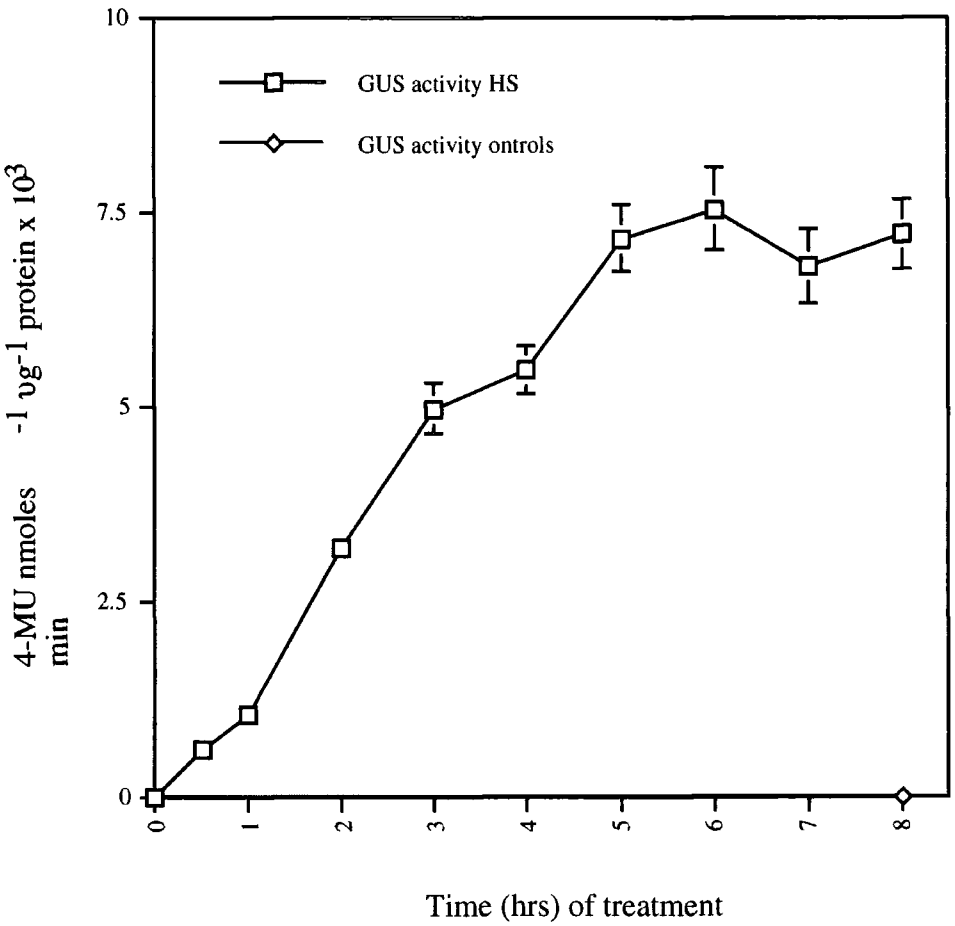


Figure 4.8 Time course induction of GUS activity in mature transgenic tobacco plant line number 14. Plants were subjected to heat shock at 40°C, leaf samples were collected at different time intervals as marked and were assayed for GUS activity.

Mature plants which were about 6 weeks old, were exposed to heat shock at 40°C over a period of 0 to 8 hrs. Leaf discs were collected at different time intervals and leaf extracts assayed for GUS activity. An early response was observed within 30 minutes when GUS levels were found to be approximately 45 times higher than the controls. GUS levels increased at a constant rate during the first 6 hours of heat shock and reached a steady state level after that (figure 4.8). Similar patterns of time course induction of GUS levels were found in transgenic *Arabidopsis* plants expressing the *Arabidopsis* hsp18.2 promoter (Takahashi et al., 1992); GUS activity was shown to have reached a maximum 4-6 hrs after exposure to 35°C and declined slowly thereafter. This expression pattern of GUS in transgenic plants is in accordance with the time course induction of hsp mRNA synthesis since the maximum RNA synthesis for most of the hsp genes occurred between 4-6 hrs (Key et al., 1985). Non-transformed heat shocked tobacco plants used as controls did not show any GUS expression.

4.1.8 Heat shock signal response in transgenic tobacco plants

Though a considerable amount of work has been carried out on heat shock proteins, very little information is available on the nature (whether localised or systemic) of the heat shock signal response. This query was addressed by designing the heat shock treatment of the transgenic plants in three different ways. In the first experiment, the whole plant body was heat shocked and the response was studied in leaf, stem and root tissue. The heat shock response in terms of GUS expression was observed in all these organs as was expected (figure 4.9 A). In the second experiment, only a single leaf of the plant was exposed to the high temperature, keeping the rest of the plant non-heat shocked (see section 2.2.2.1). The response in terms of GUS expression was studied in the heat shocked leaf, the leaves on three nodes above and below that and the stem and the root. Only the leaf exposed to the heat shock showed GUS activity (8.6×10^{-3} nmoles MU/μg protein/min), as observed in the leaves of the first experiment (figure 4.9 A) and no such response was observed in the other leaves, stem and roots. In the third experiment, only roots were heat shocked without stressing the other parts of the plant. The roots were submerged in water at 40°C in a water bath closed at the top with a thermocol sheet. In this case (figure 4.9 B) only the roots showed GUS expression

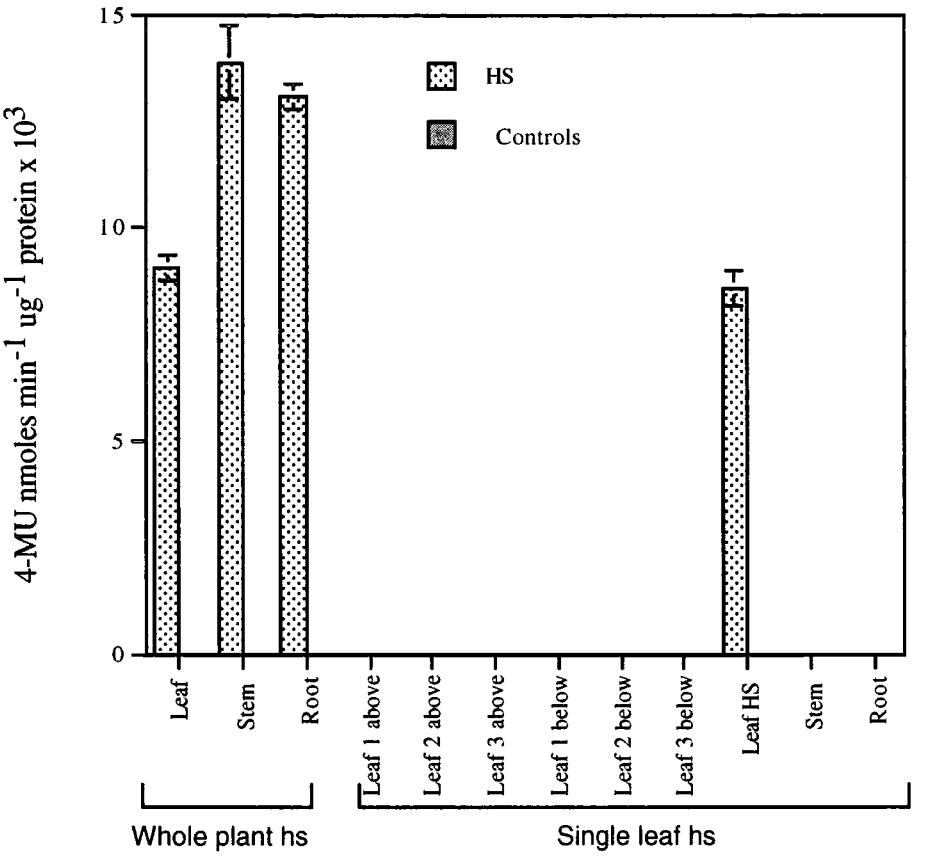


Figure 4.9 A Heat shock response in transgenic plants in term of induction of GUS activity. The whole plant or parts of the plant from transgenic line 14 were heat shocked at 40°C for 4 hrs and were assayed for GUS activity in nmoles 4-MU/min/µg protein. Leaf 1–3 above, represent leaves at three consecutive nodes above the single leaf heat shocked and leaf 1-3 below, represent the leaves at three constitutive nodes below the single leaf heat shocked.

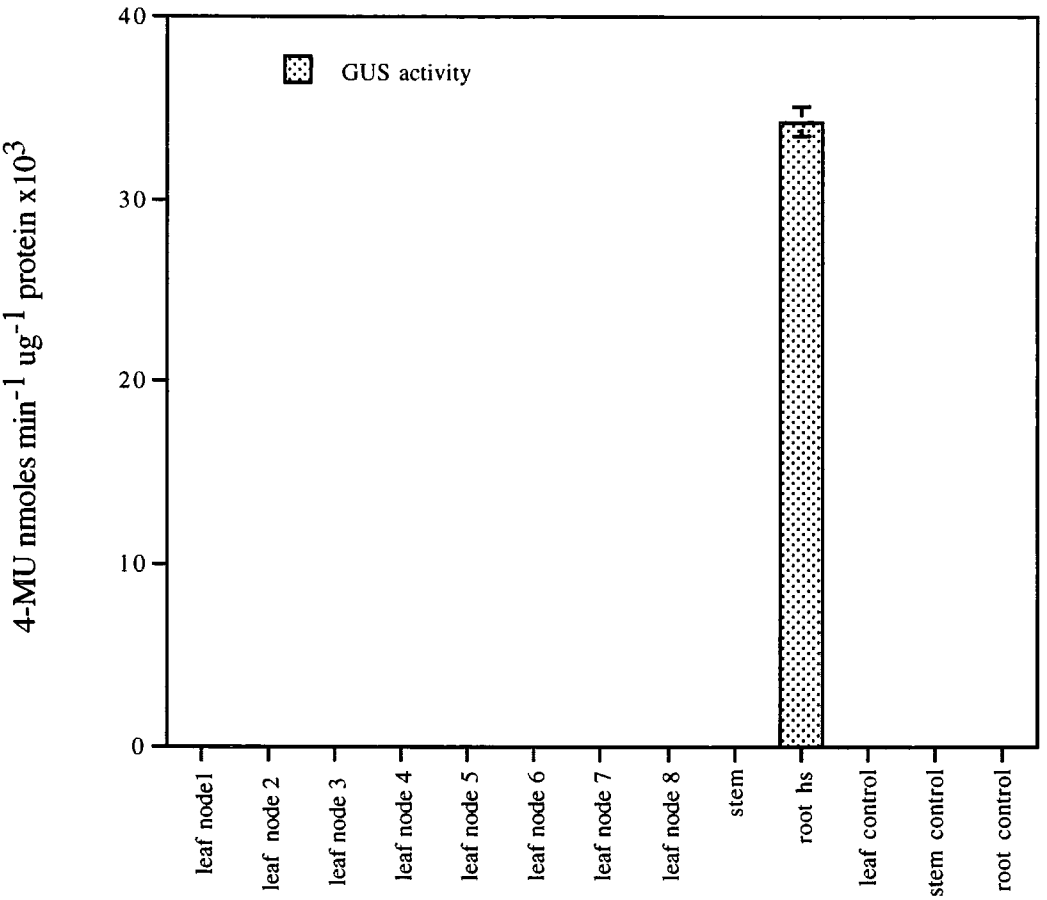


Figure 4.9 B Heat shock response in transgenic plants in term of induction of GUS activity. Only the roots of the plant from transgenic line 14 were heat shocked at 40°C for 4 hrs and were assayed for GUS activity in nmoles 4-MU/min/μg protein. Leaf node 1–8 represent leaves form each node in an ascending order from below to top and stem. Control leaf, stem and root were not subjected to heat shock.

($34.3x10^{-3}$ nmoles MU/μg protein/min). In plants which only had the roots heat shocked, GUS expression levels in roots were almost 3 times higher than the levels in leaf, stem and root of the first and second experiments. In all these three experiments, heat shock treatment was given at 40°C for 4 hrs. The transgenic plants from the same transgenic line without heat shock treatment were used as controls. No GUS activity was observed in any control experiments.

4.2 Discussion

Analysis involving the expression of chimaeric promoters in transgenic plants has greatly contributed to our understanding of specific gene expression, and the use of transgenic plants offers a unique opportunity to study the full complexity of gene regulation, both in homologous and heterologous systems (Benfey and Chua, 1989). In heterologous systems we rely on the gene reporter construct accurately indicating gene activity as is present the original host. This relies on : the promoter being receptive to the host transcriptional control factor; that the transcriptional factors are produced and activated in exactly the same way as in the original plant species; and that the product of the bacterial reporter gene is completely stable in the plant cell environment. The expression of heat shock promoters in transgenic plants is a valuable approach for quantitative and qualitative measurement of the hs response in different tissues of transgenic plants. A major question with regard to hsp synthesis during plant growth and development concerns the temporal and spatial regulation of the heat shock response. To address this question, a gusA reporter gene assembled under the control of the hsp70(LP19) gene promoter from pea was introduced into tobacco.

Most investigations in plants have concentrated on the transcriptional regulation of the genes for hsps and on defining the functions of different promoter elements. The information regarding the temporal and spatial regulation of plant hsps is limited. Tissue-specific and developmental expression of promoters of HSP18.2 from *Arabidopsis* (Takahashi et al., 1992), GmHSP17.3 B from soybean (Prandl and Schoffl, 1996), and HvHSP17 from barley (Raho et al., 1996) has been studied but no efforts have been made to study the plant hsp70 promoter in transgenic plants.

Analysis of genomic DNA from transformant number 14 containing the chimaeric promoter-reporter gene construct pHSP70(LP19)-GUS and the transgene copy number reconstruction indicate the presence of a single copy of the transgene. Generally, the presence of one to five T-DNA copies have been shown in transgenic plants transformed via the *Agrobacterium* mediated system (Raho et al., 1996). Schoffl et al. (1991), reported that transgenic tobacco plants containing one to five copies of the GmHSP17.6-L-GUS transgene, display heat-inducible GUS activity at the same level. It has also been shown for transgenic plants and animal cells that an increase in the copy number of the transgene is not positively correlated with its expression level (Schoffl et al., 1993). Segregation of T1 seeds on kanamycin into 3 resistant : 1 sensitive (table 4.2) confirmed that the transgene had integrated into the tobacco genome at a single locus in transformants 2 and 14, and at three loci in transformant number 19 where the resistant : seedling ratio was in the range of 63 : 1. As different sites of integration can affect transgene expression differently, GUS activity in a plant with the transgene at more than one locus may or may not be correlated with loci number. This was shown by variations in GUS activity level for plants with more than one transgene loci. For example, transformants 2 and 14 with the transgene at one locus were found to have GUS activity levels several fold higher than transformant number 19 which had the transgene integrated at 3 loci. Therefore, correlation between transgene expression level and loci number was not apparent. The staining of the pollen grains in T1 transformant number 14 observed in a ratio of 1 stained : 1 non-stained, as shown in table 4.3, is further confirmation of the integration of the transgene at a single locus.

As expected, the level of GUS expression varied between independent transformants having the same construct (see table 4.1). As mentioned earlier, this is probably because of the positional effects related to the insertion of T-DNA in the genome, not due to the copy number of the transgene. There is a general opinion that expression of a transgene is strongly affected by the position of the transgene in the genome but there is no correlation between gene number and level of expression (Peach and Velten, 1991). Both the level and pattern of expression can be influenced through rearrangements of the gene construct upon integration in the genome, the methylation status of the transgene and by trans-inactivation or co-suppression of transgene expression. Variations in expression of the introduced genes in transgenic plants are common (Horsch et al., 1985; Fluhr et al., 1986).

A high level of heat inducible GUS activity could be detected in most vegetative organs of transgenic tobacco at all developmental stages, whereas GUS activity was only detected in non heat shocked transgenic plants at certain developmental stages as described in section 4.1.6.2. Furthermore, on a total protein basis, quantitative differences among the different tissues were observed. In roots the GUS accumulation level was approximately 2.5 and 4 times higher than in stem and leaves, respectively. Similar tissue-specific differences in the expression level of neomycin phosphotransferase II activity driven by the *Drosophila* hsp70 promoter was reported in tobacco by Spena and Schell (1987). This tissue-specific difference is probably due to the different cell composition of the tissues analysed. The order of the heat-induced GUS accumulation level (roots>stem>leaves) as shown in figure 4.9 A&B, is in accordance with the accumulation level of hsp70(LP19) mRNA in pea (figure 3.10). This could be due to the fact that the mode of activation mechanism of tobacco HSFs are similar to pea HSFs and therefore bind to pea promoter HSEs to activate the promoter in a similar heat-induced manner as in pea. It has also been shown that heat-inducibility is conserved when reporter gene driven by the respective promoters are examined (Rieping and Schoffl, 1992; Raho et al., 1995) and heat-inducible mRNAs of the GmHSP17.3-B gene were almost identical in soybean and transgenic tobacco (Baumann et al., 1987).

A detailed deletion analysis of different hsp promoters (Czarnecka et al., 1989; Raho et al., 1995) showed that in addition to the TATA motif, multiple upstream elements contribute to the transcriptional regulation of the gene including HSEs and A-T rich elements for the amplification of the response. Furthermore, Raho et al. (1995), demonstrated that the presence of HSEs alone were not sufficient to sustain thermal inducibility. In the present study, a 1.8 kb region of the hsp70(LP19) promoter that includes all the HSEs and other regulatory sequences such as A-T rich regions, TATA motif and the first 18 amino acids of the coding region of the hsp70(LP19) polypeptide was used. It has been noticed that in case of hsp gene promoters, most of the regulatory sequences are located within 1200 bp from the transcription start. The presented results demonstrate that the hsp70(LP19) gene promoter directed gene expression in an essentially heat induced and developmentally regulated manner.

Histochemical analysis of GUS activity in all the heat shocked vegetative organs such as stem, petiole and root showed the phloem specific expression of the transgene with the exception in leaves. The vascular-specific expression particularly in xylematic components of the stem and petiole of the gusA gene driven by soybean GmHSP17.3-B and barley HvHSP17 promoter had been reported in transgenic tobacco (Prandl and Schoffl, 1996; Raho et al., 1996). Therefore, the pea hsp70(LP19)-GUS transgene expression being phloem specific, differs from the reported expression of soybean and barley promoter. It could be explained that in spite of having a similar structure of heat shock elements, the hsp70 and small hsp genes promoters have differences in their mechanism of activation and tissue-specific expression of hs genes. The possibility that HSP70 function could be related to the metabolite transfer function of some phloem cells, can not be eliminated.

In leaves the GUS activity was present in all the tissues including trichome, epidermis, mesophyll and vascular elements. This expression pattern in leaves is similar to the pattern of GUS expression in tobacco leaves expressing the soybean GmHSP17.3-B-GUS chimaeric gene (Prandl and Schoffl, 1996). The same group further reported that fully expanded but not senescent leaves showed the highest level of heat-inducible GUS activity in comparison to young leaves. Moreover, the differences in expression pattern were also noticed in different parts of the same leaf. The leaf tips and marginal regions showed higher activity than in central areas and leaf bases. Another important observation was made by Spena and Schell (1987) that the tobacco plants expressing the *Drosophila* hsp70 promoter-nptII chimaeric gene, at full maturity (usually after flowering) reduce or cease the expression of the chimaeric gene. Therefore, these results explain that the induction of hs genes and the differences in GUS expression in different tissues depend on the physiological status of the tissues and cells.

Many hsp genes are not only activated following thermal stress but are also often subject to developmental control. For example, hsp70 mRNA levels vary dramatically between organs of developing tomato fruit (Duck et al., 1989) and a plastid localised hsp70 was detected during early stages of pea seed development (Domoney et al., 1991). The mRNAs for small hsps were detected during meiotic prophase in lily (Bourchard, 1990) and maize (Dietrich et al., 1991) and during microsporogenesis in maize (Atkinson et al., 1993), tomato (Zarsky et al., 1995) and soybean (Coca et al.

1994). Furthermore, small hsps were detected in seeds of various legumes grown under field conditions (Hernadez and Vierling, 1993) and the mRNAs for a small hsps were present in tomato fruits (Fray et al., 1990).

The hsp70(LP19)-GUS fusion showed developmental expression in the pigmented region of tobacco petals which coincided with the anthocyanin synthesis. This expression is rather unique and differs from several other reports described in the literature for other heat shock promoters in plants and also the *Drosophila* hsp70 promoter in transgenic tobacco (Spena and Schell, 1987), in which no GUS activity was reported for petals. As it has been previously described in chapter 3, the pea hsp70(LP19) gene is associated with pod lignification. In this regard it could be hypothesised that either this particular gene is activated at certain stages of the lignin biosynthetic pathway or could be a *de novo* synthesis of HSP70 at this particular step. It may also be possible that somehow the signal cascade mechanism for hsp70 synthesis and for other responses like pigment synthesis and/or other secondary metabolites, involving the phenylpropanoid pathway at these stages, is common. No published data is available for the heat shock promoter reported to be expressed in petals to support this contention. A little GUS activity has been reported by Prandl et al. (1995) in petals of transgenic tobacco plants expressing the soybean GmHSP17.3-B promoter but it was exclusively after heat shock, and not as a result of developmental expression.

The observation of GUS activity in transgenic pollen grains is not a surprising fact and seems to be as result of activation of the hsp70 gene during embryogenesis. Similar results were obtained by Duck et al. (1989) in developing and mature anthers of tomato for hsp70. Cordwener et al. (1995) reported the activation of heat shock proteins during embryonic induction in *Brasicca napus* . A maize HSP90 gene mRNA was found to be strongly expressed in the absence of heat shock during the pre-meiotic and meiotic prophase stages of pollen development and in embryos (Marrs et al., 1993). The segregation of pollen grains in an approximate 1 stained : 1 non stained ratio indicated that the transgene had segregated properly during microsporogenesis. The GUS activity in pollen grains in this case can not be an artefact as reported earlier in some cases (Uknes et al., 1993) caused by the diffusion of reaction product in the staining mixture. If it is an artefact, then all the pollen grains would have shown the

blue staining. All these reports support the view that the HSP70 gene is activated during embryogenesis but the function at this stage is unknown.

