Cloning and characterization of an asparagus wound-induced gene.

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester.

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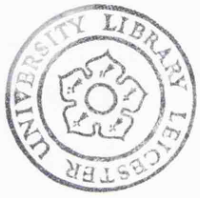

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Abstract

Following previous studies, *Asparagus officinalis* single cell suspensions were hypothesized to be a rich source of wound-inducible mRNAs. A previously isolated clone, DD1-34, was shown to hybridize to wound-inducible transcript. This sequence was used to isolate the AoPR1 (*Asparagus officinalis* Pathogenesis Related cDNA clone 1).

Data from the isolation and analysis of genomic clones hybridizing to DD1-34 probe suggested that these clones were unlikely to contain the upstream regulatory sequences of the AoPR1 gene and that the genomic arrangement of these sequences is complex. Inverse polymerase chain reactions (IPCR) were used to amplify AoPR1 genic sequences directly from the asparagus genome. Two products were cloned and sequenced, demonstrating that the correct sequences, upstream and downstream of the primers, had been amplified. The downstream IPCR product's sequence overlaps with AoPR1 coding sequence and contains an intron sequence.

The upstream IPCR product partially overlaps with the start of AoPR1 coding sequence and was successfully used in transcript mapping experiments. Translational fusions were constructed between this fragment and the β-glucuronidase (*gus*) reporter gene. GUS analysis demonstrated that this fragment, containing the AoPR1 promoter, was sufficient to drive wound-inducible transcription in transgenic tobacco. A smaller upstream fragment was insufficient to drive wound-inducible transcription. GUS expression was also detectable in tissues such as the xylem parenchyma, mature pollen and coloured regions of the petal. AoPR1-*gus* transgene expression correlates with the spatial expression patterns of phenylpropanoid biosynthesis pathway genes. The nature of the fusion suggested that the AoPR1 protein is intracellular. This is the first example of the cloning and analysis of a monocotyledon gene belonging to the `intracellular pathogenesis related protein' class. The analysis and application of AoPR1 sequences are discussed.

Aknowledgements

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To all my teachers, especially my parents.

Abbreviations

ATP	adenosine triphosphate
2-D	two dimensional
bp	base pairs
BSA	bovine serum albumin pentax V fraction
cDNA	complementary DNA
cpm	counts per minute
CTAB	hexadecyltrimethylammonium bromide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytodine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
ddATP	dideoxadenosine triphosphate
ddCTP	dideoxycytodine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dH_2O	double distilled water
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
hnRNA	heteronuclear RNA
hr	hours
IEF	isoelectric focusing

IMS	industrial methylated spirit
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobases
kD	kilodaltons
min	minutes
MOPS	3-(N-morpholino) propane-sulphonic acid
mRNA	messanger RNA
4-MU	4-methylumbelliferone
MUG	4-methylumbelliferyl glucuronide
PEG	polyethylene glycol
pfu	plaque forming unit
pI	isoelectric point
PIPES	Piperazine-N, N'-bis[2-ethane-sulphonic acid]
poly(A^+) RNA	polyadenylated RNA
PVP	polyvinylpyrolidone
QH_2O	Millipore filtered, double distilled water
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
tRNA	transfer RNA
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indoyl-β-D-glucuronide

Other abbreviations are explained in the text or are found in Sambrook *et al.*, 1989 [165]).

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Chapter 1

General introduction to defence-related gene expression

1.1 Introduction

The inability to move allows predators to graze on and cause damage to plants. Adverse weather conditions may also cause damage to plants. It is important that plants respond quickly to damage caused by predators or weather conditions in order to limit water loss from the wound site and to seal off the wound site from potential opportunistic pathogen invasion (reviewed by Bowles, 1990 [21] and Dixon and Harrison, 1990 [51]). Consequently, one of the primary responses in a wounded plant is to protect the wound site by producing physical barriers constructed from the cross-linking of monomeric molecules to provide a tough water resistant seal (discussed in sections 1.2, and 1.3. Reviewed by Bostock *et al.*, 1989 [19]). Other responses serve to deter predators from further attack and involve signalling processes that communicate the plants *status quo* to other areas and organs away from the damaged area of the plant. These systemic signals must be appropriately interpreted and result in the induction of genes to produce deterrents, for example proteinase inhibitors that inhibit insect gut proteinases making the plant indigestible, deterring the insect from grazing (discussed in sections 1.4 and 1.5). Clearly the

role of secondary messengers and signal transduction cascades acting intracellularly may have an important regulatory role in association with this response in the interpretation of the systemic signals (reviewed by Dixon and Lamb, 1990 [52]).

Apart from wounding, plants are also targets for infection by a range of pathogens. As with wounding the plant's response is both local and systemic and is directed to the containment of infection and to protection from further possible attack. This is achieved by the production of compounds that both strengthen and cause the death of cells immediately adjacent to the infected site, sealing off the site of infection and preventing further spread of the invading organism as part of the the hyper sensitive response (HR) (discussed later in this section). Other responses involve the production of hydrolytic or toxic proteins that are directed against the invading pathogen (eg. thionins, lectins, chitinases and glucanases.) at and away from the areas of infection, where they may help provide immunity from further or subsequent infection (discussed in sections 1.2 and 1.5). Most of these responses are the result of the synthesis of new proteins directed by the coordinated regulation of defence-related gene expression in response to 'wound-stress' signals transmitted around the plant.

Tobacco plants that are infected by tobacco mosaic virus (TMV) undergo a HR response and develop systemic resistance to further TMV infection in parts of the plant that had not shown symptoms of infection. This phenomena was termed as systemic acquired resistance (SAR) (Ross, 1961 and 1966 [155], [156]. Recently, it has been shown that the onset of SAR is correlated with coordinate induction of genes that belong to the pathogenesis related (PR) gene class (Ward *et al.*, 1991 [200] and Yalpini *et al.*, 1991 [205]). Following infection by TMV and also by salicylic acid treatment of tobacco, these genes are induced, both around the infection site and systemically away from the infection site in uninfected leaves (discussed later and in section 1.5, reviewed by Bol *et al.*, 1990 [10]).

Coordinate localized and systemically induced defence-related gene expression in response to wounding or pathogen attack suggests that there must be signalling mechanisms controlling these processes in the plant. Recently, there have been several defined chemical agents postulated as long distance messengers and these will be discussed later in the

introduction. It has become clear that many defence-related genes are induced by a wide variety of pathogens, by wounding and by a host of defined chemical elicitors suggesting that these stimuli may use common signal transduction mechanisms. The range of agents and stimuli that trigger defence-related gene expression is discussed next, and the individual gene classes are discussed later.

1.1.1 Gene expression at wound sites

Since many plant pathogens gain entry through wound sites, the study of the biochemistry, physiology and molecular biology of cells surrounding the wound site may provide important insights into engineering improved defence mechanisms into plants. Indeed, the response to wounding and pathogenic attack in plants is often very similar.

A wound has been defined as an external or internal injury that breaches the outer protective layers of the plant and leads to the destruction of cells in a specific area of tissue (Bostock and Stermer, 1989 [19]). Implicit in this definition is the physical rupturing of cells with concomitant loss of compartmentation. A wound may result from severe weather (wind, rain, hail, freezing), from herbivores, or during normal growth (abscission, growth cracks).

Depending on the plant species, the wound will be sealed in different ways leading to alternative wound periderm and wound barrier zone anatomy. For example, monocotyledonous plants have no meristematic activity associated with the wound site. Instead, cell autolysis and cell death occurs at the wound site, and the cells immediately adjacent to the wound surface become infused with an extensive layer of lignin and other phenols. In dicotyledonous woody and herbaceous plants, cells at the wound site also show autolysis, but in contrast to monocotyledons parenchymal cells at the wound site redifferentiate to form a lignosuberinized boundary zone and meristematic activity of cells internal to the boundary zone form a suberinized wound periderm (reviewed by Bostock and Stermer, 1989 [19]). Initially, in both types of response, there is an increase in transcriptional activity associated with high metabolic activity and up-regulation of defence-related genes. Other

structural molecules such as callose and silicon-containing deposits (Stumpf and Heath, 1985 [187]) also have a role in the formation of barriers offering resistance to infection.

1.1.2 Plant-pathogen interactions

Classical studies of plant-pathogen interactions have led to the discovery that the response of particular cultivars to particular pathogen races may differ depending on the compatability of the interaction (reviewed by Lamb *et al.*, 1989 [111] and Ellingboe, 1981 [61]). This in turn has led to the 'gene for gene' hypothesis where pathogen avirulence genes are matched by individual resistance genes in the plant cultivar. Resistance is dominant and and is thought to result from molecular recognition of the gene products of the matched avirulence/resistance genes encoded by the pathovar and cultivar respectively. If any one of the many gene for gene pair combinations between the plant and pathogen are incompatible then the plant is resistant to the pathogen and the interaction is described as an incompatible response. The incompatible reaction often involves a hypersensitive response (HR) which is characterised by the rapid death of the first infected cell and subsequent metabolic changes in cells surrounding the pathogen which ultimately leads to the death and isolation of the pathogen. The metabolic changes involved in this response often led to the accumulation of low molecular weight anti-microbial compounds which are phytoalexins. The phytoalexins are derived from precursors that include phenypropanoid intermediates, fatty acids and tepenes (reviewed by Darvill and Albersheim, 1984 [43] and Dixon, 1986 [49]).

1.1.3 Elicitor induced gene expression

More recently, it has been discovered that in some cases the same defence-related genes that are activated by the interaction of a viable pathogen with a plant can be induced by the addition of crude preparations derived from the pathogen. Analysis of the nature of the active compounds has revealed that defined cell wall oligosaccharides are particularly effective in inducing the response evoked by the pathogen (reviewed by Templeton and

Lamb, 1988 [188]). Conjecture that released plant cell wall components released as a consequence of pathogen or host mediated hydrolysis could act as endogenous elicitors proved correct when studies, such as those carried out with tomato plant cell wall extracts, identified a pectic cell wall component that acted as a proteinase inhibitor inducing factor (PIIF) (Reviewed by Ryan and An, 1988 [161] and Bishop *et al.*, 1984 [8]).

Abiotic chemically defined elicitors such as salicylic acid (Van Loon, 1988 [196]), hydrogen peroxide (Doke, 1983 [54]), glutathione (Wingate *et al.*, 1988 [204]), arachidonic acid (Bostock *et al.*, 1982 [18] and Maniara *et al.*,1984 [125]) and heavy metals (Doerner *et al.*, 1990 [53] and Hattori and Ohta, 1985 [81]) have also been used to induce defence related genes. The role, of any of these molecules in defence signaling is unclear, but it has been postulated that they perturb the normal signalling or transduction processes by mimicry or interference of the cells normal messenger processes.

1.1.4 Systemic signalling in the induction of defence-related genes

Following wounding, many genes are induced at sites away from the damaged area, for example in other leaves. Within non-wounded leaves the rapidity of induction and the abundance of detectable transcript in response to a stimulus from a wounded leaf varies considerably from gene to gene. For example, the accumulation of potato proteinase inhibitor II (PI II) transcript in a non-wounded leaf following wounding of a leaf elsewhere on the same plant is detectable in high abundance from 30 minutes to two days following wounding of the other leaf (Peña-Cortès *et al.*, 1988 [147]), whereas the the tobacco PR-1 transcript is more weakly induced following TMV infection in other non-infected leaves so that it can only be detected two days following infection (Cornelissen *et al.*, 1986 [37]). Similarly, analysis of transgenic tobacco harbouring PR-1 promoter-*gus* fusion constructs reveal induction of the reporter gene following viral attack, wounding or spraying with salicylic acid (Oshima *et al.*, 1990 [140] and Van de Rhee *et al.*, 1990 [194]). Consequently, because of the rapid, strong and systemic transcript induction, the the PI genes have been used widely in the study of the systemic 'wound signal'.

The induction of the proteinase inhibitors occurs mainly in the aerial parts of the plant in response to wounding with limited induction in lower stem or root tissue (Peña-Cortes *et al.*, 1988 [147]) (discussed in more detail in section 1.5). Work has concentrated on the identification of molecules that are mobile in the plant and that may have roles in the systemic signalling required for the induction of the PI genes in unwounded remote areas of the plant. As discussed earlier, the pectic cell wall fragment, named PIIF, has been shown to induce PI gene expression. However, this molecule is unable to move through the plant and so cannot act as a systemic signal (Ryan, 1988 [159]). Recently, a compound of low molecular mass, named super-PIIF, has been identified that is capable of mediating systemic induction of the proteinase inhibitors (Farmer *et al.*, 1989 [65]). Interestingly, the phytohormones ethylene (Ecker and Davis, 1987 [59]), abscisic acid (Peña-Cortès *et al.*, 1989 [146] and Peña-Cortès *et al.*, 1991 [148]) and the cytokinin BAP - 6-Benzylaminopurine (Kernan *et al.*, 1989 [106] have also been implicated as wound-related messengers. Volatile compounds, such as methyl-jasmonate, also induce PI gene expression implicating methyl-jasmonate in message transduction in interplant communication (Farmer and Ryan, 1990 [66]).

Physiological messages such as electrical signals (Wildon *et al.*, 1989 [203]) have also been postulated as putative wound messengers in studies using induction of the proteinase inhibitor transcripts as a systemic bioassay. Measurements of surface electrical potentials and hydraulic pressure waves in wheat leaves following localized wounding by heat (Malone *et al.*, 1991 [124]) have identified a possible hydraulic signal that travels through the xylem in response to burning.

More recent work has provided good evidence that in tobacco, salicylic acid is possibly the endogenous systemic signal responsible in the induction of genes that are associated with the systemic acquired resistance (SAR) response (Ward *et al.*, 1991 [200] and Yalpini *et al.*, 1991 [205]). As discussed earlier, in tobacco, SAR onset follows a hyper-sensitive response caused by TMV infection and correlates with the systemic induction of PR genes. Ward *et al.*, 1991 [200] postulate possible biosynthetic pathways for the production of salicylic acid from phenylpropanoid pathway intermediates. These intermediate are under the

enzymatic control of phenylalanine ammonia-lyase which plays an important role both in the hyper-sensitive response and in the production of phytoalexins (discussed in section 1.2). This model infers that links exist between the induction of phenylpropanoid pathway genes involved in the HR, and the production of a systemic signal. Salicylic acid would then act as a messenger travelling from sites undergoing the HR to remote unwounded areas of the plant and causing the induction of genes associated with SAR, such as the PR genes.

1.2 Defence genes: Their products and function

Following damage inflicted on a plant by a pathogen or predator the changes in gene expression translate to: alterations in the cells to rapidly strengthen or repair cell wall damage rapidly thus preventing water loss and further pathogen invasion; the production of chemical deterrents, digestive enzyme inhibitors, hydrolytic enzymes, toxic proteins and an array of other responses leading to a concerted beneficial response throughout the plant. Temporal and spatial control of gene expression is necessary to orchestrate the response to provide maximum protection possible depending on the rapidity of attack. For example the hyper-sensitive response may provide adequate protection from the relatively slow attack of a fungal pathogen but would be ineffective against against rapid herbivore attack.

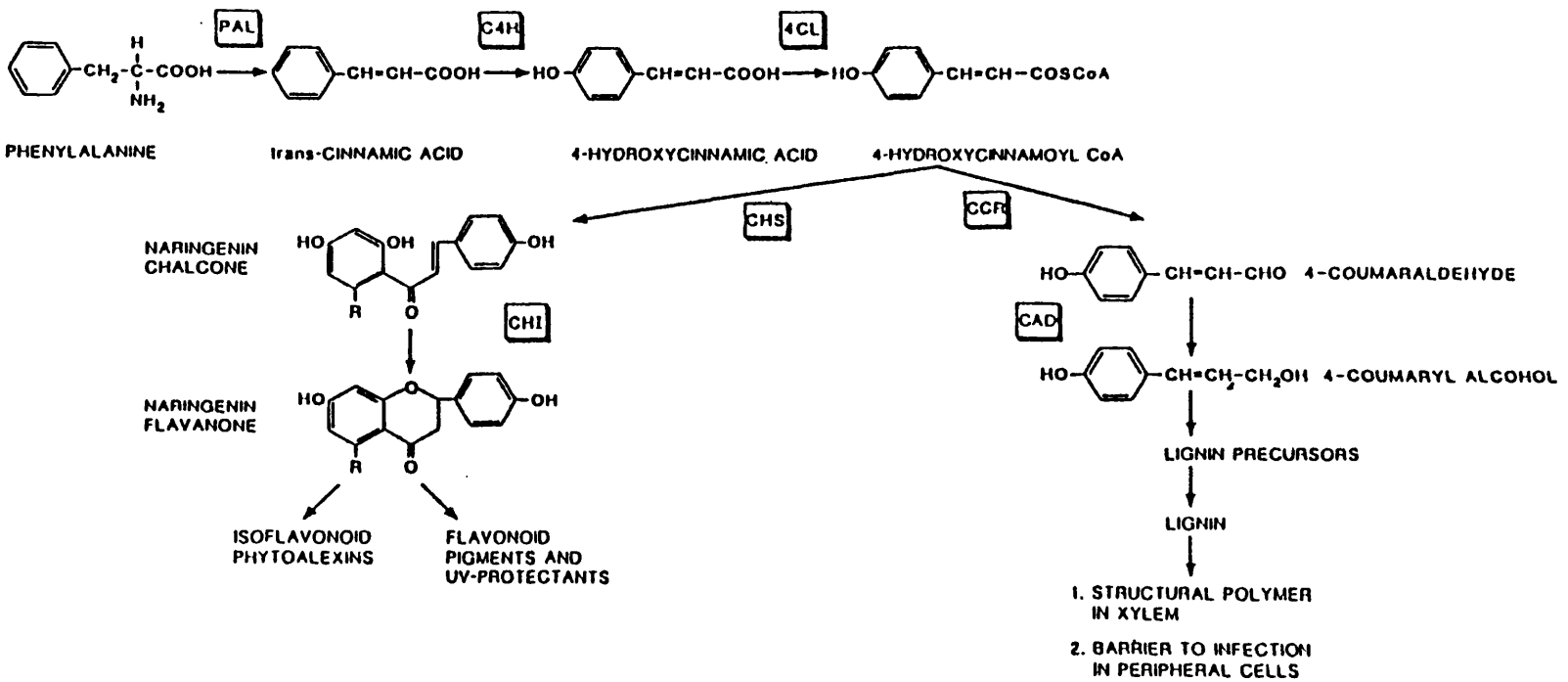
1.2.1 The phenylpropanoid biosynthesis pathway

The phenylpropanoid biosynthesis pathway is of fundamental importance to the plants survival. The low molecular weight products of this pathway produce flower pigments, insect repellents, phytoalexins, UV protectants, plant-microbe interaction signalling molecules and the monomeric constituents of the structural polymeric substances such as suberin and lignin which are important in the hyper-sensitive response (reviewed by Hahlbrock and Scheel, 1989 [76]). The enzymes catalysing steps in this pathway are encoded by

Figure 1.1. Pathway for the biosynthesis of phenylpropanoid natural products elaborated from phenylalanine.

PAL – phenylalanine ammonia-lyase; C4H – cinnamate 4-hydroxylase; 4CL – 4-coumarate-CoA ligase; CHS – chalcone synthase; CHI – chalcone isomerase; CCR – 4-coumaroyl-CoA reductase; CAD – cinnamyl-alcohol dehydrogenase.

From Lamb *et al.*, 1989.


PAL
C4H
4CL
PHENYLALANINE
trans-CINNAMIC ACID
4-HYDROXYCINNAMIC ACID
4-HYDROXYCINNAMOYL CoA
CHS
CCR
NARINGENIN CHALCONE
CHI
NARINGENIN FLAVANONE
ISOFLAVONOID PHYTOALEXINS
FLAVONOID PIGMENTS AND UV-PROTECTANTS
4-COUMARALDEHYDE
CAD
4-COUMARYL ALCOHOL
LIGNIN PRECURSORS
LIGNIN
1. STRUCTURAL POLYMER IN XYLEM
2. BARRIER TO INFECTION IN PERIPHERAL CELLS

genes under complex and tight transcriptional gene regulation and which respond to a multitude of environmental and developmental situations. The genes coding for some of the phenylpropanoid biosynthesis pathway enzymes have been cloned and studied at the molecular level and some of what is known is outlined below (fig. 1.1).

Phenylalanine ammonia lyase (PAL) genes

PAL deaminates the amino acid L-phenylalanine to yield *trans*-cinnamic acid (reviewed by Bowles, 1990 [21], Hahlbrock and Scheel, 1989 [76] and Dixon 1986, [49].) It is the first enzyme, and arguably the most interesting in terms of its gene regulation, of the phenylpropanoid pathway. Genes or cDNAs encoding the enzyme have been isolated from several plant species, including parsley (Lois *et al.*, 1989 [122]), bean (Cramer *et al.*, 1989 [41], rice (Minami *et al.*, 1989 [133]) and potato (Fritzmeier *et al.*, 1987 [71]). From studies carried out in bean several isoforms of PAL have been identified (Bolwell *et al.*, 1985 [12]). The isoforms have various Km values and are present at different abundances which led the authors to postulate that the low Km forms of PAL are specific to defence since this characteristic would enable the chanelling of scarce phenylalanine molecules into the phenylpropanoid pathway in times of metabolic stress (Bolwell *et al.*, 1985 [12] and Liang *et al.*, 1989 [116]). Three different PAL genes have been isolated from both parsley and bean (Lois *et al.*, 1989 [122] and Cramer *et al.*, 1989 [41]). These genes have been used in studies that aim to determine their mode of induction and identify those elements of their promoters that interact with *trans*-acting proteins following elicitor or light treatment (Lois *et al.*, 1989 [122]). PAL transcript assays revealed differing developmental and wound-inducible gene expression for the three bean PAL genes (Liang *et al.*, 1989a [116]). Reporter construct analysis in transgenic tobacco provided data to confirm these expression patterns (Liang *et al.*, 1989b [115]). A bean PAL 2 promoter-*gus* gene fusion showed expression of the reporter gene in specific cell types which were correlated with the production of high levels of phenylpropanoid pathway derivatives leading to known biochemical products (Bevan *et al.*, 1989 [3]). These tissues included the coloured areas of flower petals, regions where xylem differentiation and lignification was occurring and at

wound surfaces. When tobacco plants were transformed with constructs that caused over expression of the bean PAL 2 gene product a down-regulation of phenylpropanoid biosynthesis was observed with corresponding abnormal plant development (Elkind *et al.*, 1990 [60]). The author argued that this could be due to the accumulation of *trans*-cinnamic acid, an 'end product' inhibitor of PAL activity.

4-Coumarate CoA ligase

Following the production of *trans*-cinnamic acid from phenyalanine by PAL the cinnamic acid is hydroxylated, at the para-position in the aryl ring yielding 4-coumaric acid (by cinnamic acid 4-hydroxylase (CA4H)). This is then ligated to Acetyl-CoA by 4-Coumarate-CoA ligase (4CL). Two isogenes coding for this enzyme have been cloned from parsley (Douglas *et al.*, 1987) [55], fig 1.1). Gene regulation was temporally and spatially coordinate with that of that of the PAL gene with respect to induction by fungal elicitors and UV light (Lois *et al.*, 1989 [122]). GUS reporter analysis showed gene expression is directed by the parsley 4CL promoter in floral organs such as the coloured parts of petals, the nectary, pollen and the stigma surface (Hauffe *et al.*, 1991 [82]). Recent data showed that coding sequences within the 4CL 1 gene are required to confer elicitor and light transcriptional inducibility but promoter sequences alone are sufficient for the developmental regulation of the gene (Douglas *et al.*, 1991 [56]).

Following the synthesis of 4-coumaroyl-CoA the phenylpropanoid biosynthesis pathway branches. 4-coumaroyl-CoA is thus the common precursor of all phenylpropanoid derivatives including lignin and the isoflavonoids or flavonoid phytoalexins which are all end products with important anti-microbial roles in the response to pathogen attack.

1.2.2 Chalcone synthase: The flavonoid pathway

Chalcone synthase (CHS) forms naringenin chalcone by the condensation of 4-coumaryl CoA and three units of malonyl-CoA that form a new aromatic ring (reviewed Dangl *et*

al., 1989 [42]).

In parsley, there are two alleles of the gene that differ from one another by the insertion of a transposon-like element in the promoter region that does not effect UV light induction or developmental regulation of the gene (Herrmann *et al.*, 1988 [84]). In bean, there are approximately eight genes encoding CHS proteins, each of which is regulated differently in response to elicitors or wounding, perhaps in response to the production of different isoflavonoid phytoalexins (Ryder *et al.*, 1987 [162]).

DNA protection assays, promoter deletions and reporter gene assays have facilitated the identification of *cis*-acting sequences in the several plant CHS gene promoter regions that have putative regulatory roles in UV light and elicitor mediated gene responsiveness (Lipphardt *et al.*, 1988 [119], Dron *et al.*, 1988 [58], Schulze-Lefert *et al.*, 1989a [173], Schulze-Lefert *et al.*, 1989b [174] and Schmid *et al.*, 1990 [172]). The flavonoid biosynthesis pathway also leads to the production of flavonoid pigments such as the purple anthocyanin pigment and the use of CHS anti-sense constructs in petunia has led to the formation of altered phenotypes with impaired petal colour (van der Kroll *et al.*, 1988 [195]).

The suggestion by Elkind *et al.*, 1990 [60] that *trans*-cinnamate may be a regulator of PAL activity is supported by recent work that shows that cinnamate represses GUS activity in alfalfa protoplasts that have been electroporated with CHS 15 promoter-*gus* fusion constructs (Loake *et al.*, 1991 [120]).

Following the synthesis of naringenin chalcone by CHS there follows an isomerization by chalcone isomerase (CHI) to form naringenin flavone and then a cytochrome p450 dependant reaction forms an isoflavone catalysed by isoflavone synthase (IFS). The flavone and isoflavone products may then go through a further series of enzymatic mediated chemical modifications or conjugations with nucleotides such as UDP-glucose to yield a large range of phytoalexins, pigments and UV protectants.

1.2.3 The lignin pathway

From the 4-coumaroyl-CoA the enzyme 4-coumaroyl-CoA reductase (CCR) reduces the thio ester 4-coumaraldehyde, which is then further reduced by cinnamyl-alcohol dehydrogenase (CAD) to 4-coumaroyl alcohol – a direct precursor of lignin. The other lignin precursor is generated similarly from methoxylated 4-coumaroyl-CoA derived from caffeic acid or 5 hydro-ferulic acid (reviewed by Lewis and Yamamoto, 1990 [114]).

1.3 Cell wall modifying proteins

Clearly, by modifying cell walls in response to wound signals or the action of a pathogen or a pest the plant may stand a better chance of surviving an attack by physically excluding the attacking organism (reviewed by Cassab and Varner, 1988 [32] and Varner, 1989 [197]). So the next few subsections describe some of the mechanisms and genes involved in cell wall modification.

1.3.1 The extensins

The extensins are a subset of hydroxyproline rich glycoproteins which are structural proteins found in plant cell walls (reviewed by Marcus *et al.*, 1991 [126]). Other hydroxyproline rich glycoproteins include the arabinogalactan proteins (which are 90% polysaccharide), lectin-like hydroxyproline rich glycoproteins and repetitive proline rich proteins.

The extensins rapidly accumulate in the cell wall following wounding or attempted fungal invasion. As their name suggests, they undergo a great deal of post-translational modification. The hydroxyproline residues are found in repeating units of Ser(Hyp)4. The proline residue being hydroxylated by an endoplasmic reticulum associated prolyl hydroxylase (Bolwell *et al.*, 1985 [15]). Analysis of the extensins has revealed that greater than half the molecular mass is due to carbohydrate, the majority of which is proline O-linked oligoarabinoside. An associated elicitor-inducible arabinosyl transferase activity has been

described (Bolwell *et al.*, 1985 [16]). The carbohydrate groups are believed to stabilize the helical rod-like structure of the mature proteins within the cell wall which may then be immobilized by peroxidase-mediated tyrosine cross linking.

Although the genes encoding the extensins exist as multigene families, it has been suggested that in some cases alternative splicing may lead to different proteins arising from one gene (Cassab and Varner, 1988 [32]). From studies using genes from carrot, tomato and bean the induction of different families of extensin transcripts have been found to be subject to temporal and spatial control that varies according to the stimulus. In carrot, the response to ethylene leads to the accumulation of three differently sized transcripts whereas wounding leads to the induction of only two. This has been explained by suggesting ethylene causes transcription from an alternative start site in one of the genes promoters (Ecker *et al.*, 1987 [59]).

A lectin-like hydroxyproline-rich-glycoprotein from bean has also been shown to be up-regulated following addition of fungal elicitor to bean suspension cultures (Bolwell, 1987 [11]).

1.3.2 Glycine rich proteins (GRP)

These are cell wall proteins found in many plants that have a 60% glycine content. Genes encoding glycine rich proteins have been isolated from bean and petunia and show very rapid transcript induction (within 5 minutes) following wounding (Keller *et al.*, 1988 [103] and Condit *et al.*, 1987 [36]). Analysis of bean GRP promoter-*gus* reporter constructs in transgenic tobacco showed rapid gene induction following wounding in areas surrounding the wound boundary on the leaf and also developmental expression in the vascular cylinder of the stem (Keller *et al.*, 1989 and Keller and Baumgartner, 1991 [104,102]).

1.3.3 Genes induced following oxidative stress

Wounded plant tissues have been shown to release superoxide and H_2O_2 (Sekizawa *et al.*, 1987 [175] and Salin *et al.*, 1981 [163]). Superoxide radicals interact with hydrogen peroxide to form the highly toxic hydroxyl radical that can react indiscriminately with DNA, proteins, lipids and any other constituent of the cell (reviewed by Cadenas, 1989 [30]). It is not clear how these oxyradicals are formed, but the cell responds by detoxifying these radicals via the action of superoxide dismutases and catalases (reviewed by Scandalios, 1990 [168]).

It appears that superoxide dismutase (SOD) is the key enzyme providing protection against oxidative stress by converting superoxide to hydrogen peroxide which is removed by catalase and peroxidase. The superoxide dismutase enzyme exists in two main forms in plants; Cu-Zn and a Mn isoenzymes (reviewed by Scandalios, 1990 [168]). There are three Cu-Zn and one Mn superoxide dismutase isozyme in maize. The Mn isozyme is compartmentalized in the mitochondria and the Cu-Zn enzymes are either cytosolic or chloroplastic. The expression of these two genes have been correlated with general oxidative stress but as this is an outcome of the defence response they have been included in this discussion. Work at Leicester (Fioroni, 1989 [69]) resulted in the accidental discovery of an asparagus Cu-Zn superoxide dismutase that is up-regulated in mechanically isolated asparagus cells in cell suspension. Analysis of the N-terminal amino-acid sequence of this protein revealed a high homology with the maize superoxide dismutase. In tomato copper, zinc SODs are induced following treatment with ethephon - a compound that releases ethylene, by mechanical wounding and environmental stresses such as drought and light (Perl-Treves and Galun, 1991 [149]. A manganese SOD transcript from tobacco is induced following stresses such sugar metabolism in tissue culture, and in response to salicylic acid, ethylene and *Pseudomonas syringae* infection possibly as part of the systemic acquired resistance response (Bowler *et al.*, 1989 [20]).

Peroxidases utilize hydrogen peroxide in oxidative reactions that have a role in the defence reactions of the plant as well as other developmental roles (reviewed by Cassab and

Varner, 1988 [32] and Scandalios, 1990 [168]). Hydrogen peroxide produced by SOD may, for example be utilized to generate the monolignol radicals thought to be necessary as precursors to the formation of ligins and suberins mediated by cell wall associated peroxidases. Peroxidase activity is also thought to catalyse the cross-linking of other cell wall proteins such as the extensins by isotyrosine links (Cassab and Varner, 1988 [32] and Varner, 1989 [197]).

Genes for peroxidases have been cloned and analysed. The peroxidase genes cloned from tomato (Roberts *et al.*, 1988 [153]), potato (Roberts and Kolattukundy, 1989 [152]) and tobacco (Lagrimini, 1987 [109]) share high homology at the predicted protein level and have putative secretory signals and glycosylation signals. Northern analysis using tomato peroxidase probes has shown that some of the peroxidase isogenes are wound induced (Roberts and Kolattukundy, 1989 [152]).

1.3.4 Callose synthase

At the time of writing, the gene coding for this enzyme had not been cloned. The enzyme has an important function in the formation of the β-1-3-glucan structural polysaccharide called callose. The enzyme is situated in the plasma membrane and is activated in response to attempted fungal invasion or the addition of chitosan possibly in response to changes in the Ca^{2+} concentrations (Fink *et al.*, 1987 [68]). It is thought that callose synthase and cellulose synthase activity may be associated with the same enzyme complex that is able to switch synthesis of the growing glycosyl chain from polymerization on the 4' hydroxyl of the end glycosyl moity (cellulose) to the 3' hydroxyl (callose) in response to localized intracellular signals (reviewed by Delmer, 1987 [46]).