The presence of high GUS activity in the seeds of transgenic tobacco plants throughout their course of development and during germination without heat shock, indicate that HSP synthesis during these stages is a normal part of seed development and not associated with heat stress. These finding are in accordance with the data from other plant species. The HSP70 and HSP26 in barley seeds were found in control non heat shocked seeds after two days of imbibition (Kruse et al., 1993). Similar observations had been made by others. In pea, expression of small HSPs has been reported in cotyledons and axes during the course of seed development, desiccation and germination stages (DeRocher and Vierling, 1994). It has previously been reported by DeRocher and Vierling (1995), that PsHSP71.2, a cDNA corresponding to hsp70(LP19), was expressed in cotyledons and embryo axes beginning at the mid and late maturation phase. Two other homologues PsHSP71 and PsHSP70B were expressed from the beginning of seed development till 4 days after seed germination (DeRocher and Vierling, 1995).

The present results showing the GUS expression in transgenic tobacco seeds and during their germination correlate with the expression pattern of mRNA and protein in pea seeds reported by DeRocher and Vierling (1995). The localisation of GUS activity up to 14 days after germination might be due to the stability of GUS protein for long periods of time even after mRNA synthesis has stopped. Helem and Abernethy (1990) have shown that HSPs and the corresponding mRNAs were present in seeds and while the mRNAs disappear rapidly during imbibition, the proteins remain relatively stable. The *de novo* synthesis during development of mRNAs for hsps and the corresponding proteins starts a few days after germination of pea (Knack et al., 1990). Heat shock mRNAs and proteins have been found in pea seeds which did not experience heat shock in close connection with the synthesis of hsps and these developmental related hsp remain stable for some time (Vierling and Sun, 1989). This might indicate that during the early period of germination, prior to establishment of the seedling, protection against a sudden heat shock has to be granted by storage of preformed hsps.

A dramatic increase in GUS activity correlated with seed desiccation has been reported for soybean GmHSP17.3 B promoter in transgenic tobacco seeds (Prandl and Schoffl, 1996). A desiccation stage is defined as a phase of final and rapid seed dehydration preceded by a phase with relatively almost constant water content (Glau et al., 1991). During seed dehydration in pea, strong increase in mRNA levels of class II small HSPs in the cotyledons and of class I and class II small HSPs in axes were noticed (DeRocher and Vierling, 1994). Furthermore, in sunflower, levels of sHSP mRNA increase strongly during seed dehydration (Coca et al., 1994). These correlations raised the possibilities that the hsps are necessary either for the embryo to survive desiccation or for germination upon rehydration. The organ specificity of hsp gene expression during seed development suggest that hsp expression in the embryo is tightly controlled and presumably has an important role in the developing seeds (DeRocher and Vierling, 1994).

The similarity between the signal transduction pathway for desiccation and the heat stress induced expression of hsps suggests the common principle for heat stress induced gene activation via HSFs and HSEs interaction is also valid for developmental regulation (Prandl and Schoffl, 1996). Deletion analysis of soybean GmHSP17.3-B promoter showed that the elements responsible for heat inducible and developmental GUS activity are co-localised and that the HSEs containing promoter regions are essential for expression of the reporter gene under both conditions (Prandl and Schoffl, 1996). The sequences other than HSEs, such as A-T rich elements (Rieping and Schoffl, 1992), may enhance developmental expression of heat shock genes but HSEs remain crucial for induction. Therefore, the GUS gene under the control of pea hsp70(LP19) promoter could be used as a suitable reporter for the identification of developmentally regulated promoter elements in transgenic tobacco, since it is expressed during pollen development and in developing and germinating seeds and thus reflects expression of endogenous heat shock genes.

The data for the time course of the hsp70(LP19) gene promoter in terms of GUS activity during exposure of transgenic seedlings to elevated temperatures indicated a small but reproducible increase in reporter enzyme levels, reaching a maximum activity after 20 minutes. Subsequently the activity declined. The rapid response to temperature is unlikely to be a direct result of transcriptional activation, although suggestions from

other work (Key et al., 1985) would indicate that the heat shock gene activation is a rapid response. Similar results to those shown here have been reported for transgenic *Arabidopsis* seedlings. Other explainations are possible to explain this response, for example elevated temperature may cause thermal activation of GUS and also thermal deactivation/dissociation of GUS inhibitors.

In mature plants, the early response was observed after 30 minutes and increased to its maximum up to 6 hrs after heat shock. The 48 times increase in GUS levels within 30 minutes of heat shock suggests that the message could have been started earlier. As expected, in mature plants the heat shock response was delayed as compared to in seedlings. This is due to the size and complexity of the structure. A similar time course induction pattern of GUS activity driven by the *Arabidopsis* hsp18.2 promoter was reported in transgenic *Arabidopsis* and petunia by Takahashi et al. (1992). The GUS activity was reported to be at a maximum 4-6 hrs after exposure to 35°C and declined slowly thereafter. The same group also studied the pattern of accumulation of GUS protein in transgenic *Arabidopsis* and petunia under various temperature regimes. In *Arabidopsis*, maximum GUS activity after heat shock at 35°C was observed and incubation at 40°C gave almost undetectable levels of GUS activity. In petunia, the levels of GUS activity were highest at 38°C. For tobacco plants, 40°C has been shown to be the optimum temperature for maximum GUS activity (Raho et al, 1996; Prandl et al., 1995). Differing responses to different temperature conditions could be explained by the response to high temperature being species specific, some species withstanding higher temperature than others. These results suggest that the response to temperature by the promoters of the heterologous hsp genes is under the control of the host plant cells into which they are introduced.

Heat shock signal response experiments have clearly indicated that the high level of GUS activity was induced only in those regions of the plant that were exposed to heat. These results suggest that there is no endogenous signal that spreads systematically from the heat shock areas to the rest of the plant to induce the synthesis of heat shock proteins. Similar observations were made by Takahashi et al. (1992). They heat shocked a half leaf from transgenic *Arabidopsis* by emmersing into MS media maintained at 35°C for 2 hrs and observed the GUS localisation only in the heat shocked part of the leaf. The level of GUS expression was maximum in roots followed

by stem and least in leaves which correlate well with the level of hsp70(LP19) mRNA expression observed in pea root, stem and leaves as described in chapter 3. This situation stands in marked contrast to the response to wounding either by herbivory or infection by pathogens which leads to systemic induction of pathogenesis responsive (PR) or other defensive proteins in both infected and non infected parts of the plants (Farmer and Ryan, 1992; Bol et al., 1990). Therefore, the possibility that individual cells may respond independently to high temperature stress can not be ruled out.

The use of the promoter-reporter system has provided valuable information regarding the spatial and temporal expression of hsp70(LP19) gene promoter in plants. The unique expression of the transgene in petals coincided with pigment synthesis further indicates a possible role for the hsp70(LP19) gene product during anthocyanin synthesis. Finally, the absence of any systemic response to heat shock is another important discovery which will help to understand the means by which temperature increase and other stresses are perceived.

Chapter 5

Changes in expression of polyphenol oxidase (PPO) and other genes upon wounding

5.1 Results

5.1.1 Induction of PPO activity in pea leaf tissue by mechanical wounding

Local and systemic induction of PPO activity upon wounding has been demonstrated in tomato and potato plants (Constable et al., 1995; Thipyapong et al., 1995). Similar induction of PPO enzyme activity was examined in pea (*Pisum sativum*) after wounding in both wounded as well as distal unwounded leaves. Leaflets at lower nodes were mechanically wounded by crushing with pliers at several points and through the main midrib, and PPO activity was determined in both wounded and upper unwounded leaflets. An increase in PPO activity was detected in the wounded leaflets 12 hrs after wounding whereas no activity was detected in the upper unwounded leaflets. The highest levels of induced activity were reached 48 hrs after wounding when it was approximately 50-fold higher than in control unwounded plants. After 48 hrs, the activity began to decline (figure 5.1A). A similar pattern of PPO activity was observed in chickpea (*Cicer arietinum*) leaflets treated in a similar manner (figure 5.1B).

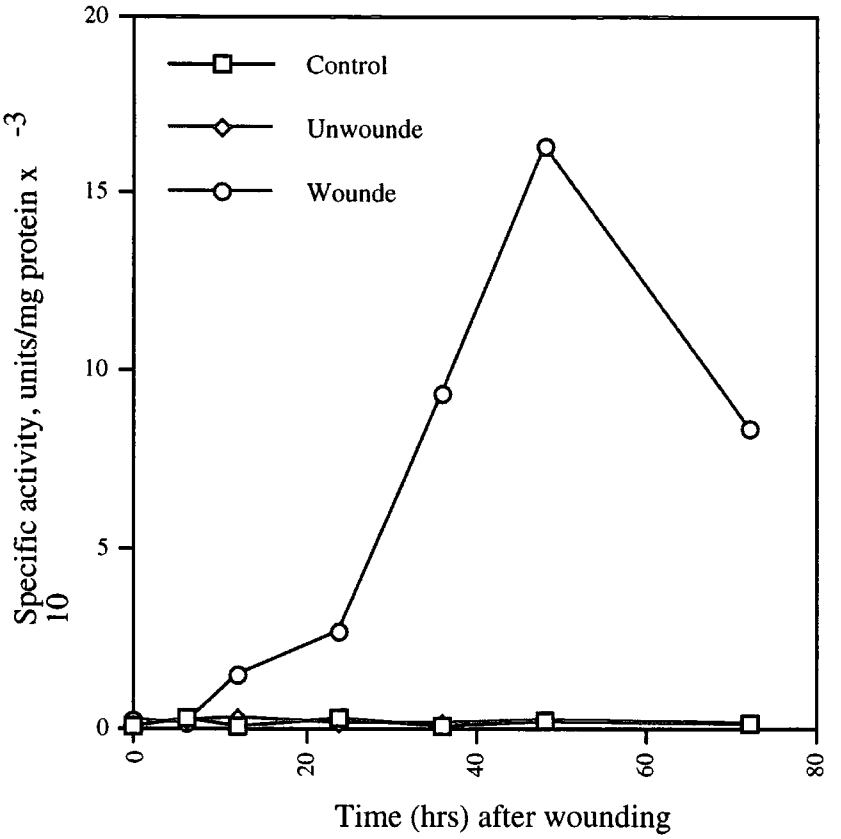
In a second experiment, PPO activity was analysed in the wounded leaflet and the adjacent opposite unwounded leaflet of the same leaf on the same plant.. Again, a significant increase in PPO activity was observed in the wounded leaflets 12 hrs after wounding and continued to increase during the next 36 hrs. No activity could be detected in the unwounded opposite leaflets and control plants (figure 5.2 A). These results demonstrate that PPO activity is only wound-inducible locally, not systemically, in pea and chickpea.

Figure 5.1 Analysis of PPO activity upon wounding of lower leaves.

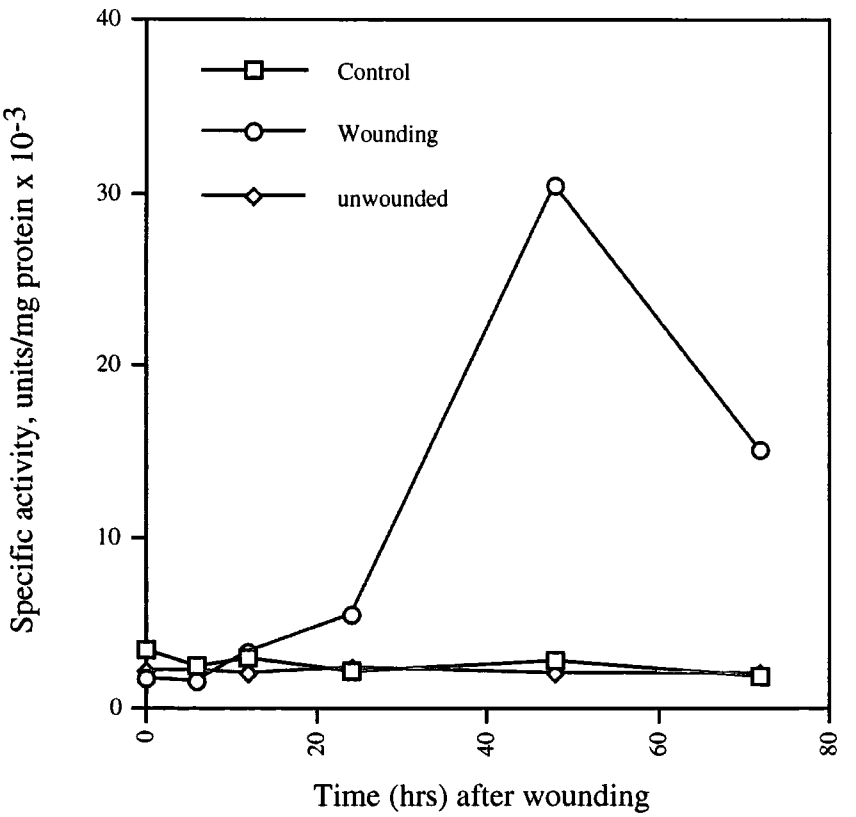
A Induction of PPO activity in pea leaves after wounding of the lower half of the plant and activity was measured in wounded and in unwounded distal leaves and control unwounded plants. Each point represents the mean of 3 assays.

B Induction of PPO activity in chickpea leaves after wounding in the same way as in pea.

(A)


Control
Unwounde
Wounde
Specific activity, units/mg protein x 10 -3
0
5
10
15
20
0
20
40
60
80
Time (hrs) after wounding

(B)


Control
Wounding
unwounded
Specific activity, units/mg protein x 10-3
0
10
20
30
40
0
20
40
60
80
Time (hrs) after wounding

5.1.2 Induction of PPO activity in pea leaves in response to methyl jasmonate

Methyl jasmonate (MeJA) is a component of the wound-inducible octadecanoid signal transduction pathway (Farmer and Ryan, 1992) and is a potent inducer of proteinase inhibitors systemically, (Farmer and Ryan, 1990) and PPO in tomato (Constable et al., 1995). Therefore, MeJa was tested for its ability to induce PPO activity in pea leaves. The pea plants were subjected to continuous exposure to MeJa vapours for 48 hrs. The leaf tissues were collected 24 and 48 hrs after exposure and extracts were assayed for PPO activity. No induction of PPO activity was found both in MeJA exposed and control plants (figure 5.2 B). This is in complete contrast with the case of PPO activity in tomato plants (Constable et al., 1995).

5.1.3 Construction of cDNA library from wounded pea leaf tissue

The results of the biochemical studies mentioned in the preceding section indicate that PPO in pea is wound-induced. Therefore, in order to isolate wound-induced polyphenol oxidase cDNA clones, a cDNA library was constructed from poly $(A)^+$ RNA isolated from pea leaf tissues after wounding. Poly $(A)^+$ RNA, used to construct the cDNA library, was isolated 48 hrs after wounding, since maximum PPO activity occurs at this time point (figure 5.1). This strategy should result in the isolation of cDNA clones corresponding not only to those genes with induction maxima around 48 hrs, but also to genes that are induced earlier and are still expressed, even at a lower level, 48 hrs after wounding.

5.1.4 Isolation of Pea Polyphenol oxidase cDNA clone

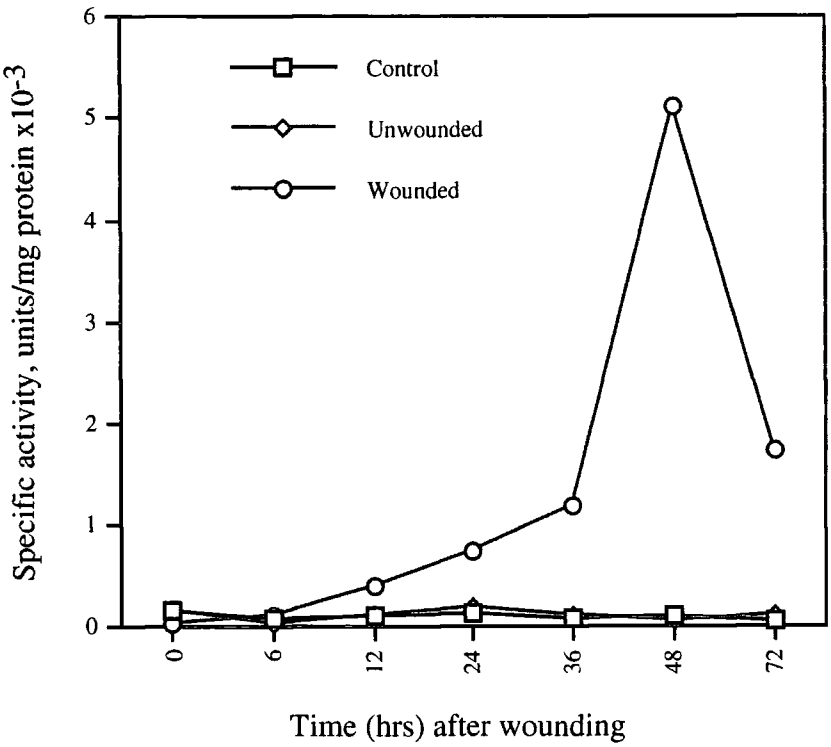
A PCR approach was used to isolate the PPO cDNA clone from the wounded pea leaf cDNA library. All the published DNA sequences for PPO genes from different plants showed the presence of two conserved copper-binding sites. Therefore, PCR primers corresponding to these sites from *Vicia faba* PPO were synthesised (Cary et al., 1992), and used to amplify the cDNA library in order to isolate the pea PPO cDNA clone. This

Figure 5.2 Analysis of PPO activity upon wounding of opposite leaflets and methyl jasmonate treatment.

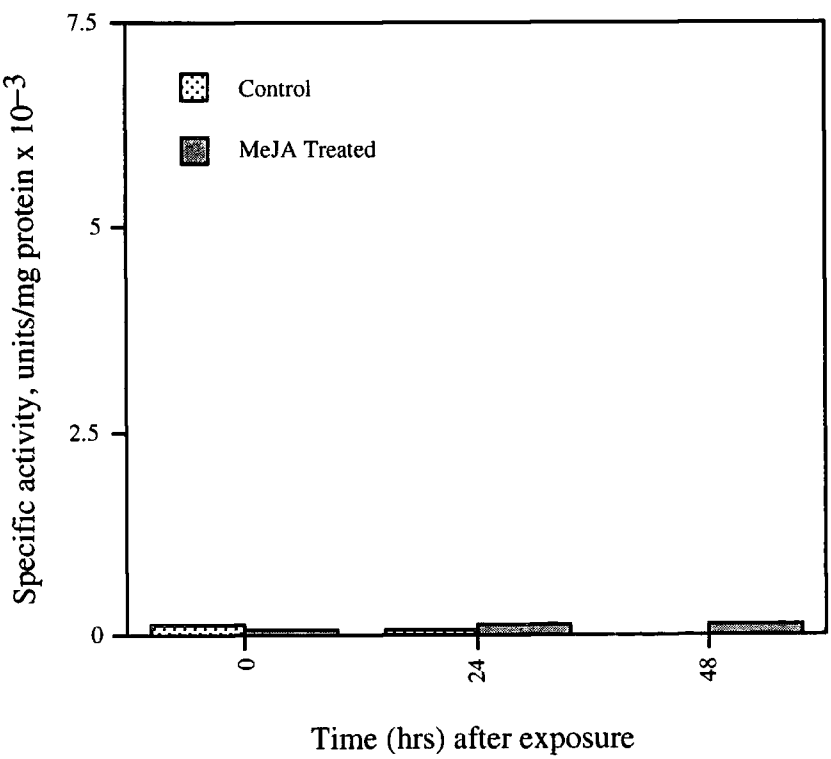
A Induction of PPO activity in pea leaves after wounding of the opposite leaflets and activity was measured in wounded and in opposite unwounded leaflets and control unwounded plants. Each point represents the mean of 3 assays.

B Induction of PPO activity in pea leaves in response to methyl jasmonate vapours.

(A)


Specific activity, units/mg protein x10-3
6
5
4
3
2
1
0
Control
Unwounded
Wounded
0
6
12
24
36
48
72
Time (hrs) after wounding

(B)


Specific activity, units/mg protein x 10−3
7.5
5
2.5
0
Control
MeJA Treated
0
24
48
Time (hrs) after exposure

strategy failed to amplify any cDNA clone corresponding to PPO, however, some non-specific amplification was observed. When these non specific amplified bands were purified and sequenced, no sequences matching to PPO sequence were found.

5.1.5 Construction of pea subtracted cDNA library

Following the unsuccessful attempt to isolate a cDNA encoding PPO from pea by a PCR approach, it was decided to construct a subtracted cDNA library to isolate the maximum number of the wound-induced mRNA transcripts along with PPO. The subtracted cDNA library was constructed by the PCR-Select™ Subtraction Hybridisation method (see section 2.2.19.1), using 2 μg poly $(A)^+$ RNA isolated from pea leaf tissue 48 hrs after wounding. After the subtractive enrichment and selective amplification, the subtracted cDNAs were cloned into PCR cloning vectors pGEM-T and/or PCR Script™ Amp SK(+) and transformed into *Epicurian coli* strain XL1-blue. Resultant white colonies were picked, grown up, and plasmid DNA was purified from them. The size of the inserts were estimated by agarose gel electrophoresis. The majority of the clones analysed had inserts between 200 and 600 bp. The small insert size was expected due to the initial digestion of cDNA with *Rsa*1 prior to adaptor ligation during subtraction (see section 2.2.19.1.).

5.1.6 Sequence analysis of subtracted cDNA clones

To identify the cDNA clones, the inserts of 21 clones were sequenced from the 5' end using an automated sequencer. Some of the cDNAs were also sequenced from the 3' end. DNA sequence data from each cDNA was corrected manually to optimise open reading frames (ORFs), using "Sequencher 3.0" (Genecodes Corp.), a sequence analysis computer software programme. The sequences obtained and the deduced amino acid sequences from them were compared to DNA and protein sequences in the EMBL/ Gene bank databases using the BLAST database search analysis. Of the 21 cDNA clones sequenced, 16 showed significant similarity to sequences within the database. Significance of homology detected was assessed by using the BLAST Smallest Sum Probability factor. Homologies with smallest sum probabilities of $>10^{-8}$ after optimisation were not considered significant. The results are summarised in table 5.1.