1.4 Proteins that have anti-invasive functions

Proteins that directly interfere with the metabolism of an attacking organism metabolism will be beneficial to the plant. These proteins could be enzyme inhibitors, hydrolytic

enzymes, lectins or have unknown biochemical function

1.4.1 Proteinase inhibitors

These are generally low molecular mass proteins that are potent inhibitors of animal and microbial proteinases but not of plant proteinases. Amongst the well studied proteinase inhibitors are the cowpea trypsin inhibitor and the solanacaceous proteinase inhibitors I and II (reviewed by Ryan, 1988 [161] and 1990 [160]). The genes encoding these proteins have been cloned and, when over expressed in transgenic tobacco plants, result in a good degree of protection against tobacco insect predators (Hilder *et al.*, 1987 [86] and Johnson *et al.*, 1989 [93]).

Molecular studies on the proteinase inhibitors have centered around the isolated wound induced genes (previously discussed in section 1.1). Initial studies showed that following localized wounding of potato plants, local induction of the proteinase inhibitor II message was detectable as was equally rapid induction of the message in leaves removed from the initially wounded leaf (Peña-Cortes *et al.*, 1988 [147]).

A Kunitz type proteinase inhibitor encoding cDNA from poplar trees has been isolated that showed systemic induction in poplar trees following wounding of remote sites (Bradshaw *et al.*, 1989 [24]). This has been used as a probe in studies which showed that the induction of this gene at sites in the plant remote from a wound site correlates with the movement of labelled sucrose molecules in the phloem, suggesting that the signal molecule mediating gene induction at remote sites in the plant may be carried in the vascular tissue (Davis *et al.*, 1991 [44]). This data agrees with the observation that induction of the proteinase inhibitors in potato mainly occurs in the aerial parts of the plant.

Promoter analysis of the potato PI II gene has shown that the whole promoter, or parts of the promoter, are able to drive transcription of *gus* or *cat* reporter genes in a wound-inducible, tuber specific and sucrose enhanced manner in transgenic potato (Keil *et al.*, 1989 [100], Sanchez-Serrano *et al.*, 1987 [166], Thornburg *et al.*, 1987 [189] and Johnson

et al., 1990 [94]). DNA binding proteins have also been identified that bind to the PI II promoter fragment in response to elicitor treatment (Palm *et al.*, 1990 [142] and Sanchez-Serrano *et al.*, 1990 [167]).

1.4.2 Endohydrolases

Enzymes that catalyse the breakdown of the pathogen cell wall polymers in response to attack by viral, bacterial or fungal pathogens are an obvious target for study (reviewed by Dixon and Harrison, 1990 [51], Dixon and Lamb, 1990 [52], Bowles, 1990 [21] and Bol *et al.*, 1990 [10]). The two most studied enzymes are the β-1-3-glucanases and the chitinases which catalyse the breakdown respectively of β-1-3-glucans and chitins found in fungal cell walls. Chitin is a cell wall structural polymer that is also found in insects. The production of these enzymes leads to an inhibition of the invasive processes of the pathogen and the release of endogenous elicitors from the plant cell wall in the form of oligosaccharides that activate other defence genes in the vicinity of the pathogen (Schulumbaum *et al.*, 1986 [170] and Dixon and Lamb 1990 [52]).

Genes for the glucanases and chitinases have been cloned from several plants including tobacco (Shinshi *et al.*, 1987 [178]), bean (Broglie *et al.*, 1986 [28]) and rice (Nishizano *et al.*, 1991 [137]). Southern analysis revealed that these genes are members of multigene families. Rapid transcript induction is observed following a number of different stimuli such as wounding, attempted fungal invasion, salicylic acid and ethylene treatment. For example, in bean cell cultures message is observable within 5 minutes of adding elicitor (Hedrick *et al.*, 1988 [83]). In cucumber and tobacco plants, the induction of these genes in response to a stimulus was observed systemically, implying a role in systemic acquired resistance (Metraux and Boller, 1986 [132], Ward *et al.*, 1991 [200]). Analysis of the bean chitinase promoter by the use of *gus* reporter analysis in transgenic tobacco, *Arabidopsis* and tomato showed rapid and strong reporter expression following fungal attack and salicylic acid treatment (Roby *et al.*, 1990 [154], Samac and Shah, 1991 [164]).

1.4.3 Lectins

These proteins have a strong binding affinity for carbohydrates. As such they may have a role in defence (reviewed by Chrispeels, 1991 [33]). Some lectins have been shown to be toxic eg. ricin, phytohemagglutinin and concavalin A. However, so far, studies have not revealed a definite biochemical role for the lectins in defence nor is it known how they accumulate following wounding. Some genes that have been isolated as wound-induced genes show homologies with carbohydrate binding domain encoding regions of lectins (Chrispeels, 1991 [33]). These include chitinases, the *win 1* and *win 2* genes isolated from potato (Stanford *et al.*, 1989 [185]) and *win 8* isolated from poplar (Parsons *et al.*, 1989 [143]). It has been suggested that chitin-binding lectins may have an inhibitory action on fungal hyphal growth by preventing the chitin molecules from cross-linking (Broekaert *et al.*, 1989 [27]).

1.5 Defence genes with unknown roles

Many proteins have been studied that are induced in response to wounding, pathogen or pest attack that as of yet have an undefined role in the defence response. Some of the genes coding for these proteins have been cloned and analysed, and in some cases this has provided evidence as to protein function.

The controlling elements associated with these genes may provide both useful promoters to drive anti pathogen/viral/pest chimeric gene constructs in transgenic crop plants and a means of dissecting the molecular mechanisms responsible for the induction of this class of genes in response to the applied stimuli (Broekaert, 1989 [27]).

1.5.1 Systemic acquired resistance (SAR) and the pathogenesis-related (PR) proteins

The PR proteins were initially identified in tobacco following tobacco mosaic virus (TMV) infection and are also known to be induced by salicylic acid. Their systemic induction correlates with the onset of SAR (as discussed in section 1.1) following the hyper-sensitive reaction from a previous TMV infection. Salicylic acid has been implicated as the systemic signal that causes the induction of PR genes at remote sites from the damaged areas as part of the SAR response. (reviewed by Bol *et al.*, 1990 [10], Yalpini *et al.*, 1991 and Ward *et al.*, 1991 [205,200]).

These 'classic' acidic proteins were originally found to accumulate in the apoplast surrounding lesions generated during the hypersensitive response triggered by infection by a necrotizing virus. Further study led to the discovery of similar basic proteins that accumulated in the vacuole. The nomenclature of these proteins was based on their migratory properties in native polyacrylamide gels. Since the discovery of more PR proteins this nomenclature has become confusing and so will be avoided here.

The function of several of the PR proteins is known and these include β-1-3-glucanases (Kauffmann *et al.*, 1987 [99]), chitinases (Legrand *et al.*, 1987 [112]), peroxidases (Bowles, 1990 [21]) and possible enzyme inhibitors (Richardson *et al.*, 1987 [151]). In addition to induction of these genes by viral infection or salicylic acid treatment the PR proteins may be induced by other stimuli. For example, using probes derived from tobacco PR chitinase and glucanase and tomato osmotin (King *et al.*, 1988 [107]) it has been shown that the protoplasting of tobacco mesophyll cells induces the corresponding transcripts in culture (Grosset *et al.*, 1990 [73]).

Amongst the well characterised PR proteins of unknown function, are the PR1 proteins. The PR1a, b and c genes isolated from tobacco have been cloned and show around 60% homology (Cornelissen *et al.*, 1986 [37]). Over expression of PR1a in transgenic plants does not result in resistance to viral pathogens, providing evidence that it may not have an anti-viral role when expressed alone but only when expressed in conjunction with other

genes required for SAR (Linthorst *et al.*, 1989 [117] and discussed in section 1.1).

PR proteins are generally accumulated extracellularly or in the vacuole following chemical treatment or pathogen infection of tobacco plants (Van den Bulke *et al.*, 1989 [192] and Mauch and Staelien, 1989 [129]). It has been suggested that the differential accumulation of chitinase in the extra-cellular spaces and the vacuole results from the expression of different isogenes (Linthorst *et al.*, 1990 [118]). Using 'riboprobes' and fluorescent antibodies in the analysis of untransformed and transgenic tobacco plants over-expressing PR1b has shown that the spatial accumulation of PR1b protein is probably controlled in a cell specific manner. If the protein is expressed in specialised cells where the vacuole acts as a large storage organelle, vacuolar accumulation occurs, whilst in other, non-specialised cells, it is exported to the apoplast (Dixon *et al.*, 1991 [48]). Other PR proteins characterised include the thaumatin-like PR proteins also known as the osmotins. The cDNAs for these genes have been cloned from tobacco and tomato that are also induced by salt stress (Cornelissen *et al.*, 1986 [38] and Payne *et al.*, 1988 [145]). The predicted proteins share sequence homology with the sweet tasting protein from *Thaumatococcus daniellii* and to a bifunctional α-amalase/proteinase inhibitor from wheat, but their precise function is not known.

1.5.2 Thionins

The thionins are small, basic proteins found in a number of monocot. and dicot. species. In barley they have been localized to the cell wall adjacent to the plasma membrane. There are between 50-100 copies of the gene in the barley genome, probably as a result of gene duplication. The thionin protein is toxic to fungi. The thionin transcript is induced following fungal attack and stress. These proteins are probably involved in the defence-response (Bohlmann *et al.*, 1988 [9]).

1.5.3 The potato wound-inducible genes

The preparation of cDNA libraries from wounded potato tuber message facilitated the isolation of some interesting clones. Some of these have been well studied at the genetic level, but to date have no assigned function.

The *win* 1 and 2 genes

A cDNA probe, complementary to wound-inducible message, was used to isolate a genomic clone that consisted of two genes in tandem coding for win 1 and win 2 proteins. Both predicted proteins contained domains similar to signal peptides and carbohydrate binding domains. Transcript regulation of *win* 1 was confined to induction in the aerial tissues of the plant 20 hours after wounding whilst *win* 2 transcript was detectable in all parts of the plant including roots and tubers (Stanford *et al.*, 1989 [185]). *Gus* reporter analysis of the *win* 2 promoter in transgenic potato confirmed these results and also showed systemic expression of the chimeric gene in vascular tissue away from the wound site. Although sequences detectable by DNA hybridization experiments indicate the presence of homologous genes in tobacco and tomato the same reporter constructs were not wound-inducible in transgenic tobacco (Stanford *et al.*, 1990 [186]).

The *wun* 1 and *wun* 2 genes

The transcripts encoded by these genes are rapidly up-regulated at the site of injury following wounding of potato tubers reaching maximal levels of steady state message from 4 to 24 hours after wounding. These transcripts also accumulate following fungal infection. Homology with the *win* genes was found in the putative signal peptide encoding region (Logemann *et al.*, 1988 [121]). Transcriptional fusions of the *wun* 1 promoter with the GUS reporter were constructed and analysed in transgenic tobacco plants. Wound inducible expression was observed in leaf, stem and root, and developmental expression was seen in the stomium of the anther and in pollen grains (Siebertz *et al.*, 1989 [179]).

1.5.4 The parsley PR genes

Cloning of elicitor-induced PR mRNAs was facilitated by the addition of an elicitor derived from *Phytopthora megasperma sp. glycinea.* (pmg) to parsley cell cultures (Somssich *et al.*, 1988 [183]). The parsley PR1 cDNA took its name as the predicted protein encoded by the cDNA clone was similar in size and charge to the tobacco PR1 protein. This is as far as the similarity extends, and there is now good evidence that the gene products fulfil different roles in the defence-response. Recently, analogues have been found in bean, potato, pea and birch (Walter *et al.*, 1990 [199]). The birch analogue was initially isolated as a pollen allergen (Breitenender *et al.*, 1989 [26]). The message in parsley is induced rapidly (within hours) and to high abundance levels by fungal invasion (Schmelzer *et al.*, 1989 [171]). There are three genes in parsley that code for almost identical predicted proteins named PR1-PR3. Unlike most of the defence related PR gene products there appears to be no signal sequence associated with the predicted proteins and it is therefore assumed that the gene products are intracellular (Somssich *et al.*, 1988 [183]). DNA footprinting analysis has revealed an inducible footprint in the parsley PR1 promoter following addition of *Phytopthora megasperma sp. glycinea* derived elicitor into parsley cell suspension cultures (Meier *et al.*, 1991 [131]). Electroporation of protoplasts with PR1 promoter-*gus* fusions suggested that expression of the parsley PR1 gene was down-regulated in parsley following protoplasting (Meier *et al.*, 1991 [131]). However, using the related PR2 promoter-*gus* gene fusions in transient assays in electroporated parsley protoplasts demonstrated that a 125 bp fragment of the promoter was sufficient to drive strong expression (van de Löcht *et al.*, 1990 [193]). Despite the knowledge gained by studies using nucleic acid sequences very little is known about the protein products.

1.6 cDNAs isolated by differential screening and related techniques

Using labelled RNA from nuclear run off experiments as a probe, many cDNAs have been isolated from the parsley elicitor-treated cell culture derived cDNA library. These have been divided into groups depending on their temporal expression profile. Some members of these groups have already been identified such as the PR1 gene (Somssich *et al.*, 1989 [181]). Other similar approaches have been undertaken with other plant systems.

Using emergent technologies such as PCR amplification coupled with hybridization subtraction should lead to the isolation of cDNA clones that are more specifically involved with interactions between a plant and attacking organisms.

1.6.1 Model systems used in the analysis of wound-induced gene expression

Depending on the type of gene analysed, different systems may offer themselves for study. If a relatively homogeneous population of wounded cells are required in order to study the effect of an abiotic elicitor on gene expression then one may choose a cell culture system that contains cells all in a similar physiological state maintained in a defined and easily controlled environment. This system would however, be inadequate to study the spatial regulation of the gene following wounding.

In most studies, researchers use a combination of systems to isolate and study gene function. The first system is one that will enrich for the type of messages that are up-regulated by the desired stimuli. In the case of transcripts that are up-regulated following the addition of fungal cell wall fragment elicitors, cell culture systems are ideal. Simple mechanical wounding, such as slicing, tubers or root storage organs have provided good systems for the isolation of these induced genes since large surface areas of responsive cells, adjacent to the wound site, are created. However, because the cells are not homogeneous the wound

response will be superimposed on senescence reactions (Hahlbrock and Scheel, 1989 [76], factors such as the age of the organ (Wielgat *et al.*, 1979 [202]) and metabolic and cytological changes induced in the wounded organ (Borchert and McChesney, 1973 [17]) will further complicate the study of gene induction in response to wounding.

Examples of this have been seen from the work carried out by several groups. Klaus Hahlbrock's group chose parsley as a model system to study flavonoid biosynthesis genes. The group found that the cultured parsley cells were easy to propagate in defined liquid medium and retained their UV inducibility of protective flavanoids or the induction of phytoalexins following treatment with pathogen-derived elicitors (Hahlbrock *et al.*, 1983 [75] and Scheel *et al.*, 1986 [169]). Similarly, Christopher Lamb's group found that suspension cultures of French bean produced large quantities of the secondary metabolites associated with defence following the addition of fungal elicitor (Dixon *et al.*, 1983 [50] and Dixon, 1986 [49]).

Sliced potato tubers were used in the analysis of the accumulation of phenolics and have been used as a source of inducible mRNAs such as those corresponding to the *win* and *wun* genes which are very highly expressed and therefore obtainable by a differential screening approach (Cottle and Kolattukudy, 1982 [39], Logemann *et al.*, 1988 [121] and Stanford *et al.*, 1989 [185]).

Whole potato plants were the only system available to study the plant-pathogen interaction involving the parasitic nematode, *Globodera rostochiens* which implicitly gave no enriched source of message as the interaction between plant and pathogen is cell specific. However using PCR technology the successful cloning of a cDNA corresponding to induced transcript was achieved by dissecting cells adjacent to infected areas (Bowles, 1991 [22]).

When questions relating to the temporal, spatial and developmental regulation of the inducible gene arise they are generally answered using whole plants that are in an environment as close to their natural state as possible. Techniques such as *in-situ* hybridization, immuno-localization may be used to determine temporal and spatial gene expression patterns following certain stimuli in the host plant. For numerical quantification RNA

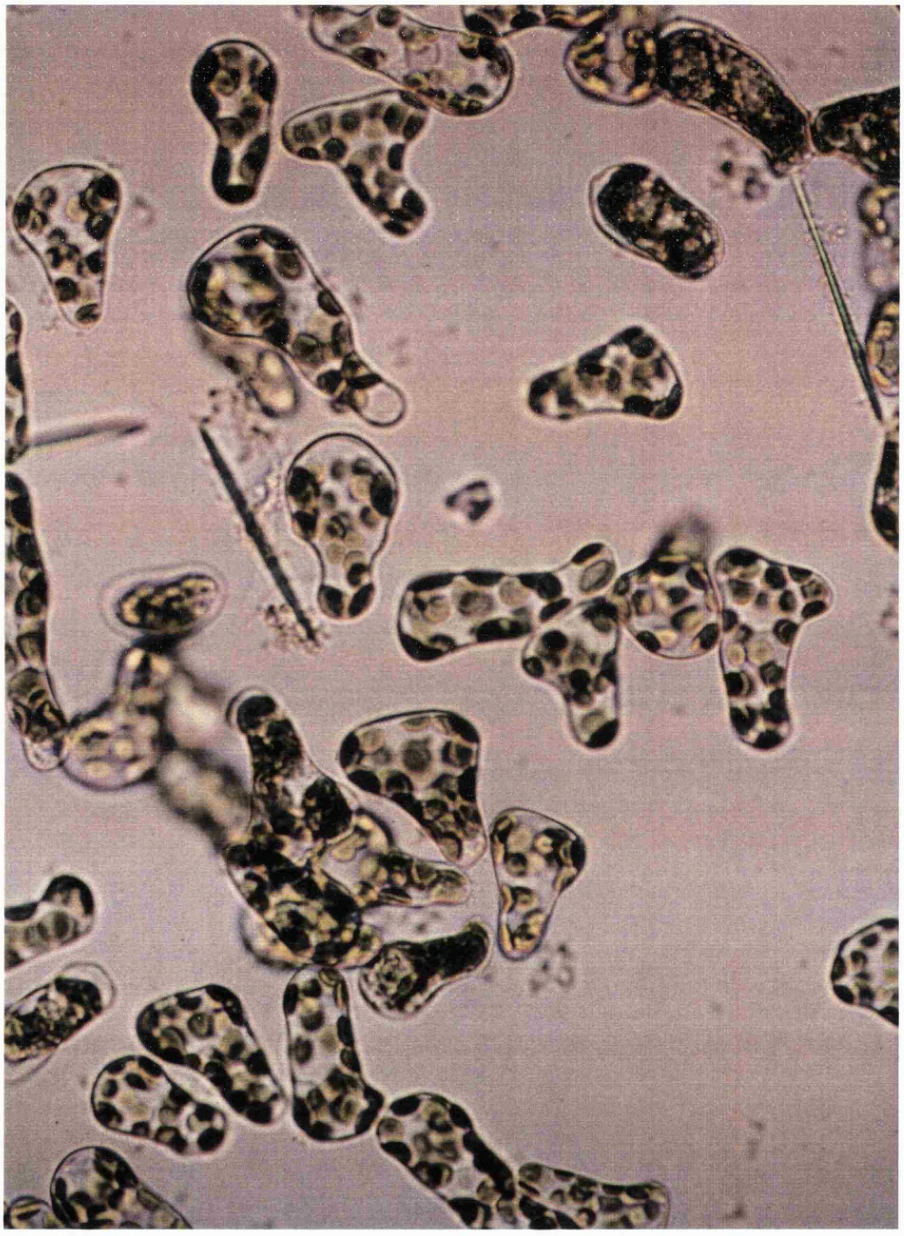
or protein abundance can be measured. However, current trends are to make reporter gene constructs to facilitate the rapid and simple analysis of the transcriptional strength and specificity of regulatory elements within genes. This technique, although extremely powerful in the elucidation of temporal and spatial expressions patterns directed by the regulatory sequences in transgenic plants, provides comparative data that may be prone to errors from abnormal expression in transgenic plants.

1.7 Scope of this thesis

Previous work carried out (Jullien and Guern, 1979 [97], Harikrishna, 1989 [78] and Harikrishna *et al.*, 1991 [79]) defined conditions that allowed the propagation of mechanically isolated *Asparagus officinalis* mesophyll cells in liquid medium. *Asparagus officinalis* is an old world monocotyledon plant and a member of the Liliacae. It is a glabrous dioecious herb with a short rhizome and has branched stems with cladodes in clusters. Mature female plants that have been pollinated by insects produce red fruit (Clapham *et al.*, 1987 [34]). Asparagus is an important vegetable crop in seventeen countries (Reuther, 1984 [150]) and in the U.S.A. state of Michigan between 1984 and 1988 asparagus netted an average value of 14,000,000 U.S.A dollars per year (Espie, 1989 [64]).

Initial studies were aimed at the identification of genes that were transcriptionally up-regulated during cell culture and which might have roles in the cellular processes involved in reactivation of cell division. The cell isolation procedure involves stripping the cladodes from six week old seedlings and isolating viable single cells following grinding in a mortar and pestle. The cells are then resuspended in a liquid medium containing inorganic salts, sucrose and phytohormones. Following mechanical separation the cells lose the ability to photosynthesize and become heterotrophic. After four to five days cell division commences and the cell culture may then be maintained for many months. The ability to generate cultures following mechanical isolation of cells has been reported for few species including peanut (*Arachis hypogaea*)(Joshi and Ball, 1968 [96]), *Zinnia elegans* (Iwaski *et al.*, 1988 [90]) and hedge bindweed (*Calystegia sepium*) (Rossini, 1972 [157]). Therefore,

Figure 1.2. Mechanically separated asparagus cells three days post isolation.



the isolation of viable cells following grinding is not possible for most plants and single cell suspension cultures are generated by a protoplasting step. Asparagus therefore offers the possibility of providing large numbers of freshly isolated viable cells that may be propagated in a liquid medium (fig. 1.2). These cells are under going many metabolic changes and are transcriptionally active (Harikrishna, 1989, [78], Paul *et al.*, 1989 [144] and Harikrishna, 1991, [79]).

Comparisons with other model systems from which wound-induced genes had been cloned, such as parsley and bean cell cultures, led to the postulation that the asparagus cell suspension may also be enriched for wound-inducible transcripts. If this proves to be the case, then asparagus may be a valuable source of monocotyledon wound-inducible transcripts.

This thesis concerns the cloning and analysis of a cDNA derived from an asparagus wound-inducible transcript, the cloning of the corresponding gene and its regulatory sequences, and the analysis of the biological function using a *gus* reporter gene in transgenic tobacco plants. The data shown relates to both the expression of this gene in its native monocotyledon plant (asparagus) and of its transcriptional regulation in a foreign dicotyledon plant where appropriate wound-inducible and developmentally regulated expression was observed

Chapter 2

Methods

All chemicals mentioned in this chapter were supplied by either Sigma, BDH, Clontech or Pharmacia unless otherwise stated. Nucleotides were acquired from Pharmacia, and labelled nucleotides from Amersham ([^{35}S] α-dATP with a specific activity of greater than 22 TBq/mmol and a concentration of 370 MBq/ml, [^{32}P] α-dCTP with a specific activity of 110 TBq/mol and a concentration of 370 MBq/ml and [^{32}P] γ-dATP with a specific activity of 110 TBq/mol and a concentration of 370 MBq/ml). Similarly, all DNA modifying enzymes were obtained from Bethesda Research Laboratories (BRL), Pharmacia, Stratagene or Boehringer Mannheim.

All methods and solutions mentioned in this section whose protocols or constituents have been omitted are as described previously (Sambrook *et al.*, 1989 [165] and Draper *et al.*, 1988 [57]).

Plant and bacterial media components are described in section 2.19

2.1 Plant DNA extraction

Modified after Murray and Thompson, 1980 [135]

Plant material was frozen in liquid nitrogen and stored at ⁻80 °C or ground up with a pre-cooled pestle and mortar under liquid nitrogen immediately. The ground up material was placed in a suitable screw cap tube and incubated with an equal volume of 2 x CTAB (100 mM Tris-Cl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 40 mM β-mercaptoethanol) at 60 °C for 15 to 30 mins with occasional shaking. After mixing, the suspension was extracted with phenol:chloroform:isoamyl-alcohol (25:24:1). The two phases were separated by centrifugation and the upper aqueous phase moved to a new tube. A tenth volume of 10% CTAB (10% CTAB, 0.7 M NaCl), pre-warmed to 60 °C, was added and the aqueous phase, re-extracted with phenol chloroform and isoamyl-alcohol. Following centrifugation, the aqueous phase was transferred to a new tube 2.5 volumes of absolute ethanol added and the nucleic acids precipitated on ice for ten mins. The nucleic acids were collected by centrifugation and then washed in 70% ethanol. Following air drying, the nucleic acid was redissolved in distilled water.

The DNA concentration was either estimated directly by agarose gel electrophoresis or the RNA was first removed by a differential precipitation and the DNA quantity analysed spectrophotometrically. The RNA removal was carried out by adding a half volume of 8 M LiCl, incubating on ice for 10 mins, and then pelleting the RNA by centrifugation at 10,000 rpm for 10 mins. The DNA was then precipitated by adding an equal volume of isopropanol and collecting the nucleic acid by a similar centrifugation step. The remaining small RNAs were removed by adding a tenth volume of 1 mg/ml RNase A and incubated at 37 °C for ten mins. After phenol chloroform extraction, a twentieth volume 4 M sodium acetate pH 6.0 and 2.5 volumes of absolute ethanol were added and the DNA collected by centrifugation. The pellet was redissolved in an appropriate volume of double distilled water.

2.2 Plant RNA extraction

Modified from Covey and Hull, 1981 [40].

Plant material was frozen in liquid nitrogen and stored at $^{-}$80 °C. The frozen material was ground up using a pre-chilled pestle and mortar. Two volumes of grinding buffer (6% 4-aminosalicylate, 1% triisopropyl naphthalene sulphonate, 6% phenol, 50 mM Tris-Cl pH 8.4) was added and the material pulverized under liquid nitrogen. The frozen material was transferred to a phenol resistant tube and allowed to thaw whilst mixing with an equal volume of phenol chloroform (1:1). Following centrifugation, the upper phase was removed and re-extracted with phenol chloroform. One twentieth volume of sodium acetate pH 6.0 and 2.5 volumes of absolute ethanol were added and the nucleic acids precipitated on ice for ten mins. The nucleic acids were collected by centrifugation at 10,000 rpm for 10 mins at 4 °C, dried on the bench and resuspended in a small volume of double distilled water, usually between 100-300 μl. Three volumes of 4 M sodium acetate pH 6.0 was added and the RNA differentially precipitated at $^{-}$20 °C for 20 mins. The RNA was then pelleted using a minifuge, washed in 70% ethanol, air dried and resuspended in double distilled water. The quality and quantity was then checked spectrophotometrically and the RNA then diluted to a final concentration of 5 mg/ml and stored at $^{-}$80 °C.

2.2.1 Spectrophotometry

A dual beam spectrophotometer (Perkin-Elmer Lambda 5 UV/VIS) was used routinely for DNA and RNA concentration determination at OD_{260} and OD_{280} and a scan was obtained between 200 and 300 nm.

2.2.2 Poly(A)$^{+}$ RNA purification

An appropriate amount of oligo-dT cellulose (5mg cellulose per mg RNA) was equilibrated in sterile 1 x loading buffer (20 mM Tris-Cl pH 7.6, 0.5 M LiCl, 1 mM EDTA and 0.1% SDS) and pipetted into a siliconised Pasteur pipette plugged with autoclaved polyallomer wool. One volume of 2 x loading buffer was added to the RNA sample, the mixture heated to 65 °C for 7 mins and then placed on ice for 5-10 mins. The RNA was then loaded onto the column in a volume of 1000 μl and the eluate reloaded 4 or 5 times. The column was

washed with 50 ml of loading buffer to remove the tRNA and rRNA. 1.5 ml of elution buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA and 0.05% SDS) at 45 °C was added in 300 μl aliquots to the column. The eluted poly $(A)^+$ RNA was collected in sterile Eppendorf tubes, quantified and precipitated with 0.2 M sodium acetate pH 6.0 and 2.5 volumes of ethanol. The poly $(A)^+$ RNA was then resuspended in double distilled water at $^-80$ °C.

2.2.3 cDNA synthesis

After Gubler and Hoffman, 1983 [74].

Starting with 2 μg of poly $(A)^+$ RNA, cDNA synthesis was carried out using a Pharmacia cDNA synthesis kit according to the protocol supplied by the manufacturer. The first strand synthesis was achieved by oligo(dT) priming and addition of FPLC pure Murine Reverse Transcriptase. RNase H was used to partially digest away the RNA strand enabling DNA polymerase to utilize the remaining RNA fragments as primers – replacing the remaining RNA strand with DNA. The newly synthesized cDNA was then purified on a Sephacryl S-300 spun column. The synthesis reaction was followed by adding 0.37 MBq of α^{32}P-dCTP and electrophoresing the products of the reaction on agarose gels, drying down and analysing the extent of the reaction by autoradiography. *Eco* RI /*Not* I adaptors were ligated onto the cDNA and the adapted cDNA then re-purified using a spun column. The adaptors were designed so that only one adaptor can ligate onto each end of the cDNA and the *Eco* RI compatible cohesive overhang is already present. The use of *Eco* RI methylase and restriction enzymes was therefore unnecessary. Once ligated to each end of the cDNA the adaptors were phosphorylated to enable efficient ligation of the cDNA into *Eco* RI digested de-phosphorylated cloning vector. This was achieved by adding ATP and T4 Polynucleotide kinase to the cDNA. The phosphorylated cDNA was then ethanol precipitated and ligated to *Eco* RI digested dephosphorylated λ Zap II from Stratagene.

2.3 DNA manipulation and modification

2.3.1 DNA ligation

The quantities of DNA and the methods used varied slightly from application to application so only a few general outlines will be given.

For general ligations of molecules with compatible cohesive termini the following conditions were used:

One to two hundred nanograms of digested dephosphorylated vector and one to two hundred nanograms of digested gel purified fragment were mixed with a fifth final volume of 5 x Ligation buffer (BRL supplied with the DNA Ligase) and 1-10 Weiss units of T4 DNA Ligase and double distilled water up to a final volume of 10-50 μl. Incubations were carried out at 37 °C 30 mins or on the bench overnight.

Blunt ended ligations were carried out similarly but with the addition of a tenth volume of 10 mM Hexamine Cobalt Chloride (Rushe and Howard-Flanders, 1985 [158]) to the reaction before adding the ligase. One or two mins after adding the enzyme a fifth volume of 100 mM NaCl was added. These conditions increase the rate of ligation of blunt termini 50 fold.

2.3.2 Restriction digests

These were carried out according to the manufacturers recommendations for each individual enzyme. Generally, a ten fold excess of restriction enzyme was used. Restriction enzyme buffers were supplied by the manufacturer.

2.3.3 End-filling or recession of restriction enzyme generated overhangs

If blunt ended cloning of a fragment with 5' overhangs was necessary then the cohesive ends could be end-filled by the addition of a tenth volume of 2 mM dNTP mix and 10 units of Klenow DNA polymerase directly post digestion. The mixture was then incubated on the bench for 30 mins and the required fragment purified.

If the fragment had 3' overhangs then the ends were digested using T4 DNA polymerase. After the restriction enzyme digestion, a tenth volume of 2 mM dNTP's and 1-10 units of T4 DNA Polymerase were added to the DNA. The reaction was allowed to proceed at 12 °C for 30 mins. At this temperature, the exonuclease activity is approximately the same as the polymerase activity, thus leading to blunt end formation.

2.3.4 Dephosphorylation of DNA

Vector DNA that was digested with a single enzyme was generally dephosphorylated prior to ligation with the fragment of interest to prevent self-ligation of the vector causing a high proportion of non-recombinants. Two to five micrograms of vector were digested with the relevant restriction enzyme. The DNA was then extracted with phenol chloroform, ethanol precipitated, dried and redissolved in a small volume of double distilled water containing a tenth volume of 10 x CIP buffer (100 mM Tris-Cl pH 8.5, 10 mM $MgCl_2$ and 10 mM $ZnCl_2$). One unit of Calf Intestinal Alkaline Phosphatase was added and the incubation allowed to proceed at 37 °C for 30 mins. The de-dephosphorylated vector was then re-extracted with phenol chloroform, ethanol precipitated, dried and redissolved in double distilled water to give a concentration of 100 ng/μl.