Table 5.1 Summary of pea subtracted cDNA clones

Clone Number	Size(bp)	Best Homology Match	Species of best match	Accession Number	Probability Factor	Inducibility (From literature)
SC3	340	S-methyl transferase (EC 2.1.1.14)	*Madagascar periwinkle*	S57636	$1.1e^{-46}$	Wounding* (upregulated)
SC7	1200	Cytochrome P450	*Arabidopsis thaliana*	U69134	$5.0e^{-39}$	Wounding*
SC9	230	HRGP	*Phaseolus vulgaris*	U18791	$7.3e^{-33}$	Wounding
SC10	229	SbHRGP3	*Glycine max*	U44838	$1.7e^{-39}$	Wounding
SC12	600	LEA76 homologue type 2 protein	*Arabidopsis thaliana*	X91912	$8.7e^{-13}$	Wounding*
SC14	230	SbHRGP3	*Glycine max*	U44838	$6.0e^{-40}$	Wounding
SC15	483	Tyrosine decarboxylase-1 (EC4.1.1.25)	*Papaver somniferum*	A55066	$5.0e^{-25}$	Pathogen
SC19	700	Chlorophyll a/b binding protein	*Petunia alba*	P13869	$1.3e^{-15}$	not known
SC20	230	Nodulin lectin	*Pisum sativum*	U31981	$8.0e^{-22}$	Wounding
SC22	367	Glycine-rich protein (GRPs)	*Dacos carota*	X58146	$2.1e^{-16}$	Wounding
SC24	240	Proline-rich protein (PRPs)	*Medicago sativa*	X99099	$1.5e^{-24}$	Wounding
SC33	230	No homology at all	———	———	———	Wounding*

SC34	331	No significant homology	————	———	———	not known
SC40	355	Seed maturation proteins	*Glycine max*	S19831	$5.0e^{-25}$	Wounding
SC51	427	RNA Helicase	*Arabidopsis thaliana*	X98130	$2.5e^{-19}$	not known
SC52	296	No significant homology	————	———	———	not known
SC57	165	Chitinase(EC3.2.1.14) / Lysozyme (EC3.2.1.17)	*Nicotiana tabacum*	S51591	$2.4e^{-08}$	Wounding*
SC58	214	Short chain alcohol dehydrogenase	*Zea mays*	A47542	$2.1e^{-14}$	Wounding*
SC59	235	No significant homology	————	———	———	not known
SC62	130	No significant homology	————	———	———	not known

* represents the wound-induced cDNA clones tested by Northern blotting.

5.1.7 Northern analysis of subtracted cDNA clones

To assess the effectiveness of the subtraction, the expression level of the mRNAs represented by the subtracted cDNA clones in wounded and unwounded pea leaves was analysed. A time course induction of mRNA transcripts upon wounding was followed. 10 μg RNA isolated from pea leaves at different time points after wounding and control non-wounded leaves was electrophoresed and blotted to nitrocellulose filters. The RNA was hybridised to the ^{32}P-labelled DNA of the different subtracted cDNA clones. For subtracted cDNA clones SC7, SC57 and SC58, RNA from leaves 6, 12, 24, 48 and 72 hrs after wounding was analysed; the probes were nearly the full length cDNAs isolated from screening of the cDNA library from wounded leaf. For the rest of the clones, RNA from leaves 24 and 48 hrs after wounding and control was analysed; the probes were the actual subtracted cDNA as shown in table 5.1. Representative results are presented here.

SC3

For clone SC3 (S-methyl transferase), a 3.34 kb mRNA was detected that hybridised with the probe both in control and wounded leaves. The level of the mRNA was increased almost 3-4 times within 24 hrs after wounding, indicating that the expression level of the corresponding gene is upregulated by wounding (figure 5.3 A).

SC12

In the case of clone SC12 (LEA76 homologue), the probe only hybridised to a 1.18 kb transcript with RNA from wounded leaves. The intensity of hybridisation was higher after 48 hrs of wounding (figure 5.3 B). Background hybridisation with 18S rRNA was observed in all RNA samples.

SC33

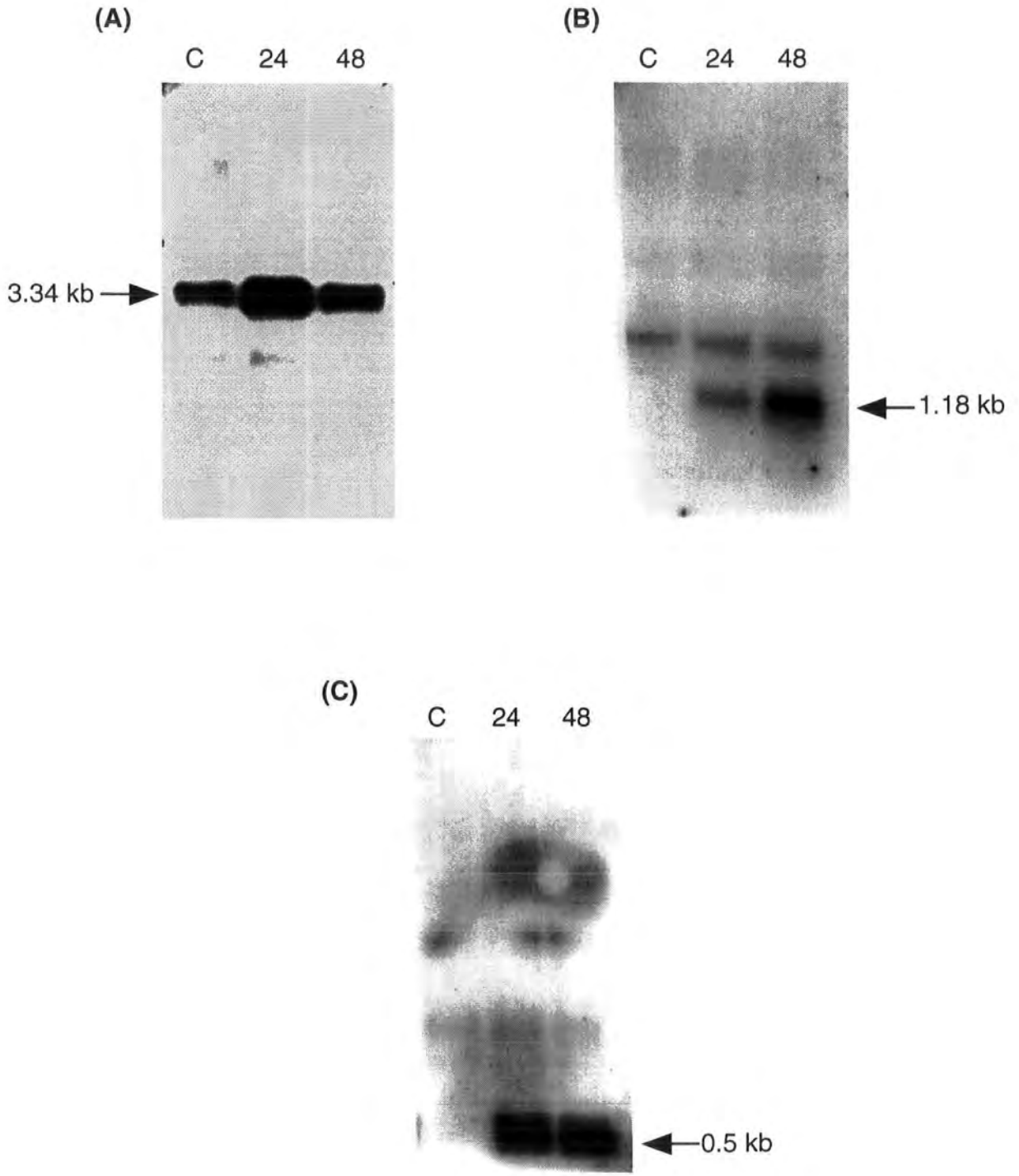
The mRNA corresponding to clone SC33 (no significant homology to sequences in database) was undetectable in control plants but was strongly induced by wounding.

Figure 5.3 Northern analysis of subtracted cDNA clones SC3, SC12, and SC33.

(𝔸) Northern analysis of the induction of SC3 gene upon wounding. Total RNA was isolated from leaves 24 hrs (lane 2) and 48 hrs (lane 3) after wounding and unwounded control (C, lane 1) plants. Each lane was loaded with 10 μg of total RNA and hybridised with ^{32}P-labelled SC3 cDNA (340 bp). Filter was washed to a final stringency of 0.1x SSC, 0.1% SDS at 45 °C for 30 minutes.

(𝔹) Northern analysis of the induction of SC12 gene upon wounding. Northern blotting and hybridisation conditions were same as in the figure legend (𝔸). Filter was hybridised with ^{32}P-labelled SC12 cDNA (600 bp).

(C) Northern analysis of the induction of SC33 gene upon wounding. Northern blotting and hybridisation conditions were same as in the figure legend (𝔸). Filter was hybridised with ^{32}P-labelled SC33 cDNA (230 bp).


(A)
C
24
48
3.34 kb
(B)
C
24
48
1.18 kb
(C)
C
24
48
0.5 kb

The probe hybridised with a small mRNA of 0.5 kb, and the intensity of hybridisation was similar with mRNA from both 24 and 48 hrs after wounding, indicating that induction of the transcript started early (figure 5.3 C).

SC7

Hybridisation to a single 2.4 kb transcript was detected when total RNA from wounded and control unwounded leaves, at different time points after wounding, was probed with the SC7 clone encoding proteins for a cytochrome P450. This transcript is expressed in control plants at a very low level and upon wounding it was significantly increased to its peak expression within 24 hrs after wounding. This induction was transient and subsequently decreased to control levels within 72 hrs after wounding (figure 5.4 A).

SC57

The expression analysis of the SC57 clone (1291 bp) encoding a chitinase/lysozyme protein, revealed an mRNA of 1.5 kb which was induced only in wounded tissues. The expression was detected as early as 6 hrs after wounding with a maximum level of expression at 12 hrs after wounding. This mRNA was present at elevated levels up to 72 hrs after wounding. (figure 5.4 B). No hybridisation was observed in control plants.

SC58

Northern analysis of RNA from wounded leaf tissue probed with SC58 cDNA (Nonmetallo-short chain alcohol dehydrogenase), identified a transcript of 1.68 kb (figure 5.4 C). The transcript was detected in SC58 mRNA as early as 12 hrs after wounding and accumulated to a highest level 48 hrs after wounding. There was no detectable message in RNA isolated from control non-wounded plants.

Figure 5.4 Northern analysis of subtracted cDNA clones SC7, SC57, and SC58.

(A) Northern analysis of the induction of SC7 gene upon wounding. Pea plants grown in soil under normal conditions were subjected to mechanical wounding. Total RNA was isolated from leaves at 6, 12, 24, 48 and 72 hrs after wounding and from unwounded control plants. Each lane was loaded with 10 μg of total RNA and hybridised with ^{32}P-labelled SC7 cDNA (1.7 kb). Filter was washed to a final stringency of 1x SSC, 0.1% SDS at 45°C for 1 hr. The number above each lane indicate the duration (hrs) after wounding and C represent the control unwounded.

(B) Northern analysis of the induction of SC57 gene upon wounding. Northern blotting, hybridisation and washing conditions were same as in the figure legend **(A)**. Filter was hybridised with ^{32}P-labelled SC57 cDNA (1291 bp).

(C) Northern analysis of SC58 gene upon wounding. Northern blotting, hybridisation and washing conditions were same as in the figure legend **(A)**. Filter was hybridised with ^{32}P-labelled SC58 cDNA (1291 bp).

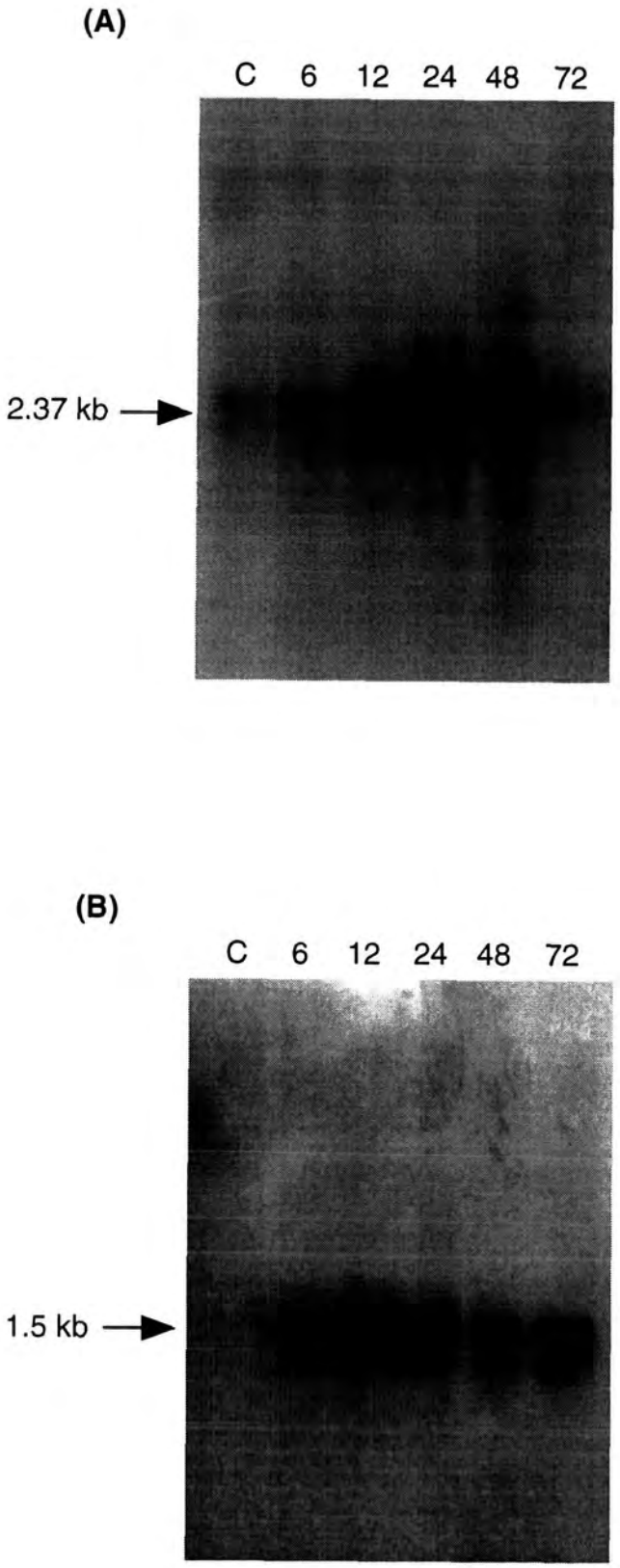
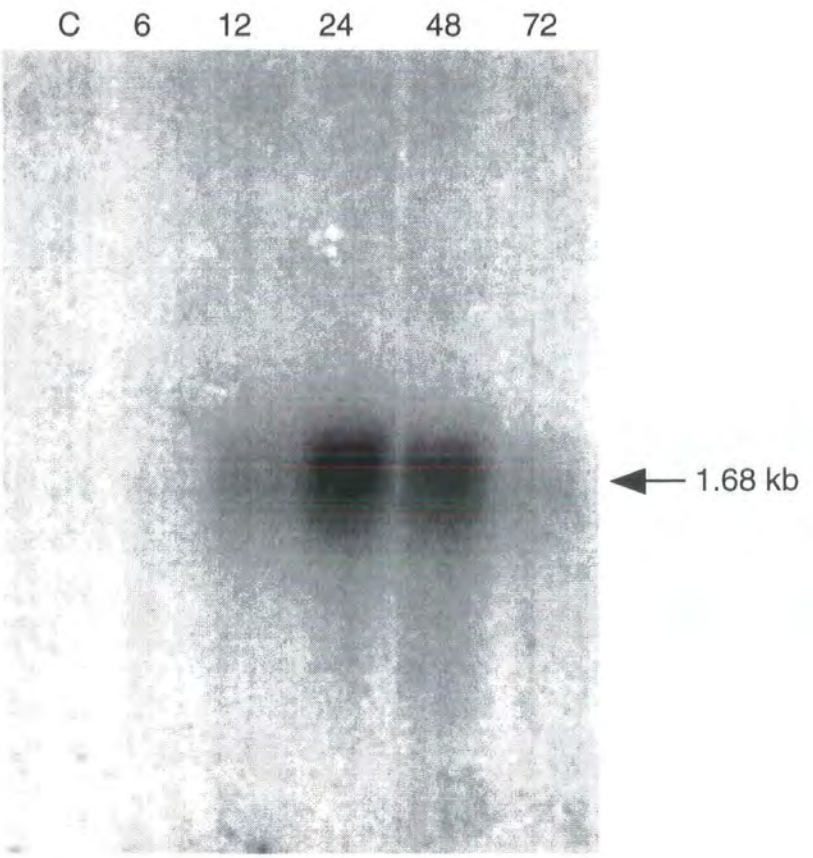


figure continued

(C)



5.1.8 Sequence analysis of PsSC10 clone

The 230 bp long subtracted cDNA clone SC10, encoding hydroxyproline-rich glycoproteins (HRGPs), was used as probe to pull out a full length cDNA from the cDNA library. The resultant clone, designated PsSC10 (PsSC stands for *Pisum sativum* subtracted clone), was sequenced fully from both ends. The sequenced insert comprised of a cDNA of 1285 bp, which contained an open reading frame (ORF) of 999 bp, encoding a putative polypeptide of 332 amino acid residues. The putative 3' untranslated region (UTR) of 268 bp and 18 bp poly (A) tail contained a single motif 'AATAAA' at nucleotide 1248–1253, corresponding exactly to the consensus eukaryotic polyadenylation signal (Proudfoot and Brownlee, 1976). (figure 5.5).

The homology search of the whole polypeptide sequence predicted by cDNA clone PsSC10, revealed significant homology with hydroxyproline-rich glycoprotein (HRGP) commonly called extensins. The highest homologies were with *Phaseolus vulgaris* HRGP (73%; Corbin et al., 1987), soybean SbHRGP3 (70%; Ahn et al., 1996), carrot extensin (59%; Chen and Varner, 1985), and tomato class 1 extensin (57%; Zhou et al., 1992). Sequence comparison of PsSC10 with other extensins indicated that PsSC10 has 8 amino acids truncation at the 5' end and is not a full length clone.

The PsSC10 amino acid sequence includes a hydrophobic signal peptide at the N-terminus. The signal peptide and the N-terminus of the putative mature protein also shared regions of high homology particularly with soybean and *Phaseolus vulgaris* extensins (figure 5.6 A). The hydrophobic nature of the signal peptide is characteristic for translocation of the protein into endoplasmic reticulum. The signal peptide cleavage site for PsSC10 can be predicted, by the consensus rule of Von Heijne (1986), to occur either between amino acid 21 and 22, or between 26 and 27. On the basis of homology with other extensin family members (figure 5.6 A), the cleavage site is predicted to be located between amino acid 26 and 27. The deduced amino acid sequence has pentapeptide repeats 'SPPPP' or its degenerate forms which are characteristic of extensin proteins. PsSC10 extensin, like its two similar counterparts in soybean and *Phaseolus vulgaris*, has two major types of repeats in the N-terminal region of the protein : 'SPPPPVHSPPPPYHYS/N' and ' SPPPPVYKYK/P' (fig 5.6B). In the

Figure 5.5 The nucleotide and deduced amino acid sequences of the PsSC10 cDNA. The nucleotide sequence is numbered from the 5' end on the left of each line. The amino acid sequence of a putative coding region is shown below the nucleotide sequences in the single-letter code. The asterisk indicates the stop codon and the single polyadenylation site is underlined.

```
ATTGCATTCACATTACTTTCTCTCACTTTGCCATCTCAAGCAAACAACTACATCTATTCATCTCCACCACCACCGGTGCACTCACCTCCTCCACCTTATC  100
 I  A  F  T  L  L  S  L  T  L  P  S  Q  A  N  N  Y  I  Y  S  S  P  P  P  P  V  H  S  P  P  P  P  Y

ATTATAGTTCACCACCACCACCAGTACACTCACCTCCTCCACCTTATCATTACAATTCACCACCACCACCGTCAGAGAAGCCATACAAATATCCTTCTCC  200
H  Y  S  S  P  P  P  P  V  H  S  P  P  P  P  Y  H  Y  N  S  P  P  P  P  S  E  K  P  Y  K  Y  P  S  P

TCCACCACCAGTTTACAAATACAAGTCACCACCTCCACCGGTATACAATTCACCTCCTCCACCATATAAGTATCCATCTCCACCGCCTCCTCCATACAAA  300
  P  P  P  V  Y  K  Y  K  S  P  P  P  P  V  Y  N  S  P  P  P  P  Y  K  Y  P  S  P  P  P  P  P  Y  K

TATCCATCACCACCACCACCTGTTTACAAGTACAACTCACCTCCTCCTCCTTACAAATATATATCTCCTCCTCCAACACCAATTTATAAATATAAGTCTC  400
 Y  P  S  P  P  P  P  V  Y  K  Y  N  S  P  P  P  P  Y  K  Y  I  S  P  P  P  T  P  I  Y  K  Y  K  S

CTCCTCCACCTGTCTATGCACCACCAATTTACAAATATAAATCTCCTCCTCCACCGGTTTATTCACCACCGGTTTACAAATATAAGTCTCCTCCTCCACC  500
P  P  P  P  V  Y  A  P  P  I  Y  K  Y  K  S  P  P  P  P  V  Y  S  P  P  V  Y  K  Y  K  S  P  P  P  P

TGTCTATTCACCACCAATTTACAAATATAAATCTCCTCCTCCACCGGTTTATTCACCACCACCAGTTTACAAATATAAGTCTCCTCCTCCACCTGTCTAT  600
  V  Y  S  P  P  I  Y  K  Y  K  S  P  P  P  P  V  Y  S  P  P  P  V  Y  K  Y  K  S  P  P  P  P  V  Y

GCACCACCAATTTACAAATATAAATCTCCTCCTCCACCGGTTTATTCACCACCGGTTTACAAATATAAGTCTCCTCCTCCACCTGTCTATTCACCACCAA  700
 A  P  P  I  Y  K  Y  K  S  P  P  P  P  V  Y  S  P  P  V  Y  K  Y  K  S  P  P  P  P  V  Y  S  P  P

TTTACAAATATAAATCTCCTCCTCCACCGGTTTATTCACCACCACCAGTTTACAAATATAAGTCTCCTCCTCCACCTGTCTATTCACCACCAATTTACAA  800
I  Y  K  Y  K  S  P  P  P  P  V  Y  S  P  P  P  V  Y  K  Y  K  S  P  P  P  P  V  Y  S  P  P  I  Y  K

ATATAAATCTTCTCCTCCACCGATTTATTCACCACCACCAGTTTACAAATATAAATCTCCTCCTCCACCAGTTTACTCGCCACCACCCATTTACAAATAT  900
  Y  K  S  S  P  P  P  I  Y  S  P  P  P  V  Y  K  Y  K  S  P  P  P  P  V  Y  S  P  P  P  I  Y  K  Y

AAGTCTCCTCCTCCACCTGTTTACTCATCACCTCCACCGGTGTATTCCCCTCCTCCACCACATTACATTTACGCATCACCTCCTCCACCATACCACTAAT 1000
 K  S  P  P  P  P  V  Y  S  S  P  P  P  V  Y  S  P  P  P  P  H  Y  I  Y  A  S  P  P  P  P  Y  H  *>.

AGATACTGGTTTATAGTAGATCCCTTGGATTTTAGGTATATATTGGAAATAAGCAGGAAGATGCATTGGCAATAATACAAGGAAGCAAAGTTTCAACGTA 1100

GTTTGTTTCAAGAAAAGCCACTCTTTTTATCTCATGTGTTTTGCTATTCTCATTGTTAGTTTGTTTGGTTTTTGAACTTAGACAGTTCTAAGGCATGTGT 1200

GTAATGTATTTTGCAACCTACTCGTTTGAAGGGATCACCAAAATTAAATAAAATATTACGTTCTACTAAAAAAAAAAAAAAAAAA
```

Figure 5.6 Putative signal peptide sequence homology and deduced amino acid sequence of PsSC10.