2.3.5 Purification of DNA fragments

DNA fragments were purified from restriction fragments cut out of agarose gels directly using the geneclean kit (BIO 101 Inc.). All solutions necessary are provided by the manufacturer. The gel slice was placed into a sterile microfuge tube and 3 volumes of 6M NaI was added and the tube incubated at 60 °C until the gel had completely dissolved. Five microlitres of the glassmilk (silica powder) solution was added, the tube mixed and incubated on ice for five mins allowing the DNA to bind to the glassmilk under high salt conditions. The glassmilk was then pelleted by brief centrifugation in a minifuge and the supernatant removed. The glassmilk was then resuspended in 300 μl of new-wash, repelleted and the washing procedure repeated twice to remove all protein, RNA and other contaminants from the DNA that was still bound to the glassmilk. The last traces of new-wash were carefully removed and the glassmilk pellet resuspended in 10 μl of double distilled water, the tube was placed in a 60 °C water bath for a min or two and the glassmilk then spun down and the eluted DNA drawn off with a pipette and placed into a clean tube. Another 10 μl of double distilled water was used to resuspend the glassmilk pellet and the elution process repeated and the eluted DNA added to the first batch of eluted DNA. A tenth of the DNA solution was used in agarose gel electrophoresis to estimate quality and quantity of DNA and the remainder used or stored at $^{-}$20 °C.

2.3.6 DNA amplification by the polymerase chain reaction (PCR)

This technique relies on the resistance of the thermophilic DNA polymerase isolated from *Thermus aquaticus* to the high temperatures that are required to melt DNA.

Most experiments were based on the suggestions in Innis *et al.*, 1990 [88] but will be briefly outlined here.

For Inverse PCR 1 μg of genomic DNA was digested with an appropriate enzyme. The DNA was then phenol chloroform extracted, ethanol precipitated and then resuspended to a concentration of 1-2 ng/2 μl in 1 x ligation buffer (50 mM Tris-Cl pH 7.4, 10 mM $MgCl_2$,

10 mM dithiothreitol and 1 mM ATP) and T4 DNA ligase added to 0.02 Weiss units/μl and allowed to circularize at 15 °C overnight. The DNA was then ethanol precipitated, dried and resuspended in 20μl double distilled water and a fifth of this used in the PCR. The reactions were made up as follows:

- 10 x PCR buffer:

  500 mM KCl
  100 mM Tris-Cl pH 8.4
  15 mM $MgCl_2$
  1 mg/ml gelatin

- 10 x dNTP stock:

  All four dNTP's at 2 mM with respect to each
  pH with NaOH to pH 7.0

4 μl of circularized DNA
5 μl 10 x PCR buffer
5 μl 10 x dNTP stock
5 μl of 10 x Primer 1 (2.5 μM)
5 μl of 10 x Primer 2 (2.5 μM)
double distilled water to give a final volume of 50μl
2.5 units of *Taq* DNA polymerase.

The reaction mix was then over layed with a few drops of mineral oil and placed in the thermal cycling machine.

A Perkin-Elmer Cetus Thermocycler was used and 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 50-55 °C for 30 seconds, extension at 72 °C for 3 mins and a final extension at 72 °C for 5 mins was performed. The mineral oil was then extracted with chloroform and an aliquot of the PCR products analysed on an agarose gel.

For standard PCR reactions the above PCR protocol was followed with minor adjustments to the number of cycles and annealing temperature.

2.4 Transcript mapping

- Hybridization buffer

  40 mM PIPES (pH 6.4)
  1 mM EDTA (pH 8.0)
  0.4 M NaCl
  80% formamide

- Nuclease-S1 mapping buffer

  0.28 M NaCl
  0.05 M sodium acetate (pH 4.5)
  4.5 mM $ZnSO_4$
  20 μg/ml single-stranded DNA (carrier DNA)
  10-100 units/ml nuclease S1

- Nuclease-S1 stop mixture

  4 M ammonium acetate
  50 mM EDTA (pH 8.0)
  50 μg/ml tRNA (carrier RNA)

- Formamide loading buffer

  80 % formamide
  10 mM EDTA (pH 8.0)
  1 mg/ml xylene cyanol FF
  1 mg/ml bromophenol blue

A single-stranded probe that is labelled only at the end that hybridizes with the RNA and hence is protected from the S1 nuclease is prepared by T4 polynucleotide kinase labelling as described below. 10 μg of RNA was added to 30 μl of hybridization buffer and mixed by pipetting. Single-stranded radio-labelled probe was added in a volume of 1 μl (approx. 0.01 pmole of a specific activity greater than 5 x 10^7 cpm/μg). The mixture was then incubated at 85 °C for 10 mins to denature the nucleic acids and then incubated overnight at the desired hybridizing temperature (40-60 °C). 300 μl of ice-cold nuclease-S1 mapping buffer was then added, mixed with the nucleic acids and digestion carried out at 37 °C for 30 mins. 80 μl of ice cold nuclease-S1 stop mixture added, the tubes allowed to cool on ice and the nucleic acids collected by adding 3 volumes of ice cold ethanol and centrifuging at 12,000 x g for 5 mins. The pellet was washed in 70% ethanol, dried and resuspended in 4 μl of double distilled water. Following the addition of 6 μl of formamide loading buffer the DNA was heated in a boiling water bath for two mins prior to loading on a denaturing polyacrylamide sequencing gel for analysis. Radio-labelled markers or a sequencing gel ladder were used to establish the size of protected fragments.

2.5 Nucleic acid electrophoresis

2.5.1 DNA agarose gel electrophoresis

Electrophoresis was carried out at 1-10 V/cm in 1 x TAE buffer (0.04 M Tris-acetate, 0.5 μg/ml ethidium bromide and 0.001 M EDTA). A 0.6 to 1% agarose gel, depending on the size range of separation required, was prepared in 1 x TAE buffer. A fifth volume of 6 x type II loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water) was added to the DNA sample prior to loading and running. Marker DNAs of known molecular mass were run along side the DNA samples to enable rapid size estimation of the DNA bands when visualized on a UV trans-illuminator (UVP Inc.). The gel was then photographed using a video camera (UVP Inc.) and video processor (Mitsubishi).

2.5.2 RNA formaldehyde gel electrophoresis

The gel was prepared by melting an appropriate amount of agarose in water, cooling to 60°C, and adding 10 x MOPS buffer (0.2 M MOPS pH 7.0, 80 mM Sodium acetate and 10 mM EDTA pH 8.0) and formaldehyde to give final concentrations of 1 x and 2.2 M, respectively. The gel was poured in a fume hood and allowed to set for 30 mins.

The RNA samples were prepared by mixing the following in a sterile microfuge tube:

RNA (up to 30 μg)	4.5 μl
5 x Formaldehyde gel-running buffer	2.0 μl
Formaldehyde	3.5 μl
Formamide	10.0 μl

The samples were incubated for 15 mins at 65 °C and chilled on ice. Two microlitres of RNA loading buffer (50% glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol FF) was added to each, and the samples run in 1 x MOPS at 3-6 V/cm. The rRNAs were used as molecular mass markers.

Following staining with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30-45 mins the gel was photographed along side a transparent ruler to enable subsequent transcript size estimation.

2.5.3 DNA sequencing gels

The following gel solutions were prepared:

- 40% Acrylamide solution:

Acrylamide (DNA sequencing grade)	380 g
N,N'-methylbisacrylamide	20 g
Double distilled water to 600 ml	

- 10 x TBE buffer:

Tris base	108 g
Boric acid	55 g
Double distilled water up to 1 litre	

- 6% Acrylamide/urea gel mix:

40% acrylamide solution	75 ml
10 x TBE	50 ml
Urea (ultrapure)	230 g
Double distilled water up to 500 mls	

The gel apparatus used was obtained from BioRad. The 20 cm x 40 cm x 0.5mm gel was poured according to their instructions having first siliconised the top plate with Sigmacote. The gel mix was prepared by pouring 80 ml of the 6% Acrylamide/urea gel mix into a plastic beaker and adding 170 μl of 25% Ammonium persulphate (made up in double distilled water) and 70 μl of TEMED (N,N,N',N'-tetramethylethylendiamine). The gel mix was then taken up into a 50 ml syringe and poured between the plates. The flat side of a sharks tooth comb was inserted and the gel allowed to polymerize. Before loading, the gel was pre-run using 1 x TBE running buffer at 2300 V until a gel temperature of 50 °C was reached. Following electrophoresis, the gel plates were dismantled and the gel transferred to a piece of 3MM Whatman paper. This was over-layed with Saran wrap and the gel dried onto the 3MM paper using a gel slab vacuum drier from BioRad. When the gel was dry, autoradiography was carried out for 1 to 2 days.

2.6 Labelling of probes

2.6.1 Labelling of double-stranded DNA probes

After Feinberg and Vogelstein, 1984 [67].

These probes were the most commonly used for work such as Southern and northern blotting and colony and plaque hybridizations. The probe DNA was generated following suitable restriction digests and was separated on a low melting-temperature agarose gel. Following photography, the amount of DNA was estimated, the gel slice containing the DNA cut out of the gel and placed into a screw cap tube. The DNA containing agarose was then melted in a 70 °C water bath and double distilled water added to give a DNA concentration of 1 ng/μl. The probe stock was then stored at $^{-}$20 °C until required. DNA fragments from standard agarose gels were also occasionally used after glassmilk purification.

The following solutions are made up and stored as stock solutions:

- Solution A:

2 M Tris-Cl pH 8.0	625 μl
5 M $MgCl_2$	25 μl
Double distilled H_2O	350 μl
β-Mercaptoethanol	18 μl
0.1 M dATP	5 μl
0.1 M dTTP	5 μl
0.1 M dGTP	5 μl

- Solution B: 2 M HEPES pH 6.0 titrated with NaOH.
- Solution C: Hexadeoxyribonucleotides evenly suspended in 3 mM Tris-Cl, 0.2mM EDTA pH 7.0 at 90 OD_{260} units/ml.

OLB was made by mixing A, B, and C in the ratio 2:5:3.

When a probe was required, the probe stock was boiled to denature the DNA and the following was placed into a separate microfuge tube:

Oligolabelling buffer (OLB)	3.0 μl
BSA (DNAse free)	0.6 μl
α^{32}P dCTP	1.0 μl
DNA polymerase I Klenow fragment	0.6 μl

Ten microlitres of boiled probe was then added to the oligolabelling mix and incubated at 37 °C for 30 to 60 mins. After this time, 85 μl of double distilled water was added. The incorporation was measured by combining 1 μl of the probe with 0.5 ml of Herring sperm DNA (500 μg/ml in 20 mM EDTA) and co-precipitating the DNA by mixing with 125 μl of 50% TCA. This mix was filtered through a Whatman GF/C disk in a filter tower and the filter washed twice with 10% TCA and twice with 100% ethanol to remove all unincorporated nucleotides. The same volume of probe was pipetted onto a second GF/C disk and the counts on each disk were measured by liquid scintillation or Cerenkov counting. The probe was then boiled for 3 mins and added to the hybridization.

2.6.2 Labelling 1st strand cDNA

For differential screening, labelled 1st strand cDNA derived from two different message populations was required. Poly (A)$^+$ RNA was prepared from the plus and the minus cell population as already described.

- 5 x RT Buffer:

Tris-Cl pH 8.3	250 mM
KCl	375 mM
$MgCl_2$	15 mM
dATP	5 mM
dTTP	5 mM
dGTP	5 mM

- Labelling reaction:

5 x RT Buffer	4 μl
Oligo $d(T)_{12-18}$ (1 mg/ml)	1 μl
DTT (100 mM)	2 μl
QH_2O	8 μl
Poly $(A)^+$ (100 ng/μl)	2 μl
α^{32}P dCTP	2 μl
Avian reverse transcriptase	1 μl

The reactions were incubated at 30 °C for 40 mins before adding 30 μl of sterile QH_2O and 50 μl of RNA degrading buffer (0.6 M NaOH and 20 mM EDTA).

The unincorporated nucleotides were removed by spun column chromatography on a Sephadex G-50 column and the incorporated counts measured by Cerenkov counting. Approximately 2 x 10^6 cpm were added to each set of filters.

2.6.3 Kinase labelling

This technique was used to generate probes labelled at one end for use in transcript mapping experiments. A suitable restriction fragment complementary to the DNA of interest was isolated and dephosphorylated using calf intestinal alkaline phosphatase. A second restriction enzyme was then used to cleave internally so that only one end of each molecule was dephosphorylated. The appropriate probe fragment was purified by agarose gel electrophoresis and end labelled using T4 polynucleotide kinase so that the protected labelled end of the probe would not be digested away in the presence of S1-nuclease.

- 10 x bacteriophage T4 polynucleotide kinase buffer

  0.5 M Tris.Cl (pH 7.6)

  0.1 M $MgCl_2$

  50 mM dithiothreitol

  1 mM spermidine HCl

1 mM EDTA (pH 8.0)

- A typical labelling reaction is shown below:

20 μl probe fragment (0.4-1.0 pmoles)
5 μl 10 x kinase buffer
4 μl [γ-^{32}P]ATP
20 μl QH_20
2 μl T4 polynucleotide kinase (20 units)

The reaction was allowed to proceed at 37 °C for 30 mins. The labelled probe was separated from unincorporated nucleotides by Sephadex G-50 spun-column chromatography. Following an ethanol precipitation, the probe was dried and the incorporated counts measured directly by Cerenkov counting.

2.7 Nucleic acid blotting

Derived from Southern, 1975 [184] and Lehrach *et al.*, 1977 [113]. DNA and RNA gels were blotted identically. The only exception being that RNA blotting equipment was kept free of contaminating RNAse by washing with detergent and dH_2O. The preparation of gels however, differs.

2.7.1 Southern blotting

DNA separated on ethidium bromide stained agarose gels was photographed on a U.V transilluminator along side a ruler. The gel was placed in 2-3 volumes of depurinating solution (0.25 M HCl) for seven mins, transferred to 2-3 volumes of denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 mins to 2 hr and then finally to neutralizing solution (3 M NaCl, 0.5 M Tris-Cl pH 7.4) for 40 mins to 2 hr. DNA transfer was carried out as below.

2.7.2 Northern blotting

Following electrophoresis, staining and photography the RNA gels require no further treatment and are blotted directly.

A tray was filled with 20 x SSC (3.0 M NaCl. 0.3 M Sodium citrate, pH 7.0) and a heavy duty sponge (Woolworth's) placed in the tray and allowed to soak up the 20 x SSC. A piece of 3MM paper cut to size was placed on the sponge and the gel placed on top, a piece of Hybond-N (Amersham) cut to size was and placed on the gel. The Hybond-N was then masked fully with cling film around the edges to prevent the 20 x SSC from passing around the edges rather than through the Hybond-N. Two more pieces of 3MM paper were placed on top of the filter and then a stack of paper towels on top. A water-bottle filled (approx. 500 g) was placed on top of the towels and the nucleic acids transferred by capillary action overnight.

2.7.3 Northern dot blots

Hybond-N filter was wetted with 10 x SSC and allowed to dry. The RNA samples were incubated at 65°C for 5 mins in three volumes of the following solution:

Formamide	500 μl
Formaldehyde (37% solution)	163 μl
10 x MOPS buffer	100 μl

The samples were then chilled on ice and 1 volume of cold 20 x SSC was added. The samples were then spotted onto the membrane in 2 μl aliquots.

2.7.4 Fixing the blots

The nucleic acids were fixed onto the Hybond-N membrane by placing the filter, nucleic acid side down, on to a UV trans illuminator for 2 mins with the UV tubes on.

2.8 Pre-hybridization and hybridization of filters

Pre-hybridization and hybridization was carried out in the same solutions. The basic solutions used are given below.

- 100 x Denhardt's solution:

  2% [w/v] BSA
  2% [w/v] Ficoll
  2% [w/v] PVP

- 20 x SSPE:

  3.6 M NaCl
  0.2 M Sodium phosphate
  0.002 M EDTA pH 7.7

- DNA Hybridization solution:

  5 x SSPE
  5 x Denhardt's solution
  0.5%[w/v] SDS
  500 μg/ml Denatured sheared herring sperm DNA
  Made in 25 ml aliquots

- RNA Hybridization solution:

  As DNA hybridization solution but made up in 50% de-ionized formamide

The filters were placed into a hybridization chamber with 25 ml of the appropriate hybridization solution and pre-hybridized for one to two hr at 65 °C for DNA blots and 42 °C for RNA blots. During this time, the probe was labelled and the incorporation checked. The probe was then boiled for three mins and then added to the chamber. Hybridization was carried out at the same temperature as the pre-hybridization for four or five hr for high copy number target sequences or overnight for low copy number sequences.

2.8.1 Washing stringency conditions

For low stringency conditions, when heterologous hybridization was required, the filters were washed at 65 °C in wash A (3 x SSC, 0.1% SDS) with several of changes of wash solution or until no counts could be detected in the wash using a hand-held Geiger counter. For higher stringency washes, wash B (0.5 x SSC, 0.1% SDS) was used in a similar way. Finally, for high stringency washes, such as those used in northern analysis and Southern analysis of plasmid DNA, wash C (0.2 x SSC, 0.1% SDS) was used.

The blots were patted dry between two sheets of Whatman 3MM paper and wrapped in Saran wrap. Autoradiography was carried out at $^{-}$70 °C using X-ray film in a cassette fitted with an intensifying screen for a period of time ranging from an hour to a week depending on the radio-activity detectable on the filter.

2.9 Construction and screening of libraries

The libraries used in this work were all made in phage λ cloning vectors.

2.9.1 Construction of genomic libraries

Using the CTAB DNA extraction method outlined earlier, DNA from asparagus was isolated with an apparent molecular mass greater than 23 Kb. It was thought that this would

be of a suitable starting size to construct a genomic library.

The λ phage EMBL 3 was used for the construction of the library. The vector is commercially available as *Eco* RI and *Bam* HI double digested phage from Stratagene Inc.. The double digestion ensures the efficient removal of the 9 kb stuffer fragment which contains genes that are not essential for the lytic cycle of λ. The *Bam* HI cohesive termini on the phage arms are compatible to *Sau* 3A generated DNA fragments. *Sau* 3A is a restriction enzyme with a tetrameric nucleotide recognition cleavage specificity and is useful for the generation of large overlapping random fragments as are required for genomic libraries.

The EMBL 3 stuffer fragment contains sequences that stop λ replication in host *E.coli* that are lysogenic to the P2 phage. This makes EMBL 3 sensitive to P2 interference, SPI^+. Once the stuffer is removed and replaced with some genomic DNA the EMBL 3 phage becomes SPI^-. This gives an improvement of the selection of recombinant vectors using the P2 lysogen P2LE392 as the host *E. coli* strain. The *in vitro* packaging reaction selects for recombinant vectors as it will only package DNA molecules that are between the sizes 36 and 49 Kb and are flanked by *cos* sites.

Using 1 unit of the enzyme *Sau* 3A, digest conditions were found, by varying digest times, that gave a suitable partial digest of the genomic DNA so that the majority of DNA had a molecular mass between 9 and 20 kb.

Ten micrograms of partially digested DNA was dephosphorylated. The dephosphorylation was then checked on an agarose gel by comparing re-ligated dephosphorylated DNA with re-ligated non dephosphorylated DNA. One microgram of dephosphorylated partially digested DNA was then ligated to 1 μg of *Bam* HI cut λ EMBL 3 arms, overnight. The ligation mixture was then packaged *in vitro* using Amersham's commercial packaging extracts.

2.9.2 Construction of cDNA libraries

For cDNA libraries, λ ZAP II (Stratagene Inc.) was used. This is a λ insertional vector as opposed to a replacement vector and can only accept inserts between 0 and 7kb in size at a maximum before the molecule is too large to package. The λ ZAP II vector contains the pBluescript vector and the cDNA inserts are cloned into the polylinker of this plasmid. As the pBluescript contains an f1 origin of replication, which allows the synthesis of single-stranded DNA phagemids, the pBluescript recombinant can be obtained directly in plasmid form without any need to extract λ DNA and subclone the fragment. This is achieved by super infection of a host cell containing a λ ZAP II clone of interest with R408 helper phage

Synthesis of cDNA with adaptors has been described earlier. The cDNA with *Eco* RI/*Not* I adaptors was ligated to *Eco* RI digested dephosphorylated λ ZAP II vector from Stratagene Inc.. The ligation mixture was packaged with the Amersham packaging extracts according to their instructions and an aliquot plated out.

2.9.3 Plating out phage libraries

A single colony of the relevant *E.coli* host was picked into L-broth supplemented with 0.2% maltose, 10 mM $MgSO_4$ with the appropriate antibiotics and grown overnight in a shaking 37 °C incubator. The following morning, 1 ml of the overnight culture was added to 50 ml of pre-warmed L-broth containing 0.4% maltose, 10 mM $MgSO_4$ and the relevant antibiotics and grown in the 37°C shaker to an $O.D_{650} = 0.5$. The culture was placed on ice, spun at 3000 rpm for 10 mins at 4 °C and the pellet resuspended in 25 ml of ice-cold 10 mM $MgSO_4$.

The library was diluted to the desired titre with SM:

NaCl	5.8g
$MgSO_4.7H_2O$	2 g

1 M Tris-Cl pH 7.5 50ml

2% gelatin solution 5 ml

made up to a litre with double distilled water autoclaved and stored at room temperature.

An aliquot of phage stock or library was added to 150 μl of fresh plating cells and incubated at room temperature for 2 mins. Nine ml of molten top agar (NZY broth, 0.7% agarose and 10 mM $MgSO_4$) at 45°C was added to the phage/cell incubation, quickly mixed and poured onto a Petri dish containing NZY agar. The plates were inverted and incubated overnight at 37 °C. The plaques were visible as cleared areas on a lawn of bacteria.

2.9.4 Plaque lifts and library screening

Once the plates had grown, they were placed at 4 °C for an hour to harden the top agar. Circles of Hybond-N, cut to the same size as the plates, were placed onto the plates for a min and were keyed onto the plates using a needle and marker pen. So that the holes on the filter matched the marks drawn onto the plate. The filter was then peeled off the plate as carefully as possible, avoiding slippage using a pair of millipore tweezers. Trays containing Whatman 3MM paper soaked in denaturing solution and neutralizing solution were prepared and the filter placed phage side up on the denaturing paper for three mins then on the neutralizing paper for three mins. Any pieces of agar that had stuck to the filter were removed by rinsing the filter in 3 x SSC. The filter was then air dried. UV cross linking, pre-hybridization, hybridization, washing and autoradiography were carried out as previously described for Southern blotting.

When probing with an random hexamer primed labelled double-stranded DNA probe only one filter for each plate was required but for differential screening with labelled 1^{st} strand cDNA replica filters were made and probed separately with the plus probe and the minus probe. Following auto-radiography, the differentially expressed clones could be identified that hybridized only to one of the probes and not the other.

Positive clones were cored out of the plate and placed into a microfuge containing 1 ml of SM and a drop of chloroform to stop bacterial growth. This was titred and rescreened. Generally a single plaque was taken from a re-plated clone to ensure the isolation of a single plaque-pure clone. A small amount of this stock can be mixed with 200 μl of plating cells, media and incubated in the 37 °C shaker overnight to make a high titre phage stock of the clone.

2.9.5 Amplification of libraries

Generally, in order to ensure a reasonable possibility of isolating a positive clone, the whole genomic library must be screened (as discussed in chapters 3 and 4) Consequently, approximately a million recombinants, for asparagus, have to be screened. Once the initial screen had been done and the positives picked, the library is recovered in an amplified form by pouring 5-10 mls of SM onto each plate and the plates rotated slowly on a platform overnight. The following morning the SM was be pipetted off into a polypropylene tube and the debris removed by centrifugation. Once the supernatant had been pipetted into a new tube a few drops of chloroform were added and the amplified library stored in the fridge.

For cDNA libraries 300,000 independent clones were amplified and the remainder of the library stored unamplified.

2.9.6 Excision of pBluescript from λ ZAP

Two hundred microlitres of both the positive clone stock and fresh plating cells were placed in a 50 ml polypropylene tube with 10 μl of R408 helper phage (10^7 pfu) and incubated on the bench for 15 mins. 5 mls of NZY media were added and the tube placed in the 37 °C shaker for 4-6 hr.

The tubes were then placed in a 70 °C water bath for 20 mins to kill the cells and the λ ZAP phage. The rescued phagemid, which survives this temperature contains the single-

stranded pBluescript vector with the relevant insert.

The rescued phagemid was allowed to infect fresh plating cells by adding 20-200 μl of the heat treated rescued phagemid stock to 150 μl of plating cells, incubating on the bench for 5 mins, and spreading out on NZY-agar containing 100 μg/ml ampicillin. The plates were incubated overnight at 37 °C and the recombinant clones, now in double-stranded plasmid form, were amplified.

2.9.7 Medium scale phage preparation

Firstly, the phage was amplified in liquid culture by mixing 200 μl of plating cells and phage stock in an Eppendorf tube and incubating on the bench for 5 mins. This was then added to 50 mls of NZY media supplemented with 0.4% maltose and 10 mM $MgSO_4$ in a sterile 250 ml conical flask. The flask was then covered with foil and placed in a 37 °C shaker overnight. The following day, the culture had generally lysed and fibrous debris was visible. If this had occurred, the process was repeated using an aliquot of the non-lysed culture as a high titre phage stock.

To disrupt any unlysed cells, 1 ml of chloroform was added to the culture and the flask returned to the shaker for 15 mins. The debris was removed by centrifugation and the clear supernatant placed in a clean polypropylene tube. 0.5 μl of 60,000 u/ml DNase I and 5 μl of RNase I (10 mg/ml) was added and the tube returned to the shaker for a further 30 mins. The phage lysate was transferred to a polypropylene tube capable of withstanding high g-force and an equal volume of 20% PEG 6000, 2M NaCl made in SM was mixed into the lysate and the tube placed on ice for an hour. The phage was then collected by centrifugation at 10,000 rpm at 4°C for 15 mins in a Sorval RC5-B. All the supernatant was thoroughly removed and the phage pellet resuspended in 0.5 ml of SM and placed into microfuge tubes. To each tube, 5 μl 0.5 M EDTA and 5 μl of 10% SDS were added and the tubes incubated at 70°C for 15 mins. Two phenol chloroform extractions were performed and the phage DNA precipitated by adding a twentieth volume 4M sodium acetate pH 6 and 2.5 volumes of absolute ice-cold ethanol. The DNA was collected by

centrifugation, washed in 70% ethanol, air dried and resuspended in 100 μl of double distilled water. The DNA was then digested with suitable restriction enzymes and the approximate concentration estimated by agarose gel electrophoresis.

Following identification of homologous restriction fragments by Southern blotting, restriction fragments were isolated from the agarose gel, glassmilk purified and cloned into appropriately treated pBluescript vector.

2.10 Cloning and manipulation of plasmids

The favoured strain of host *E.coli* for the majority of cloning was XL1-blue. This is a Rec A^- male strain with a selectable tetracycline resistance marker on the F episome. It also contains other mutations with beneficial effects on the copy number and blue/white colour selection.

2.10.1 Transformation of *E.coli*

One ml from an overnight culture of XL1-blue *E.coli* was used to inoculate 50 ml of L-broth and the culture grown to an $O.D_{600}$ of 0.5 in the shaking 37 °C shaker. The cells were pelleted at 4 °C by centrifugation at 3000 rpm for 5 mins, and the media pipetted off. The cells were resuspended gently in 25 ml of ice-cold 50 mM $CaCl_2$ and left on ice for an hour, collected by centrifugation at 3000 rpm, 4 °C for five mins the supernatant removed and the bacterial pellet resuspended in 5 ml of 50 mM $CaCl_2$ 20% glycerol. The cells were then pipetted into 100 μl aliquots in Eppendorf tubes which were then flash frozen in liquid nitrogen. The competent cells were stored at $^-$80 °C until required.

Ligated DNA was transformed by adding 10 μl of 10 x TCM (100 mM Tris-Cl pH 7.5, 100 mM $CaCl_2$, 100 mM $MgCl_2$) to up to 100 μl of the ligation mix and adding a 100 μl aliquot of competent cells. The transformation mix was incubated on ice for 20 mins. The tube was placed in a water bath at 42 °C for exactly 2 mins to heat shock the

cells, 1 ml of L-broth was then added and the cells were incubated at 37 °C for an hour in order to express the antibiotic resistance genes carried on the newly received cloning vector. Following expression the cells were collected by centrifugation, the spent media removed and the cells resuspended in 100 μl of L-broth. 1, 10 and 89 μl of these cells were plated out on L-agar containing the relevant concentration of the selectable antibiotic, and if blue/white colour selection for recombinants is possible, 80 μg/ml X-gal and 20 mM IPTG. The plates were incubated at 37 °C overnight. The transformed cells formed colonies. When using colour selection, colonies containing recombinant vector plus insert appear white due to the interruption of the Lac Z gene and non recombinants appear blue.

2.10.2 Small scale plasmid preparations

Modified from Birnboim and Doly, 1979 [7]

- Solution I:

  50 mM glucose
  25 mM Tris-Cl pH 8.0
  10 mM EDTA pH 8.0

- Solution II:

  0.2 N NaOH
  1% SDS

- Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

1.5 mls of an overnight culture grown in L-broth plus antibiotic in the 37 °C shaker was spun down in in the minifuge for three mins. The spent broth was removed and the

bacterial cell pellet resuspended in 100 μl of solution I. 200 μl of solution II was added and the contents gently mixed by inversion. Following the addition of 150 μl of solution III, the tubes were shaken briefly to mix the solutions. The newly formed precipitate was removed by centrifugation and the supernatant pipetted to a new tube carefully avoiding any carry over of the precipitate. The crude plasmid DNA was then precipitated by adding 2.5 volumes of absolute ethanol, mixing and centrifuging at room temperature for 5 mins. The ethanol was pipetted off and the pellet washed in 70% ethanol, air dried and resuspended in 50 μl of double distilled water. Five to ten microlitres were then used in restriction analysis.

2.10.3 Large scale plasmid preparations

500 ml of culture was grown and the cells harvested by centrifugation at 10,000 rpm at room temperature for 10 mins in a Sorval RC-5B centrifuge using GSA polypropylene bottles. The broth was poured off and the pellet resuspended in 18 ml of solution I. To lyse the bacteria, 40 ml of solution II was added and the tube bottle inverted. The genomic DNA and protein was precipitated by the addition 20 ml of solution III when the bottles were shaken to mix the contents. The white precipitate was collected by centrifugation at 10,000 rpm for 5 mins and the supernatant filtered through polyallomer wool into clean vessels. An equal volume of isopropanol was mixed into the contents and the nucleic acids collected by centrifugation at 10,000 rpm for 10 mins. The supernatant was discarded and the remaining traces removed with a pipette. The pellet redissolved in 3 ml of double distilled water. The nucleic acid solution was transferred to a siliconised corex tube and 3 ml of 8 M LiCl was added and the tube vortexed. The precipitated large RNAs were removed by centrifugation at 10,000 rpm, 4 °C for 10 mins and transferring the supernatant to a fresh tube. The DNA was then precipitated by adding an equal volume of isopropanol, mixing and then centrifuging at 10,000 rpm for 10 mins. Following redissolving the pellet in 500 μl of double distilled water and transfer to a minifuge tube, 20 μl of 10 mg/ml RNase I was added and the tube incubated at 37 °C for 30 mins. 500 μl of 1.6 M NaCl containing 13% (w/v) PEG 6000 was mixed into the DNA solution and the

DNA collected by centrifugation at 4 °C for 10 mins. The supernatant was discarded and the pellet resuspended in 300 μl of double distilled water, phenol chloroform extracted twice and then ethanol precipitated by adding 100 μl of 10 M ammonium acetate and 2 volumes of absolute ethanol. The DNA was recovered by centrifugation for 5 mins, washed in 70% ethanol, air dried and resuspended in 500 μl of double distilled water. Following analysis by UV spectrophotometry, the DNA was redissolved to a concentration of 1 mg/ml and stored at $^{-}$20 °C.

2.11 DNA sequencing

Dideoxynucleotide sequencing was carried out using the Sequenase 2.0 kit from USB inc. The manufacturer provided all solution mixes and sequencing was carried out according to the manufacturers recommendations.

Sequenase 2.0 is obtained from the expression of a genetically engineered bacteriophage T7 DNA polymerase gene. Some of the advantages over other enzymes that are suitable for sequencing are that it has extremely low 3' to 5' exonucleolytic activity, it is highly processive leading to the synthesis of very long chains and it has a very high rate of polymerization. The limiting factor to obtaining sequence is generally the resolution of the denaturing gel system.