A. Homology between leader sequences of HRGP extensin polypeptides. The leader sequence and first six amino acid of the putative coding sequence of PsSc10 were compared to sequences in a composite protein sequence database (SWISS-PROT database) using CLUSTAL W (1.7) homology search programme at ExPASy molecular biology server home page. Conserved amino acid residues are indicated by (*) and similar amino acid residues by (:). Dashes (–) indicate the gaps introduced to maximise alignment.

B. Deduced amino acid sequence of PsSC10 arranged to show its Ser-Hyp repeats and linker amino acids.

A

```
PSSC10                    -IAFTLLSLTLPSQ--A:NNYIYS
PvHRGP        MGFPMASLILSTLALTLISL-FPSQTLA:DNYIYS
SbHRGP3       MGSPMASLTL-TIALTIISLTLPSQTLA:DNYIYS
                         :*:*::** :***  * :*****
```

B Ser-Hyp repeats with linkers

IAFTLLSLTLPSQANNYIYS - Putative signal peptide

```
SPPPPVHSPPPPYHYS
SPPPPVHSPPPPYHYN
SPPPP  SEKP YKYP
SPPPPVY      KYK
SPPPPVY        N
SPPPP       YKYP
SPPPPP      YKYP
SPPPPVY      KYKN
SPPPP       YKYI
SPPP     TPIYKYK
SPPPPVYAPP IYKYK
SPPPPVYSPP VYKYN
SPPPPVYSPP IYKYK
SPPPPVYSPPPVYKYK
SPPPPVYAPP IYKYK
SPPPPVYSPP VYKYK
SPPPPVYSPP IYKYK
SPPPPVYSPPPVYKYK
SPPPPVYSPP IYKYKS
SPPP IYSPPPVYKYK
SPPPPVYSPPPIYKYK
SPPPPVYS
SPPPPVY
SPPPP      HYIYA
SPPPP       YH
```

PsSC10 extensin repeats, valine is present at the 6th position, whereas tyrosine is found in this position in soybean or *Phaseolus vulgaris* extensins. In the C-terminal region a single major repeat 'SPPPPVYS/APPV/IYKYK' is present. In the amino acid sequence, at two sites, Ala-Hyp_2 motifs were present. The presence of alanine-hydroxyproline motifs is a characteristic of monocot extensins.

5.1.9 Sequence analysis of PsSC15 clone

The 483 bp subtracted cDNA clone SC15, shown to encode tyrosine decarboxylase (TyDC), was used to screen the cDNA library in order to isolate the full length clone. The insert in the isolated clone, designated PsSC15, was sequenced completely. The insert fragment was 1578 bp long, including a 3' UTR of 198 bp, plus a poly (A) tail of 29 bp long. It contained an "ORF" of 1348 bp, encoding a putative polypeptide of 442 amino acid residues (figure 5.7). The consensus eukaryotic polyadenylation signal 'AATAAA' was not found; the best matches 'AATATT' and 'AATATA', were present at positions 1419–1423 and 1533–1538 bp.

Interestingly, clone PsSC15 contained three internal premature TAA translation termination codons in the anticipated "ORF" at nucleotide positions 182–184, 377–379, and 419–421. Subsequent to these three premature stop codons, the ORF continues to a termination codon in a position similar to that find in *P.somniferum* TyDC1 (Facchini et al., 1994); and Parsley TyDC2 (Kawalleck et al., 1993) (figure 5.9). To remove the presence of any ambiguity, sequences were overlapped by using the restriction fragments to make a series of subclones. The restriction map and sequencing strategy are shown in figure 5.8. The overlapped sequences still had the three internal stop codons and thus the mRNA corresponding to PsSC15 could not encode a functional protein. A premature translation stop codon has also been reported in the TyDC4 gene of *P.somniferum* by Facchini et al., 1994.

The polypeptide was found to be homologous to tyrosine decarboxylase proteins, which have been assigned a role in pathogen resistance (Kawalleck et al., 1993). Sequence alignment of the protein encoded by PsSC15 cDNA, with known aromatic amino acid decarboxylases showed 45% identity to TyDC1 from *P.somniferum*

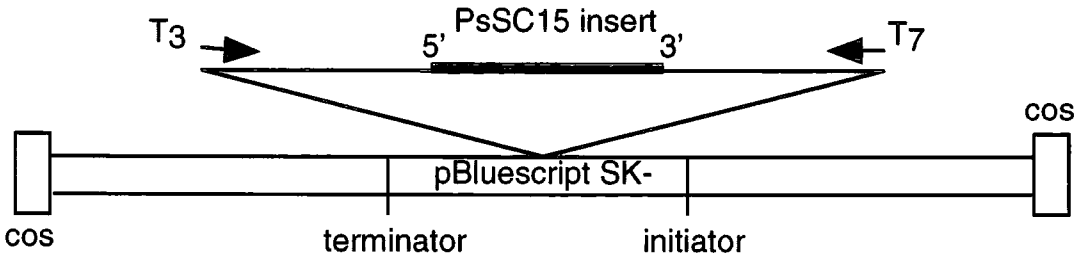
Figure 5.7 The nucleotide and deduced amino acid sequences of the PsSC15 cDNA. The nucleotide sequence is numbered from the 5' end on the left of each line. The amino acid sequence of a putative coding region is shown below the nucleotide sequences in the single-letter code. The asterisk indicates the stop codon and the putative polyadenylation signals are underlined.

```
AAAAATCAAATCAACAAGAATCATGAATCCTCTAGACCTTGAAGAATTCAAAATACAAGGCTACATGATGATTGGTTTTCTCACAGATTACTACAAAAAC   100
  K  I  K  S  T  R  I  M  N  P  L  D  L  E  E  F  K  I  Q  G  Y  M  M  I  G  F  L  T  D  Y  Y  K  N

ATTGAAAACTATCCTGTTTTAAGCAAAGTAGAACCTGGTTACCTAGAAAAAATACTACCATCTTCACCTCTGTTTCAACCATAATCCATTGAATCAATAC   200
  I  E  N  Y  P  V  L  S  K  V  E  P  G  Y  L  E  K  I  L  P  S  S  P  L  F  Q  P  *  S  I  E  S  I

ATGAAGATGTTCAACAACATATAATCCCTGGAATCACATATTGGGTACAACATGTGAGGCTATTTTGTGAAGATTGGTTGCTACAAGAGATGAAAATCTT   300
H  E  D  V  Q  Q  H  I  I  P  G  I  T  Y  W  V  Q  H  V  R  L  F  C  E  D  W  L  L  Q  E  M  K  I  F

TCCCAAATTGGAAAAGAAAATATTGGAAAACTTGTTGTGTATTGTTCTGATCAAACACATAGTGCACTTCAAAAAGTAACTCAAATTGTTGGAATTCATG   400
  P  K  L  E  K  K  I  L  E  N  L  L  C  I  V  L  I  K  H  I  V  H  F  K  K  *  L  K  L  L  E  F  M

CACAAAATTTTAGAGTCATAAAAACTAAAGGGTCCAATTTTTTTTAATTTGTCTCATGAATCTTTTCTTTCTACCATTCTTTTAGATGTTGAAAATGGTT   500
  H  K  I  L  E  S  *  K  L  K  G  P  I  F  F  N  L  S  H  E  S  F  L  S  T  I  L  L  D  V  E  N  G

TGGTTCCTTGTTACTTATGTGCAACGGATGGCACCACCGCAACAAATGTTATTGATCCTATAAAGTTATTGTGTAATATGGCAATTGAGTATGATATTTG   600
L  V  P  C  Y  L  C  A  T  D  G  T  T  A  T  N  V  I  D  P  I  K  L  L  C  N  M  A  I  E  Y  D  I  W

GGTTCATGTTGATGCTGCTTATGCAGGTTCAACTTGTATTTGTTCTGAATATAGACATTACATTGATGGAATTGAAGATGTAAATTCTTTTAGTTTTAAT   700
  V  H  V  D  A  A  Y  A  G  S  T  C  I  C  S  E  Y  R  H  Y  I  D  G  I  E  D  V  N  S  F  S  F  N

TCTCATAAATGATTTTTGACTAACTTAGCTTGTTGTTGTCTTTGGGTTAAAGATCTCACTGCTTTGACAAAATCACTATCAACATATCCTGAATTCTTGC   800
  S  H  K  *  F  L  T  N  L  A  C  C  C  L  W  V  K  D  L  T  A  L  T  K  S  L  S  T  Y  P  E  F  L

AGAACATTAATTCTGATTTAAAGGAAGTGATTAATTACAAAGATTGGCAAATACCCTTGAGTAGGAAATTTAATTCACTCAAACTATGGATTGTTATTCG   900
Q  N  I  N  S  D  L  K  E  V  I  N  Y  K  D  W  Q  I  P  L  S  R  K  F  N  S  L  K  L  W  I  V  I  R

AAGCTACGGCGTTGAAAATCTCAAGAAGTTTCTGAGGAATCATGTGGAAATGGCTAAAACATTTGAAGAACTGGTGAGGAAAAATGAGAGGTTTGAAATT  1000
  S  Y  G  V  E  N  L  K  K  F  L  R  N  H  V  E  M  A  K  T  F  E  E  L  V  R  K  N  E  R  F  E  I

ATTGTGCCGACAAGATTCGCTTTGGTTTTCTTTAGGATTTCTCCATCAGCAATTAACATTGATAATGGTAGTGAAGATTGTTATTATATTGGAAAAAAGA  1100
  I  V  P  T  R  F  A  L  V  F  F  R  I  S  P  S  A  I  N  I  D  N  G  S  E  D  C  Y  Y  I  G  K  K

TGAATCATGGATATTTGATGAATGAAGTGGATCGTAAATTGCTAGATTCAGTTAATGGTTATGGTAAGGCTTACATTACTTATTTTGAGGTTGATGGAGC  1200
M  N  H  G  Y  L  M  N  E  V  D  R  K  L  L  D  S  V  N  G  Y  G  K  A  Y  I  T  Y  F  E  V  D  G  A

TTTTGTTATCAGATGTTCTATTGGTTCAACTTTAACAGAGGAGCATCATGTGAATATGACATGGAAATTGTTGCAAGAACATGCAAGTTTTCTATTAGGT  1300
  F  V  I  R  C  S  I  G  S  T  L  T  E  E  H  H  V  N  M  T  W  K  L  L  Q  E  H  A  S  F  L  L  G

AAAAGATTGGATTTAGAGAACAAGCAAGCTTGTGAAACTCAATCTGGGTGAAGCGTTTCATCTCAAACTGTTCATTTTCTGTCTATTGTCTCTATCTCTG  1400
  K  R  L  D  L  E  N  K  Q  A  C  E  T  Q  S  G  *>.....

TACTCATCAAATGTAGAAAATATTATGTAATTTTCATCTGTTATTGCATCAAGCAAGCGTGTGTATGTGTGTGAGAGGGGGAGAGGGTGTAAGTACTTGT  1500

TATGAAATTTTCTTTGATTGTTACTTAAAAGGAATATATGTGATTAATTAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 5.8 A The cloned cDNA insert, PsSC15, in pBluescript SK- within the Lambda ZAP II vector.

B Physical map and sequencing strategy for cDNA insert, PsSC15, excised in the pBluescript phagemid by co-infection with helper phage. The unique restriction enzyme sites for subcloning are indicated. Filled boxes represent the coding sequence region. The sequenced fragments and the direction of the sequencing are indicated by arrows. Restriction enzyme sites are as follows: Bg : BglII; E : EcoRI; H : HindIII, X : XhoI.

(A)


T3
5'
PsSC15 insert
3'
T7
cos
pBluescript SK-
cos
terminator
initiator

(B)

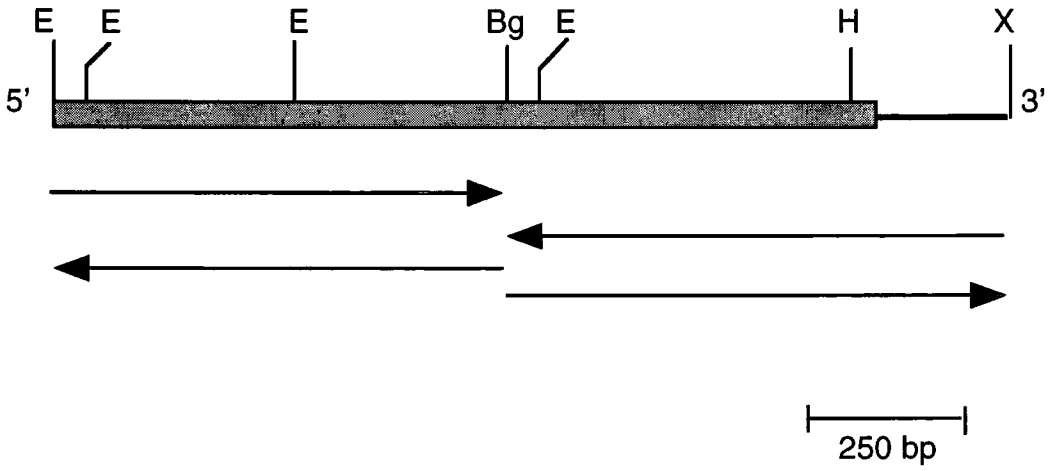
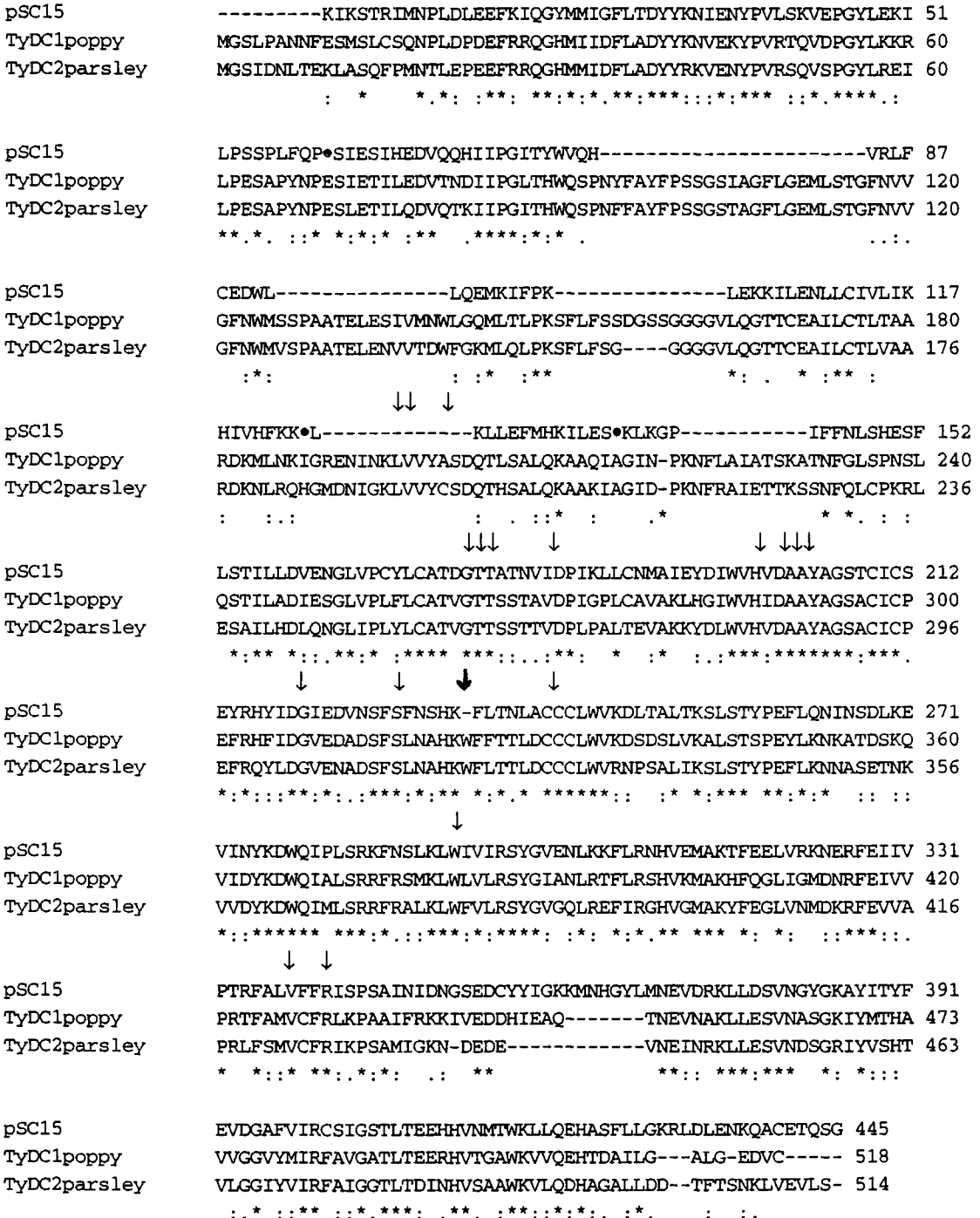

E
E
E
Bg
E
H
X
5'
3'
250 bp

Figure 5.9 Comparison of the deduced amino acid sequences of PsSC15 encoding tyrosine decarboxylase with *P.somniferum* TyDC1 (Facchini et al., 1994), parsley TyDC2 (Kawalleck et al., 1994). Conserved amino acid residues are indicated by (*) and similar amino acid residues by (:). Dashes indicate the gaps introduced to maximise alignment. Conserved amino acid positions found in all pyridoxal phosphate-dependent decarboxylases are marked by vertical arrows. The arrow in bold indicates the putative pyridoxal phosphate-binding site. Solid circles represent the premature internal stop codons.


pSC15 ---------KIKSTRIMNPLDLEEFKIQGYMMIGFLTDYYKNIENYPVLSKVEPGYLEKI 51
TyDC1poppy MGSLPANNFESMSLCSQNPLDPDEFRRQGHMIIDFLADYYKNVEKYPVRTQVDPGYLKKR 60
TyDC2parsley MGSIDNLTEKLASQFPMNTLEPEEFRRQGHMMIDFLADYYRKVENYPVRSQVSPGYLREI 60
pSC15 LPSSPLFQP•SIESIHEDVQQHIIPGITYWVQH----------VRLF 87
TyDC1poppy LPESAPYNPESIETILEDVTNDIIPGLTHWQSPNYFAYFPSSGSIAGFLGEMLSTGFNVV 120
TyDC2parsley LPESAPYNPESLETILQDVQTKIIPGITHWQSPNFFAYFPSSGSTAGFLGEMLSTGFNVV 120
pSC15 CEDWL----------LQEMKIFPK----------LEKKILENLLCIVLIK 117
TyDC1poppy GFNWMSSPAATELESIVMNWLGQMLTLPKSFLFSSDGSSGGGGVLQGTTCEAILCTLTAA 180
TyDC2parsley GFNWMVSPAATELENVVTDWFGKMLQLPKSFLFSG----GGGGVLQGTTCEAILCTLVAA 176
pSC15 HIVHFKK•L----------KLLEFMHKILES•KLKGP----------IFFNLSHESF 152
TyDC1poppy RDKMLNKIGRENINKLVVYASDQTLSALQKAAQIAGIN-PKNFLAIATSKATNFGLSPNSL 240
TyDC2parsley RDKNLRQHGMDNIGKLVVYCSDQTHSALQKAAKIAGID-PKNFRAIETTKSSNFQLCPKRL 236
pSC15 LSTILLDVENGLVPCYLCATDGTTATNVIDPIKLLCNMAIEYDIWVHVDAAYAGSTCICS 212
TyDC1poppy QSTILADIESGLVPLFLCATVGTTSSTAVDPIGPLCAVAKLHGIWVHIDAAYAGSACICP 300
TyDC2parsley ESAILHDLQNGLIPLYLCATVGTTSSTTVDPLPALTEVAKKYDLWVHVDAAYAGSACICP 296
pSC15 EYRHYIDGIEDVNSFSFNSHK-FLTNLACCCLWVKDLTALTKSLSTYPEFLQNINSDLKE 271
TyDC1poppy EFRHFIDGVEDADSFSLNAHKWFFTTLDCCCLWVKDSDSLVKALSTSPEYLKNKATDSKQ 360
TyDC2parsley EFRQYLDGVENADSFSLNAHKWFLTTLDCCCLWVRNPSALIKSLSTYPEFLKNNASETNK 356
pSC15 VINYKDWQIPLSRKFNSLKLWIVIRSYGVENLKKFLRNHVEMAKTFEELVRKNERFEIIV 331
TyDC1poppy VIDYKDWQIALSRRFRSMKLWLVLRSYGIANLRTFLRSHVKMAKHFQGLIGMDNRFEIVV 420
TyDC2parsley VVDYKDWQIMLSRRFRALKLWFVLRSYGVGQLREFIRGHVGMAKYFEGLVNMDKRFEVVA 416
pSC15 PTRFALVFFRISPSAINIDNGSEDCYYIGKKMNHGYLMNEVDRKLLDSVNGYGKAYITYF 391
TyDC1poppy PRTFAMVCFRLKPAAIFRKKIVEDDHIEAQ-------TNEVNAKLLESVNASGKIYMTHA 473
TyDC2parsley PRLFSMVCFRIKPSAMIGKN-DEDE----------VNEINRKLLESVNDSGRIYVSHT 463
pSC15 EVDGAFVIRCSIGSTLTEEHHVNMTWKLLQEHASFLLGKRLDLENKQACETQSG 445
TyDC1poppy VVGGVYMIRFAVGATLTEERHVTGAWKVVQEHTDAILG---ALG-EDVC----- 518
TyDC2parsley VLGGIYVIRFAIGGTLTDINHVSAAWKVLQDHAGALLDD--TFTSNKLVEVLS- 514

(Facchini et al., 1994); 44% identity to TyDC2 from Parsley (Kawalleck et al., 1993); 39% to tryptophan decarboxylase (TrpDC) from *C. roseus* (DeLuca et al., 1989), and 37% to dihydroxyphenylalanine decarboxylase (dopaDC) from *Drosophila melanogaster* (Morgan et al., 1986). The best match homology of PsSC15 with *P.somniferum* TyDC1 and Parsley TyDC2 has been shown in figure 5.9. PsSC15 protein also contains conserved amino acid positions found in all pyridoxal phosphate dependent decarboxylases which are marked in figure 5.9. A putative pyridoxal phosphate-binding site found in all aromatic amino acid decarboxylases was also found in PsSC15. The amino acid sequence comparison with these tyrosine decarboxylases suggested that pSC15 is not a full length cDNA and that its encoded polypeptide has a 9 amino acid truncation at the N-terminal.

5.1.10 Sequence analysis of PsSC57 Clone

A 1295 bp long cDNA clone designated PsSC57 was isolated during the cDNA library screening using the subtracted cDNA clone SC57 (165 bp) which showed high homology with a chitinase/lysozyme on the database. This cDNA clone contained an "ORF" of 1086 bp, encoding a polypeptide of 361 amino acids, 182 bp 3' "UTR" plus a 23 bp poly (A) tail (figure 5.10). The cDNA has two polyadenylation signal sequences 'AATAAA' beginning at nucleotides 1187 and 1229, which are 76 and 34 nucleotides upstream from the poly (A) tail, respectively.

A homology search using the whole polypeptide sequence predicted by PsSC57 revealed significant homology with chitinase/lysozyme enzymes which are involved in plant resistance to pests and pathogens. The highest homologies were with tobacco chitinase class V protein (48.6%; Melcher et al., 1994), and tobacco chitinase/lysozyme Pz cDNA (48.2%; Heitz et al., 1994) (figure 5.11). However, the amino acid sequence alignments are characterised by the presence of blocks of highly conserved regions. The polypeptide predicted by PsSC57 does not have a consensus seven amino acid C-terminal vacuolar targeting peptide. Also the N-terminal signal peptide cleavage site conserved between S_{25} and Q_{26} in the chitinase class V and Pz chitinase/lysozyme (figure 5.11) was not found in the predicted polypeptide encoded by PsSC57. The sequence comparison with these homologous sequences indicated that PsSC57 is not a

Figure 5.10 The nucleotide and deduced amino acid sequences of the PsSC57 cDNA. The nucleotide sequence is numbered from the 5' end on the left of each line. The amino acid sequence of a putative coding region is shown below the nucleotide sequences in the single-letter code. The asterisk indicates the stop codon and the single polyadenylation site is underlined.

```
AAAAAGCATTCATTCCTCCCGATTTCCACACTTCTCATCATTCTCCAACTACAATTCTCCTCCACAAACGCTGCCGTTAAAGGTGGCTATTGGTATTCTG  100
 K  K  H  S  F  L  P  I  S  T  L  L  I  I  L  Q  L  Q  F  S  S  T  N  A  A  V  K  G  G  Y  W  Y  S

ATAGTGGCCTTGCAGTTTCTGACATTAATCCCTCTTATTTCACTCACCTGTTCTGTGCATTTGCTAACCTTGATTCTAACACCAACAGAGTCACAATTTC  200
 D  S  G  L  A  V  S  D  I  N  P  S  Y  F  T  H  L  F  C  A  F  A  N  L  D  S  N  T  N  R  V  T  I  S

TTCTGCAAACGCAGCTAGATTCTCAACCTTTACTCAAACCGTCCAAGCAAAGAGTAGTTCAGTGAAAACCCTTTTATCAATTGGTGGTGGTGAAGGTCCT  300
   S  A  N  A  A  R  F  S  T  F  T  Q  T  V  Q  A  K  S  S  S  V  K  T  L  L  S  I  G  G  G  E  G  P

ACTTTGGCAAACAAATTTGCCAGTATGGCTAGCCAAGCTAGTAGCCGAAAATCGTTCATAGACTCTTCAATCCAGCTAGCCAGAAGTAATAACTTCAATG  400
 T  L  A  N  K  F  A  S  M  A  S  Q  A  S  S  R  K  S  F  I  D  S  S  I  Q  L  A  R  S  N  N  F  N

GTCTTGATCTTGACTGGGAATATCCATCCACAGACACAGACAAGACCAACTTTGGTTTGCTCATCAAGGAGTGGAGAGCTGCAGTGGCGAAAGAGTCTAG  500
 G  L  D  L  D  W  E  Y  P  S  T  D  T  D  K  T  N  F  G  L  L  I  K  E  W  R  A  A  V  A  K  E  S  S

CAGTTCCGGGAAGGCGGTACTGTTGTTAACTGCTGCAGTGGGTGGCTCTGATCAGATCACCGGATTGAAGTACTATCCAGGTCAGGATATTGCAAACAAC  600
   S  S  G  K  A  V  L  L  L  T  A  A  V  G  G  S  D  Q  I  T  G  L  K  Y  Y  P  G  Q  D  I  A  N  N

TTGGACTGGGTCAATGTAATGACTTATGACCTTTTCATCTCAGATAGTTATCCAACATCGACACAGCCACCTGCTCCTTTGAAAAACCCAACTGGCCAGT  700
 L  D  W  V  N  V  M  T  Y  D  L  F  I  S  D  S  Y  P  T  S  T  Q  P  P  A  P  L  K  N  P  T  G  Q

TCAGTGTAGATGAAGGCATCACAAAATGGATAGGATTAGGAGTGCCGAAAAACAAACTAGCACTGGGATTACCCGCCTATGGTTACAAGTGGAGTTTGTC  800
 F  S  V  D  E  G  I  T  K  W  I  G  L  G  V  P  K  N  K  L  A  L  G  L  P  A  Y  G  Y  K  W  S  L  S

AGATCCTAATAAACATGGACTTTTTGATAAAGCTACCCAGGGACTTGGGGCAGTGAAGTACAAGGATATAAAAAATGCTGGGGCGCAGGTTGTGTATAAT  900
   D  P  N  K  H  G  L  F  D  K  A  T  Q  G  L  G  A  V  K  Y  K  D  I  K  N  A  G  A  Q  V  V  Y  N

TCCACATATGTTACAAATTATGCCTTCAAAGGCACAGATTGGTATGGATACGATGATACTCAGAGTATATCTGCCAAGGTTTCTTATGCCAAGCAAAATG 1000
 S  T  Y  V  T  N  Y  A  F  K  G  T  D  W  Y  G  Y  D  D  T  Q  S  I  S  A  K  V  S  Y  A  K  Q  N

GATTGTTGGGATATTTTTTTTTGGCATATTGAACAAGACAGCAACTGGGCTCTTTCTTCAACAGCTTCTCAAACACTGGGGGCCTAAAGGATTGTAGACAG 1100
 G  L  L  G  Y  F  F  W  H  I  E  Q  D  S  N  W  A  L  S  S  T  A  S  Q  T  L  G  A  *>.............

AGTATGCTGCAAAGAGAATAAAGATGGAGGGTTATCTATATTATAGTAAAGTTATGGCCTTTCTAGTTTATATCCTCTCGTGTAATAATAAAAAATAAGA 1200

ATGAGATATTAAAGGATCTATCTAAAGTAATAAAATTAAAATCTTGCTTTGCTTCTCTTGATCTAATCAAAAAAAAAAAAAAAAAAAAA
```

```
pSC57           ---KKHSFLPISTLLIILQLQFSSTNAAVKGGYWYSDSGLAVSDINPSYFTHLFCAFANL 57
PzChi/ly        MANSVTLFSIIFSCFLLRQLVCTNSQNVIKGGYWFKNSGLALNNIDSTLFTHLFCAFADL 60
chi-V           MANSVTLFAIIFSCFLLQQLVCTNSQ-NVKGGYWFKDSGLALNNIDSTLFTHLFCAFADL 59
                   .   *  * : ::: **  :.::  :*****:.:****:.:*:.: *********:*

pSC57           DSNTNRVTISSANAARFSTFTQTVQAKSSSVKTLLSIGGGEGPTLANKFASMASQASSRK 117
PzChi/ly        NPQSNQLIISPENQDSFSQFTSTVQRKNPSVKTFLSIAGGRADTTA--YGIMARQPNSRK 118
chi-V           NPQLNQLIISPENQDSFRQFTSTVQRKNPSVKTFLSIAGGRANSTA--YGIMARQPNSRK 117
                :.: *:: **. *   *  **.*** *..****:***.**.. : *  :. ** *..***

pSC57           SFIDSSIQLARSNNFNGLDLDWEYPSTDTDKTNFGLLIKEWRAAVAKESSSSGKAVLLLT 177
PzChi/ly        SFIDSSIRLARQFGFHGLDLDWEYPLSATDMTNLGILLNEWRTAINMEARNSGRAALLLT 178
chi-V           SFIDSSIRLARQLGFHGLDLDWEYPLSAADMTNLGTLLNEWRTAINTEARNSGRAALLLT 177
                *******:***. .*:********* : :* **:* *::***:*:  *: .**:*.****

pSC57           AAVGGSDQITGLKYYPGQDIANNLDWVNVMTYDLFISDSYPTSTQPPAPLKNPTGQFSVD 237
PzChi/ly        AAVSYSPRVNGLNYPVESVARNLNWINLMAYDFYGPNWSPSQTNSHAQLFDPVNHISGS 237
chi-V           AAVSNSPRVNGLN-YPVESLARNLDWINLMAYDFYGPNWSPSQTNSHAQLFDPVNHVSGS 236
                ***. * ::.**: ** :.:*.**:*:*:*:**:: .:  *:.*:. * * :*..:.* .

pSC57           EGITKWIGLGVPKNKLALGLPAYGYKWSLSDPNKHGLFDKAT--QGLGAVK-----YKDI 290
PzChi/ly        DGINAWIQAGVPTKKLVLGIPFYGYAWRLVNPNIHDLRAPAAGKSNVGAVDDGSMTYNRI 297
chi-V           DGINAWIQAGVPTKKLVLGIPFYGYAWRLVNANIHGLRAPAAGKSNVGAVDDGSMTYNRI 296
                :**. **  ***.:**.**:* *** * * :.* *.*   *:  ..:***.     *: *

pSC57           KN----AGAQVVYNSTYVTNYAFKGTDWYGYDDTQSISAKVSYAKQNGLLGYFFWHIEQD 346
PzChi/ly        RDYIVQSRATTVYNATIVGDYCYSGSNWISYDDTQSVRNKVNYVKGRGLLGYFAWHVAGD 357
chi-V           RDYIVESRATTVYNATIVGDYCYSGSNWISYDDTQTVRNKVNYVKGRGLLGYFAWHVAGD 356
                ::    : * .***:* * :*.:.*::* .*****::  **.*.* .****** **:  *

pSC57           SNWALSSTASQTLGA------ 361
PzChi/ly        QNWGLSRTASQTWGVSSQEMK 378
chi-V           QNWGLSRTASQTWGVSFQEMK 377
                .**.** ***** *.
```

Figure 5.11 Comparison of the deduced amino acid sequences of PsSc57 with tobacco Pz chitinase/lysozyme (Heits et al., 1994), and tobacco class V chitinase (Melchers et al., 1994). Conserved amino acid residues are indicated by (*) and similar amino acid residues by (:). Dashes indicate the gaps introduced to maximise alignment.

full length cDNA and that its encoded polypeptide has a few amino acid truncation at the N-terminus.

5.1.11 Sequence analysis of PsSC58 cDNA clone

A full length cDNA clone, designated PsSC58, encoding a protein similar to nonmetallo-short-chain alcohol dehydrogenase was isolated from the cDNA library using subtracted cDNA clone SC58 as a probe. The PsSC58 cDNA insert consists of 1011 bp with an ORF of 817 bp encoding a putative polypeptide of 271 residues from an initiation codon at nucleotide 48–51 to a termination codon at nucleotide position 861–864 (figure 5.12). The 3' UTR was 129 bp long, in addition to a poly (A) tail of 18 bp. The 3' UTR contained two 'AATAAA', motifs corresponding to the consensus eukaryotic polyadenylation signal at nucleotide positions 906–911 and 965–970.

Analysis of the deduced amino acid sequence of the PsSC58 protein revealed extensive homology with a family of nonmetallo-short-chain alcohol dehydrogenases (figure 5.13). The highest homologies (% identity) were with the amino acid sequences deduced from a cowpea drought-induced cDNA clone CPRD12 (67%; Iuchi et al., 1996), tomato GAD3 (48.5%; Jacobson and Olszewski, 1996), gama grass (*Tripsacum dactyloides*) short-chain alcohol dehydrogenase (47.5%; Li et al., 1997) and the maize sex determination gene *TASSELSEED2* (46.3%; Delong et al., 1993). The PsSC58 protein also has sequence similarity (44%) with tobacco TFHP1 which has been identified as a DNA binding protein that interacts with a promoter region of horseradish peroxidase (Kawaoka et al., 1994). The predicted polypeptide from PsSC58 has a conserved motif 'GAGGIG' corresponding to an NAD-or NADP- binding site (GxxGxG motif; Jany et al., 1984) at amino acid position 24–29. Comparison of the amino acid sequence predicted by PsSc58 with CPRD12 (see above) indicates that PsSc58 is a full length cDNA.

Figure 5.12 The nucleotide and deduced amino acid sequences of the PsSC58 cDNA. The nucleotide sequence is numbered from the 5' end on the left of each line. The amino acid sequence of a putative coding region is shown below the nucleotide sequences in the single-letter code. The asterisk indicates the stop codon and two putative polyadenylation sites are underlined.

```
ACACAACTTAACTTCTCTTCGTCTTCCAGAAACGTGAAAAACTCACCCATGGCAACCGTTCCTTTGGCCTCAGCTGTTCTAAGAAGGCTTGAGGGTAAGG  100
.............................................<M  A  T  V  P  L  A  S  A  V  L  R  R  L  E  G  K

TGGCTCTGATCACCGGTGGTGCTGGCGGCATTGGCGAAGCCACCGCAAGACTCTTCTCCAACCATGGAGCTAAAGTCGTAATAGCTGACCTCCAAGACGA  200
V  A  L  I  T  G  G  A  G  G  I  G  E  A  T  A  R  L  F  S  N  H  G  A  K  V  V  I  A  D  L  Q  D  D

CAAAGGTCACTCCATTTGCCAGGAGTTACACAAATCATCTGCCTCTTATGTTCACTGCGACGCGACAAAGGAAGAAGACGTAGAAACCGCCGTGAACACG  300
   K  G  H  S  I  C  Q  E  L  H  K  S  S  A  S  Y  V  H  C  D  A  T  K  E  E  D  V  E  T  A  V  N  T

GCGGTTTCCACTTACGGTAAACTCGACATCATGATAAACAACGCTGGCATATCCGGTGCGAATAACACCAACATACTTGAAAACACGTTATCTGAATTTC  400
 A  V  S  T  Y  G  K  L  D  I  M  I  N  N  A  G  I  S  G  A  N  N  T  N  I  L  E  N  T  L  S  E  F

AACAAGTGGTTAACGTGAACTTGGTTGGTGTCTTTCTTGGAACAAAACATGCTGCAAGAGTAATGATCCCTGCTCGACGAGGTAGCATAATTAACACAGC  500
Q  Q  V  V  N  V  N  L  V  G  V  F  L  G  T  K  H  A  A  R  V  M  I  P  A  R  R  G  S  I  I  N  T  A

TAGTATTGCTGGAAACATAGGTGGTACGATGAATCATGCTTACACAAGTTCAAAGCACGCCGTTGTGGGGCTGACGAGAAACACGGCGGTCGAGCTTGGA  600
   S  I  A  G  N  I  G  G  T  M  N  H  A  Y  T  S  S  K  H  A  V  V  G  L  T  R  N  T  A  V  E  L  G

CCGTTCGGTATTCGGGTGAATTGTGTGTCGCCATATATTGTTCTCACGCCGATGTCTAAGAAATACTTGGAGCTAGATGATGATGGGATTCTAGGGTTTT  700
 P  F  G  I  R  V  N  C  V  S  P  Y  I  V  L  T  P  M  S  K  K  Y  L  E  L  D  D  D  G  I  L  G  F

ATTCTAACCTAAAGGGTACTAATCTTTTGCCAAATGATGTGGCTGAAGCTAATCTGTACTTGGGAAGTGATGAATCTAAGTATGTAAGTGGTCACGATCT  800
Y  S  N  L  K  G  T  N  L  L  P  N  D  V  A  E  A  N  L  Y  L  G  S  D  E  S  K  Y  V  S  G  H  D  L

CGCTGTTGATGGAGGATGTTCGGTTGTGAACAATGGATTTTGTGTCTTTGGACGATCTGTCTGAAACTATGTGTTCTTTGATGTGTGTTTAGGCCATGAA  900
   A  V  D  G  G  C  S  V  V  N  N  G  F  C  V  F  G  R  S  V  *>.................................

ACTATAATAAAGTAGTTTTCTTATAAACTTAGTTGATTGAAGTATTTTCCTGGGAAGAGTCCTAAATAAAAAAGTATATTTATGTTGGGTGTCAAAAAAA 1000

AAAAAAAAAAA
```

```
                                    <----->
TOBTFHP         --------------EGKVAIITGAASGIGEASARLFVEHGARVVVADIQDELGQKVVDSI 46
GAD3            --------------EGKVAIITGAASGIGEASARLFVEHGARVVVADIQDELGQKVVDSI 46
PsSC58          MATVPLASAVLKRLEGKVALITGGAGGIGEATARLFSNHGAKVVIADLQDDKGHSICQEL 60
CPRD12          MANGSVLSPVVKRLEGKVAIITGGASGIGEATARLFSQHGAHVVVADIQDDVGLSLCNEL 60
                        *****:***.*.*****:**** :***:**:**:**: * .: :.:

TOBTFHP         GSDKASYRHCDVTDEKQVEETVAYAVEKYGTLDIMFSNVGT--LNFCSVLDMDVLAFDET 104
GAD3            GSDKASYRHCDVTDEKQVEETVAYAVEKYGTLDIMFSNVGT--LNFCSVLDMDVLAFDET 104
PsSC58          HKSSASYVHCDATKEEDVETAVNTAVSTYGKLDIMINNAGISGANNTNILENTLSEFQQV 120
CPRD12          K--SAIYVHCDVTKEEDIEKCVDTAVSKFGKLDIMFNNAGTGDEFKKSILDNTKSDFERV 118
                   .* * ***.*.*:::*  *  **..:*.****:.*.*       .:*:     *:..