2.11.1 Single-stranded template production

Single-stranded template DNA suitable for sequencing was produced by rescuing phagemid from a clone containing the relevant insert in pBluescript. pBluescript contains an origin of replication recognized by proteins that can produce single-stranded DNA. These proteins include those involved in the packaging of the DNA into phagemid particles and other proteins required for the successful production of phagemids and are encoded by a helper phage genome. The helper phage is a M13 derivative and is poor at replicating itself but replicates phagemid DNA efficiently. It carries a kanamycin resistance gene allowing

selection of cells carrying the helper phage. The plasmid of interest, in a male host strain, is infected with helper phage which enters the cell through the sex pilli. The helper phage is then expressed and produces single-stranded phagemid from the pBluescript clone.

An overnight culture of the clone containing the DNA to be sequenced, grown in ampicillin and tetracycline (tet ensures maleness of bacterial strain), was used to inoculate 10 ml of NZY containing the same antibiotics. The culture was grown to an $O.D_{600}$ of 0.5. 1 ml of this was placed into a new tube. 10^9 pfu of the helper phage M13K07 were added and the tube returned to the 37 °C shaker for an hour. Following this time, 9 ml of NZY was added containing kanamycin at a final concentration of 100 μg/ml. The culture was allowed to grow at 37°C overnight with shaking.

The next day, the cells were spun down at 4,000 rpm for 10 mins and the supernatant placed into a new tube. A ninth volume of 40% PEG 6000 and 5M sodium acetate pH 7.0 was mixed in and the phage allowed to precipitate for 10 mins on ice. The phagemid was then recovered by centrifugation (10,000 rpm in a Sorval RC-5B or or full speed in a minifuge) for 10 mins. As much of the PEG supernatant as possible was removed and the pellet resuspended in 100 μl of double distilled water. The solution was extracted twice with phenol chloroform. A twentieth volume of 4 M sodium acetate pH 6.0 and 2.5 volumes of absolute ethanol were added. The tubes were then chilled to $^-20$ °C for 10 mins and the single-stranded DNA collected by centrifugation for 10 mins. The pellet was washed in 70% ethanol, air dried and and resuspended in 20 μl of double distilled water. An aliquot was run on an agarose gel to check quality and quantity.

Usually 1-7 μl (0.5-1 μg) of this was used in the annealing step with 10 ng a suitable synthetic oligonucleotide primer. Annealing was carried out by placing the tubes containing template DNA, primer and a fifth volume of 5 x sequencing buffer (USB sequenase 2.0 kit) in a total volume of 10 μl into a boiling water bath for 3 mins and then transferring the tubes to a beaker containing boiling water and allowing the beaker to cool to room temperature.

2.11.2 Sequencing double-stranded templates

Occasionally, plasmid DNA was used as the template. The plasmid was prepared from a 50 ml overnight culture grown in NZY broth with the relevant antibiotics. The same relative proportions of solutions I, II and III were used, as in the large scale plasmid preparations. The PEG precipitation step was omitted as PEG has a detrimental effect on template quality but all other steps were carried out in a suitable scaled down volume.

The quality and quantity of the plasmid was estimated by both spectrophotometry and agarose gel electrophoresis.

Ten micrograms of plasmid DNA was denatured by adding double distilled water to a final volume of 18 μl and then adding 2 μl of 2M NaOH, incubating at room temperature for 5 mins and then neutralizing by adding 4 μl of 5M ammonium acetate pH 4.8 (pH with glacial acetic acid) and adding 3 volumes of absolute ethanol. The tubes were placed in the $^{-}$80 °C freezer for 10 mins and the denatured DNA recovered by centrifugation at 4 °C for 10 mins. The pellets were washed in 70% ethanol, air dried and resuspended in 14 μl of double distilled water. The DNA solution was then divided into two new tubes and 20 ng of the appropriate primer added to each to allow the sequencing of both strands of DNA. Annealing was carried out as single-stranded template and sequencing carried out according to the Sequenase 2.0 manufacturers recommendations.

2.12 *Asparagus officinalis* growth and culture

2.12.1 Asparagus cell isolation

Asparagus seedlings were grown by Graham Benskin in the Leicester University botanical gardens in a glasshouse. Six week old plants were sent to the laboratory and 10 g of cladodes were stripped off the plants and surface sterilized in 10% bleach for 20 mins. They were then washed thoroughly in sterile tap water and ground up in a sterile pestle

and mortar with a small amount of sterile tap water. The resulting cell suspension was filtered through a sterile 64 μm mesh filter and the cells collected by centrifuging at 800 rpm in a Sorval RT6000B for 3 mins. The water was pipetted off and replaced with fresh sterile tap water. The process then repeated and the cells resuspended to a concentration 4 x 10^5 intact cells/ml in asparagus medium. The intact cell concentration had initially been found by counting the cell number using a haemocytometer. The fraction of viable cells was calculated from the proportion of cells that had a disrupted cytoplasm. The cell suspension was then placed in 10 ml aliquots in 90 mm Petri dishes sealed with Nescofilm or in 50 ml aliquots in 250 ml conical flasks sealed with a cotton bung and foil. The cells were incubated in the dark at 25 °C on a rotating platform, set at 40 rpm. The cells were checked for infections before harvesting by their appearance as viewed down a microscope. Once the cells had been harvested by centrifugation at 1,000 rpm for 10 mins, the spent medium was removed and the cells used directly, or flash frozen in liquid nitrogen and stored at $^-$80 °C.

2.12.2 Growth of asparagus for wounding studies

Asparagus seeds were surface sterilized in 100% IMS for 3 mins and washed in sterile tap water. The water was replaced with more sterile water and the seed allowed to imbibe for 3 hr. The seeds were then scattered on sterile water-soaked vermiculite in a foil covered sterile washing up bowl. The seedlings were then placed at 25 °C in the dark until the hypocotyl had grown to a hight of 5 cm (approximately two weeks post-germination). The seedlings were then harvested and cut in sterile conditions into relevantly sized sections and placed on filter paper soaked with sterile water in Petri dishes. The dishes were sealed with Nescofilm and the seedling sections left for the appropriate time in the relevant conditions. The seedling sections were then harvested, flash frozen in liquid nitrogen and stored at $^-$80 °C.

2.13 *Agrobacterium* methods

The strain of *Agrobacterium tumefaciens* used in all manipulations was LBA4404 (Hoekema *et al.*, 1983 [87]). This carries chromosomal rifampicin resistance and is propagated on NZY media containing 100 μg ml of this antibiotic. Growth was carried out at 25 °C as the Ti plasmid is not stable above this temperature.

2.13.1 Conjugation of recombinant plasmids into *Agrobacterium*

Once constructs had been made in the relevant pBin19 based vectors (Bevan, 1984 [4]), pBI101 or ROK2 in XL1 host *E.coli* 100 μl of overnight culture of *A. tumafaciens* LBA4404, the XL1 *E.coli* containing the construct of interest and HB101 *E.coli* containing pRK2013 (Ditta *et al.*, 1980 [47]) helper plasmid were spread on a NZY plate containing no antibiotics and the plate incubated overnight at 25 °C. (The pRK2013 helper plasmid has a wide host origin of replication and provides all the relevant factors to mobilize the conjugative transfer of the binary vector from *E.coli* to *A.tumefaciens*). The following day, a bacterial loop full of cells were streaked on to NZY plates containing 100 μg/ml rifampicin and kanamycin and grown at 25 °C until single colonies appear. This allows for the selection *Agrobacterium* transformed with the binary plasmid that contains a kanamycin resistance marker.

Single colonies are picked and grown with shaking at 25 °C for two days in NZY containing rifampicin and kanamycin each at 100 μg/ml. This stock is then used to transform plants after first checking for the presence and structure of the binary plasmid by Southern blot analysis.

2.13.2 Isolation of total nucleic acids from *Agrobacterium*

1.5 ml of the *Agrobacterium* cultures were spun down in a microfuge for 5 mins. The broth was removed and the cells resuspended in 300 μl of double distilled water. 100 μl of 5%

Sarkosyl and 150 μl of 5 mg/ml pronase E was added and mixed. The tube was incubated at 37 °C for an hour. The solution was extracted with phenol chloroform 3 times and the supernatant transferred to a new tube. A twentieth volume of 6 M sodium acetate pH 6.0 and 3 volumes of ethanol were added and the nucleic acid precipitate collected by centrifugation for 10 mins at 4 °C. The pellet was washed in 70% ethanol, air dried and resuspended in 50 μl of double distilled water.

10 μl of the *Agrobacterium* DNA was digested with appropriate restriction endonucleases and the products run out on agarose gels. Southern blots were made and suitable radio-labelled DNA probes used to analyse the DNA.

2.14 Tobacco leaf disk transformation

Mature *Nicotiana tabacum* SR1 plants supplied from the Leicester University botanical gardens were used for leaf disk transformation.

Just fully expanded leaves were surface sterilized in 10% bleach for 15 mins. The leaves were thoroughly rinsed in sterile tap water. Working on a sterile ceramic tile the leaf was cut into 0.5-1 cm squares avoiding the mid-ribs and the leaf edges.

A few disks were not dipped into the *Agrobacterium* in order to provide controls. The remainder were dipped into a 50 fold dilution of the relevant transformed *Agrobacterium* strains. The disks were then placed onto MSD4 x 2 plates. The plates were sealed with Nescofilm and incubated at 25 °C at low light intensity (2000-3000 lux) for 2 days until *Agrobacterium* growth was just visible.

The leaf disks were then transferred to fresh MSD4 x 2 plates containing 100 μg/ml kanamycin and 400 μg/ml augmentin (kanamycin selects for transformants and augmentin is a bacterial static antibiotic that prevents the growth of *Agrobacterium*). Half the control disks were placed on these plates and the other half transferred to fresh MSD4 x 2 plates supplemented with augmentin. This provided controls to check that the kanamycin

concentration is sufficient to kill non-transformed plants and that the MSD4 x 2 media is functioning correctly to produce shoots from the disks. The plates were incubated in the same conditions as before.

After a few weeks shoots appeared on the leaf disks. The shoots were cut from the disk and any callus removed. The shoots were placed in plastic pots containing MS0 media with the same concentration of antibiotics used previously.

After a further 1-2 weeks the majority of shoots produced roots. These plants were carefully removed from their pots and transplanted into Fisons potting compost. These plants were transferred to the transgenic plant growth room. Once the plants began to flower, a paper bag was placed over the flowers to prevent cross-pollination.

2.15 Screening transgenic plants for GUS activity

The *E.coli* β-glucuronidase gene (*gus*) has been developed as a reporter gene for the transformation of plants. The (*gus*) product is a stable enzyme that can hydrolyse fluorometric and colourometric β-glucuronide analogs allowing localization and quantitation of plant promoter expression (Jefferson *et al.*, 1987 [92]).

2.15.1 Protein quantitation for GUS experiments

Protein concentration was calculated using the Bradford method (Bradford, 1976 [23]).

- Bradford Solution

  600 mg/l Serva blue (Coomasie stain)
  2% Perchloric acid

The precipitate was allowed to settle overnight and the Bradford reagent filtered twice through Whatman No. 1 paper.

The assay was carried out in a microtitre plate with 10 μl of protein sample, 100 μl of Q water and 100 μl of Bradford solution. Bovine serum albumin standards were prepared and the microtitre plate read by a Dynatech MR5000 microtitre plate reader. A programme was written into the plate reader to calculate the protein concentration of the samples directly.

2.15.2 Fluorometric assay for GUS activity

- 200 mM $NaPO_4$ pH 7.0 This is made by mixing two stock solutions. *Stock A:* 0.2 M NaH_2PO_4 (31.2 g/l). *Stock B:* 0.2 M Na_2HPO_4 (28.39 g/l). For pH 7.0, combine 39 ml of A with 61 ml B.
- GUS extraction buffer

  50 mM $NaPO_4$ pH 7.0
  10 mM EDTA
  0.1% Triton X-100
  0.1% Sarkosyl
  10 mM β-mercaptoethanol

- 1 mM 4-methyl umbelliferone
- GUS fluorometric assay buffer. 1 mM methyl umbelliferyl glucuronide made in GUS extraction buffer.
- 200 mM Na_2CO_3

A small amount of plant material (approx 1g) was ground up in an Eppendorf tube using a micro-homogenizer in 200 μl of GUS extraction buffer and the tube stored on ice. 200 μl of 200 mM Na_2CO_3 were pipetted into the wells of a microtitre plate. Enough wells were filled to correspond to the number of time points required for each plant sample. Reactions were carried out at 37 °C.

Reactions were started by adding 10 μl of the plant extract to 500 μl of GUS fluorometric assay buffer that had been pre-warmed to 37 °C and immediately pipetting 20 μl of the mixed reaction into one of the filled wells in the microtitre plate. This was the time=0 reaction point. At regular time intervals this process was repeated. A few wells were left blank to serve as controls. The fluorescence was then measured, directly in the plate, in a Perkin-Elmer fluorimeter, exiting at 365 nm and measuring emmission at 455 nm. Dilutions of the 1 mM 4-methyl umbelliferone were used on the plate to allow a standard concentration against fluorescence curve to be calculated.

2.15.3 Histochemical localization of GUS activity in plant sections

- 0.5 M MES pH 5.6 (pH adjusted with NaOH)
- Fixation Solution

  0.3% Formaldehyde
  10 mM MES pH 5.6
  0.3 M Mannitol
- X-GLUC solution. 2 mM X-GLUC in 50 mM Na_2PO_4 pH 7.0. X-GLUC was made up in DMF at 100 mg/ml and stored at $^-$20 °C.

Sections were hand cut, placed in the fixation solution and were vacuum infiltrated for a min. The sections were left at room temperature for 45 mins, washed several times in 50 mM $NaPO_4$ pH 7.0, then transferred to X-GLUC solution and incubated for times from 1 hour up to overnight. The sections were then rinsed in 70% ethanol and observed.

2.16 Transient assays using pollen

Mature tobacco pollen was a gift from David Twell. The ballistic particle gun used was as manufactured by DuPont. The transient transformations were carried out as described elsewhere (Twell *et al.*, 1989 [190]).

2.16.1 Preparation of tungsten particles

Tungsten particles from DuPont were suspended at 0.05 g/ml in ethanol and stored at $^{-}$20 °C in 0.5 ml aliquots. When ready for use the particles were spun down and resuspended in 0.5 ml of distilled water. This washing procedure was repeated twice. Finally, the particles were resuspended and 25μl aliquots of the particle suspension were added to clean microfuge tubes. Five to seven micrograms (1 mg/ml)of reporter construct DNA was added followed by 25 μl of 1 M $CaCl_2$ solution. The tubes were then vortexed and 10 μl of 0.1 M spermidine (free base) added. Following incubation in ice for ten mins the particles were resuspended by sonication for 1-2 seconds at full power on a horn type sonicator. Two microlitres of the resuspended particles were then pipetted onto the macro-projectile.

2.16.2 Firing procedure

The sample was placed in the chamber. The stopping plate, macro projectile, blank 2.2 charge and the firing assembly were correctly placed. The chamber was evacuated down to 25 imperial inches of Mercury and the charge fired. The vacuum was released and the sample removed and placed into a 25 °C incubator for 6 hr.

2.17 Luciferase assays

These were carried out essentially according to Ow *et al.*, 1986 [141].

The tissue was ground up in 0.5 ml of luciferase extraction buffer (100 mM phosphate buffer pH 7.5, prepared by mixing 82 ml of 1 M K_2HPO_4 with 18 ml of 1 M KH_2PO_4 and diluting to a litre with dH_2O, 1 mM Dithiothreitol), the debris were spun down for 5 mins in a microfuge and the supernatant removed to a fresh microfuge tube. 50 μl of the protein extract are added to the bioluminescence photometer reaction cuvette followed by 100 μl of ATP buffer (50 mM HEPES pH 7.8, 20 mM $MgCl_2$ and 10 mM ATP). The machine

then adds 100 μl of 0.5 mM D-luciferin (Analytical Luminescence laboratory) made up in 100 mM sodium citrate and measures the light emitted.

2.18 Vectors and *E.coli* strains

- λZAP II: An engineered lambda insertional vector from Stratagene. This vector contains the pBluescript phagemid. This vector has a number of unique cloning sites that can accept DNA up to 7 Kb in length. The phagemid allows automatic excision of the clone in plasmid form when cells are co-infected with a M13 homolog helper phage.

- The pBluescript vectors: These are all phagemids from Stratagene. They have been constructed to allow *in vitro* RNA transcription of cDNA inserts from bacteriophage T3 and T7 promoters, the rescue of single-stranded DNA suitable for sequencing, as well as the normal attributes expected from modern bacterial cloning vectors, such as a large multicloning site, blue white colour selection for recombinants, high copy number and a β-lactamase selection gene.

- XL1-Blue: recA1, endA1, gyrA96, thi, hsdr17, supE44, relA1, lac, [F' proAB, $LacI^q$ ZΔM15, Tn10(tet^R)]. An uncharacterized mutation enhances the α-complementation to give a more intense blue colour on plates containing X-gal and IPTG.

- LE392: hsdR514, hsdM, supE44, supF58, lacY1 or Δ(lacIZY)6, galK2, galT22, metB1, trpR55.

- P2392: LE392 (P2 lysogen)

2.19 Bacterial media

- LB medium (Luria-Bertani Medium)

  For 1 litre:

To 950 ml of deionized H_2O, add:

bacto-tryptone	10 g
bacto-yeast extract	5g
NaCl	10g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 M NaOH (0.2 ml). Adjust the volume of the solution to 1 litre with deionized H_2O. Sterilize by autoclaving for 20 mins at 15 lb/sq.in with slow cooling.

- NZY medium

For 1 litre:
To 950 ml of deionized H_2O, add:

Diffco Casein hydrolysate	10 g
NaCl	5g
Diffco bacto-yeast extract	5g
$MgSO_4.7H_2O$	2g

Complete and autoclave as for LB.

For solid media add 15 g of Diffco bacto-agar per litre prior to autoclaving for plates or 7 g of bacto-agar per litre for top agar.

2.20 Plant media

- MS0 media. A Media based on MS salts (Murashige and Skoog 1962 [134]).

Component	Concentration (mg/l)
$CaCl_2.2H_2O$	440.000
NH_4NO_3	1650.000
KNO_3	1900.000
KI	0.830
$CoCl_2.6H_2O$	0.025
KH_2PO_4	170.000
H_3BO_3	6.200
$Na_2MoO_4.2H_2O$	0.250
$MgSO_4.7H_2O$	370.000
$MnSO_4.4H_2O$	22.300
$CuSO_4.5H_2O$	0.025
$ZnSO_4.4H_2O$	8.600
$FeSO_4$	27.850
Na_2EDTA	37.250
Glycine	2.000
Inositol	100.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
Thiamine HCl	0.100
Sucrose	30,000.000

Adjust the pH to 5.8 with 0.1 M HCl. The above components with the exception of sucrose are marketed in preweighed packets by Flow Laboratories Ltd.

- MSD4 x 2

  MSO medium with the following additions:

NAA	0.1 mg/l
6-BAP	1.0 mg/l
Sucrose	30,000.0 mg/l
pH 5.8	

- Asparagus Medium

modified from Nagata and Takebe, 1971 [136]

Component	Concentration (mg/l)
NH_4NO_3	825.000
KNO_3	925.000
$CaCl_2.2H_2O$	220.000
$MgSO_4.H_2O$	1233.000
KH_2PO_4	680.000
Na_2EDTA	37.300
$FeSO_4.7H_2O$	27.800
H_3BO_3	6.200
$MnSO_4.4H_2O$	22.300
$ZnSO_4.7H_2O$	10.580
KI	0.830
$NaMoO_4.2H_2O$	0.250
$CuSO_4.5H_2O$	0.030
Mannitol	30,000.000
Sucrose	10,000.000
Myo Inositol	100.000
Thiamine HCl	1.000
NAA	1.000
6-BAP	0.300

Adjust pH to 5.8 with KOH. Just prior to use add 3.4 ml of filter sterilized glutamine (2.35 g/100 ml) per 80 ml of medium.

Chapter 3

The analysis of the DD1-34 clone and homologous genomic clones

3.1 Introduction to previous work and experimental processes

A schematic shows the relationships between proteins, oligonucleotides and clones described in this chapter in Appendix I

In previous studies by Harikrishna [78] a protein (named DD1) was characterised, which is highly up-regulated in mechanically isolated asparagus cells. This target protein was identified through an extensive study of mRNA population changes during asparagus cell suspensions using two dimensional gel separations of *in vitro* translated message products using poly(A)$^+$ derived from freshly isolated cell suspensions at different times post initiation (Harikrishna, 1989 and Harikrishna *et al.*, 1991 [78], [79]). The DD1 protein was purified and microsequencing carried out to determine its primary structure. Using the amino acid sequence data, a 128 fold degenerate, oligomeric nucleotide was synthesized that corresponded to the predicted DD1 transcript. A clone was then isolated from a cDNA library, constructed us-

ing message from asparagus suspension cultures, via its sequence homology to the oligonucleotide (Fioroni, 1989 [69]). The isolated clone was named DD1-34. It was postulated that the gene product may have a role in the process of 'dedifferentiation' and reactivation of the asparagus cell division cycle.

Northern analyses provided good evidence that the corresponding message is up-regulated following cell isolation. Antibody raised against the DD1 protein cross reacted with a β-galactosidase-DD1 fusion protein generated from the pBluescript DD1-34 recombinant clone and hybrid-released message led to the translation of a protein of the same molecular mass as the observed DD1 protein.

Thus, the antibody and DD1-34 DNA probes showed that the message and protein were temporally regulated coordinately following mechanical isolation of asparagus cells (Fioroni, 1989 [69]).

3.1.1 General conclusions regarding asparagus as a model system

Although initially interested in the aspects of cell cycle reactivation and cellular 'dedifferentiation' Fioroni, 1989 [69] and Harikrishna, 1989 [78] realised that asparagus cells were subjected to mechanical damage during the initiation of cell suspension cultures. This suggested that there would be a reasonable probability of obtaining cDNAs derived from wound induced transcripts if mechanically isolated cells were used as a source of RNA. Parallels with the work of Lamb using bean suspension cultures and Hahlbrock using parsley cell cultures could also be drawn (reviewed by Hahlbrock and Lamb, 1989 [76]). However, a major difference was that the bean and parsley systems were completely 'dedifferentiated' and well established dividing cultures, whereas the work with asparagus cells concentrated on analysing message population differences observable from a few hours to a few days post cell isolation when the first cell division occurred. *In vitro* translation products of some asparagus up-regulated transcripts were detectable months after culture initiation, whereas other transcripts were abundant only transiently following culture initiation (Harikrishna, 1989 [78]). The transcript populations that were transiently up-regulated in a time period between 3 hours and 5 days were argued by Dr Fioroni to have a

Figure 3.1. Restriction map of the DD1-34 clone showing restriction fragments used as probes.

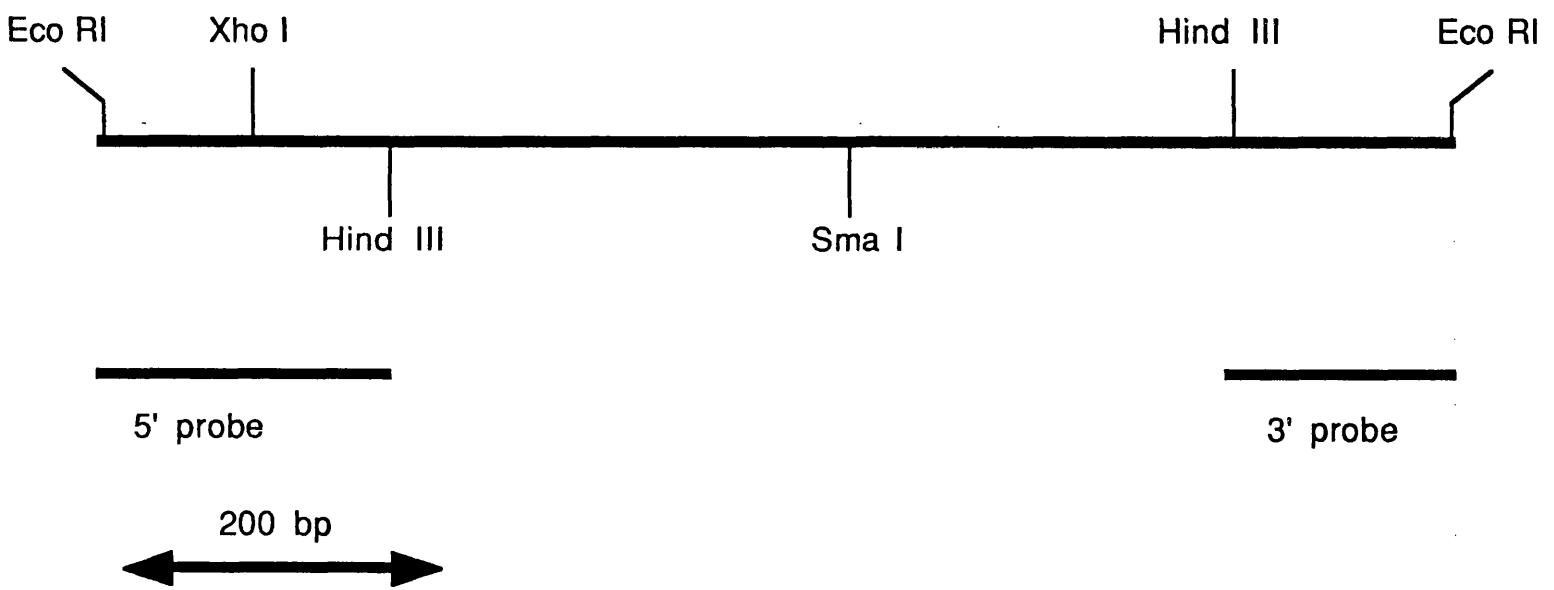
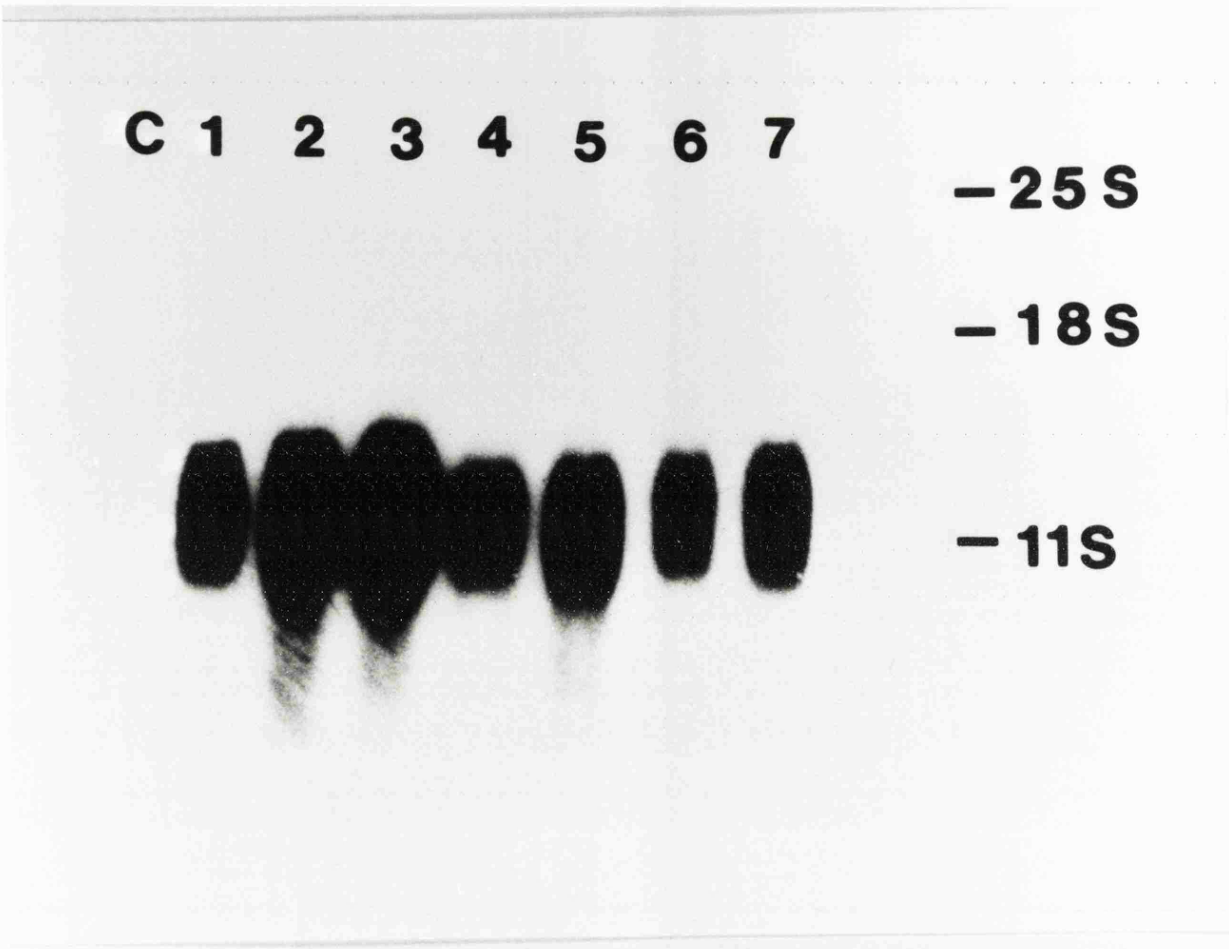
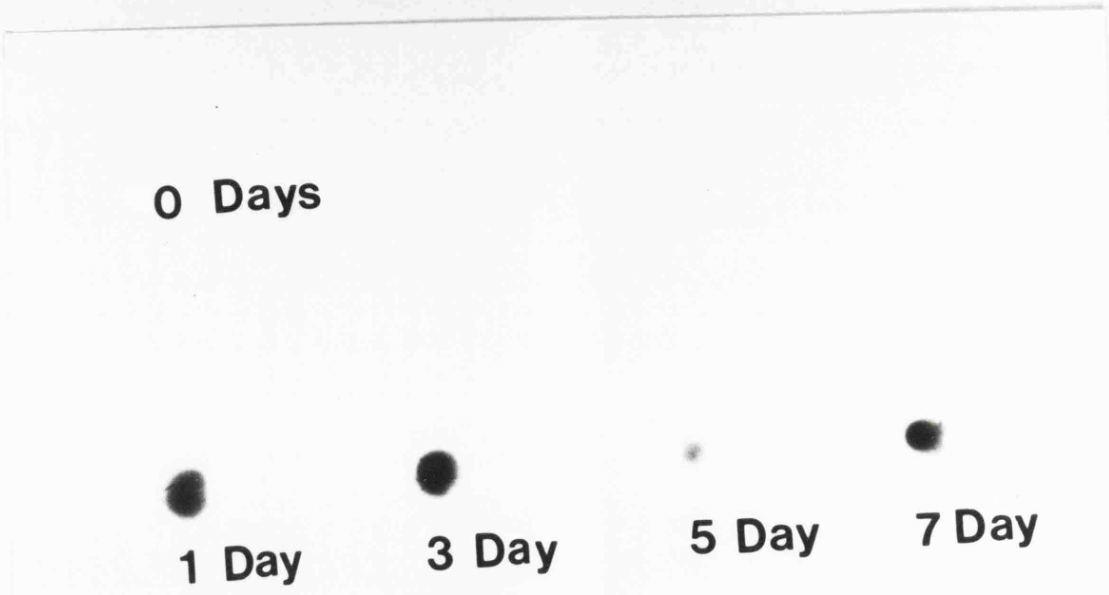

Eco RI
Xho I
Hind III
Eco RI
Hind III
Sma I
5' probe
3' probe
200 bp

Figure 3.2a. Northern analysis of total RNAs hybridized to the DD1-34 insert. 10 μg RNA per lane.

Lane C	cladode.
Lanes 1-7	cell suspensions 1-7 days post isolation.

Figure 3.2b. Northern dot blot of total RNAs that were isolated from two week old dark grown asparagus seedlings sliced into 5 mm sections and aged in sterile conditions for 1-7 days post wounding and hybridized to the DD1-34 insert. 10 μg RNA per dot.


C 1 2 3 4 5 6 7
25S
18S
11S


0 Days
1 Day
3 Day
5 Day
7 Day

fair probability of belonging to the wound-inducible class of transcripts. Conversely, those transcript populations that were stably and persistently induced probably belonged to the class of transcripts that are induced in response to changes in the cells metabolism due to long term culture effects such as those involved in the differentiation of cell state in passing from a photoautotrophic to a heterotrophic mode (Fioroni, 1989 [69]).

Unfortunately, due to lack of time the analysis of this new cDNA clone and the elucidation of its DNA sequence was not completed by Dr Fioroni. Consequently, canonical proof that the predicted protein encoded by the cDNA matched the DD1 microsequence was not obtained.