TOBTFHP         MAIN-VRIALAVKHAAKVMVDKKIRGSIICNASLEGILAGAASLAYIASKHAVVGIIKAA 163
GAD3            MAIN-VRIALAVKHAAKVMVDKKIRGSIICNASLEGILAGAASLAYIASKHAVVGIIKAA 163
PsSC58          VNVNLVGVFLGTKHAARVMIPAR-RGSIINTASIAGNIGGTMNHAYTSSKHAVVGLTRNT 179
CPRD12          ISVNLVGPFLGTKHAARVMIPAR-RGCIINTASVAGCIGGGATHAYTSSKHALVGLTKNT 177
                : :* *   *..****:**:  : **.** .**: * :.*  . ** :****:**: : :

TOBTFHP         ARELGPHGIRVNGVSPYGIATPLVTKAYGLDAALLEEAIYGN-GHLKGVKLSTMHVAQSA 222
GAD3            ARELGPHGIRVNGVSPYGIATPLVTKAYGLDAALLEEAIYGN-GHLKGVKLSTMHVAQSA 222
PsSC58          AVELGPFGIRVNCVSPYIVLTPMSKKYLELD----DDGILGFYSNLKGTNLLPNDVAEAN 235
CPRD12          AVELGQFGIRVNCVSPFAIVTPLLNKYFNLD----EEGVRKTYMNLKGWYPVPNDVAEAA 233
                * *** .***** ***: : **: .*   **    ::.:     :***    . .**::

TOBTFHP         LFLASDESAYTSGQNLAVDGGLSSILKLQ------- 251
GAD3            LFLASDESAYTSGQNLAVDGGLSSILKLQ------- 251
PsSC58          LYLGSDESKYVSGHDLAVDGGCSVVNNGFCVFGRSV 271
CPRD12          LYLASDESKFVSSHNLVIDGGLINSNVGFPMFEM-- 267
                *:*.**** :.*.::*.:***
```

Figure 5.13 Comparison of the deduced amino acid sequences of PsSC58 cDNA clone encoding nonmetallo-short chain alcohol dehydrogenases, with Cowpea CPRD12 (Iuchi et a., 1996), and tobacco TFHP1 (Kawaoka et al., 1994). Conserved amino acid residues are indicated by (*) and similar amino acid residues by (:). Dashes indicate the gaps introduced to maximise alignment. Broken line with arrows indicate putative amino acids involved in the NAD- or NADP-binding site (GxxGxG motif, Jany et al., 1984).

5.1.12 Screening of young potato tuber cDNA library with tomato PPO cDNA clone

The main objective of this work was to isolate a PPO cDNA clone. When attempts using subtraction hybridisation failed, it was decided to screen a potato cDNA library as PPO is constitutively expressed in potato tubers (Thipyapong et al. 1995). A tomato PPO clone (750 bp) encoding the C-terminal end of the polypeptide was kindly donated by Prof. Lifschitz (Technion-Israel Institute of Technology, Haifa, 32000, Israel). The screening of young potato tuber cDNA library (kindly provided by Dr. David Bown, Durham University), resulted in the isolation of a 1471 bp long PPO cDNA clone which was designated ypPPO (young potato tuber polyphenol oxidase).

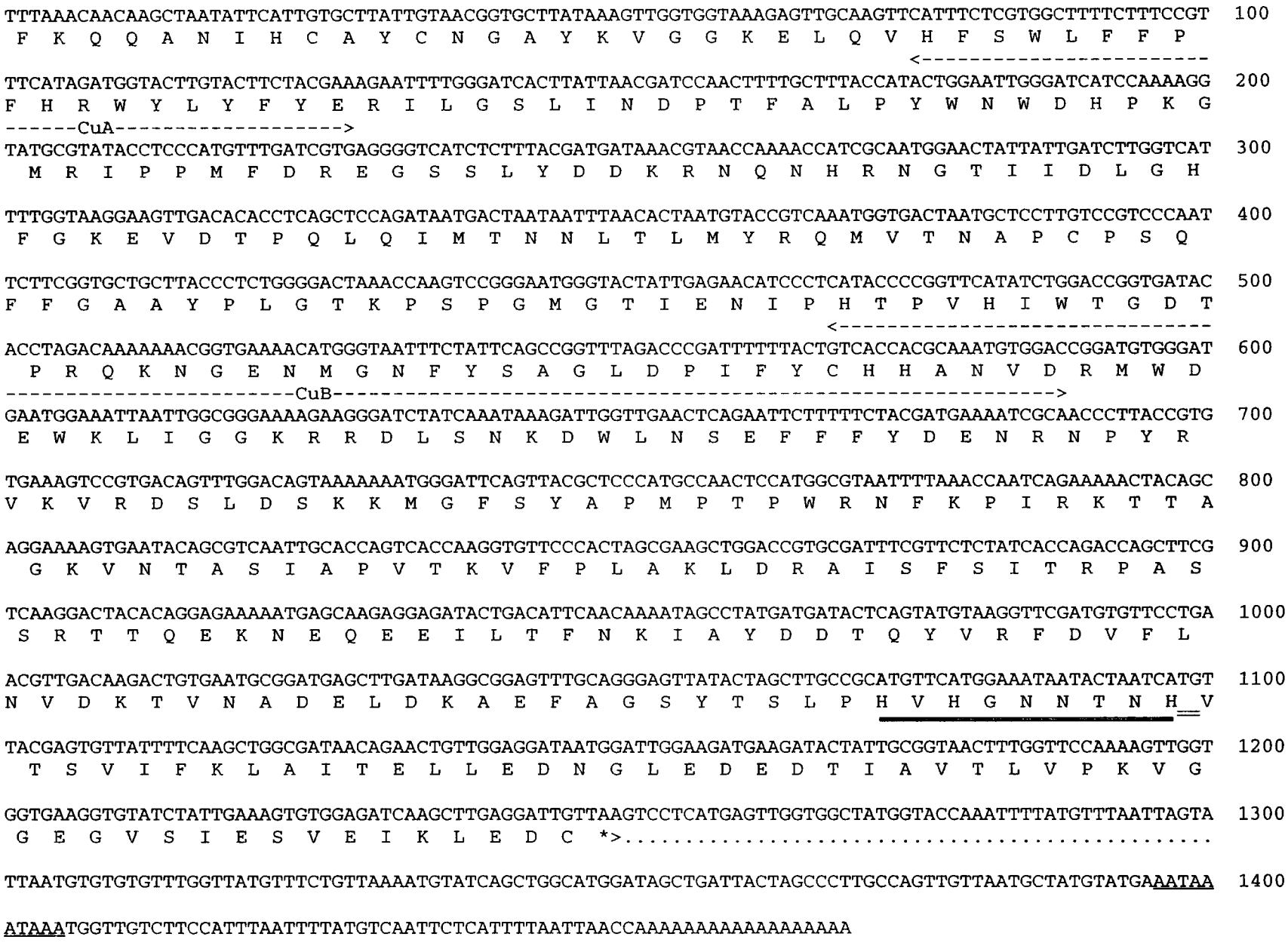
5.1.13 Sequence analysis of ypPPO clone

The 1471 bp cDNA insert fragment had an ORF 1251 nucleotides long that encoded a polypeptide of 471 amino acid residues. The 3' UTR was 201 bp, in addition to a poly (A) tail 19 bp long; it contained a single consensus eukaryotic polyadenylation signal motif 'AATAAA' at nucleotide position 1396–1401 (figure 5.14). The predicted polypeptide included two conserved His-rich copper-binding sites designated as CuA, encoded by nucleotides 75–129, and CuB (nucleotides 467–587). The polypeptide also has an additional His-rich region "HVHGMNTNH" encoded by nucleotides 1071–1098.

5.1.14 Screening of wounded pea leaf cDNA library using ypPPO cDNA as probe to isolate pea PPO clone

In order to isolate a pea PPO cDNA clone, duplicate plaque lifts of $5x10^4$ pfu from a cDNA library from wounded pea leaf tissue (see section 5.1.2) was screened using ypPPO cDNA as a probe. During primary screening, no hybridisation was detected. Following many unsuccessful attempts, it was decided to check whether PPO cDNAs are represented in the library from tissues 48 hrs after wounding. The whole cDNA library was PCR amplified using T3 and T7 primers and hybridised with ^{32}P-labelled

Figure 5.14 The nucleotide and deduced amino acid sequences of the ypPPO cDNA clone. The nucleotide sequence is numbered from the 5' end on the left of each line. The amino acid sequence of a putative coding region is shown below the nucleotide sequences in the single-letter code. The asterisk indicates the stop codon and the single polyadenylation site is underlined. Two consensus copper-binding domains are indicated by arrows marked CuA and CuB. Dark underlined amino acids represent an additional His-rich region.


TTTAAACAACAAGCTAATATTCATTGTGCTTATTGTAACGGTGCTTATAAAGTTGGTGGTAAAGAGTTGCAAGTTCATTTCTCGTGGCTTTTCTTTCCGT 100
F K Q Q A N I H C A Y C N G A Y K V G G K E L Q V H F S W L F F P
<----------
TTCATAGATGGTACTTGTACTTCTACGAAAGAATTTTGGGATCACTTATTAACGATCCAACTTTTGCTTTACCATACTGGAATTGGGATCATCCAAAAGG 200
F H R W Y L Y F Y E R I L G S L I N D P T F A L P Y W N W D H P K G
------CuA---------->
TATGCGTATACCTCCCATGTTTGATCGTGAGGGGTCATCTCTTTACGATGATAAACGTAACCAAAACCATCGCAATGGAACTATTATTGATCTTGGTCAT 300
M R I P P M F D R E G S S L Y D D K R N Q N H R N G T I I D L G H
TTTGGTAAGGAAGTTGACACACCTCAGCTCCAGATAATGACTAATAATTTAACACTAATGTACCGTCAAATGGTGACTAATGCTCCTTGTCCGTCCCAAT 400
F G K E V D T P Q L Q I M T N N L T L M Y R Q M V T N A P C P S Q
TCTTCGGTGCTGCTTACCCTCTGGGGACTAAACCAAGTCCGGGAATGGGTACTATTGAGAACATCCCTCATACCCCGGTTCATATCTGGACCGGTGATAC 500
F F G A A Y P L G T K P S P G M G T I E N I P H T P V H I W T G D T
<----------
ACCTAGACAAAAAAACGGTGAAAACATGGGTAATTTCTATTCAGCCGGTTTAGACCCGATTTTTTACTGTCACCACGCAAATGTGGACCGGATGTGGGAT 600
P R Q K N G E N M G N F Y S A G L D P I F Y C H H A N V D R M W D
----------CuB----------
GAATGGAAATTAATTGGCGGGAAAAGAAGGGATCTATCAAATAAAGATTGGTTGAACTCAGAATTCTTTTTCTACGATGAAAATCGCAACCCTTACCGTG 700
E W K L I G G K R R D L S N K D W L N S E F F F Y D E N R N P Y R
TGAAAGTCCGTGACAGTTTGGACAGTAAAAAAATGGGATTCAGTTACGCTCCCATGCCAACTCCATGGCGTAATTTTAAACCAATCAGAAAAACTACAGC 800
V K V R D S L D S K K M G F S Y A P M P T P W R N F K P I R K T T A
AGGAAAAGTGAATACAGCGTCAATTGCACCAGTCACCAAGGTGTTCCCACTAGCGAAGCTGGACCGTGCGATTTCGTTCTCTATCACCAGACCAGCTTCG 900
G K V N T A S I A P V T K V F P L A K L D R A I S F S I T R P A S
TCAAGGACTACACAGGAGAAAAATGAGCAAGAGGAGATACTGACATTCAACAAAATAGCCTATGATGATACTCAGTATGTAAGGTTCGATGTGTTCCTGA 1000
S R T T Q E K N E Q E E I L T F N K I A Y D D T Q Y V R F D V F L
ACGTTGACAAGACTGTGAATGCGGATGAGCTTGATAAGGCGGAGTTTGCAGGGAGTTATACTAGCTTGCCGCATGTTCATGGAAATAATACTAATCATGT 1100
N V D K T V N A D E L D K A E F A G S Y T S L P H V H G N N T N H V
TACGAGTGTTATTTTCAAGCTGGCGATAACAGAACTGTTGGAGGATAATGGATTGGAAGATGAAGATACTATTGCGGTAACTTTGGTTCCAAAAGTTGGT 1200
T S V I F K L A I T E L L E D N G L E D E D T I A V T L V P K V G
GGTGAAGGTGTATCTATTGAAAGTGTGGAGATCAAGCTTGAGGATTGTTAAGTCCTCATGAGTTGGTGGCTATGGTACCAAATTTTATGTTTAATTAGTA 1300
G E G V S I E S V E I K L E D C *>.....
TTAATGTGTGTGTTTGGTTATGTTTCTGTTAAAATGTATCAGCTGGCATGGATAGCTGATTACTAGCCCTTGCCAGTTGTTAATGCTATGTATGAAATAA 1400
ATAAATGGTTGTCTTCCATTTAATTTTATGTCAATTCTCATTTTAATTAACCAAAAAAAAAAAAAAAAA

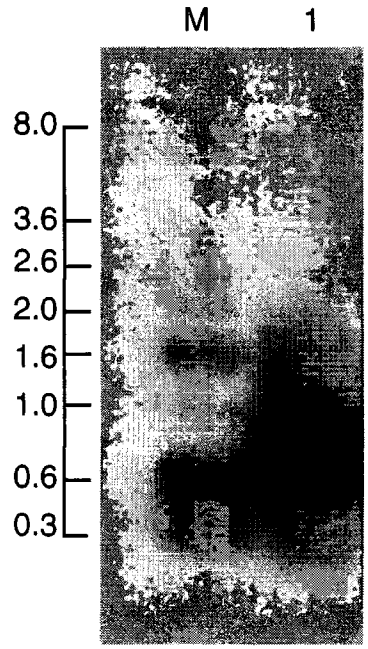


Figure 5.15 Southern blot hybridisation of cDNA from wounded pea leaf cDNA library. The whole cDNA library was amplified and 20 µl of amplified PCR product was electophoresed and blotted on nitrocellulose filter. Filter was probed with ^{32}P-labelled ypPPO cDNA and washed to a final stringency of 0.1x SSC, 0.1% w/v SDS for 30 minutes. Lane M represent standard size marker and lane 1 as amplified cDNA from pea cDNA library.

ypPPO probe after electrophoresis and blotting on nitrocellulose filter. Strong hybridisation of the whole library in the form of a smear was detected (figure 5.15). The hybridisation signal indicated that PPO message existed in the library. Failure to hybridise the plaques during screening could have been due to some technical problems, but at this stage further screening was abandoned.

5.2 Discussion

Plants respond to wounding in a complex way involving coordinated action of many secondary pathways which lead to the activation or *de novo* synthesis of many defensive gene products. These gene products provide resistance to plants in many ways, for example by deterring the pests and pathogens, reinforcing the cell wall to provide a tough barrier to intruders or by producing antinutrient proteins. Polyphenol oxidase (PPO) has long been known to be induced by wounding and pathogens in many different plant species at both local and systemic levels (Thipyapong et al., 1995). The induction pattern of this enzyme in pea and chickpea, in response to wounding, was studied and the isolation of a PPO cDNA clone was attempted as a major part of this project.

The presented data indicates the transient accumulation of PPO in response to wounding in pea and chickpea leaves. In both cases, more than a 12-fold increase in PPO activity was observed in the wounded leaves 48 hrs after wounding. No systemic induction was observed in the unwounded parts of the plants. Methyl jasmonate, a strong systemic inducer of PPO in tomato and potato also failed to induce any PPO activity in pea. Therefore, these results suggests that PPO is not involved in any systemic response in *Pisum sativum*. This is in complete contrast with reports of systemic induction of PPO both by wounding and treatment with MeJA in other plants such as potato (Constable et al. 1995), and tomato (Thipayapong et al., 1995). The systemic induction of PPO activity in potato and tomato was reported to be many fold higher than the activity induced in the wounded and proximal unwounded leaves.

Transgenic tomato plants overexpressing the prosystemin gene, a precursor of a mobile wound signal called systemin, showed extraordinarily high levels of PPO and

proteinase inhibitor (PIs). Supplying systemin or MeJA to wild type plants through cut stems induced activity of both PPO and PIs in leaves (Constable et al., 1995). Similarities in the characteristics of induction of PIs and PPO strongly suggest that both of these responses utilise the octadecanoid signal transduction pathway. Contrary to tomato and potato, the situation in pea appeared to be different. There is no systemic induction of PPO activity and the failure of MeJA to induce PPO activity is, therefore, not surprising and further confirms the absence of a systemic signal. These results led to two speculations. First, it is assumed that induction of PPO activity in wounded leaves in both pea and chickpea is not due to the activation of an octadecanoid pathway but more likely it is simply due to loss of PPO latency by damage caused to cells. PPO is localised in chloroplasts on the thylakoid membrane, separated from its phenolic substrates that are sequestered into vacuoles. The oxidation of phenolics happens only when the enzyme and substrate lose their compartmentation. There is considerable evidence that PPO is not active as a phenol oxidase in chloroplasts , but is limited as a phenol oxidase by latency or lack of substrate (Meyer and Biehl, 1982). Secondly, it is also equally possible that wound signalling may involve branched interconnecting pathways (Ellard-Ivey and Douglas, 1996). Particularly, the fact that PPO is induced locally by wounding independent of jasmonate synthesis (or exogenous application of MeJA), points to the existence of separate wounding-signalling systems. Studying of the induction pattern of PPO and proteinase inhibitors in pea and other legumes in response to ABA, linolenic acid and systemin may give some further evidence to support the above assertion.

The systemic induction of PPO in other plants favours a defensive role in plant-pest interactions. Felton et al. (1989), has demonstrated a strong correlation between PPO levels in tomato leaves and reduction in growth of *Heliothis zea* feeding on the tomato foliage. Tomato plants expressing antisense prosystemin were found to be extremely susceptible to tobacco hornworm (*Manduca sexta*) larvae attack (Orozco-Cadenas et al., 1993). Moreover, tomato plants overexpressing PPO showed a significant amount of resistance towards *Lacnobia decemlineata* (Steffens et al., 1994). Such results indicate that the products of PPO activities, which disrupt digestion in the insect gut (Felton et al., 1992), and the pathway for its induction are major components of plant defense. On the basis of comparison of these results with the findings of other groups, it is argued that induction of PPO is not an active defence mechanism at least in the case of

pea and chickpea. However, the fact that PPO failed to accumulate systemically in pea and chickpea leaves in response to wounding and MeJA treatment, does not mean that PPO is not involved in defense reactions. The quinones produced as a result of PPO and phenolic compound interaction, due to mechanical injury or to cellular disruption from disease, are very reactive, and could act as good candidates for involvement in protection from other organisms at the wound site.

A pea cDNA library was screened in order to isolate cDNA clones corresponding to PPO. Unfortunately, this strategy was hampered due to a lack of specific probes. Probing the PCR amplified pea cDNA library with the ypPPO cDNA clone isolated from young potato tuber cDNA library, showed a strong hybridisation, thus indicating that cDNA clones corresponding to PPO exist in the pea library. The failure to hybridise any phage plaques is suggested to be the result of technical problems and this needs further experimentation.

To learn more about the wound-induced genes in plant defense, it is important to increase our knowledge about the different proteins that accumulate in tissues stressed by wounding or other factors. Although, the main aim of this study was originally to isolate a PPO gene, later interest was generated to isolate other transcripts which are expressed differentially in response to wounding to obtain a wound-inducible promoter. The transgenic approach, to provide self-defense to plants against invading pests and pathogens by expressing an antimetabolic protein, is a novel approach but is restricted due to the lack of availability of highly active inducible promoters. The constitutive synthesis of defensive compounds has the disadvantage that chemicals are present throughout the ontogeny of the organism and thus allow certain organisms to break through this chemical defense by slowly adapting resistance to these compounds. The induced synthesis approach is that defensive chemicals are biosynthesised only after the plant is attacked. Therefore, in order to isolate the maximum number of transcripts induced by wounding, a subtractive hybridisation approach was used and a number of cDNA clones representing wound-induced genes have been identified. Of the 21 subtracted cDNA clones sequenced, 16 clones showed significant homology with sequences on the database, and 13 out of these 16 cDNA clones showed homology to wound or pathogen induced genes on the protein/DNA database. Five cDNA clones showed no significant homology to any sequences in the database and therefore some

of these represent the transcripts of novel genes which could be wound or pathogen induced.

Expression analysis by Northern hybridisation of the genes corresponding to the cDNAs (see section 5.1.6) indicated the increased expression of these genes in response to wounding. Some of these genes are weakly expressed under normal growth conditions, with transcript levels increasing upon wounding (e.g. SC3, and SC7), and some are only expressed after wounding (e.g. SC12, SC33, SC57 and SC58). This implies that these genes are stress responsive and are differentially regulated in response to a variety of controlling elements. Sequence analysis of these genes has indicated homologies with genes that have roles in plant defense against invading pathogens and pests and other environmental stresses. Therefore, it is likely that the proteins encoded by these genes may be involved in the defense response. The promoters of the genes which are totally wound-induced, particularly the novel subtracted cDNA clone SC33, could be very useful to induce the expression of foreign genes in transgenic plants in response to insect or pathogen attack. The success of the subtraction approach was quite significant indicating that PCR Select Subtraction Hybridisation is a powerful technique for isolating differentially expressed transcripts.

The PsSc10 cDNA clone has been shown to encode a HRGP extensin and has a high homology with extensins found in two other legume plants, soybean and *Phaseolus vulgaris* (see table 5.1). Extensins are encoded by multigene families which are known to be transcriptionally activated by wounding, pathogen infection and elicitor treatment, in many plants (Jose and Peugdomenech, 1993). It has been hypothesised that the synthesis of extensins in response to wounding may strengthen the cell wall by cross-linking extensin monomers, and may also agglutinate invading bacteria by acting as a polycation (Lech et al., 1982). In the pea subtracted cDNA library, three cDNAs designated SC9, SC10, and SC14 were found to be identical and showed homology with soybean and *Phaseolus vulgaris* extensins, indicating the possibility that pea has a multigene family of extensins. Therefore, the presence of extensins encoded by a multigene family in pea could further support the idea that different extensin proteins are expressed in different tissues to fulfil different structural roles.

The regulation of genes encoding extensins in response to various environmental stresses including wounding, pathogen infection and elicitor treatment, appears to be complicated. Even in a given plant, members of the extensin gene family may be regulated in different ways. For example, in tomato, three classes of extensin polypeptides have been described (Showalter et al., 1991; Zhou et al., 1992). Only two of these classes are found to be wound-inducible. Increased levels of extensin transcripts in response to wounding in many plants has been shown. In tomato, class 1 and class 2 extensins are accumulated locally after wounding (Showalter et al., 1992); in carrot, two transcripts of 1.5 and 1.8 kb size, accumulate differentially as a result of wounding (Tierney et al., 1988); the *Brassica napus* gene, extA, normally expressed in root, was induced dramatically in leaf, stem and petiole upon wounding (Shirsat et al., 1996). Therefore, wound-induced accumulation of extensins seems to be a feature of cell wall protein gene expression.

Comparisons of the sequences among the related proteins reveals both unique and conserved regions in the PsSC10 gene product. Numerous direct and inverted repeats were found throughout the entire polypeptide predicted by PsSc10. Although, not much is known about PsSC10 at this stage and it is premature to assign a particular function, the sequence homology of PsSc10 with other extensins with a role in plant defenses against pathogens would indicate that PsSC10 encoded extensin may has a similar function. The precise localisation of cells expressing pea extensin gene PsSC10 may give clues to understanding its role in the cell wall.

The predicted putative polypeptide of cDNA clone PsSC15 shows a high homology with both plant and animal aromatic amino acid decarboxylases, with a specificity towards tyrosine, tryptophan and dihydroxyphenylalanine (dopa). Plant aromatic amino acid decarboxylases exhibit a high substrate specificity (Facchini et al., 1994), whereas the animal aromatic amino acid decarboxylases accept a broader range of substrates such as L-dopa, L-tyrosine, L-tryptophan, L-phenylalanine, and L-histidine (Noe et al., 1984). This Pea TyDC is the first aromatic amino acid decarboxylase cDNA isolated from legumes although the specificity of its product towards different aromatic amino acids has yet to be found out. The remarkable degree of amino acid sequence identity, and the conserved distribution of α-helics and β-sheets, among all characterised aromatic amino acid decarboxylases suggests that they may be

evolutionary related (DeLuca et al., 1989). Indeed, sequence comparisons of pyridoxal phosphate-dependent decarboxylases in general, suggest a common evolutionary origin.

The occurrence of tyrosine decarboxylase/ diphenylalanine decarboxylase (TyDC/DODC) activity in the latex of the opium poppy was suggested as evidence for its involvement in the biosynthesis of alkaloids such as isoquinoline and phenanthrenes (Stadler et al., 1988). In addition to being the first committed step in isoquinoline alkaloid biosynthesis, TyDC genes show a rapid transcriptional induction by pathogens and elicitors which has been implicated in the defense response (Kawalleck et al., 1993, Trezzini et al., 1993). The significance of the TyDC gene in pea is not yet understood. High levels of tyramine, a product of TyDC/DODC activity, have been found in cell walls of wounded plant tissues (Borg-Oliver and Monties, 1993). Tyramine and its derivatives, such as feruloyltyramine, are good substrates for peroxidases and following oxidative polymerisation, tyramine or its derivatives may function to reinforce cell walls, making them less susceptible to penetration by pathogens (Hohlfled et al., 1995). Therefore, these observations, along with the fact that TyDC genes have been isolated from plants that do not produce isoquinoline alkaloids, such as *Arabidopsis thaliana* (Trezzini et al,. 1993), parsley (Kawalleck et al., 1993), and in pea indicate that TyDC genes may have some additional roles in plants beyond providing alkaloid precursors.

The presence of 3 premature stop codons in the coding region of the PsSC15 gene as marked in figure 5.7, is an unusual phenomenon. Occurrence of a premature stop codon has also been reported by Facchini et al.(1994), in one of the genomic clones designated TyDC4, in opium poppy. It is presumed that the PsSC15 clone either represents a pseudogene or may not encode a functional protein. Further studies of the structure and regulation of the PsSC15 gene may help to elucidate the mechanism by which wounding or other related stresses co-ordinately regulate this gene.

cDNA clone PsSC57 encodes a protein that shares equal homology with two tobacco PR proteins i.e. Chi-V protein (48.6%) and Pz (zinc chelating PR protein) chitinase/lysozyme (48.2%). Genes encoding the enzyme chitinase and chitinase/lysozymes are important for the protection of plants against pests and

pathogens. As shown in figure 5.4 B, Northern analysis of PsSC57 gene transcripts indicated that this gene is differentially regulated in response to wounding. The rapid and transient increase in PsSC57 gene expression suggests that PsSC57 might be a part of the active defense mechanism of the pea plant. Despite having a high homology, the PsSc57 gene differs from the Pz chitinase lysozyme gene. Pz chitinase/lysozyme was not expressed in response to wounding (Heitz et al., 1994), but is expressed in response to TMV infection and mRNA message was detected as early as 1-2 days post inoculation with a maximum turnover after 6 days. Therefore, in contrast to the Pz mRNA accumulation profile, the induction of PsSc57 mRNA is much faster. Similarly, a strong induction of Chi-V mRNA has been shown in response to TMV infection, ethaphon treatment and wounding but the wounding-induced accumulation of mRNA in leaves was much less than that in TMV and ethaphon treated leaves (Melcher et al., 1994). Both these genes (Chi-V and Pz zinc chelating PR precursor) are capable of hydrolysing chitin oligomers and have been shown to possess significant antifungal activity against a number of fungi, alone or in combination with other hydrolases (Melchers et al., 1994; Heitz et al., 1994).

Chi-V and Pz chitinase/lysozyme both contain a putative 7 amino acid C-terminal propeptide that directs the protein to the vacuole and may thus be classified as intracellular class 1 PR genes. Although PsSC57 has high homology with Chi-V and Pz chitinase/lysozyme, it differs from these two genes as it lacks the C-terminal propeptide. Therefore, it is assumed that the protein encoded by PsSC57 is not a vacuolar protein and is suggested to be an extracellular PR protein. Neuhaus et al. (1991) demonstrated that a sequence of seven amino acids located at the C-terminus of the tobacco class-1 chitinase was necessary for correct targeting to the vacuole. However, to reach a firm conclusion, the PsSC57 gene needs further study to clarify this. Over expression of the gene encoding PsSC57 in transgenic plants or blocking of protein synthesis by an antisense mRNA approach, may shed light on its function in defense or normal developmental processes.

The amino acid sequence of the putative PsSC58 protein revealed high homology with nonmetallo-short-chain alcohol dehydrogenases (figure 5.13), but not to alcohol dehydrogenases of the type reported to be induced by desiccation, cold, and hypoxia (Dolferus et al., 1994). The family of nonmetallo-short-chain alcohol dehydrogenases

includes mammalian 15-hydroxyprostaglandin dehydrogenases, prokaryotic glucose dehydrogenases, and insect alcohol dehydrogenases. The maize gene, *TASSELSEED2*, involved in sex determination of flowers (Delong et al., 1993), CPRD12 of cowpea with a presumed function in draught tolerance (Iuchi et al., 1996), and a gibberelin induced GAD3 gene of tomato (Jacobsen and Olszewski, 1996) also belong to the nonmetallo-short-chain alcohol dehydrogenases. All these proteins have the conserved motif of the NAD or NADP-binding site. These NAD or NADP-linked oxidoreductase enzymes are involved in many aspects of metabolism including glucose, fatty acids, and steroid pathway metabolism (Persson et al., 1991). A similar NAD or NADP-binding motif was found in PsSC58 protein. The PsSC58 protein also has significant homology with a wound-induced TFHP1, a DNA-binding protein of tobacco (figure 5.13). From the sequence comparisons alone, it is not possible to predict a specific biochemical function for the putative PsSC58 protein. Further analysis of PsSC58 encoded protein is necessary to examine whether the PsSC58 protein has DNA-binding activity.

The PsSC58 gene was induced significantly by wounding. None of the proteins homologous to PsSC58 has been shown to be wound-induced. Therefore, it is the first member of the nonmetallo-short-chain alcohol dehydrogenase family, shown to be wound-induced. Under wounding stress, the PsSC58 protein might be involved in the degradation of sugars and lipids to produce ATP. CPRD12, the protein with highest homology to PsSC58 has been shown to be induced in response to draught, salt and exogenous application of ABA but was not induced by either MeJA or SA (salicylic acid). Wounding response was not studied in CPRD12. Recent studies have demonstrated that a number of compounds may be involved in the signal transduction pathways leading to the induction of proteinase inhibitors and other wound-induced genes (Farmer and Ryan, 1992). These include oligosaccharides, systemin, phytohormone ABA, MeJA, and SA. Hildmann et al. (1992) have shown that a number of wound-inducible genes are responsive to the hormone ABA and that these genes are not induced by wounding in a mutant which is deficient in ABA. This has led to the proposal that ABA may be central to the molecular mechanism by which wounding induces these genes (Hildmann et al., 1992). Therefore, to draw a functional similarity with CPRD12, it is necessary to study the induction of PsSC58 in response to other stresses to clarify the functional role of PsSC58 under different stress conditions.

The PCR-Select Subtraction hybridisation strategy was a successful attempt to isolate differentially expressed cDNA clones. The expression of these cDNA clones under different stress conditions may help to understand possible links between different stress responses. A full characterisation of these cDNA clones may identify potential candidates to improve resistance of crop plants to disease, pests and abiotic stresses.

Chapter 6

Conclusions and future prospects

A differentially expressed cDNA clone designated LP19, encoding the C-terminal of hsp70 gene, was isolated from a pea pod cDNA library representing poly $(A)^+$ RNA from line L59 pods at 4-6 DAF. The gene corresponding to LP19 was isolated and designated as hsp70(LP19). The transcriptional regulation of the hsp70 gene family is known to be very complex and its members have been included in the family of molecular chaperones. The stress-70 proteins are required for a wide range of processes, including protein folding, unfolding, oligomerisation, subcellular localization and proteolytic removal. The pea hsp70(LP19) gene has shown a complex pattern of expression, as it is expressed not only in response to heat shock but also during normal developmental events in the plant, including pod lignification (Dhankher et al., 1997) and seed maturation (DeRocher and Vierling, 1995). The hsp70(LP19) mRNA, unlike other plant hsp70 genes (Kalinski et al., 1995; Neumann et al., 1994), failed to express under other environmental stresses such as wounding and salt stress.

Expression of hsp70(LP19) gene is closely linked to pod lignification, it is highest in pods of L59 and FF which both have a differentiated lignified endocarp, reduced in L58 with partially lignified endocarp and undetectable in L1390 in which the endocarp is not lignified. The isolation of hsp70(LP19) mRNA transcripts with heterogeneous 3' UTR, indicate the further complexity of the hsp70(LP19) gene regulation. The exact role of the presence of heterogeneous 3' UTR is not known at this stage but it is assumed that hsp70(LP19) gene expression may be regulated in the different cells by some mechanism involving the generation of heterogeneous 3' UTR and subsequent RNA processing. The role of hsp70(LP19) in pod lignification and seed development is consistent with the proposed roles for hsp70s in differentiation and development (Lindquist, 1986; Zimmermann et al., 1989).

Southern analysis of genomic DNA indicated the presence of hsp70(LP19) in all the experimental lines. Therefore, it is concluded that the absence of pod lignification in

L1390 is not due to the change in hsp70 gene expression but it is possibly playing a role in change in developmental pattern caused by the gene corresponding to classical loci p and v. A conclusive proof of the involvement of the hsp70(LP19) gene in development of the lignified endocarp phenotype could be obtained by localisation of the corresponding mRNA in lignifying tissues in the pods of the experimental pea lines. A time course induction of hsp70(LP19) mRNA in developing pods would identify the exact time of induction, and whether it coincided with the onset of lignification. Furthermore, studies on the induction of hsp70(LP19) gene by heat shock in pea line L1390 which lacks the differentiated lignified endocarp may establish a link between hsp70(LP19) expression and pod lignification.