3.2 Approaches taken in the analysis of DD1-34

It was necessary to sequence the DD1 clone to obtain the final data required to show the cDNA was indeed the correct clone corresponding to the synthetic oligonucleotide sequence and the protein microsequence data. Elucidation of what stimuli would induce the transcript was also an aim. Concurrently, a genomic library was constructed in order to isolate corresponding genomic clones to provide more data on the structure of the gene.

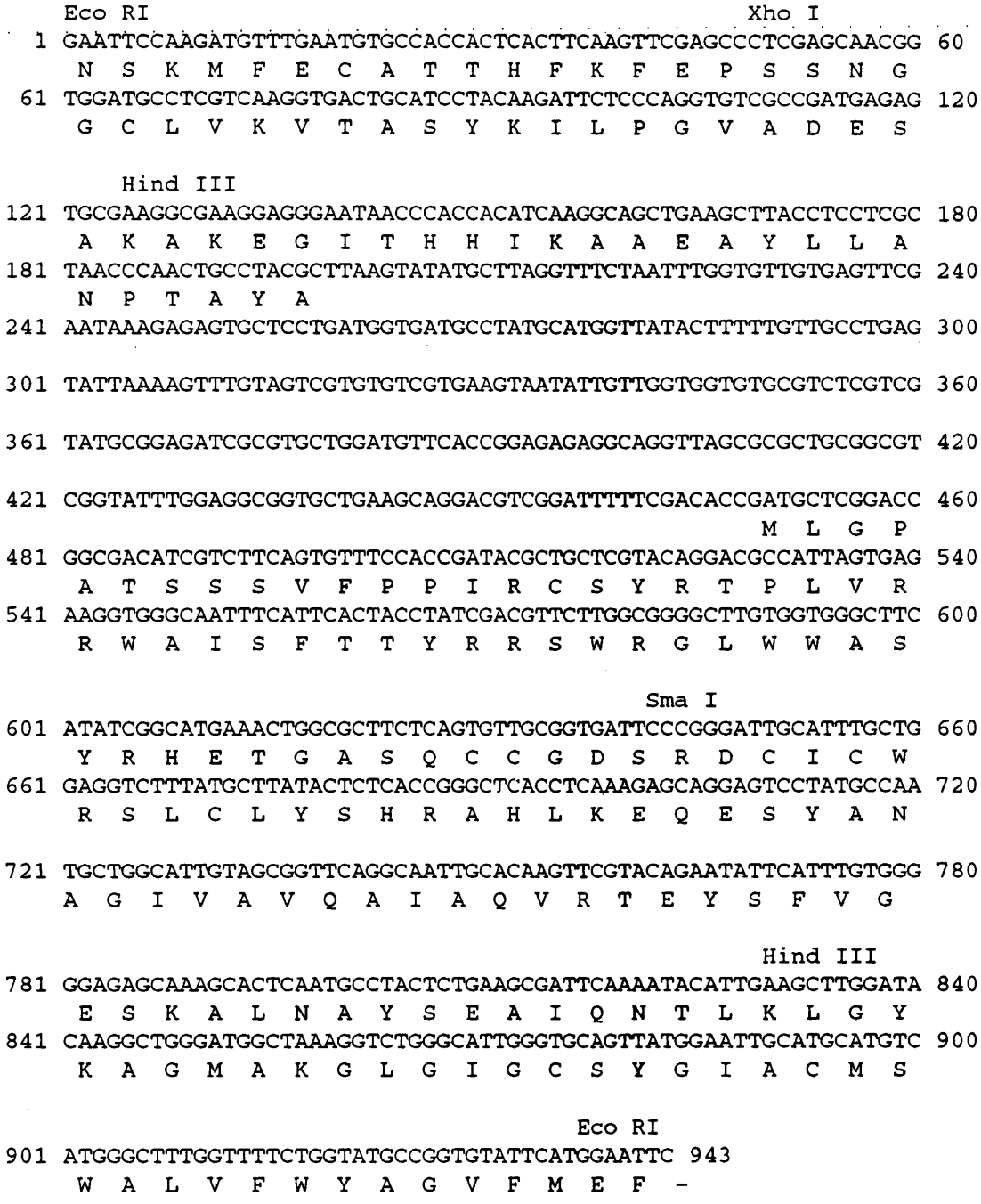
3.2.1 Restriction endonuclease analysis of the DD1-34 clone

Standard restriction endonuclease digestions, using hexameric sequence recognition enzymes, resulted in the construction of the restriction map shown in fig. 3.1.

3.2.2 Northern analysis of transcript using DD1-34 as a probe

Using RNA purified from cells in tissue culture in northern analysis showed that the radio-labelled DD1-34 clone hybridized to transcripts approximately 900 bp in length. The abundance of the hybridizing mRNA increased during tissue culture to a maximum steady state around two to three days post culture initiation. The size

Figure 3.3. DNA sequence of the DD1-34 insert with predicted significant open reading frames shown below and restriction sites above.


Eco RI
Xho I
1 GAATTCCAAGATGTTTGAATGTGCCACCACTCACTTCAAGTTCGAGCCCTCGAGCAACGG 60
N S K M F E C A T T H F K F E P S S N G
61 TGGATGCCTCGTCAAGGTGACTGCATCCTACAAGATTCTCCCAGGTGTCGCCGATGAGAG 120
G C L V K V T A S Y K I L P G V A D E S
Hind III
121 TGCGAAGGCGAAGGAGGGAATAACCCACCACATCAAGGCAGCTGAAGCTTACCTCCTCGC 180
A K A K E G I T H H I K A A E A Y L L A
181 TAACCCAACTGCCTACGCTTAAGTATATGCTTAGGTTTCTAATTTGGTGTTGTGAGTTCG 240
N P T A Y A
241 AATAAAGAGAGTGCTCCTGATGGTGATGCCTATGCATGGTTATACTTTTTGTTGCCTGAG 300
301 TATTAAAAGTTTGTAGTCGTGTGTCGTGAAGTAATATTGTTGGTGGTGTGCGTCTCGTCG 360
361 TATGCGGAGATCGCGTGCTGGATGTTCACCGGAGAGAGGCAGGTTAGCGCGCTGCGGCGT 420
421 CGGTATTTGGAGGCGGTGCTGAAGCAGGACGTCGGATTTTTCGACACCGATGCTCGGACC 460
M L G P
481 GGCGACATCGTCTTCAGTGTTTCCACCGATACGCTGCTCGTACAGGACGCCATTAGTGAG 540
A T S S S V F P P I R C S Y R T P L V R
541 AAGGTGGGCAATTTCATTCACTACCTATCGACGTTCTTGGCGGGGCTTGTGGTGGGCTTC 600
R W A I S F T T Y R R S W R G L W W A S
Sma I
601 ATATCGGCATGAAACTGGCGCTTCTCAGTGTTGCGGTGATTCCCGGGATTGCATTTGCTG 660
Y R H E T G A S Q C C G D S R D C I C W
661 GAGGTCTTTATGCTTATACTCTCACCGGGCTCACCTCAAAGAGCAGGAGTCCTATGCCAA 720
R S L C L Y S H R A H L K E Q E S Y A N
721 TGCTGGCATTGTAGCGGTTCAGGCAATTGCACAAGTTCGTACAGAATATTCATTTGTGGG 780
A G I V A V Q A I A Q V R T E Y S F V G
Hind III
781 GGAGAGCAAAGCACTCAATGCCTACTCTGAAGCGATTCAAAATACATTGAAGCTTGGATA 840
E S K A L N A Y S E A I Q N T L K L G Y
841 CAAGGCTGGGATGGCTAAAGGTCTGGGCATTGGGTGCAGTTATGGAATTGCATGCATGTC 900
K A G M A K G L G I G C S Y G I A C M S
Eco RI
901 ATGGGCTTTGGTTTTCTGGTATGCCGGTGTATTCATGGAATTC 943
W A L V F W Y A G V F M E F -

of the hybridizing transcript was approximately equal to that of the DD1-34 insert (fig 3.2a). To test whether the clone may have resulted from a wound-inducible transcript, total RNA was isolated from asparagus seedlings chopped into 5 mm sections and incubated on sterile damp Whatmann 3MM paper for various time points following wounding. This RNA was used in northern dot blot analysis which demonstrated that the transcript was indeed induced in wounded seedling sections (fig. 3.2b).

3.2.3 Sequence analysis of DD1-34

From subclones generated using the restriction endonuclease recognition sites within the DD1-34 clone DNA, sequence was obtained using single stranded DNA rescued from pBluescript derived subclones. Sequence data was confirmed by sequencing both stands of the clone at least once. The sequence data is presented in fig. 3.3 and shows the presence of restriction enzyme sites and significant open reading frames. Using the sequence data homology, searches were carried out with the oligonucleotide sequence used in the library screening process. our expectation was to find sequence with 100% identity to one of the possible sequence combinations of the degenerate oligonucleotide present in the DD1-34 sequence. The search revealed at best only a 65% identity between the DD1-34 sequence and the oligonucleotide sequence between the 478th and 494th bp of the DD1-34 sequence (data not shown). Furthermore, the cDNA did not contain a predicted open reading frame that could encoded the predicted protein sequence of the DD1 protein microsequence data. Of more importance, was the discovery that there was no single complete open reading frame, but two incomplete open reading frames, one which started within the coding sequence and one with no apparent stop codon. This data highlighted inconsistencies with previous findings of Dr Fioroni. For example, according to the sequence data the DD1 clone did not match the expected sequence required to encode the protein sequence as predicted by the microsequence. Data base sequences searches revealed no known homologues in the NBRF protein sequence and EMBL DNA sequence data bases at the time. These data caused some confusion and at this point further work

Figure 3.4. Southern blot of digested asparagus genomic DNA hybridized to the 5 probe generated from the DD1-34 clone (as shown in fig. 3.1). Digests were as follow:

Lane 1 *Xho* I and *Hind* III

Lane 2 *Xho* I

Lane 3 *Xba* I and *Hind* III

Lane 4 *Xba* I

Lane 5 *Bgl* I and *Hind* III

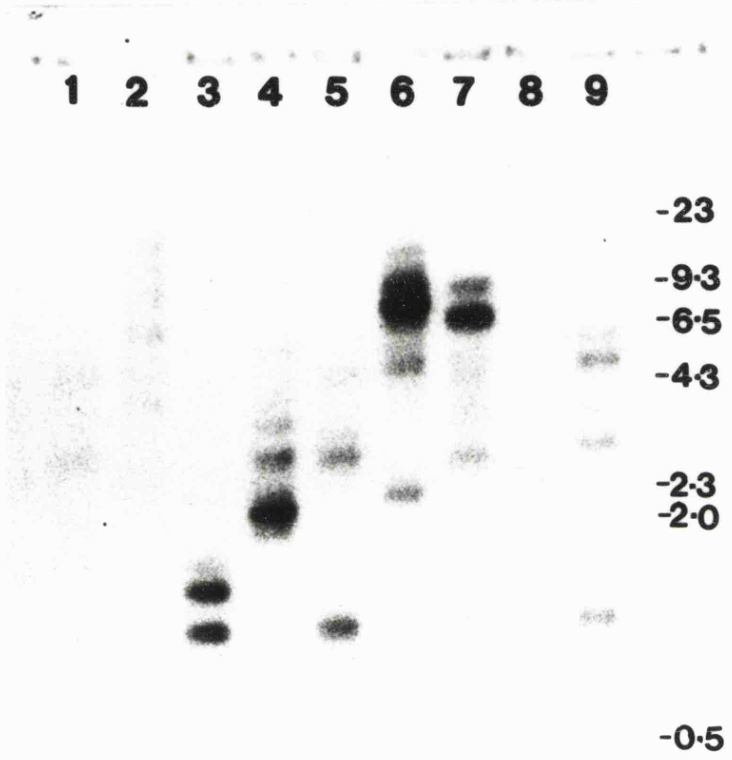
Lane 6 *Bgl* I

Lane 7 *Eco* RI

Lane 8 *Eco* RI and *Hind* III

Lane 9 *Hind* III

Molecular mass markers are shown in kb sizes to the right. 10 μg DNA per lane.


1 2 3 4 5 6 7 8 9
-23
-9·3
-6·5
-4·3
-2·3
-2·0
-0·5

on this clone was suspended in favour of analysing genomic clones homologous to the DD1 clone in order to try and collect data to clarify the uncertainties raised.

3.3 Isolation and analysis of genomic homologues to the DD1-34 clone

To study gene structure, genomic DNA fragments that include sequences flanking the exonic sequences must be isolated and cloned. The most direct method is to isolate high molecular mass genomic DNA and cleave it in such a way as to yield a series of overlapping random fragments of an appropriate size range to accommodate the cloning vector used.

Clarke and Carbon, 1976 [35] derived an equation that determines the mathematical probability of having any given DNA sequence in a genomic library

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

where P is the derived probability, f is the fractional proportion of the genome in a single recombinant and N is the necessary number of recombinants. It can be found by using this equation that if larger fragments of genomic DNA are inserted into the cloning vectors then fewer recombinants need to be screened.

Using standard DNA isolation procedures genomic DNA no larger than an approximate 100 kb average size could be obtained.

The general rule that isolated DNA in the region of 100–150 Kb in size is suitable for molecular cloning into λ replacement vectors, but DNA of twice this average size is required for the construction of cosmid libraries meant that it was prudent to construct a replacement λ vector library using dephosphorylated *Sau* 3A partially digested genomic DNA fragments cloned into EMBL 3 λ arms.

3.4 Genomic Southern analysis

Before proceeding with genomic cloning it is prudent to carry out genomic DNA blot hybridization experiments. This gives information on the copy number and organization of genomic sequences within the genome. The DD1-34 5' probe revealed many hybridizing sequences within the asparagus genome (fig. 3.4). Interestingly an *Eco* RI fragment of approximately 900 bp was found to hybridize to the 170 bp DD1-34 5' probe (fig 3.1) which correlates with the 900 bp DD1-34 clone.

From the data obtained through hybridization experiments it was predicted that there would be a possibility of cloning several different genomic homologues from a genomic library.

3.5 Screening of the genomic library

Following packaging of the library, an aliquot was plated out which indicated an estimate of the library size was 500,000 recombinants. Dr Elizabeth Paul had recently estimated the asparagus genome size to be around 1.2 pg (Paul *et al.*, 1989 [144]). Assuming in the worst case, an average insert size of 10 kb for the library, then this implied that there was greater than a 95% probability of the library containing any desired DNA sequence.

The whole of the library was plated out on P2LE392 *E.coli*, screened and 20 initial hybridizing plaques were identified. These were put through two more rounds of purification which resulted in nine separate isolates being identified that contained sequence which hybridized to the DD1-34 clone.

3.6 Analysis of the genomic clones

At this point, given that the first open reading frame identified in the sequence of the DD1-34 clone was incomplete, it was felt important to characterize the 5' regions to the DD1-34 hybridizing regions of any genes contained within the genomic clones. This was deemed important as it was hoped that by studying these regions

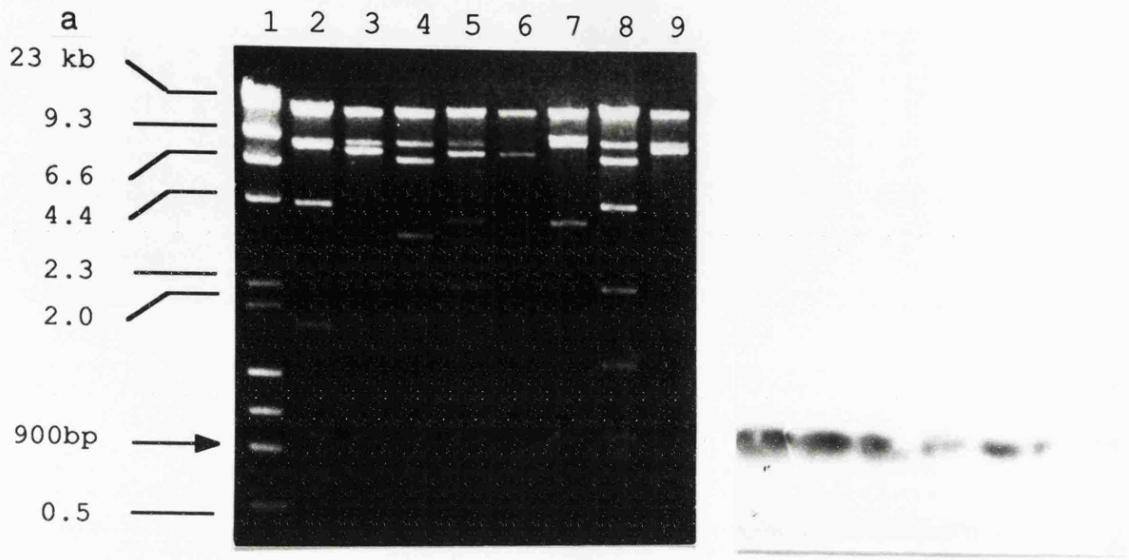
Figure 3.5a. *Eco* RI digests of genomic clones isolated by hybridization to the DD1-34 insert, with Southern hybridization to the DD1-34 5' probe shown to the right. Hybridizing fragments are shown by arrows.

Lane 1 λ *Hind* III and ϕX174 *Hae* III markers.
Lanes 2–9 genomic clones 1–8

Figure 3.5b. *Hind* III digests of genomic clones isolated by hybridization to the DD1-34 insert, with Southern hybridization shown to DD1-34 5' probe shown to the right. Hybridizing fragments are shown by arrows.

Lane 1 λ *Hind* III marker.
Lanes 2–9 genomic clones 1–8

Approximately 1 μg DNA per lane. Some hybridizing fragments were lost during photographic reproduction.


a
1 2 3 4 5 6 7 8 9
23 kb
9.3
6.6
4.4
2.3
2.0
900bp
0.5

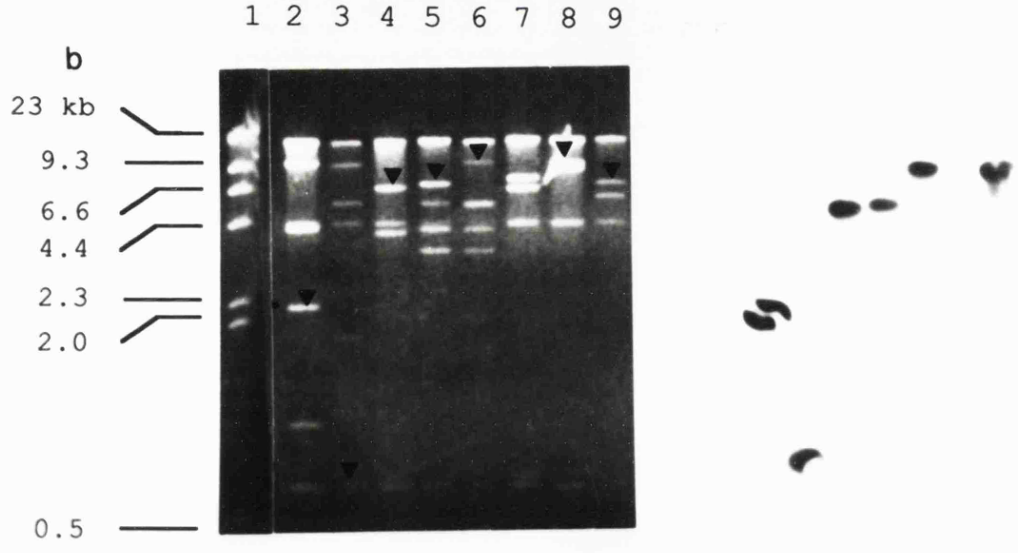
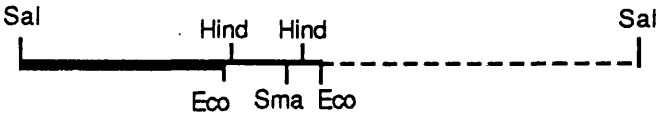
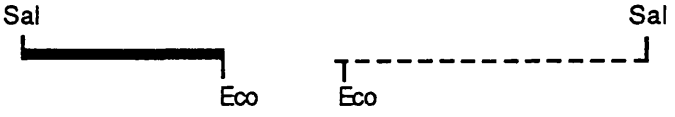

1 2 3 4 5 6 7 8 9
b
23 kb
9.3
6.6
4.4
2.3
2.0
0.5

Figure 3.6. pBluescript derived subclones of genomic clones 1, 3 and 8.

pBluescript Genomic clone 3 subclones

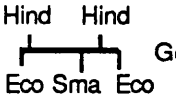


Genomic clone 3 6 Kb Sal



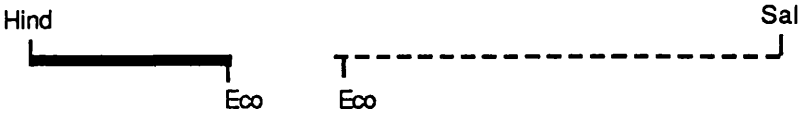
Genomic clone 3 2 kb Eco Sal upstream

Genomic clone 3 3kb Eco Sal downstream



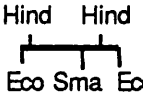
Genomic clone 3 Eco 900 bp

pBluescript Genomic clone 1 subclones



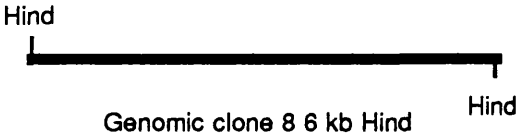
Genomic clone 1 2 kb Eco Hind upstream

Genomic clone 1 4 kb Eco Sal downstream

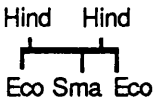


Genomic clone 1 Eco 900 bp

pBluescript Genomic clone 8 subclones



Genomic clone 8 6 kb Hind

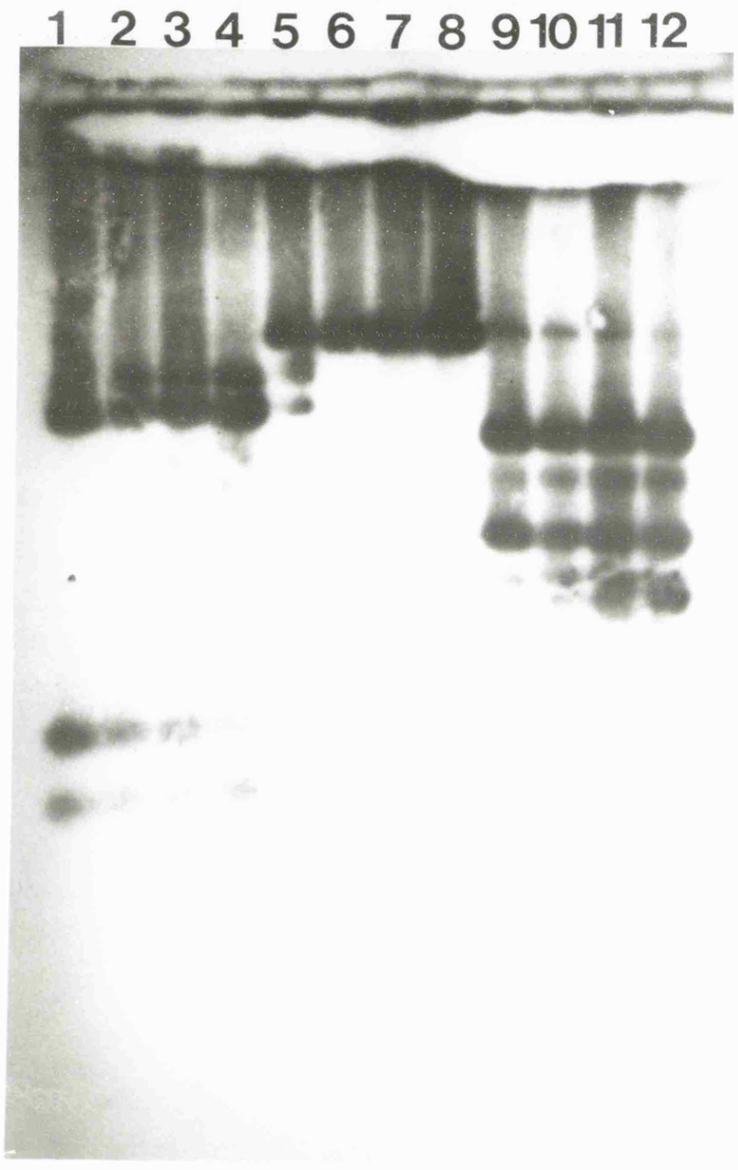


Genomic clone 8 900 bp Eco

Figure 3.7. Southern blot of the DD1-34 and *Eco* RI 900 bp derived genomic subclones following *Eco* RI digestion and digestion with tetrameric nucleotide recognizing restriction endonucleases hybridized with the DD1-34 insert. Restriction digests were as follows.

Lane 1	DD1-34 digested with *Alu* I
Lane 2	900 bp *Eco* RI subclone derived from genomic clone 1 digested with *Alu* I
Lane 3	900 bp *Eco* RI subclone derived from genomic clone 3 digested with *Alu* I
Lane 4	900 bp *Eco* RI subclone derived from genomic clone 8 digested with *Alu* I
Lane 5	DD1-34 digested with *Hae* III
Lane 6	900 bp *Eco* RI subclone derived from genomic clone 1 digested with *Hae*III
Lane 7	900 bp *Eco* RI subclone derived from genomic clone 3 digested with *Hae* III
Lane 8	900 bp *Eco* RI subclone derived from genomic clone 8 digested with *Hae* III
Lane 9	DD1-34 digested with *Sau* 3A
Lane 10	900 bp *Eco* RI subclone derived from genomic clone 1 digested with *Sau* 3A
Lane 11	900 bp *Eco* RI subclone derived from genomic clone 3 digested with *Sau* 3A
Lane 12	900 bp *Eco* RI subclone derived from genomic clone 8 digested with *Sau* 3A

Approximately 1 μg DNA per lane.


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

an upstream continuation of the first open reading frame predicted by the DD1-34 clone would be identified that would encode predicted protein sequence identical to that obtained by the DD1 protein microsequence. The identification and purification of promoter regions was also desirable.

Phage DNA preparations, restriction digests and Southern blotting experiments were carried out. The results obtained indicated that each individual clone contained an identical 900 bp *Eco* RI fragment that hybridized to the 170bp DD1-34 5' probe but when *Hind* III digests of the genomic clones were used in Southern analysis different sized fragments hybridized (fig. 3.5).(Differences in the intensities of hybridizing fragments is probably due to uneven blotting and transfer.) Restriction maps confirmed that the mapped genomic inserts had different sized restriction fragments (fig. 3.6). These data were complex and attempts to correlate hybridizing cloned genomic restriction fragments to those hybridizing restriction fragments from genomic DNA were hampered by the large number of hybridizing fragments observed in each genomic DNA digest.

However, hybridizing fragments from the genomic clones were subcloned into pBluescript for further analysis.

3.6.1 Sequence analysis of genomic clones

The 900 bp Eco RI fragments are identical

Initial DNA hybridization analysis of the genomic clones suggested that all the genomic clones contained a 900 bp fragment, apparently identical to the DD1-34 clone. These 900 bp *Eco* RI fragments were easily subcloned. The DD1-34 clone and subclones containing the 900 bp fragment generated from genomic clones 1, 3 and 8 were used in Southern analysis following digestion with tetrameric sequence recognizing restriction endonucleases (fig. 3.7). These data showed that the restriction enzyme fragments hybridizing with the DD1-34 900 bp probe were identical. The sequence of the 900 bp fragment derived from genomic clone 3 was obtained and shown to be identical to that of the DD1-34 clone (Appendix II).

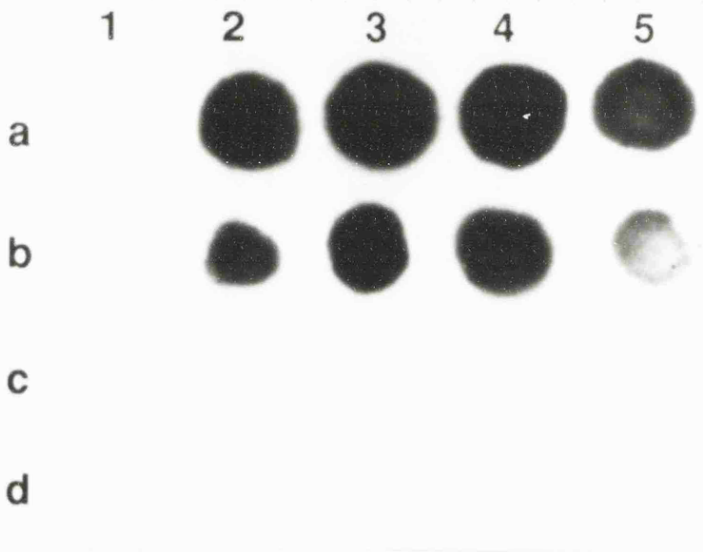
Figure 3.8. RNA dot blot probed with 5' and 3' probes from the DD1-34 clone (fig. 3.1). RNAs were isolated from asparagus cell suspensions 0–4 days post isolation (columns 1–5).

Row a 10 μg of total RNA/dot hybridized to the 5' probe.

Row b 5 μg of total RNA/dot hybridized to the 5' probe.

Row c 10 μg of total RNA/dot hybridized to the 3' probe.

Row d 5 μg of total RNA/dot hybridized to the 3' probe.


1
2
3
4
5
a
b
c
d

The 5' regions to the 900 bp Eco RI fragments of the genomic clones do not cross hybridize

While these data were being collated a 2 Kb *Eco* RI, *Hind* III generated fragment from genomic clone 1 that was 5' to the 900 bp *Eco* RI was used in hybridization experiments with the other genomic clones. The results of this experiment proved negative indicating the possibility that the genomic clones were different from one another and were derived from sequences in the asparagus genome that are unrelated with the exception of a small 900 bp region. This theory correlated with the large number of hybridizing sequences observable in the asparagus genomic Southern.

3.6.2 Northern hybridizations using a split DD1-34 clone

Restriction and cross-hybridization analysis of the cloned genomic sequences and the large number of hybridizing sequences present within the asparagus genome suggested that the DD1-34 gene sequence was complex and explanations for the evolution of the gene were not easy. The sequence data of both the 900 bp fragment from genomic clone 3 and the DD1-34 cast doubt as to the structure and derivation of the DD1-34 clone and it was decided that this may be resolved by using probes derived from regions containing the two possible open reading frames. The DD1-34 5' probe was used to represent the region of the DD1-34 clone that encoded the first predicted open reading frame. A new 3' probe comprised of a 130 bp *Hind* III *Bam* HI restriction fragment was isolated from a different subclone used previously in obtaining sequence data of the DD1-34 clone and corresponded to the second incomplete open reading frame predicted by the sequence (fig. 3.1 and fig. 3.3).

Using replica filters prepared from total RNA isolated from different aged asparagus cell suspensions as target transcript containing populations it was discovered that only the 5' probe hybridized with RNA from the cell population showing the characteristic induction of transcript, whilst the 3' probe failed to hybridize to the RNA (fig. 3.8).

3.7 Conclusions and conjecture concerning the identity of the DD1-34 clone

It was now known that the DD1-34 clone was probably not derived from a single transcript as different parts of the clone appeared not to hybridize to the same transcript. A possible explanation was that the clone consisted of an insert containing more than one cDNA molecule – one part derived from a hybridizing transcript and the other from a transcript that is present in the mRNA population of asparagus cell culture at extremely low abundance. Bearing in mind the sequence identity between the DD1-34 clone and the corresponding hybridizing sequence from genomic clone 3, another possible explanation was formulated. If there had been contaminating genomic DNA in the RNA preparation that was not removed in the purification of poly(A^+) RNA used in the preparation of cDNA, there would be a chance of cloning a small *Eco* RI genomic fragment. This would depend on the the efficiency of the protective methylation of *Eco* RI sites carried out before ligating on *Eco* RI linkers to facilitate the cloning of cDNA inserts into the vector, as excess linker molecules are removed prior to ligation by digestion with *Eco* RI. This explanation relies on improbable technical failings in the differential precipitation of RNA to remove DNA, the purification of polyadenylated RNA and the protective methylation procedure. There is also very little chance of this sequence being represented in a relatively small library. Other explanations may be arrived at by considering the possibility of cloning incompletely processed mRNA or hnRNA derived cDNA. Why there should be so many hybridizing sequences present in the asparagus genome may be explainable in terms of retrotransposon or transposon activity or the presence of multicopy pseudogenes. However, the fact remained that the DD1-34 clone contains sequence that hybridizes to wound-inducible transcript, thus instead of pondering why these data had been obtained and investigating the phenomenon further, the DD1-34 clone was abandoned and a new cDNA clone corresponding to the mRNA hybridizing region of the DD1-34 clone sought.

Chapter 4

Construction and screening of a cDNA library and the analysis of resultant clones

4.1 Introduction

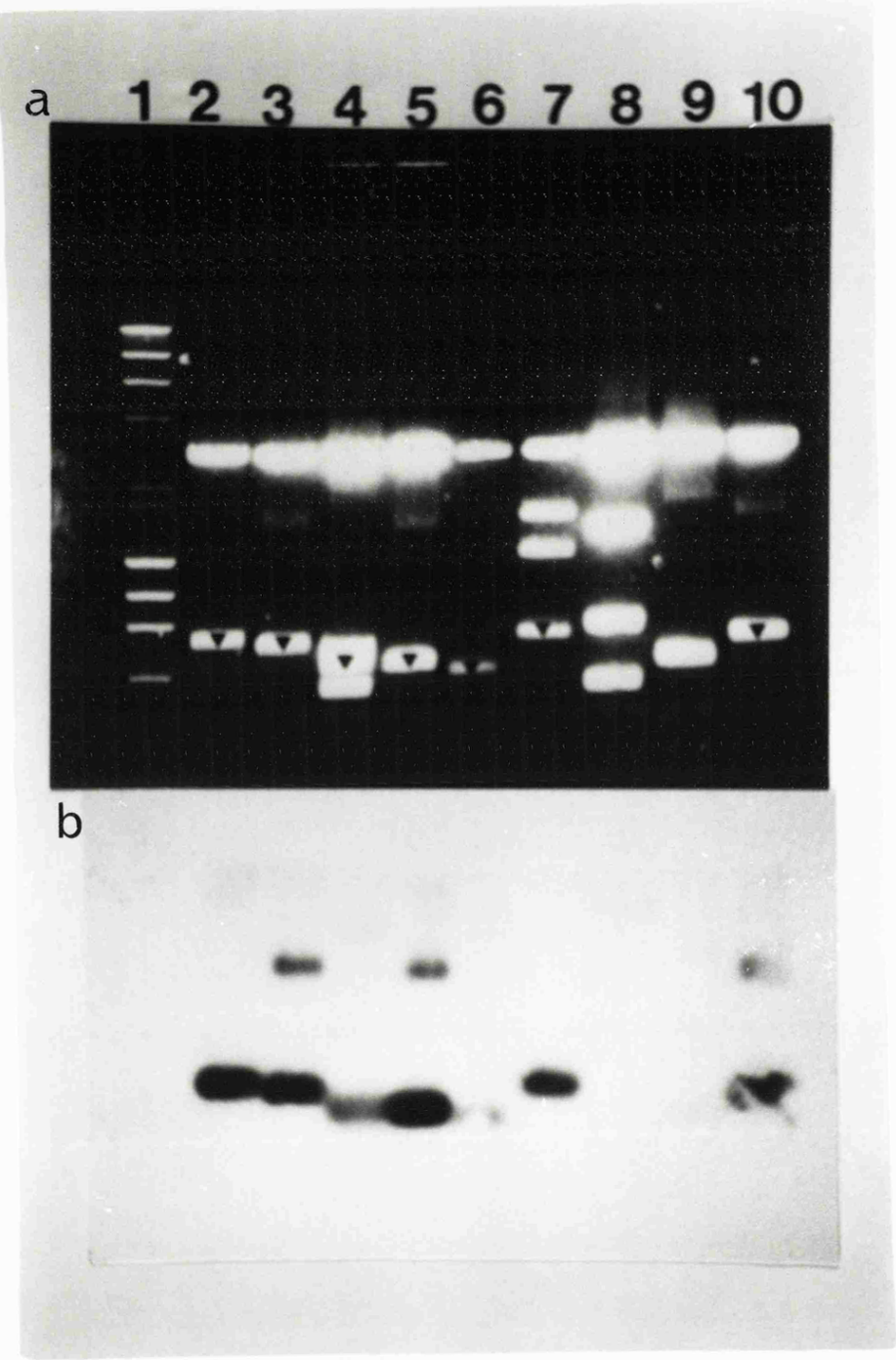
Following the analyses of the DD1-34 clone it was decided to construct a new cDNA library in the insertion vector λZap II from Stratagene. This vector has the advantages of blue/white colour selection of recombinant clones and it contains the pBluescript vector sequences. These sequences allow the automatic subcloning of cDNA recombinants in this plasmid following super-infection with a filamentous single stranded helper phage.

The cDNA synthesis was achieved using poly(A^+) RNA purified from total RNA pooled from asparagus cells that had been in suspension from 1-3 days. Northern analysis showed that during this period the transcript hybridizing to the DD1-34 5' probe was most abundant in culture. cDNA synthesis was carried out according to a modified method of Gubler and Hoffman (Gubler and Hoffman, 1983 [74]) using a Pharmacia cDNA synthesis kit. *Eco* RI/*Not* I adapters were used to avoid methylases and endonucleases in the cloning steps. A small amount of labelled dCTP was added

Figure 4.1a. *Eco* RI digests of WIP cDNA clones isolated by hybridization to the DD1-34 5' probe, with Southern hybridization to the DD1-34 5' probe shown below in figure 4.1b. Hybridizing fragments are shown by arrows.

Lane 1	λ *Hind* III and ϕX174 *Hae* III markers.
Lanes 2–10	cWIP cDNA clones 1–9

Approximately 1 μg DNA per lane.


a
1 2 3 4 5 6 7 8 9 10
b

to the synthesis mixture to trace the reaction and some of the second strand cDNA was run out on an agarose gel to check the size distribution of the products. Following ligation of the cDNA into the vector and *in vitro* packaging a small aliquot of the library was plated out on X-gal IPTG plates to estimate the size of the library and the proportion of recombinants. The library contained approximately 1.2 x 10^6 phage and the proportion of nonrecombinants was below 1%.

4.1.1 Screening for cDNA clones homologous to DD1-34

From information gained in northern hybridization experiments, as described in chapter 3, it was clear that the transcripts that hybridized to the DD1-34 clone were of fairly high abundance in the message population used to construct the library. Based on this knowledge, it was thought sufficient to screen twelve thousand clones. An aliquot of the cDNA library was amplified, the resultant plaques transferred to nylon membranes and hybridized to the 5' probe generated from the DD1-34 clone. Eighty hybridizing clones were identified from which 15 were picked at random and purified through two more rounds of screening. From this, nine clones passed the selection process and the recombinant plasmids were rescued. Plasmid mini-preparations were performed and the DNA digested with *Eco* RI. The digests were separated on an agarose gel, blotted onto a filter and probed with the DD1-34 5' probe (fig. 3.1). The results are shown in fig. 4.1.

Seven clones hybridized strongly to the *Eco* RI fragments and one had a weakly hybridizing insert. From these data it could be estimated that hybridizing clones represented approximately 0.3% of the library screened by deviding the number of hybridizing clones by the total number screened. It could be seen that about a third of the clones had one or more *Eco* RI fragments that did not hybridize to the probe, suggesting that these clones contained more than one cDNA.

From northern analysis the transcript size was estimated at 900 bp so clones containing inserts as close as possible to this were required. Two clones that contained single *Eco* RI fragments that were larger than the other hybridizing fragments were chosen as candidates for further analysis. These were named cWIP 1 and cWIP 2 (

Figure 4.2. DNA sequence of the cWIP1 insert with predicted protein translation shown below and restriction sites above.

```
  1 ATGAGTTCAGGGAGCTGGAGCCACGAGGTCGCTGTCAATGTCGCCGCAGGACGGATGTTC 60
    M  S  S  G  S  W  S  H  E  V  A  V  N  V  A  A  G  R  M  F
 61 AAGGCGGCAATGCTCGACTGGCACAACCTCGGCCCTAAGATTGTGCCTGACTTTATTGCC 120
    K  A  A  M  L  D  W  H  N  L  G  P  K  I  V  P  D  F  I  A
121 GGTGGCTCAGTGGTGTCTGGAGATGGATCTGTAGGAACCATCCGAGAGATCAAGATCAAC 180
    G  G  S  V  V  S  G  D  G  S  V  G  T  I  R  E  I  K  I  N

                                                  Acc I
181 AATCCTGCTATACCTTTCAGCTATGTGAAGGAACGCCTGGATTTCGTAGACCATGACAAG 240
    N  P  A  I  P  F  S  Y  V  K  E  R  L  D  F  V  D  H  D  K
241 TTCGAGGTGAAGCAGACCCTCGTGGAAGGTGGAGGTTTAGGTAAGATGTTTGAATGTGCC 300
    F  E  V  K  Q  T  L  V  E  G  G  G  L  G  K  M  F  E  C  A
301 ACCACTCACTTCAAGTTCGAGCCCTCGAGCAACGGTGGATGCCTCGTCAAGGTGACTGCA 360
    T  T  H  F  K  F  E  P  S  S  N  G  G  C  L  V  K  V  T  A
361 TCCTACAAGATTCTCCCAGGTGTCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACC 420
    S  Y  K  I  L  P  G  V  A  D  E  S  A  K  A  K  E  G  I  T

                       Hind III
421 AACCACATGAAGGCAACCGAAGCTTACCTCCTAGCCAACCCAACTGCCTACGTTTAAATA 480
    N  H  M  K  A  T  E  A  Y  L  L  A  N  P  T  A  Y  V

481 TAGTGATTGTGTTTCTTTGCGTGAAGTGCTTGTGAGTTTGAATAAGGAGATTGGTTATGA 540

      Hind III
541 GGAAGCTTGATGGGGTCATACATAGTTAGTTTATGTTGAATGATCAGCCTTTTTTGTGTG 600

601 AAGTACTTGGGAGTTTGAATAAGGAGACTGAATATGAGAAAGATTGATGGAGTTATCGTT 660

661 CATGTTGAATGATCAGCCTTATCAGTTTGTAACAGTGTCGAATGATCAGTCTTATCAGTT 720

721 TGTAATGGTGGCTTCAA 737
```

Figure 4.3. Sequence homology between the DD1-34 and the cWIP sequence.

```
           Quality:  197.3              Length:    262
             Ratio:  0.774                Gaps:      3
Percent Similarity: 92.095   Percent Identity: 92.095

DD1-34.1 x Cwip.Seq           ..

                .         .         .         .         .
      8 AAGATGTTTGAATGTGCCACCACTCACTTCAAGTTCGAGCCCTCGAGCAA 57
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    283 AAGATGTTTGAATGTGCCACCACTCACTTCAAGTTCGAGCCCTCGAGCAA 332
                .         .         .         .         .
     58 CGGTGGATGCCTCGTCAAGGTGACTGCATCCTACAAGATTCTCCCAGGTG 107
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    333 CGGTGGATGCCTCGTCAAGGTGACTGCATCCTACAAGATTCTCCCAGGTG 382
                .         .         .         .         .
    108 TCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACCCACCACATCAAG 157
        |||||||||||||||||||||||||||||||||| ||||||| |||
    383 TCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACCAACCACATGAAG 432
                .         .         .         .         .
    158 GCAGCTGAAGCTTACCTCCTCGCTAACCCAACTGCCTACGCTTAAGTATA 207
        ||| | |||||||||||||| || ||||||||||||||| |||| ||||
    433 GCAACCGAAGCTTACCTCCTAGCCAACCCAACTGCCTACGTTTAAATATA 482
                .         .         .         .         .
    208 .TGCTTAGGTTTCTAATTTGGTG......TTGTGAGTTCGAATAAAGAGA 250
         || ||  ||||||  ||  |||      ||||||||| ||||||  ||||
    483 GTGATTGTGTTTCT..TTGCGTGAAGTGCTTGTGAGTTTGAATAAGGAGA 530
                .
    251 GTGCTCCTGATG 262
         || |  ||| |
    531 TTGGTTATGAGG 542
```

WIP for wound-induced protein) and were both about 750 bp in length and could possibly have contained almost full length cDNAs.

Restriction enzyme mapping experiments showed that the cWIP 1 and 2 clones were derived from homologous transcripts but the cDNA's were inserted into the cloning site in different orientations (data not shown). As a check that the adaptors had ligated correctly onto the cDNAs a *Not* I digest was carried out using the clones. This yielded identical restriction fragments to the *Eco* RI digest indicating that the adaptors were correctly in place.

4.2 Sequence analysis of the cWIP 1 clone

Sequence analysis was carried out on subclones generated from the cWIP 1 clone such that both strands of the DNA were sequenced. Using computational analysis, a predicted open reading frame was found and a restriction map generated (fig. 4.2). The predicted open reading frame starts from the very first base pair and is a methionine, the first amino acid of a polypeptide. This data suggested that cWIP 1 clone contained no sequence corresponding to the 5' untranslated region of the transcript but may contain the full polypeptide encoding sequence.

Sequence comparisons with the DD1-34 clone revealed areas of strong homology between the central parts of the cWIP 1 cDNA and the 5' region of the DD1-34 clone. The region of homology was in the sequence that encoded the predicted open reading frame such that there was approximately 90% identity over a predicted 60 amino acid region between cWIP 1 and DD1-34 and a corresponding 90% identity in the nucleotide sequence over 250 bp (fig. 4.3). Other than this, there was no other homology between these sequences.

The cWIP 1 clone encoded a predicted open reading frame of 474 bp that was more extended in the upstream regions in comparison to that obtained by the DD1-34 clone, but there was insufficient evidence to indicate that the cWIP 1 clone contained the complete coding sequence. There was also a 263 bp untranslated region but no poly(A) tail on the cWIP 1 clone, so it was concluded that this was not a full length

Figure 4.4. Southern blot of *Eco* RI digested λ genomic clones and the 5 kb pBluescript subclone derived from the 2 kb *Eco* RI, *Sal* I fragment from genomic clone 3 (figure 3.6) hybridized to the cWIP insert.

Lanes 1–3 λ genomic clones 1–3.
Lane 4 5 kb pBluescript subclone derived from the 2 kb *Eco* RI, *Sal* I fragment from genomic clone 3.
Lanes 5–9 λ genomic clones 4–8

Approximately 1 μg of DNA per lane.

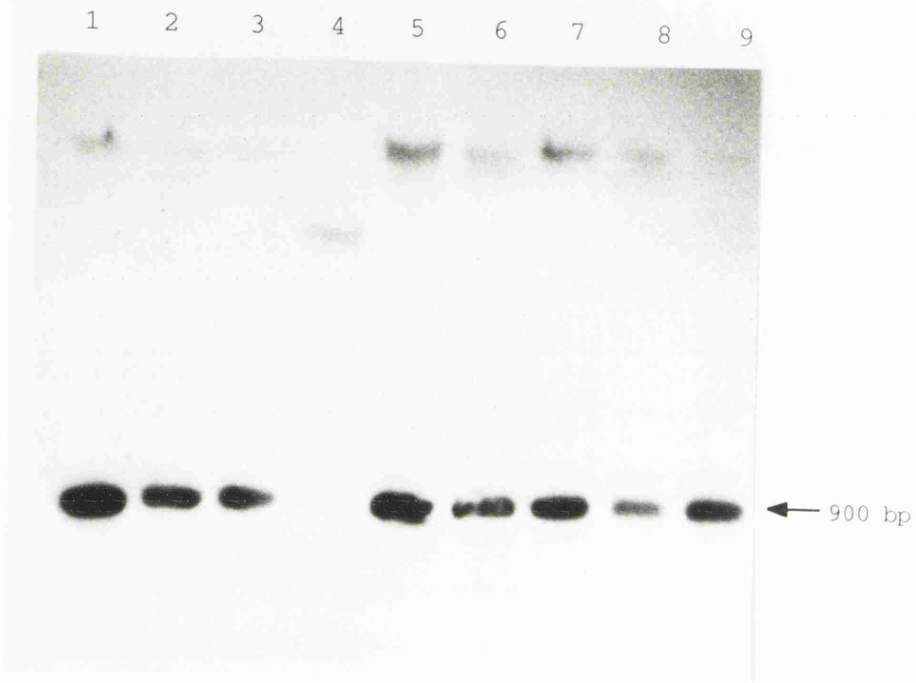

1
2
3
4
5
6
7
8
9
900 bp

Figure 4.5a. Southern blot of digested asparagus genomic DNA hybridized to a 220 bp 5' probe generated from the cWIP1 clone following digestion with *Acc* I (fig. 4.2). Digests were as follow:

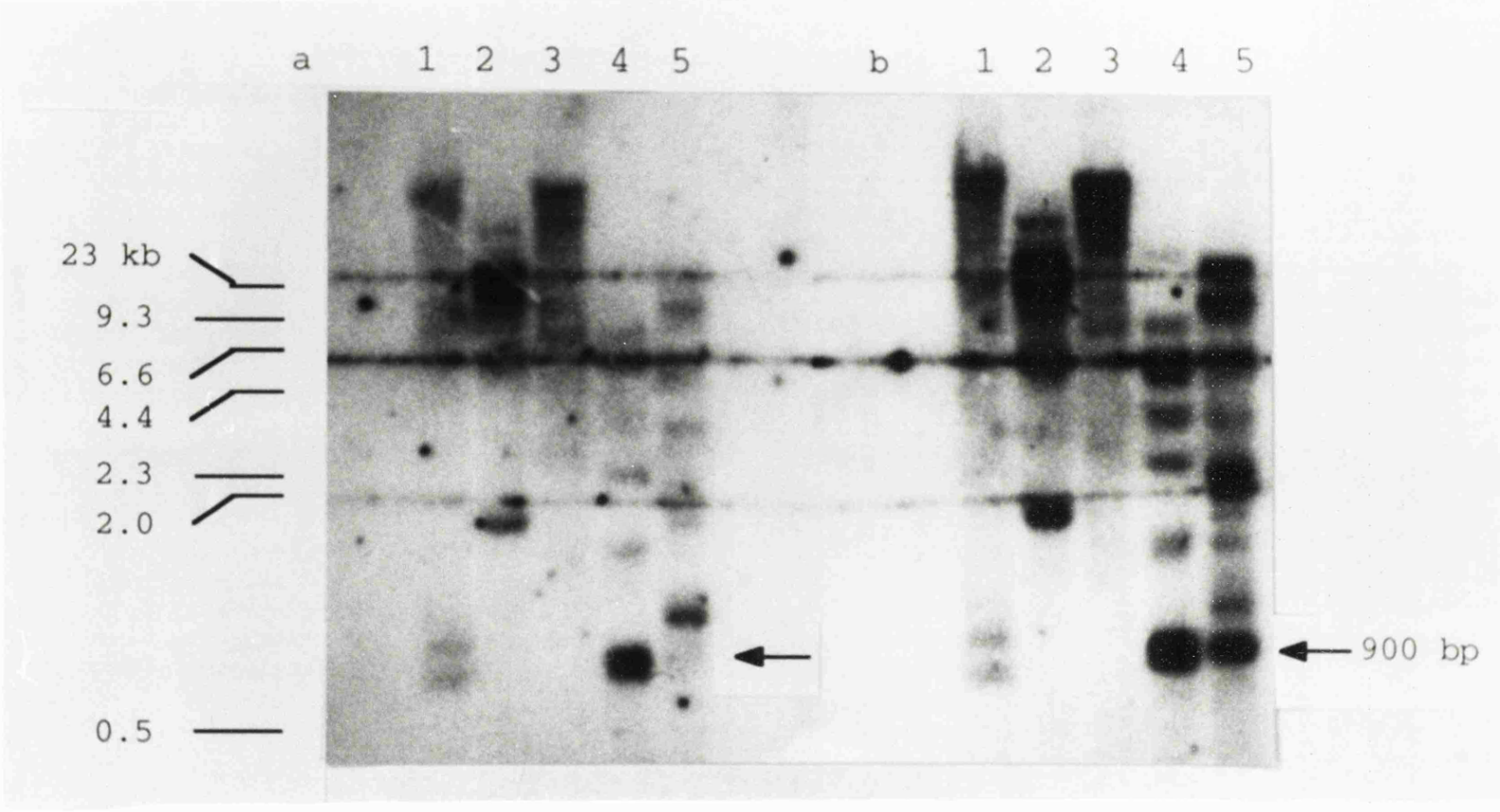
Lane 1 *Bst*X I
Lane 2 *Bgl* I
Lane 3 *Bam* HI
Lane 4 *Hin*d III
Lane 5 *Eco* RI

Molecular mass markers are shown in kb sizes to the right.

Figure 4.5b. Southern blot of digested asparagus genomic DNA hybridized to the full cWIP insert. Digests were as follow:

Lane 1 *Bst*X I
Lane 2 *Bgl* I
Lane 3 *Bam* HI
Lane 4 *Hin*d III
Lane 5 *Eco* RI

The arrows indicate the presence or absence of a hybridizing 900 bp *Eco* RI fragment that corresponds to the 900 bp *Eco* RI fragments in the genomic clones. Approximately 10 μg of DNA per lane.


a
1
2
3
4
5
b
1
2
3
4
5
23 kb
9.3
6.6
4.4
2.3
2.0
0.5
900 bp

cDNA clone. The predicted protein encoded by the cDNA has a molecular mass of 16.9 kDa and a near neutral isoelectric point.

4.2.1 Relationship between the cWIP 1 clone and genomic sequences

Southern blots were prepared using *Eco* RI digests of the genomic clones and these were hybridized with probes consisting of the complete 730 bp cWIP 1 insert and a cWIP 5' probe generated from a 230 bp *Eco* RI *Acc* I digests of the clone (fig. 4.2).

As expected from the sequence homology, the 900 bp *Eco* RI fragment contained in all the genomic clones hybridized to the full cWIP 1 probe (fig. 4.4). The cWIP 1 5' probe did not hybridize with any of the genomic clones.

Using both the full cWIP 1 probe and the cWIP 1 5' probe Southern analysis of asparagus genomic DNA revealed hybridizing sequences that were identical to those seen using the DD1-34 probe (fig. 4.5 and fig 3.4)). However, of note was the disappearance of the hybridizing *Eco* RI 900 bp fragment when the cWIP 1 5' probe was used. These data are in agreement with the observation that the cWIP 1 5' probe does not hybridize to the 900 bp *Eco* RI fragments contained within the genomic clones. The data also provides good evidence that the cWIP 1 clone, although homologous to regions contained within the DD1-34 and genomic clones, is derived from sequences not closely linked to the sequences encoded by the genomic clones in the asparagus genome.

The cWIP 1 5' probe was used to re-screen the genomic library but gave no hybridizing plaques. A new library (1 million plaque forming units) was constructed and screened but this also produced no results. This suggested that the genomic libraries constructed may have been under-represented in certain sequences within the genome as the overall size should have been sufficient to yield hybridizing genomic clones. Alternatively, the estimate of the asparagus genome size was incorrect. Indeed in a recent publication the size of the asparagus genome is estimated at 2.1 pg (Bennett and Smith, 1991 [2]) which is over twice the size estimated by Paul *et al.*, 1989 [144].

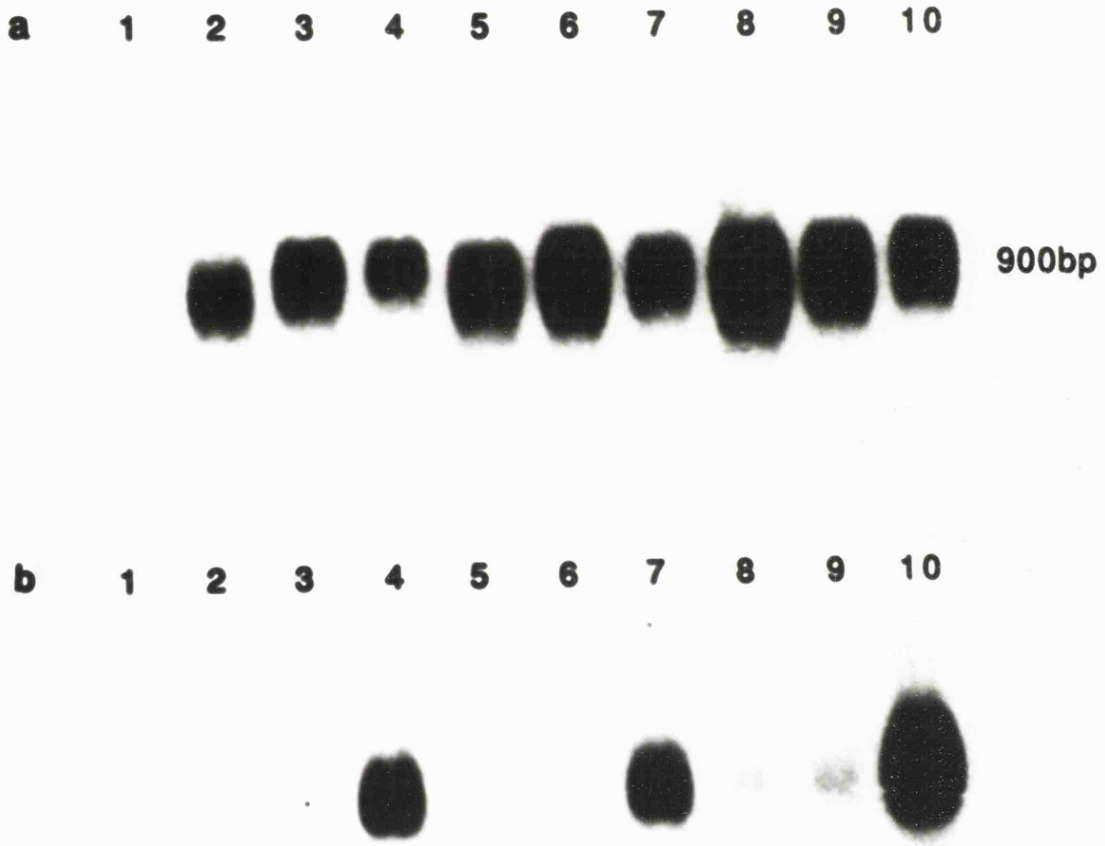
Figure 4.6a. Northern analysis of transcript following cell isolation and wounding. Total RNAs hybridized to the cWIP1 insert. RNAs were isolated from the following:

Lane 1	unwounded two week old dark grown asparagus seedlings.
Lanes 2, 5 and 8	asparagus cell suspensions 1, 2 and 3 days post isolation respectively.
Lanes 3, 6 and 9	two week old dark grown asparagus seedlings cut into 2 mm sections and incubated in sterile conditions for 1, 2 and 3 days post slicing respectively.
Lanes 4, 7 and 10	two week old dark grown asparagus seedlings cut into 10 mm sections and incubated in sterile conditions for 1, 2 and 3 days post slicing respectively.

Figure 4.6b. Northern analysis of transcript abundance at the wound site. Total RNAs hybridized to the cWIP1 insert. RNAs were isolated from the following:

Lane 1	unwounded two week old dark grown asparagus seedlings.
Lanes 2, 5 and 8	5 mm length sections of two week old dark grown asparagus seedlings 5 mm away from the wound site incubated in sterile conditions for 1, 2 and 3 days respectively.
Lanes 3, 6 and 9	2.5 mm length sections of two week old dark grown asparagus seedlings 2.5 mm away from the wound site incubated in sterile conditions for 1, 2 and 3 days respectively.
Lanes 4, 7 and 10	2.5 mm length sections of two week old dark grown asparagus seedlings that include the wound site incubated in sterile conditions for 1, 2 and 3 days respectively.

10 μg of total RNA was loaded per lane.


a 1 2 3 4 5 6 7 8 9 10
900bp
b 1 2 3 4 5 6 7 8 9 10

4.2.2 Northern analysis using cWIP 1 as a probe

From preliminary data it was known that transcript corresponding to sequences within DD1-34 was up-regulated during the initiation of mechanically isolated cell cultures and in chopped up seedlings. To show that the cWIP1 cDNA also hybridized to wound-induced transcript a series of northern experiments were performed.

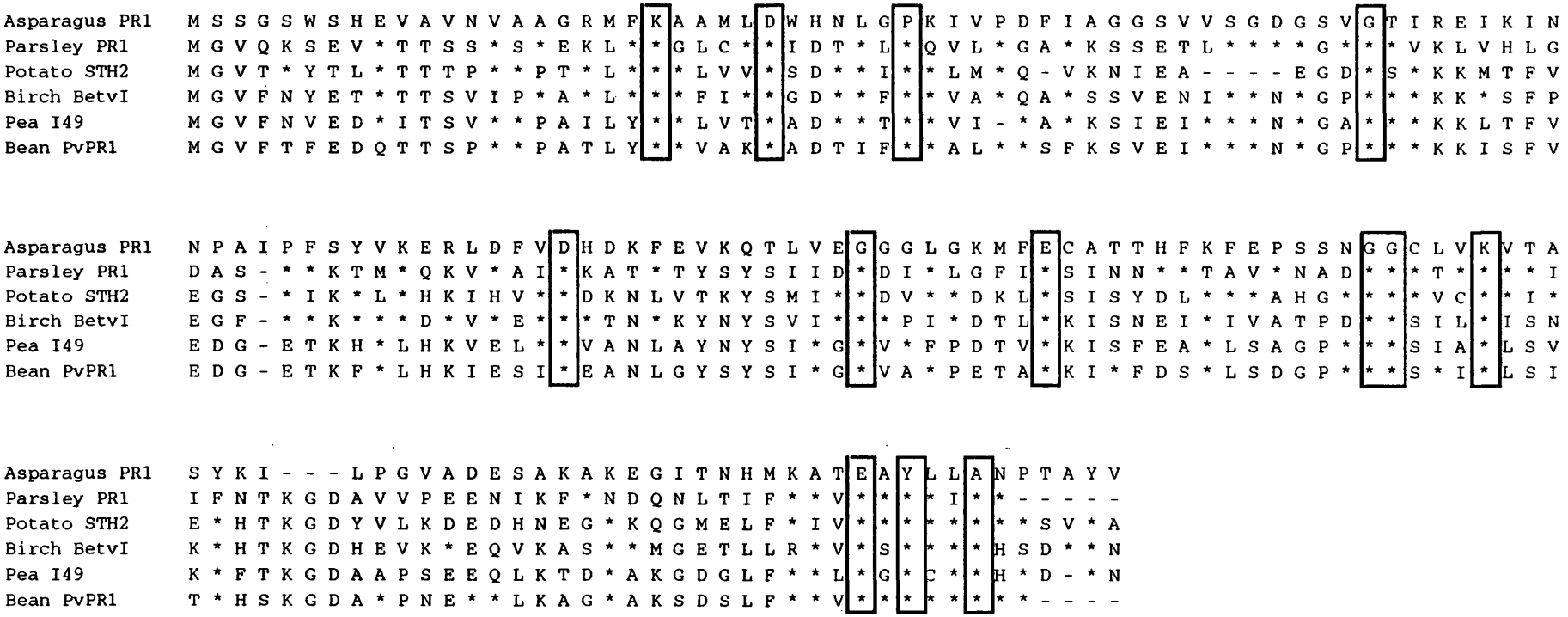
RNA was isolated from different aged cell cultures and from seedlings that had been chopped into different lengths and left for varying time periods on sterile damp filter paper. Northern gel blots were prepared and probed with the cWIP 1 insert. The autoradiograph demonstrated induction of hybridizing message with time for both chopped seedling and cell culture RNA. The approximate message levels seen for the chopped up seedlings was in the same order of magnitude as the cell culture induced message (fig. 4.6a).

Differences were seen in the relative amounts of message hybridizing to the probe between RNA populations isolated from the chopped up seedlings for any given time point depending on the size of seedling fragment used: the smaller the seedling fragments, the greater the relative intensity. This suggested that the transcript may be inducible only at, or close to, the site of damage. To test this theory RNA was isolated from sections of asparagus seedlings that had been cut into uniform lengths, left for appropriate times and then sliced into different portions with sections that included the wound site, sections that were adjacent to the wound site and sections that were at a distance to the wound site. The northern blot demonstrated that only RNA extracted from tissue that contained a wound site contained hybridizing transcript (fig. 4.6b).

4.2.3 Homologies between cWIP 1 and other sequences

Using computer aided database searches similarities were found both at the DNA sequence and predicted protein levels to cWIP 1. The most significant of these is the similarity between predicted proteins. Similarly sized predicted proteins encoded by cDNAs from a pea disease resistance protein, PI49 (27% identity, Fristensky *et al.*,

Figure 4.7. Alignment of predicted amino acid sequences of the AoPR1 (cWIP1) homologues belonging to the new class of PR protein as described by Walter *et al.*, 1990. Gaps have been introduced to facilitate alignments and are represented as dashes, residues of absolute identity to the corresponding AoPR1 residues are shown as asterices and regions that are absolutely conserved are boxed.