The polypeptide encoded by hsp70(LP19) gene has no unusual features distinguishing it from other plant hsp70s. However, an unusual feature of the transcribed sequences is that this gene lacks an intron, whereas other plant hsp70 genes have one or more introns, with the exception of the hsp70 gene from soybean (Roberts and Key, 1991). The 5' flanking end of the pea hsp70(LP19) gene is also like that of other hs genes in that it contains multiple heat shock elements (HSEs), and other enhancer elements such as A-T rich sequences. All the heat shock gene promoters from different organisms have a similar kind of structural organisation. All contain multiple HSEs which are prerequisite for the binding of the heat shock transcription factors (HSFs) to activate the transcription which led to the synthesis of hsp genes. On this model, the pea hsp70(LP19) gene promoter has all the necessary *cis*-acting sequences to account for the heat-induced expression observed.

The transgenic tobacco plants expressing the transgene pHSP70(LP)-GUS, indicated the tissue-specific and developmental regulation of hsp70(LP19). Histochemical analysis of GUS activity revealed a phloem-specific expression of the transgene in all the heat shocked vegetative organs studied, with the exception in leaves, where a variable pattern of expression was noticed. Although, a similar kind of expression pattern was observed in the leaves of transgenic tobacco plants expressing soybean GmHSP17.3-B promoter, the exact reason for this exceptional expression pattern in leaves is not known and require further experimentation. The phloem-specific expression of the transgene is different from other heat shock promoters.

The high levels of GUS detected in non-heat shocked transgenic plants at certain specific developmental stages further indicate that hsp70(LP19) gene is developmentally regulated. A high level of GUS activity in seeds of transgenic tobacco plants throughout their course of development and during germination without heat shock is in accordance with the developmental expression of hsp70(LP19) mRNA in pea as reported by DeRocher and Vierling, (1995). A unique pattern of GUS expression was observed in petals which coincided with the onset of the anthocyanin synthesis. The expression of the transgene in pollen grains is also in agreement with the expression of other hsp genes at this particular stage (Cordwener et al., 1995; Marrs et al., 1993). The segregation of the pollen grains during microsporogenesis indicate that expression in pollen grains can not be an artefact. No correlation was found between the transgene loci number and GUS expression levels. In transformants number 2 and 14 in which the transgene has integrated at a single locus, GUS expression levels were several folds higher than the transformants number 19 where transgene has integrated at three loci. These findings extend further support to the contention that transgene expression level is related to the position of the transgene integration into the genome and not to the transgene loci number.

Time course induction of the hsp70(LP19) gene promoter in terms of GUS activity indicated the fast response of the heat shock. However, in mature plants, the response was delayed as compared to seedlings. This was supposed to be due to the difference in the size and complexity of the tissue structure. The absence of the systemic signal of the heat shock response, which is completely different from the response of wounding caused by pests and pathogens, suggests that individual cells respond to high temperature independently. The hsp70(LP19) gene promoter may be useful to direct phloem-specific expression of genes in plants after heat shock, if linked to appropriate coding sequences.

A transient increase in PPO activity observed in both pea and chickpea, in response to wounding, was similar to the response observed in potato and tomato (Constable et al., 1995; Thipyapong et al., 1995). However, unlike potato and tomato, pea plants did not show any systemic induction of PPO activity. This finding suggests that systemic signalling responses may not be as identified in plants as has been thought.

As has been discussed already, an approach to plant protection using gene products whose expression has induced by pests and pathogens, is restricted due to the lack of availability of highly active inducible promoters. An attempt was made to isolate wound-induced genes and their promoters by applying a subtractive hybridisation approach. Most of the isolated subtracted cDNA clones showed homology with genes that have roles in plant defense against pests and pathogens. The Northern analysis of subtracted cDNA clones indicates *de novo* synthesis of mRNAs of some genes and upregulation of others in response to wounding. Therefore, the promoters of these genes particularly SC33, PsSC57 and PsSC58, are good candidates for exploitation as inducible promoters to express defensive genes.

The overall impact of this research work should be significant. The results have thrown light on the functional significance of heat shock proteins and demonstrated that hsp70(LP19) gene is both stress-induced and developmentally regulated. It has also indicated an important area of future research to establish a functional link between tissue lignification and hsp70 expression. Such studies may explain if hsp70 expression is associated with pod lignification and does it play a direct role in pod lignification or play an indirect role by changing the developmental pattern caused by other genes associated with pod lignification.

The work on wounding response has indicated that the wounding response in plants is a very complex process. Not all the plants respond to wounding systemically and thus the systemic response is not conserved in all plant species. It has been observed that wounding, like other abiotic stresses, causes the alteration of expression of many genes and not just a few as was suggested earlier. Therefore, the transgenic approach to transfer of genes that encode protective proteins into agriculturally important crops to enhance the resistance against pests and pathogens should not rely on a single gene but instead will rely on a multigenic approach.

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Short communication

Characterisation of a pea hsp70 gene which is both developmentally and stress-regulated

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Abstract

A pea pod cDNA library was screened for sequences specific to lignifying tissue. A cDNA clone (pLP19) encoding the C-terminal region of a hsp70 heat shock protein hybridised only to pod mRNA from pea lines where pod lignification occurred. Expression of pLP19 was induced by heat shock in leaves, stems and roots of pea and chickpea plants. Four different poly(A) addition sites were observed in cDNAs derived from the same gene as pLP19. This gene was fully sequenced; unlike most hsp70 genes, it contains no introns. The 5′-flanking sequence contains heat shock elements and other potential regulatory sequences.

Abbreviations: HSE heat shock element; HSP, heat shock protein.

Like all organisms, plants respond to a rapid elevation in environmental temperature by transcriptional activation of a set of genes that encode protective proteins called heat shock proteins (HSPs). The HSPs exhibit highly ubiquitous and conserved features, and are proposed to be essential for cell survival [18]. The major classes of HSP are distinguished by their molecular weights: hsp110, hsp90, hsp70, hsp60 and low-molecular-weight (LMW) hsp (reviewed in [28]). Although HSP are produced in response to high temperature, certain specific HSPs are also found at a significant level in normal unstressed cells (constitutive expression), or are produced at a particular stage of the cell cycle or during development in the absence of stress [7, 17, 18, 20]. It has been suggested [25] that HSPs (and the related heat shock cognate or HSC proteins) are involved in the folding, assembly and disassembly of proteins and protein containing complexes, both during normal growth and after heat shock [4, 14, 30]. Many of the agents and treatments that enhance the activity of the HSP genes are known to cause unfolding of pre-existing proteins or to induce the synthesis of unfolded polypeptides [10, 29]. Thus the accumulation of unfolded peptides could be the signal for increased expression of HSP genes.

The hsp class designated hsp70 is found in both eukaryotes and prokaryotes, and represents a highly conserved multigene family, whose genes are expressed under a variety of physiological conditions. The diversity of hsp70 genes is partly accounted for by the presence of distinct hsp70 homologues in cytoplasm [32], in the lumen of ER [21] and the matrix of chloroplasts and mitochondria [2, 6, 19]. The hsp70s are highly homologous and show a high level of sequence conservation, with at least 50% identity at the amino acid level [3]. Several plant cytoplasmic hsp70s have been characterised: in *Arabidopsis* [32], petunia [31], maize [27], soybean [26] and tomato [16]. The genes for the hsp70s of plants are activated by a variety of stresses in addition to heat shock [33]. For example, wounding, water deficit, abscisic acid, heavy metals and cold can induce the synthesis of the

The composite nucleotide sequence reported for the HSP70(LP19) gene and the cDNAs derived from it has been submitted to the EMBL Nucleotide Sequence Database under the accession number X99515.

hsp70s [15, 22, 31]. Other reports have shown that these genes can also be expressed during normal plant development [9, 33]. The present paper describes a cytoplasmic pea hsp70 gene whose mRNA transcripts are specifically expressed in lignifying tissues during pod development, as well as in heat shock.

Isolation and sequence analysis of pLP19 cDNA clones

Pea (*Pisum sativum* L.) plants of lines L59, L58 and L1390 (Pea Gene Bank Catalogue, Weibullsholm Institute, Sweden, 1989; kindly supplied by the Weibullsholm Institute, Sweden) and cv. Feltham First (FF) were used as the basis for this investigation. These lines show different phenotypes in lignification of the pod endocarp. Pods of L59 and FF have a lignified parchment layer (genotype *PV*); L58 has a partially lignified layer (genotype *Pv*) and the lignified parchment layer is absent in L1390 (genotype *pv*). A cDNA library from developing pods (4–6 days after flowering; DAF) of pea line L59 was constructed and differentially screened as previously described [8]. A differential screen of 8×10^4 plaques of the amplified L59 lignified pod cDNA library was performed according to a method based on that of Olszeski *et al.* [24], using [α-^{32}S]dCTP and [α^{32}P]dCTP-labelled cDNAs synthesised from 4–6 days after flowering (DAF) pod mRNAs [8] from L59 and L1390, respectively. Clones, including pLP19, were selected on the basis of consistent preferential hybridisation to a total cDNA probe from the lignified pods (L59) when compared to a probe from the non-lignifying pods (L1390), following a secondary and tertiary screen.

The pLP19 insert was characterised by DNA sequencing. The cDNA fragment was 1072 bp in length, and contained an open reading frame of 813 nt encoding a polypeptide of 271 aa, a 3′ non-coding region of 256 nt, plus a poly(A) tail of 18 nt. The polypeptide, which was truncated at the N-terminus, was homologous to the C-terminal region of the hsp70 family of proteins; the highest similarity scores were with the plant hsp70s, with homology to a soybean hsp70 [36] being as high as 89.7% (data not shown). The sequence of LP19 is included in the composite sequence shown in Figure 1. The clone pLP19 was subsequently used to rescreen the pea (L59) pod cDNA library , and seven further homologous cDNA species (designated pLP19/1, pLP19/2, pLP19/3, pLP19/4, pLP19/6, pLP19/7 and pLP19/9; pLP19/3 and pLP19/7 were identical in sequence, as were pLP19/1 and pLP19/2) were isolated. DNA sequencing showed that all these cDNAs, including pLP19, could be the products of a single gene. All the cDNAs showed truncation of the N-terminal sequence of the encoded polypeptide although two were almost full length (see Figure 1). None of the cDNAs showed evidence of truncation at the 3′ end, since all had poly(A) tails of varying lengths. However, the sites of addition of the poly(A) tail differed among the different clones. Clones pLP19/1,2 have the longest 3′-untranslated region (UTR), of 285 bp; the original clone, pLP19 has a 3′-UTR of 258 bp; pLP19/4, pLP19/6 and pLP19/9 have a 3′-UTR of 252 bp and pLP19/3,7 have the shortest 3′-UTR, of 226 bp (see Figure 1). The position of poly(A) addition is thus highly variable in these transcripts. The 3′-UTR sequence lacks a consensus poly(A) addition signal sequence, AATAAA, the best match, AATTTA, occurring at 236 bp after the stop codon.

Expression of mRNA species corresponding to pLP19 in developing pods

Dot blot analysis of total RNA from developing pods (4–6 DAF) of L59, L58, L1390 and FF, hybridised with ^{32}P-labelled pLP19 cDNA, confirmed differential expression of the mRNA corresponding to pLP19 in lignifying tissue (Figure 2A). Strong hybridisation was observed with pod RNA from L59, L58 and FF, where partial or complete lignification of the endocarp takes place. Hybridisation to total RNA from pods of L1390, where no endocarp lignification occurs, was not above the background hybridisation to *Escherichia coli* ribosomal RNA. This result was confirmed by northern blot analysis of total pod RNA from the experimental pea lines. Probing with labelled pLP19 cDNA showed hybridisation to an mRNA species of estimated size 2.6 kb in L58, L59 and FF, where pod lignification takes place, but no hybridisation was detected in L1390, where pod lignification does not occur (Figure 2B). The observed hybridisation signal was significantly lower in L58, than in either L59 or FF; L58 exhibits partial rather than complete pod lignification.

Expression of mRNA species corresponding to pLP19 under stress conditions

Total RNA from heat shocked leaves, roots and stem tissues was analysed by northern blot, and probed with the hsp70 cDNA clone pLP19. In each case, hybridisation to a single band of 2.6 kb was observed, with

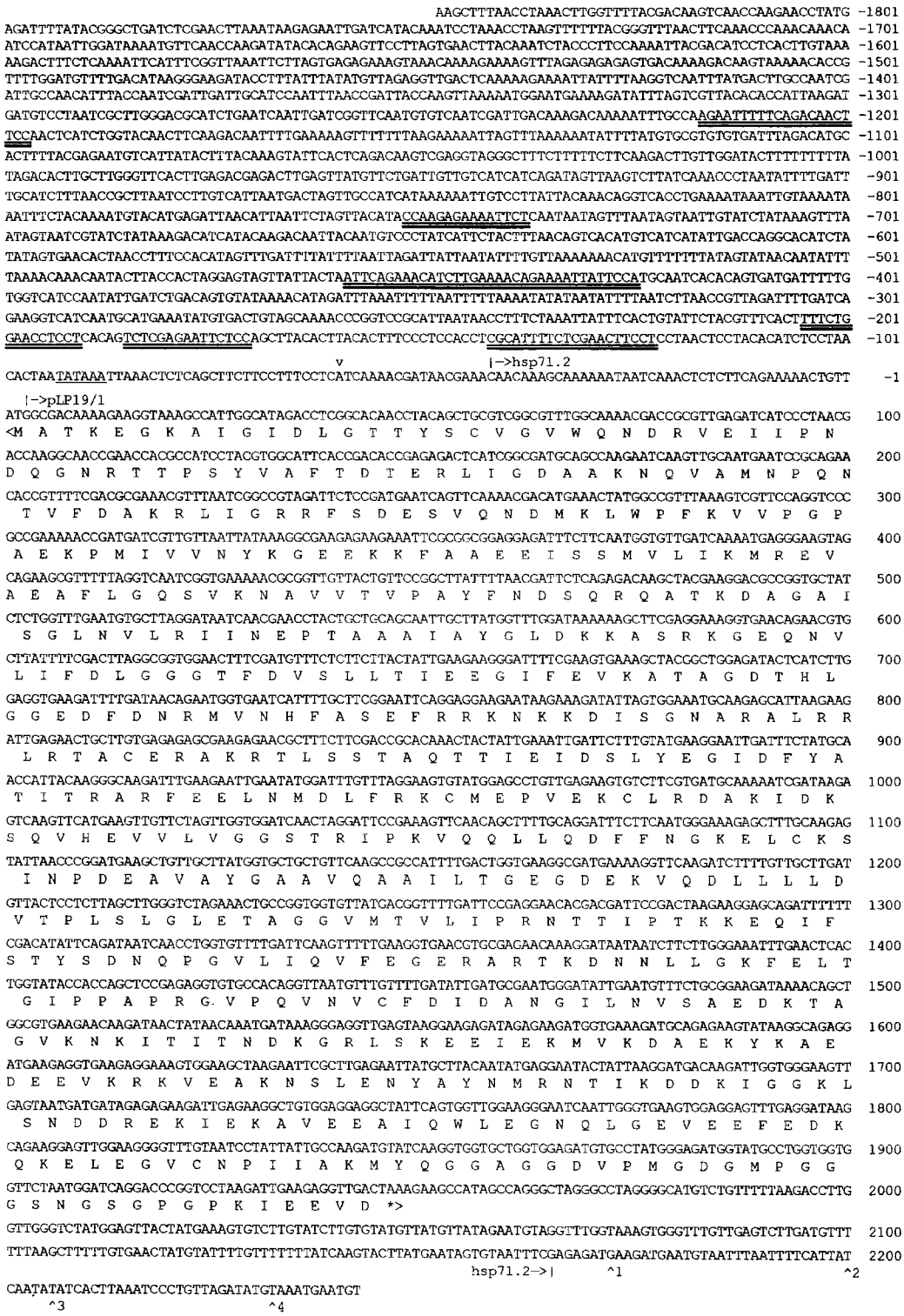

Figure 1. Composite sequence for cDNA species pLP19, pLP19/1,2,3,4,6,7,9, and PsHSP71.2, and the pea gene hsp70(LP19). DNA sequencing was performed by the dideoxy-termination method adapted for automated sequencing, according to protocols supplied by Applied Biosystems and using an Applied Biosystem Model 373A DNA Sequencer. Both strands of the DNA were sequenced, and fragments were overlapped. Numbering is from the first base of coding sequence. The TATA box location (underlined), and the putative transcription start (denoted by 'v' above the sequence) were identifed by using consensus sequences, and by analogy with similar hsp70 genes. Sequences in the 5′-flanking region in agreement with the consensus for heat shock elements are double-underlined. The start and finish of cDNA PsHSP71.2 [11] (which was not polyadenylated) are denoted by |->hsp71.2 ... hsp71.2->|. The start of cDNAs pLP19/1,2 is similarly denoted. Polyadenylation addition sites are indicated by '^' below the sequence: ^1, pLP19/3,7; ^2, pLP19/4,6,9; ^3, pLP19; ^4, pLP19/1,2.

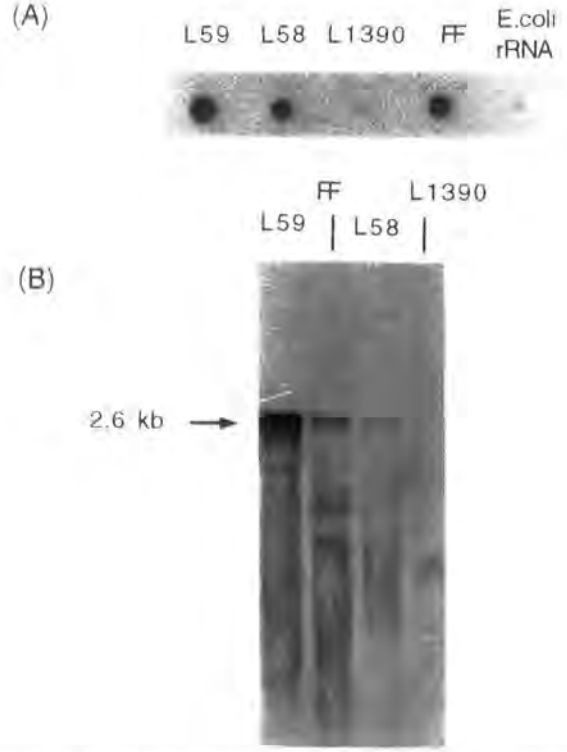

Figure 2. Expression of hsp70 gene corresponding to cDNA pLP19 in developing pods from different pea lines. Total RNA was prepared from 4–6 DAF pods from each of the experimental pea lines, L59, L58, L1390 and FF by the 'hot SDS' method [8]. The embryos and main veins were excised prior to RNA extraction. **A.** Dot blot of total RNA from pods of pea lines (and *E. coli* negative control) probed with cDNA pLP19. Total RNA (5 μg) from pods at 4-6 DAF of pea lines L59, L58, L1390 and FF were dot blotted onto a nitrocellulose filter (Schleicher and Schuell type BA85) using a HybriDot Manifold (Bethesda Research Laboratories, Scotland) according to the manufacturer's instructions. The filters were hybridised for 16–18 h at 65 °C using Hybosol solution (1.5× SSPE, 3% PEG, 7% SDS, 250 μg ml^{-1} heparin and 100 μg ml^{-1} denatured, sonicated herring sperm DNA), containing 2–10 ng ml^{-1} of [α-^{32}P]dCTP-labelled pLP19 DNA probe. Filters were washed to a final stringency of 0.1× SSC, 0.1% SDS at 65 °C. **B.** Northern blot of RNA from pods of different pea lines probed with cDNA pLP19. Total RNA (10 μg) from pods at 4–6 DAF of L59, L58, L1390 and FF were separated on formaldehyde-agarose (1.5%) gels. The gels were blotted onto nitrocellulose filters, and probed with pLP19 cDNA labelled with [α-^{32}P] dCTP by random priming. Hybridisation was preformed for 16 h at 42 °C in 15 ml of 50% formamide, 2× Denhardt's solution, 5× SSC, 0.1% SDS and 200 μg ml^{-1} denatured sonicated herring sperm DNA. The filters were washed to a final stringency of 0.1× SSC, 0.1% SDS at 65 °C prior to autoradiography.

no detectable hybridisation to RNA from control tissues. As a proportion of total RNA, the level of pLP19 mRNA was greatest in root tissues, with lower accumulation in stem and very low accumulation in leaves (Figure 3A). Similar results were observed when the pLP19 probe was used to detect the presence of the corresponding mRNA species in heat shocked and control chickpea plants (Figure 3B); again mRNA levels were highest in root tissue, lower in stem and least in leaves. Analysis of total RNA extracted from pea and chickpea plants under salt stress, or after wounding, by northern blotting, did not show any detectable expression of sequences hybridising to pLP19 (data not presented).

Analysis of pea genomic DNA for genes corresponding to pLP19

Genomic DNA from pea lines L59, L58 and L1390 was analysed in a Southern blotting experiment in order to establish the presence of gene(s) corresponding to the pLP19 cDNA. After washing to high stringency (0.1× SSC, 0.1% SDS, 65 °C), the pLP19 probe hybridised to a single band of ca. 12 kb in the *Bam*HI digests, and to a single band of ca. 1.6 kb in the *Hin*dIII digests, in all three pea lines, at an intensity of hybridisation suggesting that a single gene fragment was detected (Figure 4).

Isolation and analysis of a gene corresponding to pLP19

A pea genomic library (generously provided by Dr D.P. Bown, Durham University) was used to isolate an hsp70 gene. The library was constructed with pea DNA extracted from leaves and stipules [13], partially digested with *Sau*3AI, cloned into Promega's λGEM-12 vector. The library was screened by the plaque hybridisation procedure using pLP19 cDNA labelled with [α^{32}P]dCTP as a probe. Filters were washed to a final stringency of 0.1× SSC, 0.1% SDS at 65 °C. Positive plaques were taken through secondary and tertiary rounds of plaque purification and screening, resulting in the isolation of three independent genomic clones. Restriction maps suggested that these three clones all contained the same hsp70 gene; in all three cases a 1.6 kb *Hin*dIII fragment that hybridised to the cDNA probe was present, corresponding to the 1.6 kb *Hin*dIII fragment observed in genomic DNA. The genomic clone designated λOPD7 was subcloned, and the region containing the hsp70 gene was sequenced fully. This gene was designated hsp70(LP19). The complete nucleotide sequence and predicted amino acid sequence of pea hsp70(LP19) is shown in Figure 1. Comparison of nucleotide sequences of the cDNA and genomic clones shows that there is no intron present

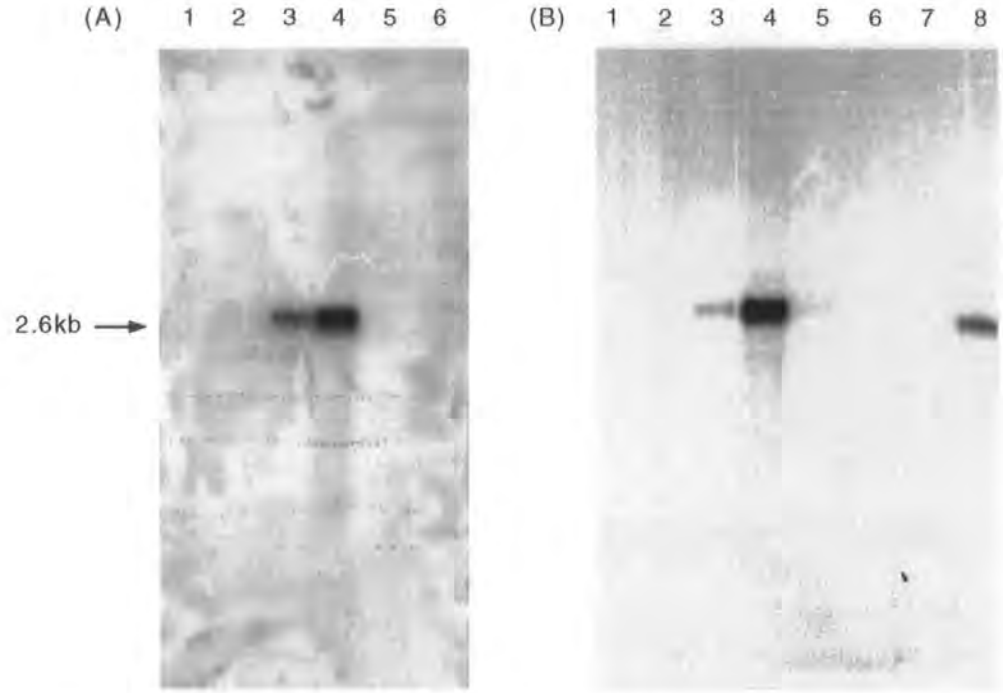

Figure 3. Expression of hsp70 genes corresponding to cDNA pLP19 in heat-shocked and stressed tissues of pea and chickpea. Ten-day-old plants (grown in vermiculite) were heat-shocked at 42 °C for 4 h in growth chambers maintaining high humidity. For salt stress treatment, 8-day-old plants were kept in salt solution (500 mM NaCl) for 2 days before harvesting. Plants were mechanically wounded by crushing the leaves with pliers at several points, including the major veins. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until required Total RNA was purified from tissues as designated by acid guanidinium thiocyanate-phenol-chloroform extraction, separated by agarose gel electrophoresis, subjected to northern blotting, and probed with cDNA pLP19 as described in the legend to Figure 2B. **A.** Expression in pea (line FF). Lanes 1, 3 and 5 contain RNA isolated from leaf tissue; lanes 2, 4 and 6 contain RNA isolated from root tissue. RNA in lanes 1 and 2 was isolated from control plants (normal growing conditions), in lanes 3 and 4 from heat-shocked plants, and in lanes 5 and 6 from salt-stressed plants. **B.** Expression in chickpea. Lanes 1, 3 and 5 contain RNA isolated from leaf tissue, lanes 2, 4 and 6 contain RNA isolated from root tissue, and lanes 7, 8 and 9 contain RNA isolated from stem tissues. RNA in lanes 1, 2 and 7 was isolated from control plants (normal growing conditions), in lanes 3, 4 and 8 from heat-shocked plants, and in lanes 5, 6 and 9 from salt-stressed plants.

in the gene. The coding and 3′-flanking regions of this gene were identical to the pLP19 cDNAs, establishing that hsp70(LP19) is the gene from which the cDNAs are derived.

The polypeptide predicted by pea hsp70(LP19) has high amino acid sequence homology with other cytoplasmic hsp70s from soybean (95%), petunia (87%), maize (84.5%), tomato (88%), carrot (84.5%), yeast (72.3%), man (64.2%) but is less homologous to pea plastid (48%) and mitochondria (49.8%) localised hsp70s [7]. The amino acid sequence of hsp70(LP19) is strongly conserved, when compared to other hsp70s, throughout the ATP binding domain and peptide binding domain, but diverges in the carboxy-terminal region. However, the carboxy-terminal of hsp70(LP19) has the sequence 'GPKIEEVD', which is similar to the consensus for other hsp70s; the last four amino acids form a motif 'EEVD', which is conserved throughout all plant and animal hsp70s. The amino acid sequence predicted by pea hsp70(LP19) does not have an amino terminal leader sequence characteristic for import into ER, mitochondria or chloroplast, which further supports the hypothesis that pea hsp70(LP19) is a cytoplasmic hsp70.

Sequence analysis of 5′-flanking region of hsp70(LP19)

Analysis of the 5′-flanking end of this genomic clone revealed the presence of several perfect and imperfect matches to consensus heat shock promoter elements (HSEs), represented by 5 bp 'nGAAn' inverted repeats, either head to head or tail to tail in orientation [12], present upstream of the presumptive TATA box. The first consensus heat shock element, HSE I, is 30 bases upstream of the TATA box. The other elements HSE II,

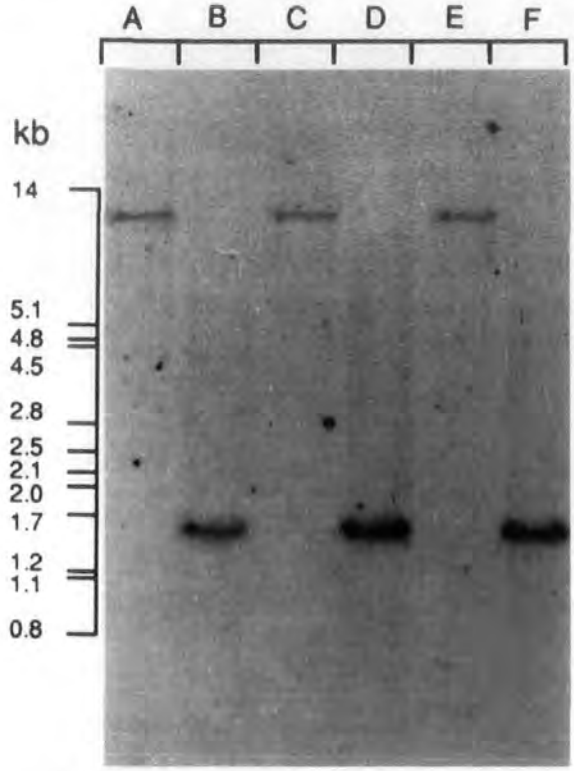

Figure 4. Genes corresponding to cDNA pLP19 are present in pea lines with both lignified and non-lignified pods. 20 μg genomic DNA from different pea lines (isolated by standard techniques [13]) was loaded in each lane as follows: lanes A, B, L59 (lignified pods); lanes C, D, L58 (partially lignified pods); lanes E, F, L1390 (unlignified pods). DNA in lanes A, C and E was digested with *Bam*HI; DNA in lanes B, D and F was digested with *Hin*dIII. After separation by agarose gel electrophoresis, DNA fragments were blotted, and probed with pLP19 labelled with [α-^{32}P]dCTP by random priming. Hybridization was performed for 16 h at 65 °C in 5× SSC, 5× Denhardts' solution, 0.1% SDS and 200 μg ml^{-1} denatured, sonicated herring sperm DNA. The filter was washed to a final stringency of 0.1× SSC, 0.1% SDS at 65 °C and autoradiographed.

HSE III, HSE IV, HSE V and HSE VI are positioned at −78 to −92, −97 to −111, −331 to −364, −643 to −657, and −1102 to −1122, relative to the TATA box respectively, as shown in Figure 1. All the HSEs have three to six 5 bp units except HSE V which has only two 5 bp repeats. In addition to HSEs, there are four A-T-rich regions, ranging from 20 to 50 A-T bases, positioned at −260 to −300, −468 to −507, −734 to −757, and −1063 to −1111, from the TATA box, and two sites having simple GAGA repeats at positions, −1430 to −1439 and −1458 to −1466.

Relationship of hsp70(LP19) to other hsp70 genes

A recent paper by DeRocher and Vierling [7] described three cDNAs encoding cytoplasmic hsp70 homologues in pea, which are induced to different extents in leaves by heat shock, and are expressed in zygotic, or zygotic and maternal tissues in developing seeds. The nucleotide and the predicted amino acid sequence of one of these cDNAs, designated PsHSP71.2, is contained within the pea hsp70(LP19) gene sequence described in the present paper; the 5′ and 3′ termini of this cDNA are marked on the gene sequence in Figure 1. In agreement with results presented here, expression of PsHSP71.2 was only detected in leaves in response to heat shock, and was not present under normal growth conditions. Roots and stems were not examined in the earlier paper. DeRocher and Vierling [7] also detected expression of PsHSP71.2 in the cotyledons and axes of developing pea seeds, during the later stages of development (mid-maturation onwards); these tissues were not examined in this paper. Differential expression of mRNA species corresponding to the three cDNAs isolated by DeRocher and Vierling [7] was considered to be 'consistent with the hypothesis that there are functional distinctions between cytoplasmic hsp70s'.

The polypeptide encoded by the pea hsp70(LP19) gene has no unusual features distinguishing it from other plant hsp70s. However, an unusual feature of the transcribed sequence is that this gene lacks an intron, where other plant hsp70s have one or more introns, with the exception of the hsp70 gene from soybean [26]. This gene is the most similar to pea hsp70(LP19) in encoded amino acid sequence, and probably represents the soybean homologue of hsp70(LP19). Despite this, no significant homologies were observed between the two genes in non-coding sequence, as compared by dot-plot and aligning software (data not presented).

The 5′-flanking end of the pea hsp70(LP19) gene is like that of the other hs genes in that it contains multiple heat shock elements (HSEs), with one or more elements present in the 5′-flanking region immediately upstream of the TATA box. The transcriptional activation of heat shock genes requires the activity of two or more binding sites for heat shock factor (HSF) trimers, and these sites act synergistically to activate transcription (reviewed by Fernandez *et al.* [11]). On this model, the pea hsp70(LP19) gene promoter has the necessary *cis*-acting sequences to account for the heat-shock-induced expression observed. In addition, further potential regulatory elements are present, in the form of A-T-rich sites, which act as enhancers for the

thermo-inducibility of gene expression [12]. Proximal A-T-rich sites contribute more to enhancement when compared to distal ones, and the pea hsp70(LP19) gene has two sites, of ca. 40 bases each, within 500 bases of the TATA box. There are also two distal sites having simple GAGA repeats, which are close to each other, and could represent the site for binding of the 'GAGA' factor which acts to 'open' the promoter for access by HSFs.

Expression of hsp70(LP19)

The pea hsp70(LP19) gene clearly has a complex pattern of expression, as it is expressed not only in response to heat shock, but also during normal developmental events in the plant, including pod lignification and seed maturation. Expression of the gene is closely linked to pod lignification; it is highest in pods of L59 and FF which both have a differentiated endocarp with lignification, reduced in L58 with partially lignified endocarp, and undetectable in L1390 which does not have a differentiated lignified endocarp. The gene is also expressed when lignification is taking place [8]. Analysis of genomic DNA by Southern blotting shows that the hsp70(LP19) gene is present in all the lines, and thus the differences in mRNA accumulation must be due to differences in the transcriptional activity of this gene. It is unlikely that the change in hsp70 gene expression is a causative factor in the absence of pod lignification in L1390 (lignification in other tissues in this line is not affected), but it may play a role in the change in developmental pattern caused by the genes corresponding to the classical loci *p* and *v*. The role of pea hsp70(LP19) in pod endocarp lignification is consistent with other proposed roles for hsp70s in differentiation and development [17, 33].

The complexity of regulation of the hsp70 gene family is further indicated here with the isolation of hsp70(LP19) mRNA transcripts with heterogeneous 3′-untranslated regions (3′-UTRs). The presence of poly(A) 'tails' on these cDNAs shows that they cannot be artefacts. The expression of hsp70(LP19) may be regulated in the differentiating cells by some mechanism involving the generation of heterogeneous 3′-UTRs and subsequent RNA processing. The 3′-UTR of hsp83 in *Leishmania* is involved in temperature-dependent regulated decay [1].

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