Asparagus PR1 M S S G S W S H E V A V N V A A G R M F K A A M L D W H N L G P K I V P D F I A G G S V V S G D G S V G T I R E I K I N
Parsley PR1 M G V Q K S E V * T T S S * S * E K L * * G L C * * I D T * L * Q V L * G A * K S S E T L * * * * G * * * V K L V H L G
Potato STH2 M G V T * Y T L * T T T P * * P T * L * * * L V V * S D * * I * * L M * Q - V K N I E A - - - - E G D * S * K K M T F V
Birch BetvI M G V F N Y E T * T T S V I P * A * L * * * F I * * G D * * F * * V A * Q A * S S V E N I * * N * G P * * * K K * S F P
Pea I49 M G V F N V E D * I T S V * * P A I L Y * * L V T * A D * * T * * V I - * A * K S I E I * * * N * G A * * * K K L T F V
Bean PvPR1 M G V F T F E D Q T T S P * * P A T L Y * * V A K * A D T I F * * A L * * S F K S V E I * * * N * G P * * * K K I S F V
Asparagus PR1 N P A I P F S Y V K E R L D F V D H D K F E V K Q T L V E G G G L G K M F E C A T T H F K F E P S S N G G C L V K V T A
Parsley PR1 D A S - * * K T M * Q K V * A I * K A T * T Y S Y S I I D * D I * L G F I * S I N N * * T A V * N A D * * * T * * * * I
Potato STH2 E G S - * I K * L * H K I H V * * D K N L V T K Y S M I * * D V * * D K L * S I S Y D L * * * A H G * * * * V C * * I *
Birch BetvI E G F - * * K * * * D * V * E * * * T N * K Y N Y S V I * * * P I * D T L * K I S N E I * I V A T P D * * S I L * I S N
Pea I49 E D G - E T K H * L H K V E L * * V A N L A Y N Y S I * G * V * F P D T V * K I S F E A * L S A G P * * * S I A * L S V
Bean PvPR1 E D G - E T K F * L H K I E S I * E A N L G Y S Y S I * G * V A * P E T A * K I * F D S * L S D G P * * * S * I * L S I
Asparagus PR1 S Y K I - - - L P G V A D E S A K A K E G I T N H M K A T E A Y L L A N P T A Y V
Parsley PR1 I F N T K G D A V V P E E N I K F * N D Q N L T I F * * V * * * * I * * - - - - - -
Potato STH2 E * H T K G D Y V L K D E D H N E G * K Q G M E L F * I V * * * * * * * * S V * A
Birch BetvI K * H T K G D H E V K * E Q V K A S * * M G E T L L R * V * S * * * * H S D * * N
Pea I49 K * F T K G D A A P S E E Q L K T D * A K G D G L F * * L * G * C * * H * D - * N
Bean PvPR1 T * H S K G D A * P N E * * L K A G * A K S D S L F * * V * * * * * * * * - - - -

1988 [70]), parsley PR1 protein, PcPR1 (32% identity, Somssich *et al.*, 1988 [183]), a potato PR protein, pSTH2 (34% identity, Matton *et al.*, 1989 [128] and Matton and Brisson, 1990 [127]), a birch pollen allergen, *BetvI* (36% identity, Breitender *et al.*, 1989 [26]) and bean elicitor induced transcripts, PvPR1 and 2 (29% identity, Walter *et al.*, 1990 [199]). The predicted proteins were aligned using the Clustal programme (Higgins and Sharp, 1989 [85]) in an order that gave the fewest branches in the cluster. Only the residues showing absolute conserved homology are boxed (fig. 4.7). Asparagus is the only monocotyledon member of this family and as such shows a lower degree of identity to the other members of the family in comparison to the identities shown amongst themselves. For example the potato predicted protein, STH2 shares 47% identity to the birch, *BetvI* predicted protein and the parsley predicted protein, PR1 shares 39% identity with the potato STH2 predicted protein.

The cDNA's from bean and parsley were isolated from established cell suspension cultures and were derived from inducible transcripts following the addition of fungal elicitors to the cell suspensions. Interestingly, the birch cDNA was cloned as the major pollen allergen encoding cDNA by using human allergen-induced antibodies to screen an expression library. These predicted proteins all belong to a ubiquitous class of conserved pathogenesis-related proteins including pollen allergens as described by Walter *et al.*, 1990 [199]. For this reason the cWIP 1 clone was more appropriately renamed cAoPR1 for *Asparagus officinalis* pathogenesis-related protein coding cDNA clone 1. These predicted proteins share no homology with the 'classical' PR-1 proteins identified in tobacco.

4.2.4 An identity for the DD1 protein

The protein microsequence used in the design of the degenerate oligonucleotide used in the cloning procedure of DD1-34 was screened against the protein sequence data base, which had been recently updated and revealed greater than 80% identity over the full 28 amino acid microsequence, with the maize Cu-Zn superoxide dismutase predicted protein sequence (Cannon *et al.*, 1987 [31]). This degree of homology left little doubt that the DD1 protein was a Cu-Zn superoxide dismutase whose abun-

dance increased dramatically following initiation of cell suspension culture. As mentioned previously no homology was found between the predicted proteins encoded by cAoPR1, DD1-34 and the microsequence. Similarly no homology was found between cAoPR1, DD1-34 and the maize SOD-1 clone at the nucleotide sequence or predicted protein level.

4.3 Conclusions about the cAoPR1 (cWIP 1) clone

From the initial cloning experiments it could be concluded that transcript homologous to the cAoPR1 clone was abundant, representing approximately 0.3% of the message population used in the construction of the library. Northern analysis data confirmed this showing that hybridizing transcript was rapidly and strongly up-regulated in both cell culture suspensions and in chopped up seedlings. In chopped up seedlings, the appearance of hybridizing transcript following slicing was observed to be associated with tissues close to the wound site suggesting that the AoPR1 gene is most strongly activated at the wound surface. Other homologous clones isolated were not studied further due to lack of time. It may be possible that some of these clones were derived from transcript coded by a separate but highly homologous gene to the AoPR1 gene. Sequence analysis of these clones may validate this possibility.

Southern blotting experiments revealed the presence of a large number of hybridizing sequences in the asparagus genome. There was a region in the cAoPR1 clone that cross hybridized with the DD1-34 clone and the genomic clones isolated. Using a small fragment derived from the 5' region of the cAoPR1 clone as a probe no hybridizing sequences were detected in the DD1-34 or genomic clones. Genomic Southern blots probed with this fragment did not detect a 900 bp *Eco* RI fragment that had been previously associated with the DD1-34 clone and the genomic clones (fig. 4.5). This suggested that the gene encoding the transcript from which the cAoPR1 clone was derived, although containing regions of homology to the genomic clones was not closely linked on the asparagus genome to any of the genomic sequences cloned. These experiments offered no further evidence to the elucidation of the structure and function of the sequences contained within the DD1-34 and ge-

nomic clones. However, these data provided evidence that the hybridizing transcript observed in chopped-up seedlings and cell suspension cultures was not derived from sequences encoded by the genomic clones and as such these genomic clones would be an unlikely source of the promoter region mediating the induction of transcript.

A further attempt to screen an asparagus genomic library, using the 5' probe derived from the cAoPR1 clone, failed to identify any hybridizing plaques. Although the library size was believed to be sufficient to give a good chance of obtaining corresponding genomic clones the following may explain failure. Firstly, in reality the library was not large enough and unluckily did not contain any corresponding sequences (as discussed in 4.2.1), or secondly the library was not equally representative of all parts of the asparagus genome. It has been shown that plant genomic DNA is highly methylated and that methylated DNA is resistant to digestion by certain restriction enzymes (Shapiro, 1976 [176]). *In vitro* packaging extracts used in the construction of phage libraries have also been shown by Stratagene Inc. to package methylated DNA less efficiently than unmethylated DNA. For these and similar reasons certain genes may be under represented in a genomic library.

Sequence analysis of the cAoPR1 clone revealed an open reading frame encoding a predicted 16.9 kDa protein with a neutral isoelectric point. This predicted protein was homologous to other pathogenesis related proteins that were derived from fungal elicitor-induced transcripts of parsley and bean established cell suspension cultures. The fact that these clones were obtained from mechanically isolated cell suspensions provides circumstantial evidence that asparagus cell suspensions may be an enriched source of wound-inducible transcripts. *In situ* analysis has shown induction of the parsley PR1 transcript around sites of attempted fungal invasion (Schmelzer *et al.*, 1989 [171]. Analysis of sequence obtained from the corresponding parsley genomic clones suggests that the encoded proteins are probably intracellular (Somssich *et al.*, 1988 [183]). Similar cDNA's have been isolated from pea (Fristensky *et al.*, 1988 [70]), potato (Matton and Brisson, 1989 [128]) and birch (Breitender *et al.*, 1989 [26]). These data suggest that the asparagus homologue may also have a role in response to pathogen invasion which in monocotyledonous

species, may differ, in respect to both the extent and rapidity of the production of wound-boundary associated molecules, to that observed in dicotyledonous species. (Personal communications from Drs M. Walter and I. Somssich state that the bean and parsley homologous PR genes are also induced following mechanical wounding, suggesting that AoPR1 gene induction following wounding is not anomalous for this family of PR genes.) To examine the possibility that the monocotyledon AoPR1 transcription is initiated in a wound-inducible manner, perhaps with a slightly altered expression pattern to its homologous dicotyledon transcripts, it was decided to isolate the AoPR1 promoter for analysis in transgenic tobacco plants using reporter genes. This would also be a pilot experiment to test whether or not asparagus may provide monocotyledon-derived regulatory gene sequences that would function in dicotyledons as well as monocotyledons.

The discovery that the DD1 protein was probably a Cu-Zn superoxide dismutase protein via homology with the predicted maize protein was interesting and implies that the asparagus cell cultures are undergoing metabolic oxidative stresses. As a final check that the DD1-34 clone was totally unrelated to the protein microsequence that showed homology to the maize Cu-Zn superoxide dismutase all open reading frames encoded by this clone were analysed for homology to the Cu-Zn superoxide dismutase sequence. As expected there were no predicted proteins encoded by the DD1-34 clone that were homologous to Cu-Zn superoxide dismutase. The conclusions of these data are not in agreement with the earlier data that the DD1-34 lac Z fusion protein cross reacted with polyclonal antibodies raised against the DD1 protein (Fioroni, 1989 [69]).

The cAoPR1 clone is not a full length cDNA but comparison of its predicted open reading frame with those predicted by homologous clones from other species suggests the clone contains all the peptide encoding sequence.

From the data obtained through experiments analysing AoPR1 transcript regulation in chopped asparagus seedlings and the sequence data relating to the cAoPR1 clone it was apparent that the AoPR1 gene was wound induced and encoded a small protein of unknown function. The AoPR1 cDNA represents the first monocotyledon member

of the new class of pathogenesis-related protein family as described by Walter *et al.*, 1990 [199]. The obvious progression of this work required further analysis of AoPR1 gene regulation. Promoter-reporter genes provide a rapid and simple way of studying gene expression in transgenic plants. However, in order to commence these studies, it was first necessary to isolate the AoPR1 promoter.

Chapter 5

Amplification and characterization of genomic sequences encoding the AoPR1 message

5.1 Introduction to the inverse polymerase chain reaction

Having failed to isolate a genomic clone corresponding to the cAoPR1 clone by the conventional approach of screening a genomic library a different approach was taken. This involved the direct amplification of the required sequences from the genome by the exploitation of the inverse polymerase chain reaction (IPCR) (Ochman *et al.*, 1988 [138]). This technique allows the amplification of sequences that flank known sequences. Genomic DNA is digested with a restriction enzyme that does not cleave within the sequence of interest. The cleaved DNA is then diluted and ligated under conditions that favour intramolecular ligation to form monomeric circles. These circles are then added to the PCR reaction mix as template. The oligonucleotide primers are designed in such a way that when they anneal to the target template

Figure 5.1. Sequence of the AoPR1 cDNA clone showing sequences that the synthetic primers used in polymerase chain reactions hybridize to. The point of the arrow represents the 3' terminus of the primer. A representation of the inverse PCR technique is shown below.

```
  1  ATGAGTTCAG GGAGCTGGAG CCACGAGGTC GCTGTCAATG TCGCCGCAGG

 51  ACGGATGTTC AAGGCGGCAA TGCTCGACTG GCACAACCTC GGCCCTAAGA
                                <-------- EXT 1. --------
101  TTGTGCCTGA CTTTATTGCC GGTGGCTCAG TGGTGTCTGG AGATGGATCT
      -------- IPCR 1. -------->
151  GTAGGAACCA TCCGAGAGAT CAAGATCAAC AATCCTGCTA TACCTTTCAG

201  CTATGTGAAG GAACGCCTGG ATTTCGTAGA CCATGACAAG TTCGAGGTGA

251  AGCAGACCCT CGTGGAAGGT GGAGGTTTAG GTAAGATGTT TGAATGTGCC

301  ACCACTCACT TCAAGTTCGA GCCCTCGAGC AACGGTGGAT GCCTCGTCAA

351  GGTGACTGCA TCCTACAAGA TTCTCCCAGG TGTCGCCGAT GAGAGTGCGA
          <-------- POL 1. --------
401  AGGCGAAGGA GGGAATAACC AACCACATGA AGGCAACCGA AGCTTACCTC

451  CTAGCCAACC CAACTGCCTA CGTTTAAATA TAGTGATTGT GTTTCTTTGC

501  GTGAAGTGCT TGTGAGTTTG AATAAGGAGA TTGGTTATGA GGAAGCTTGA

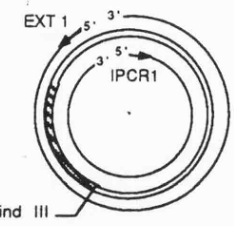
551  TGGGGTCATA CATAGTTAGT TTATGTTGAA TGATCAGCCT TTTTTGTGTG

601  AAGTACTTGG GAGTTTGAAT AAGGAGACTG AATATGAGAA AGATTGATGG

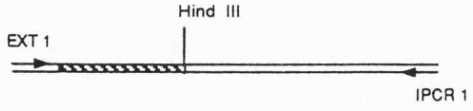
651  AGTTATCGTT CATGTTGAAT GATCAGCCTT ATCAGTTTGT AACAGTGTCG

701  AATGATCAGT CTTATCAGTT TGTAATGGTG GCTTCAA
```

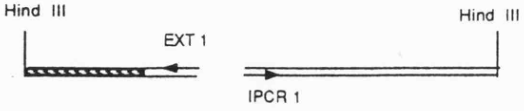
Digest genomic DNA, recircularise at low DNA concentration, anneal PCR primers and start PCR reaction.



Amplify for 30-35 cycles and obtain specific IPCR product



Resolve IPCR product by digesting with the identical restriction enzyme used in the initial digestion of genomic DNA.

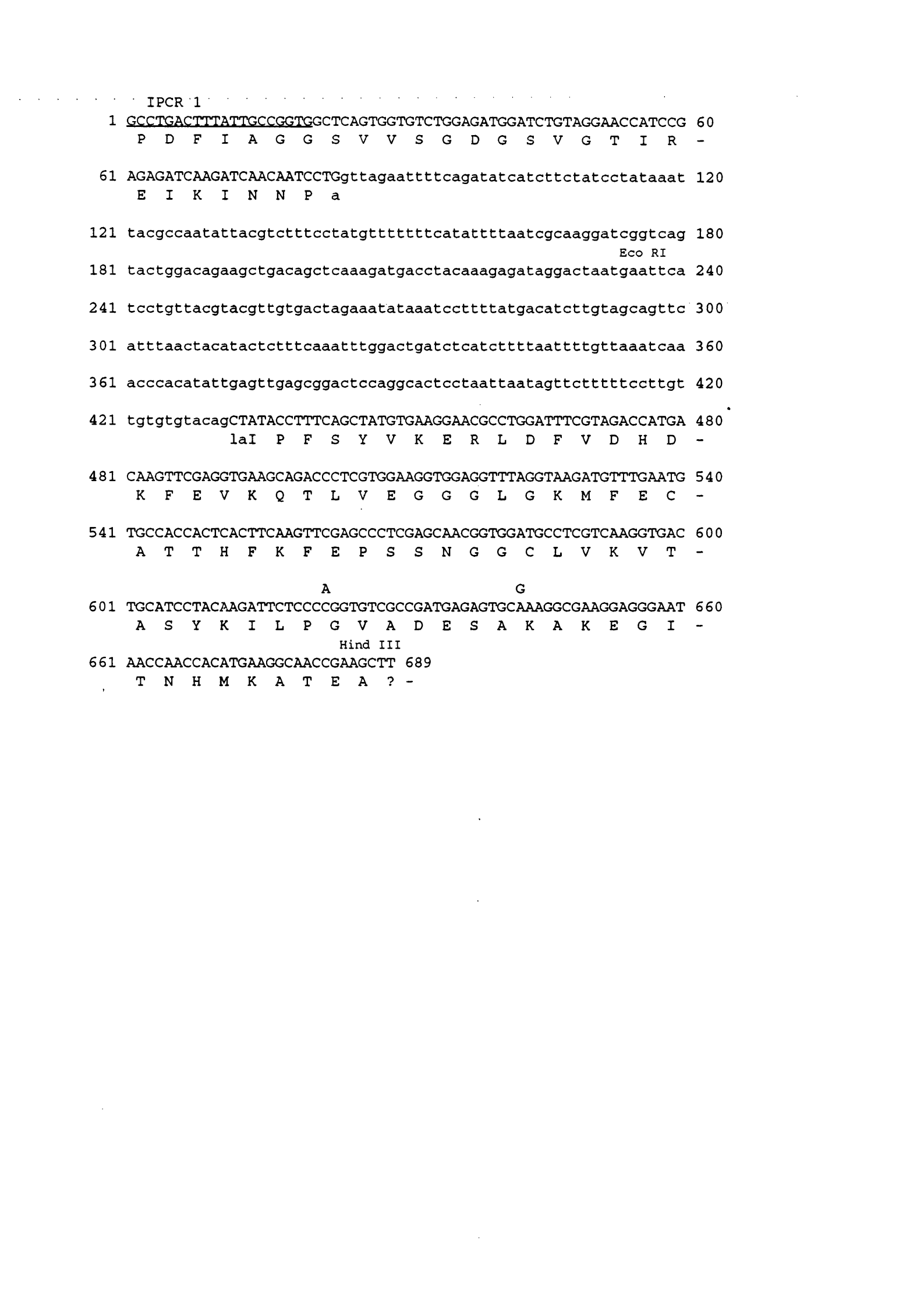


circles they lie only a few base pairs apart and their 3' termini are facing opposite directions. For general PCR, the primers are designed so that the opposite happens; the primers anneal hundreds to thousands of base pairs apart with their 3' termini facing one another. After the primers have specifically annealed to the template circles the temperature is raised to the 72 °C optimum temperature required by the thermo-stable *Taq* DNA polymerase to extend the annealed primers. Following the complete extension of each strand the circle is melted by heating the DNA strands at 95 °C and the new templates are now available for more primer annealing, extension and melting steps such that the major product of the amplification is a head-to-tail arrangement of sequences flanking the target sequence. The precise junction between the upstream and downstream sequences is marked by a restriction enzyme site for the enzyme used in the initial digestion of the genomic DNA. This can be used in the separation of the head-to-tail molecules.

5.2 Amplification of 5' sequences flanking the AoPR1 coding region

From the sequence of the cAoPR1 clone two 20mer oligonucleotides were designed that would be useful for the IPCR reaction (fig. 5.1). The EXT 1. primer which hybridizes to the sense strand of the cDNA between positions 71 and 91 reads 5' CGAGGTTGTGCCAGTCGAGC 3' and the IPCR 1. primer which hybridizes to the antisense strand of the cDNA between positions 104 to 124 reads 5' GCCT-GACTTTATTGCCGGTG 3'. Asparagus genomic DNA was cleaved initially with either *Hind* III and *Eco* RI restriction enzymes and circularized. Twenty five cycles of amplification were carried out and the products run out on an agarose gel. There were many PCR products, some of which were probably nonspecific. The experiment was repeated including control template that had not been digested and template that had been digested but not ligated. These experiments suggested that products of approximately 900 bp, 700 bp and 250 bp were abundant nonspecific products. Southern blot analysis of the products using the cAoPR1 5' probe revealed that

Figure 5.2. DNA sequence of the AoPR1 IPCR coding region and intron with the predicted intron shown in lower case letters. The predicted protein translation is shown below. Restriction enzyme sites and sequence discrepancies with the AoPR1 cDNA sequence are shown above. The IPCR 1 primer site is underlined.


IPCR 1
1 GCCTGACTTTATTGCCGGTGGCTCAGTGGTGTCTGGAGATGGATCTGTAGGAACCATCCG 60
P D F I A G G S V V S G D G S V G T I R -
61 AGAGATCAAGATCAACAATCCTGgttagaattttcagatatcatcttctatcctataaat 120
E I K I N N P a
121 tacgccaatattacgtctttcctatgttttttcatattttaatcgcaaggatcggtcag 180
Eco RI
181 tactggacagaagctgacagctcaaagatgacctacaaagagataggactaatgaattca 240
241 tcctgttacgtacgttgtgactagaaatataaatccttttatgacatcttgtagcagttc 300
301 atttaactacatactctttcaaatttggactgatctcatcttttaattttgttaaatcaa 360
361 acccacatattgagttgagcggactccaggcactcctaattaatagttctttttccttgt 420
421 tgtgtgtacagCTATACCTTTCAGCTATGTGAAGGAACGCCTGGATTTCGTAGACCATGA 480
laI P F S Y V K E R L D F V D H D -
481 CAAGTTCGAGGTGAAGCAGACCCTCGTGGAAGGTGGAGGTTTAGGTAAGATGTTTGAATG 540
K F E V K Q T L V E G G G L G K M F E C -
541 TGCCACCACTCACTTCAAGTTCGAGCCCTCGAGCAACGGTGGATGCCTCGTCAAGGTGAC 600
A T T H F K F E P S S N G G C L V K V T -
A G
601 TGCATCCTACAAGATTCTCCCCGGTGTCGCCGATGAGAGTGCAAAGGCGAAGGAGGGAAT 660
A S Y K I L P G V A D E S A K A K E G I -
Hind III
661 AACCAACCACATGAAGGCAACCGAAGCTT 689
T N H M K A T E A ? -

there were IPCR products that hybridized to this probe. Other enzymes such as *Xho* I, *Xba* I and *Bgl* I were used to try and generate different IPCR fragments, but only nonspecific fragments were obtained as judged from hybridization experiments.

5.3 Cloning and analysis of AoPR1 IPCR generated fragments

The IPCR fragments hybridizing to the cAoPR1 probe, obtained by either *Hind* III or *Eco* RI digestion, self-ligation and subsequent amplification of asparagus DNA were approximately 1.1 kb and 1.3 kb in size, respectively. To isolate these IPCR products from the nonspecific products they were sliced out of agarose gels and used as a template stock for another 5-10 cycles of amplification. Following this procedure, a single product was generally obtained but occasionally contaminants of similar molecular mass were present on agarose gels. The fragments were treated with T4 DNA polymerase to remove any overhangs and the products were cleaved with the appropriate enzymes (*Hind* III for the 1.1 kb fragment generated from *Hind* III digested, self-ligated and amplified DNA and *Eco* RI for the 1.3 kb fragment generated from *Eco* RI digested, self-ligated and amplified DNA) and cloned as blunt/sticky fragments into appropriately prepared plasmid vector. (The restriction enzyme digests of the amplified products served two purposes: firstly, the amplified linear molecule is resolved into two fragments containing contiguous DNA sequence following digestion of the amplified product by the same restriction enzyme used in the initial digestion of the genomic DNA (fig. 5.1) Secondly, the digestion generates molecules with cohesive and blunt ends facilitating cloning.) The cloning efficiency of these fragments was reduced compared to that observed from the cloning of similarly sized blunt fragments generated by restriction enzyme digests. The first product cloned was a 672 bp blunt/sticky fragment and was named AoPR1-intron. Sequencing of this DNA fragment revealed that this DNA is located at a downstream region relative to the primer binding sites (fig. 5.2). There was also a putative intron present within this sequence that spanned the regions of sequence homology

Figure 5.3. DNA sequence of the AoPR1 IPCR promoter and coding region with the predicted protein translation shown below. The numbering of the bases is based on the putative transcriptional start site that was determined by nuclease-S1 mapping (shown by an underlined bold character). A putative TATA box is shown as an underlined, italicised sequence. Motifs similar to Loake and Lois boxes (Loake *et al.*, 1991 and Lois *et al.*, 1989) are shown as bold characters. Repeated sequences are underlined. Direct repeats, restriction enzyme sites and nucleotide discrepancies with the AoPR1 cDNA sequence are shown above the sequence. The predicted AoPR1 amino acid sequence is shown below. The EXT 1 primer binding site at the end of the sequence is also underlined.

Eco RI

-982 GAATTCAGGGGTAAGTTTGCAAATATCAAGATTTGGGGGGGCCAAATCTACAAATATGAA -923

-922 ATATTTGAGAGGTATGTATGCAAAAACCCCTATAAAATTTCCCTCAGGACTAGACCATCG -863

-862 TGGTTAAATGATCAAGTGCCTACTTGGCAGAATTTCTTTCGAGCAGCCTCCTCCTACAAG -803

-802 TTGCATTTGTTGCGCTTACGATAATTGTCAAAGAAGTAGGTAAAATAAAGACATGATCAC -743

-742 TAATATTAAGGATAAGATTAAAAATAAGTCCAGGATTAACCGGTCGGCCCATCAATTACT -683

-682 TGCTGACCTTTGTTGCCGTCCCACGACTTCCATTTTCTAACCGTCCATTTTTCATTTGTT -623

-622 TTTAGCTATATTTAATATTAATGGGATATAAATTATAAACATTCCTCCTCCCAAAAAAAT -563

-562 AAGTTTAAGTAATACTGCAATAGACAGTGTTTTAAGCCATGTAATTCAGTAAAAGTTCTT -503

-502 TTTTATTCTGA**ACCTAGCC**CTAAAAAGGCCATGCGGGTAATTAGTTCAGTCAACTGAATA -443

Repeat 1 Repeat 2 Hind

-442 TACAACGTTTTGAACCAAAGTTAACATGTACAGGCCAATAGAAGTTATTTGACCGTAAGC -383

III Repeat 1

-382 TTAGTCTCTACATTCATTCAACGTTCTTGAATCAAAGTGACCTGTACAGGCCAATAGAAG -323

Repeat 2 Hind III Xba I

-322 TT**ACCTGACC**GTAAGCTTAGTCTCTACATTCATTCCTCTGAGACGATATTCTAGAAGCCT -263

-262 GCTTTCAAGTCTAAAAGGCACAATCTTTTTTCCTCACCACTTGTTGAGGTACTTATGATT -203

-202 TTAAAGATGAAACATTTTTTTTTACTTTTCCCCTTTAATTTCTTTGATTTTTTTTTTTCTT -143

-142 GGTAGTTGGAAGTACTTTTCATACCCTAGAAAATCCACTGTTGATCTTTGAAATATCAGC -83

-82 AATCTTTGAAATAATATCAGCAACCACGACA**CCTACC**ATTCTCAAATTCACTC*TATAAAA* -23

+1

-22 GGGTAAACCTTTGCTTACCTCT**A**TGCTCACTCACAAGGAGAACAAACACTCATCGGTGCT 37

T

38 ACATAACAACAGAGAGGAAACATGAGTTCAGGGAGCTGGAGCCACGAGGTCGCTGCCAAT 97

M S S G S W S H E V A A N

Taq I

98 GTCGCCGCAGGACGGATGTTCAAGGCGGCAATGCTCGACTGGCACAACCTCG 157

V A A G R M F K A A M L D W H N L

EXT 1 primer

Figure 5.4a. Southern blot of digested asparagus genomic DNA hybridized to a 700 bp 5' probe generated from the AoPR1 IPCR promoter and coding region clone following digestion with *Xba* I and *Eco* RI (fig. 5.7). Digests were as follow:

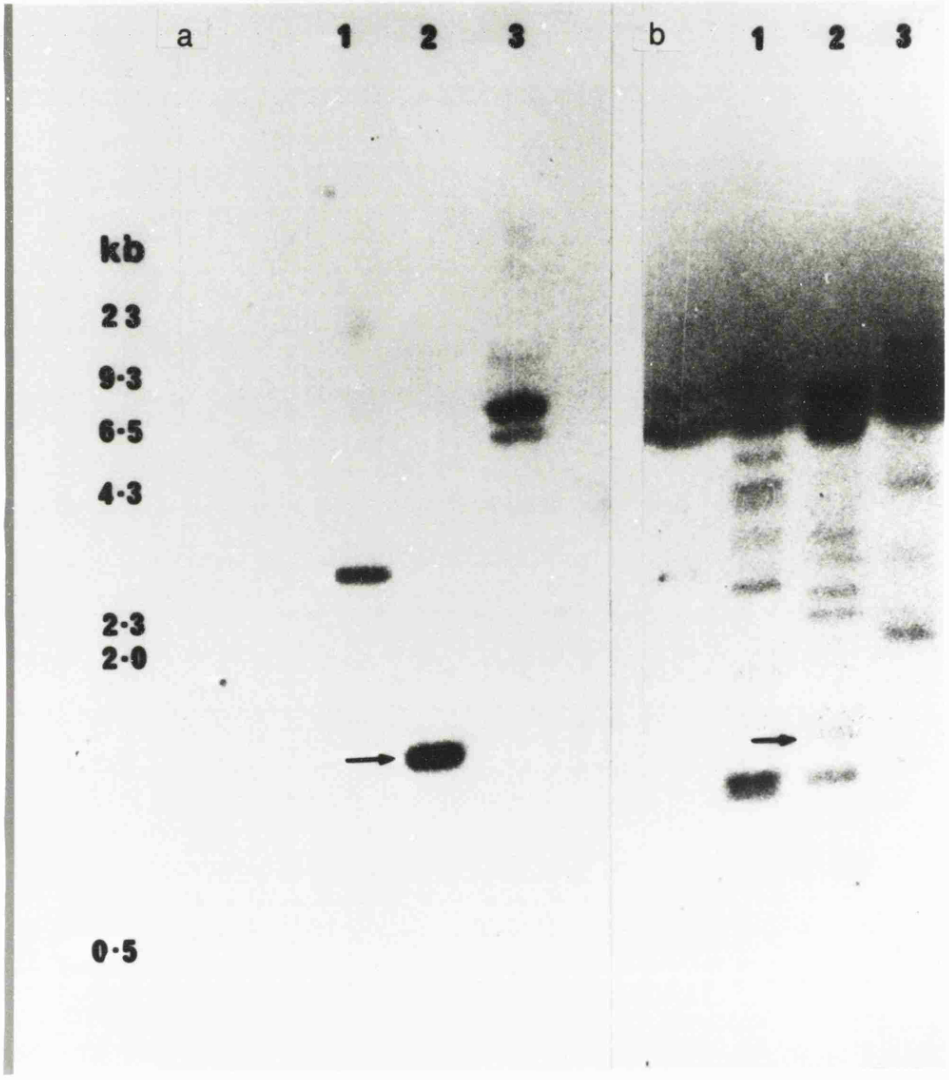
Lane 1 *Hin*d III
Lane 2 *Eco* RI
Lane 3 *Bam* HI

Molecular mass markers are shown in kb sizes to the left.

Figure 5.4b. Southern blot of digested asparagus genomic DNA hybridized to the AoPR1 cDNA. Digests were as follow:

Lane 1 *Hin*d III
Lane 2 *Eco* RI
Lane 3 *Bam* HI

The arrows indicate the presence of a hybridizing 1.3 kb *Eco* RI fragment that corresponds to the 1.3 kb product obtained following *Eco* RI digestion, ligation and application of the IPCR technique on asparagus DNA as in the text. Approximately 10 μg of DNA were loaded per lane.


a
1
2
3
b
1
2
3
kb
23
9·3
6·5
4·3
2·3
2·0
0·5

between the IPCR fragment and the cAoPR1 clone. The putative intron was 347 bp long and started and ended with the GT-AG acceptor donor consensus sequences (Breathnach and Chambon, 1981 [25]). More importantly, it contained an internal *Eco* RI site which allowed the orientation of the IPCR product derived from the *Eco* RI digest. It was then apparent that the *Eco* RI derived IPCR product contained a 250 bp fragment located downstream from the primer binding site and a 1100 bp fragment that was upstream relative to the primer binding site and should therefore contain the AoPR1 gene promoter.

5.4 Analysis of upstream regions of the AoPR1 gene

To confirm that the 1100bp fragment was derived from upstream regulatory regions of the AoPR1 gene, sequence analysis was necessary. The 1100 bp fragment was cloned into a pBluescript plasmid vector and sequenced in its entirety in both directions. The generated clone was named AoPR1 Eco IPCR. The sequence data revealed a 91 bp overlapping sequence with the 5' region of the cAoPR1 cDNA containing only one T to C transition at position 94 that altered the codon meaning from a valine to an alanine. This data provided good evidence that this was indeed the amplified upstream region of the AoPR1 gene. Sequence data is shown in fig. 5.3.

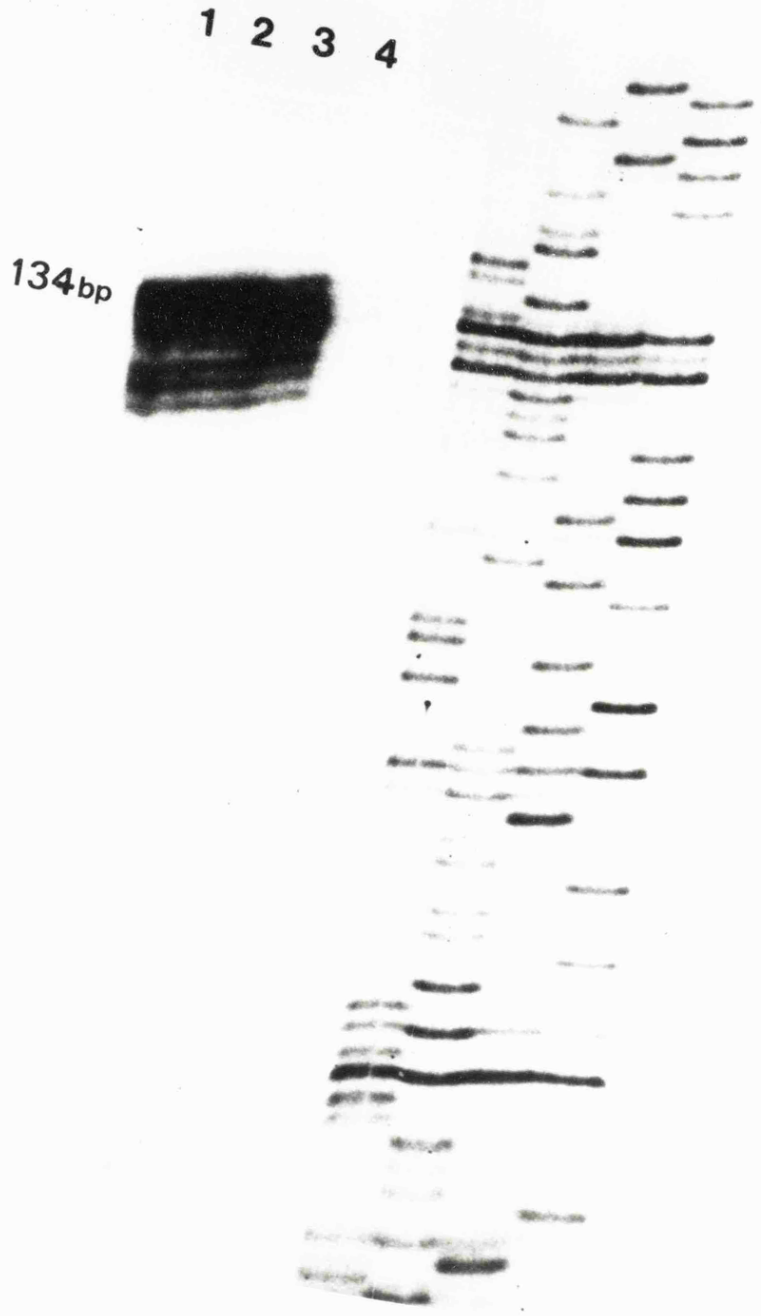
5.4.1 Southern blotting experiments

Genomic Southern blots were carried out using identical filters of asparagus genomic DNA digests. Filters were probed with the AoPR1 cDNA probe and a 700 bp probe derived from an *Xba* I digest of AoPR1 Eco IPCR that contained no overlapping sequence with the cDNA (fig 5.3 and fig. 5.7). The autoradiograph revealed the characteristic large number of hybridizing sequences to the cDNA probe but relatively few hybridizing sequences were observable with the *Xba* I generated AoPR1 Eco IPCR probe (fig. 5.4). However, bands that hybridized with both the cDNA and the IPCR probe were observable. Of particular importance was the 1.3 kb

Figure 5.5. S1-transcript mapping used to identify the probable transcriptional start site of the AoPR1 gene. An appropriate end labelled DNA fragment (as described in the text) was hybridized to RNAs from the following sources;

Lanes 1, 2 and 3	two week old dark grown asparagus seedlings cut into 2 mm sections and incubated in sterile conditions for 1, 2 and 3 days post slicing respectively.
Lane 4	unwounded two week old dark grown asparagus seedlings.

Post hybridization the S1-nuclease digestion was performed and the products separated on a denaturing gel with a sequencing ladder run along side as a molecular mass marker. The largest protected fragment corresponds to a 134 bp.


1 2 3 4
134bp

Eco RI fragment that corresponds approximately in size to the total IPCR product obtained from the *Eco* RI digested asparagus DNA. There was no detectable hybridization observed to the 900 bp *Eco* RI fragment that was detected using the cAoPR1 probe. As expected, hybridization was not detected using the complete AoPR1 Eco IPCR fragment in cross hybridizations with both the genomic clones previously isolated and the DD1-34 clone. Re-screening genomic libraries with this probe was also unsuccessful.

5.4.2 Transcript mapping experiments

Transcript mapping not only determines the boundaries between transcribed and non transcribed regions of genes but can also give estimates of transcript abundance. The method relies on the principle that DNA/RNA hybrids are protected from digestion by single stranded exonucleases.

Using the AoPR1 Eco IPCR insert, a probe was prepared from a 846 bp *Taq* I fragment which was end labelled following a T4 polynucleotide kinase reaction. This was then cleaved with *Xba* I, generating a 403 bp probe with only the anti-sense strand labelled at the 5' end (fig. 5.3). This probe was hybridized to total RNA from wounded asparagus seedlings at 50 °C and subsequently treated with S1-nuclease. The products were run along side end-labelled markers or sequencing ladders on a 8% polyacrylamide sequencing gel.

The autoradiographs showed several protected fragments of similar size and the largest protected fragment in each case was 134 bp (fig. 5.5). This was taken to be the major product as it is possible that the nuclease had partially digested the ends that may be more prone to transient melting at lower temperatures, giving rise to the smaller observed products. Comparison of the amount of protected transcript in chopped-up seedling RNA that had been left for varying lengths of time with that in seedling material flash frozen without ageing, agreed with the observations of the northern analysis as shown in fig. 4.6a.

Figure 5.6. PCR reaction performed on DNA from members of the Liliaceae using the IPCR 1. and POL 1. primers (fig. 5.1).

Lane 1 1 kb marker (BRL) .

Lane 2 asparagus (*Asparagus officinalis*).

Lane 3 butchers broom (*Ruscus aceleatus*).

Lane 4 solomon's seal (*Polygonatum multiflorum*).

Lane 5 peruvian lily (*Altroemeria aurea*).

Lane 6 *Simenthis planifolia*

Products were observed at approximately 600 bp.

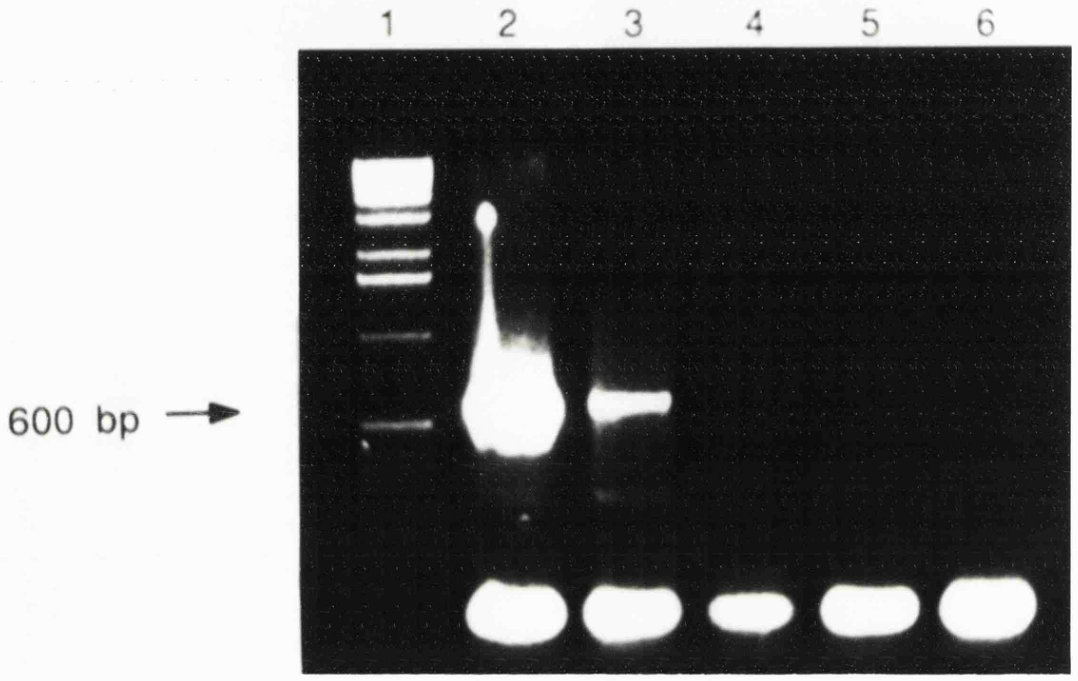
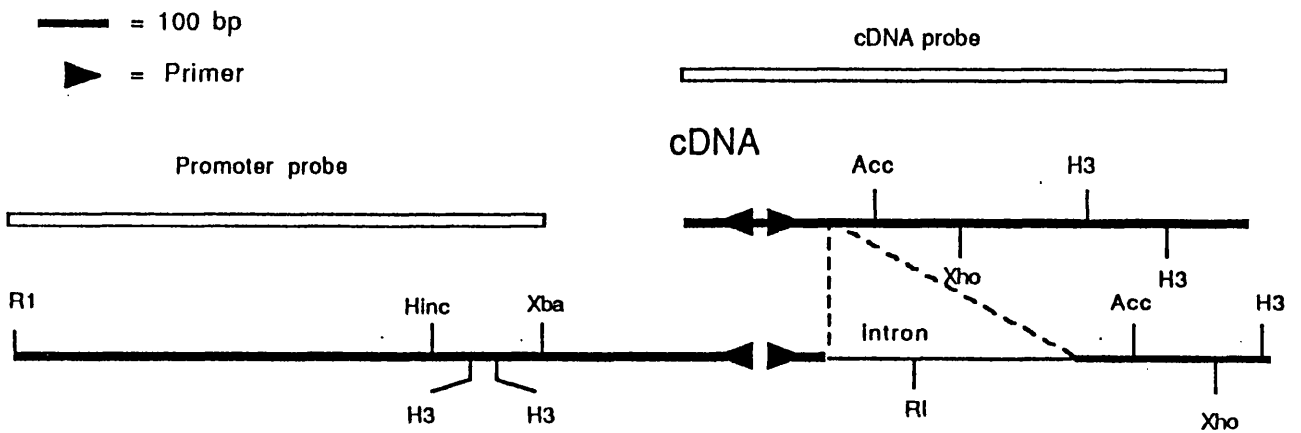

1
2
3
4
5
6
600 bp

Figure 5.7. Gene map of the AoPR1 gene based on data obtained from the cAoPR1 cDNA and IPCR products obtained. Restriction fragments used as probes used are also shown



Fragments obtained from Eco RI digested asparagus DNA post amplification and digestion with Eco RI

Fragments obtained from Hind III digested asparagus DNA post amplification and digestion with Hind III

5.5 Amplification of AoPR1 related sequences from other plants.

Hybridization was not observed between the AoPR1 cDNA and DNA from several different plants species including other members of the Liliaceae (following a low stringency wash in wash B as described in section 2.8.1). The polymerase chain reaction offers a more sensitive alternative to Southern hybridization in the identification of similar sequences in other species with large genomes. For this reason a new primer, POl. 1, which reads 5' GGGAGAATCTTGTAGGATGC 3' and anneals to the sense strand of the cDNA between positions 358 and 378, was designed for use in conjunction with the IPCR 1. primer described earlier this chapter. When used in a standard PCR experiment with asparagus genomic DNA these primers should anneal to sequences that are either side of the putative intron and a 602 bp fragment that includes the intron is amplified (fig. 5.6).

Genomic DNA from asparagus, butchers broom (*Ruscus aculeatus L*), Solomon's seal (*Polygonatum multiflorum L*) and other monocotyledonous plants belonging to the Liliaceae that had been used previously in Southern blotting and shown no hybridizing sequences present, following low stringency washes, within their genomes were used in the amplification experiment. A product of around 620 bp was observed from asparagus and a similar sized, but less abundant product was obtained from butchers broom but no other products were visible from any other plant species (fig. 5.6) suggesting sufficient homology exists only between butchers broom and asparagus to amplify genic sequences. This data supports the classification that *Asparagus* and *Ruscus* are closely related within the Liliaceae (Clapham *et al.*, 1987 [34]).

5.6 General conclusions regarding the IPCR products

From initial experiments it was apparent that the IPCR method was amplifying sequences that cross hybridized with the cAoPR1 probe. These fragments were purified from non-specifically amplified products and parts of the specific IPCR

fragments were cloned into plasmid vectors and sequenced. This analysis allowed a putative gene map to be produced as shown in fig. 5.7.

From the sequence of a 672 bp *Hind* III generated fragment a 347 bp intron was predicted that started in the 63rd codon of the predicted open reading frame. The putative intron contained the consensus GT-AG donor and acceptor sites (Breathnach and Chambon, 1981 [25]) and an AT rich region which is required for efficient splicing (Godall and Fillipowicz, 1989 [72]). There were two predicted mutations on the basis of sequence overlap comparisons with the cAoPR1 cDNA of an A to C transversion and a G to A transition that did not effect the codon meaning (fig.5.2). The rate of error observed for the number of amplification cycles used was within the error rate predicted by Keohavong *et al.*, (1989 [105]).

Analysis of the 1132 bp *Eco* RI generated product showed a 91 bp overlap with sequence contained in the cAoPR1 clone. The only base pair mismatch in this overlapping sequence was a T to C transition changing a predicted valine to an alanine in the 12th codon of the predicted protein. When a specific end labelled probe was used in transcript mapping experiments it was found that from the protected fragment size a transcriptional start site could be predicted. The putative transcriptional start site was 59 bp 5' to the first predicted ATG that was present in both the cDNA and the IPCR sequence. A putative TATA box, with the sequence TATAAA, was present 29 bp 5' to this ATG. These sequences and distances agree with the general features of plant promoters as described by Joshi, 1987 [95]). The sequence around the first ATG also correlated with the general observations of Kozak, 1985 [108] and so these data agree with the previous supposition that the cAoPR1 clone contains the entire coding sequence.

When using the complete 1.1 kb *Eco* RI generated IPCR product to screen the EMBL database no extensive sequence homology with other sequences were not found. However, homology to published sequence boxes implicated important in the regulation of other wound induced genes were found by direct searching. The sequences ACCTGACC and ACCTAGCC, found at -320 bp and -491 bp are similar to the sequences ACCTTGCC and ACCTAACC that interact with DNA-binding

protein factors in the potato PI-II promoter and the maize CHS promoter respectively (Palm *et al.*, 1990 [142] and Lois *et al.*, 1989 [122]. These sequences are in turn similar to a recently reported sequence motif implicated important in the regulation of transient expression of chalcone synthase 15 promoter CAT-reporter constructs in electroporated alfalfa protoplasts by phenylpropanoid pathway intermediates (Loake *et al.*, 1991 [120]). This sequence reads CCTACC$(N)_7$CT and therefore contains CCTACC which is present between -51 and -56 in the AoPR1 promoter (fig 5.3). This sequence does not include the 3' CT sequence. However, this dinucleotide sequence is probably not absolutely required for biological response to the phenylpropanoid pathway intermediates (as determined by deletion experiments G. Loake personal communication).

Southern hybridization analysis using a 700 bp *Xba* fragment from the 5' region of the AoPR1 Eco IPCR clone gave a simple banding pattern in the asparagus genome, indicative of a single or low copy number sequence (fig. 5.4). This probe did not hybridize to the same *Eco* RI restriction fragments as the DD1-34 and genomic clones. A 1.3 kb *Eco* RI fragment that hybridized to this probe corresponds well to the IPCR product obtained supporting the assumption that the correct upstream region had been cloned from genic sequences that were unlinked to the DD1-34 sequence.

All IPCR sequences were compared to the DD1-34 clone, the homologous genomic clones and the maize SOD-1 cDNA but no new sequence homology was found. This provided very good evidence that the AoPR1 gene was not related to a superoxide dismutase gene.

Knowing that there is a inherent error frequency in the amplification of sequences by PCR of around 1 bp every 300 bp it can be assumed that the sequence data obtained must contain errors at a frequency equal to this. Due to lack of time, in the sequence analysis of the PCR products only one clone was sequenced. Ideally 3 or more clones from different PCR reactions should be cloned and sequenced. In this way sequence in one clone that deviates from that contained in the other two clones can be found, allowing more accurate sequence data to obtained. Another approach

is to directly sequence the products obtained from the amplification reaction using a series of synthetic oligonucleotide primers. Possible PCR errors occurring in the earlier stages of the amplification may then be identified as more than one band of identical molecular mass appearing on the sequencing gel.

The inverse PCR technique had been successfully applied to the cloning of the AoPR1 gene sequences and at the time this represented one of the first examples of monocotyledonous gene sequences to be cloned by this technique. The sequence, Southern hybridization and S1-nuclease data supported the idea that the regulatory region of the AoPR1 gene had been isolated and it remained to test the putative AoPR1 regulatory sequences for biological function by reporter analysis in transgenic plants.

Chapter 6

Construction of AoPR1-gus reporter genes and their analysis in transgenic tobacco.

6.1 Introduction

Following the isolation of upstream regions of the AoPR1 gene by IPCR there was good evidence from sequence analysis and S1 transcript mapping experiments (fig. 5.5) that the AoPR1 Eco IPCR clone was sufficient to direct transcription of the AoPR1 gene. To test this theory, an approach was taken that involved the construction of promoter/reporter gene fusions. The *E.coli* β-glucuronidase gene (*gus*) was chosen as a reporter gene (Jefferson *et al.*, 1986 [91]). The activity of this enzyme is easily assayed using either the histochemical stain X-gluc which is a colourless chromogenic substrate yielding a blue precipitate following GUS-mediated hydrolysis, or MUG that yields the fluorescent molecule 4-MU following GUS-mediated hydrolysis which may be measured quantitatively with the aid of a fluorimeter. This AoPR1 promoter-reporter gene fusion would then provide data regarding temporal and spatial gene expression. Tobacco was chosen as the plant to be initially transformed as the process of *Agrobacterium* mediated tobacco transformation is simple, repeatable

and generates transgenic plants over a relatively short time period. Foreseeable draw backs were that tobacco is not closely related to asparagus and this could pose problems regarding the likelyhood of a monocotyledon-derived promoter functioning in a dicotyledonous plant, as the accurate expression of a monocotyledonous gene in a dicotyledon may depend on the specific gene transferred and the plant species transformed (reviewed Weising *et al.*, 1988 [201]). For example, insufficient transcription (Ellis *et al.*, 1987 [62]) and aberrant processing (Keith and Chua, 1986 [101]) were also observed in the case of the maize *AdhI* gene in transgenic tobacco.

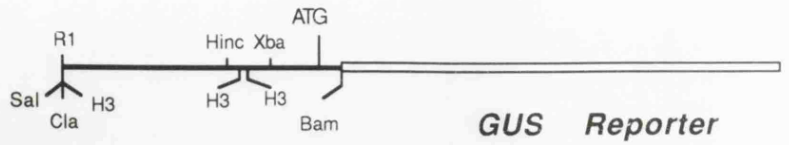
At the time of constructing the reporter genes the transcript mapping data had not been obtained and so it was thought wiser to construct translational fusions rather than transcriptional fusions which would have involved conjecture as to the transcriptional start site. For ease, restriction sites present within the polylinker sequences or within the AoPR1 promoter were used in the construction of the translational fusions. This meant that approximately the first 30 amino acids of the translational fusions would be coded for by the AoPR1 sequence. If these sequences coded for a signal peptide sequence the fusion protein may be exported. When the GUS protein is exported it becomes glycosylated at a cryptic site and is rendered functionally inactive (Iturriaga *et al.*, 1989 [89]). This was not considered a great risk as there were data for the parsley homologues that suggested that the encoded proteins were intracellular (Somssich *al.*, 1986 [182]) and there were no predicted regions within the 30 amino acids that displayed predicted hydrophobic domains characteristic of signal peptides (von Heijne, 1985 [198]).

Expression data relating to the parsley, bean and birch pollen allergen homologues (outlined in chapter 4 and in Walter *et al.*, 1990 [199]) and the northern analysis of wound-induced transcript detected by the AoPR1 cDNA clone (fig. 4.6) suggested that the promoter directs expression of the reporter following wounding and also suggested that expression may be observed in pollen and in other tissues of the plant.

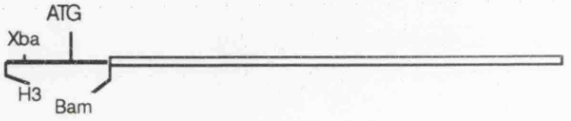
Many of the classical PR and defense genes are induced by the chemical elicitor salicylic acid (Ward *et al.*, 1991 [200] and Yalpini *et al.*, 1991 [205]). Tobacco PR1a

Figure 6.1. AoPR1-*gus* reporter translational constructs shown with Southern analysis of transformed *Agrobacterium tumefaciens* strain LBA4404, harbouring these constructs hybridized with the AoPR1 IPCR promoter and coding region probe.

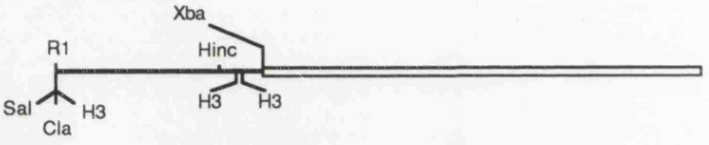
Lanes 1, 2, 11 and 12	pBI101.1 AoPR1-*gus* Full 1.1 kb digested with *Bam* HI and *Sal* I
Lanes 3, 4, 9 and 10	pBI101.1 AoPR1-*gus* Full 5'D 0.4 kb digested with *Bam* HI and *Hind* III
Lanes 5, 6, 7 and 8	pBI101.1 AoPR1-*gus* Full 3'D 0.7 kb digested with *Xba* I and *Sal* I
Lane 13	untransformed *Agrobacterium tumefaciens*



pBI101.1 AoPR1-GUS Full 1.1 kb

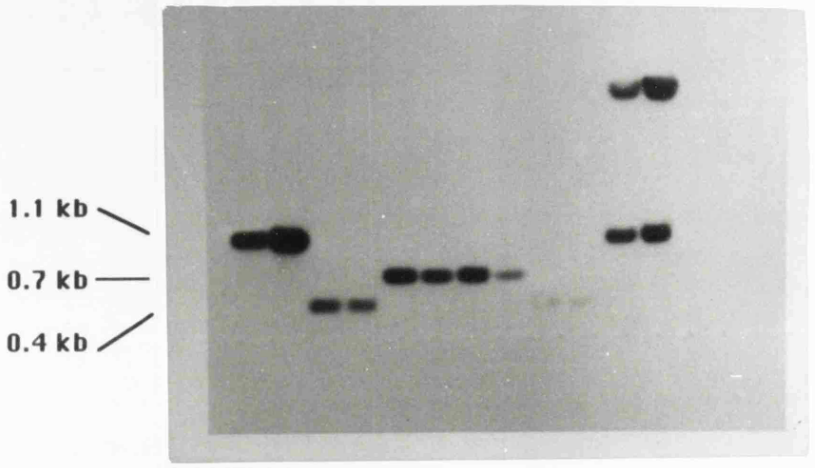


pBI101.1 AoPR1-GUS 5'D 0.4 kb



pBI101.1 AoPR1-GUS 3'D 0.7 kb

1 2 3 4 5 6 7 8 9 10 11 12 13



promoter-*gus* reporter expression revealed that this promoter was able to direct expression around mechanical wound sites and systemically after vacuum infiltrating and incubating with 2 mM salicylate (Ohshima *et al.*, 1990 [140]). Although the AoPR1 predicted protein is not related to tobacco PR1a protein at the sequence level there are similarities in protein size and the respective gene expression profiles; both showing wound inducibility with similar temporal patterns of transcript abundance which suggests the possibility that the AoPR1 promoter might be responsive to salicylic acid.

6.2 Construction of the AoPR1-*gus* fusions

Using standard molecular cloning techniques an AoPR1 *Bam* HI *Sal* I fragment containing the full promoter and 30 amino acids of AoPR1 predicted coding sequence was removed from the recombinant pBluescript vector and this was ligated into identically cut pBI101.1 vector (Jefferson *et al.*, 1987 [92]). pBI101.1 is a binary vector that contains the *gus* reporter gene downstream from a cloning site, followed by a NOS terminator region that directs efficient polyadenylation of the *gus* message. Upstream from this region is a NPT II gene cassette that allows selection of transformed plants on media containing the antibiotic kanamycin. These sequences all lie in the Ti plasmid derived left and right borders that both assist and define the region of DNA transferred into the plant's genome. Other sequences of note contained in the vector include a kanamycin resistance cassette allowing selection of transformed bacteria on media containing kanamycin, a wide host range origin of replication and sequences that facilitate the conjugative transfer of the vector between *E.coli* and *Agrobacterium*. The recombinant pBI101.1 plasmid was named pBI101.1 AoPR1-*gus* Full 1.1 kb (fig. 6.1). This plasmid was used in the construction of AoPR1-*gus* 5'D 0.4 kb. The deletion of 5' sequences distal to the predicted TATA box was created by digesting pBI101.1 AoPR1-*gus* Full 1.1 kb with *Hind* III releasing the distal sequences and then recircularizing the deleted plasmid. As a control, pBI101.1 AoPR1-*gus* 3'D was constructed. This plasmid resulted from an *Xba* I *Eco* RI 700 bp fragment cloned into appropriately digested pBI101.1. pBI101.1

AoPR1-*gus* 3'D 0.7 kb contained sequences distal to the predicted TATA box but was missing the putative TATA box and transcriptional start site (fig. 6.1) and was expected to give no GUS activity.

6.2.1 *Agrobacterium* transformation

Following the construction of the *gus* fusion vectors the presence of the correct inserts were confirmed by Southern blotting hybridization experiments and predictably sized restriction enzyme fragments were found to hybridize to labelled AoPR1 IPCR Eco probe.

The plasmids were introduced into LBA4404 *Agrobacterium tumefaciens* via conjugative transfer mediated by the pRK2103 containing strain of *E.coli* that contains all the required factors to enable tri-parental matings to take place. Transformed agrobacteria were selected for on media containing rifampicin and kanamycin and re-streaked twice on selective media to obtain pure single clones. The rifampicin resistance marker is carried chromosomally and the kanamycin resistance marker is carried on the binary vector.

Total agrobacteria DNA preparations were prepared and Southern blots carried out to confirm that the constructs were harboured within the Agrobacteria transconjugants (fig. 6.1).

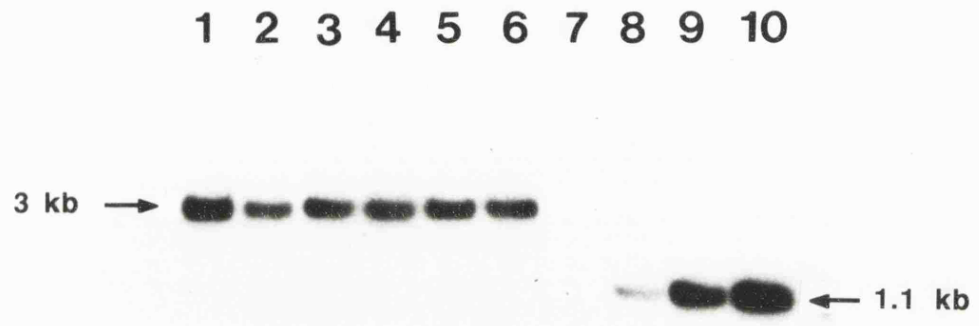
6.3 Tobacco leaf disk transformation

Transformed tobacco plants were generated by standard techniques. There were 10 individual kanamycin resistant plants harbouring the AoPR1-*gus* full 1.1kb construct, 7 individual kanamycin resistant AoPR1-*gus* 5'D 0.4kb plants and 9 individual kanamycin AoPR1-*gus* 3'D 0.7 kb plants. Once the plants had produced sizable roots in kanamycin containing medium they were transferred to compost filled pots and maintained in the transgenic plant growth rooms.

Figure 6.2. Southern blot of *Eco* RI digested genomic DNA isolated from different transgenic tobacco lines hybridized to the AoPR1 IPCR promoter and coding region probe.

Lanes 1–6	transformed tobacco lines 1, 3, 7, 12 and 14 harbouring AoPR1-*gus* 1.1 kb Full construct.
Lane 7	untransformed tobacco.
Lanes 8–9	1, 5 and 10 times T-DNA copy number reconstructions.

Approximately 10 μg of DNA was loaded per lane.


1 2 3 4 5 6 7 8 9 10
3 kb
1.1 kb

6.3.1 Initial transformation data

DNA extractions were performed on six putative transformants containing the AoPR1-*gus* 1.1 kb Full constructs from plant lines designated 1, 3, 7, 12, 14 and 16. Untransformed tobacco DNA was used as a negative control and as carrier DNA for 1, 5 and 10 times T-DNA copy number reconstructions. Ten micrograms of DNA was digested with *Eco* RI which should release a fragment of approximately 3 kb containing the AoPR1 promoter, *gus* reporter and nos poly (A) signal. The 2n size of the tobacco genome is approximately 7.8 pg (Bennett and Smith, 1991 [2]. For ten microgrammes of DNA this corresponds to approximately 1.3 x 10^6 copies of the genome. For a 1 x reconstruction using a 6 kb plasmid containing the AoPR1 promoter, approximately 4 pg is required. The reconstructions consisted of the 1.1 kb AoPR1 promoter fragment alone plus untransformed *Eco* RI digested tobacco DNA. Following Southern blotting and hybridization with the AoPR1 promoter fragment the autoradiogram suggested that the putative transformants contained the predicted 3 kb *Eco* RI fragment that hybridized with the AoPR1 promoter labelled probe. Untransformed tobacco showed no detectable hybridization to the AoPR1 promoter sequences. By comparisons with the reconstructions it appeared that the transformants contained between 1 and 4 copies of the construct (fig. 6.2).

6.4 Analysis of wound-induced *gus* expression driven by the AoPR1 promoter in representative lines

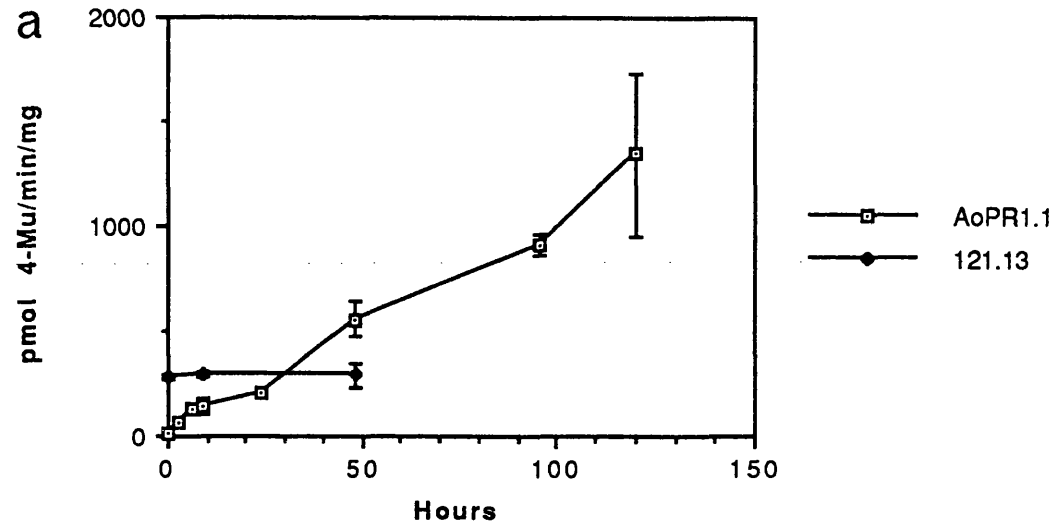
The mature kanamycin resistant plants were initially checked for GUS activity following wounding. This was achieved by stabbing leaf disks with laboratory fine forceps 15 times per square centimeter and incubating the leaf disks on sterile, damp filter paper for 24 hours on the bench. Protein was then extracted in GUS extraction buffer and the tissue assayed fluorometrically over a time course of 2 hours.

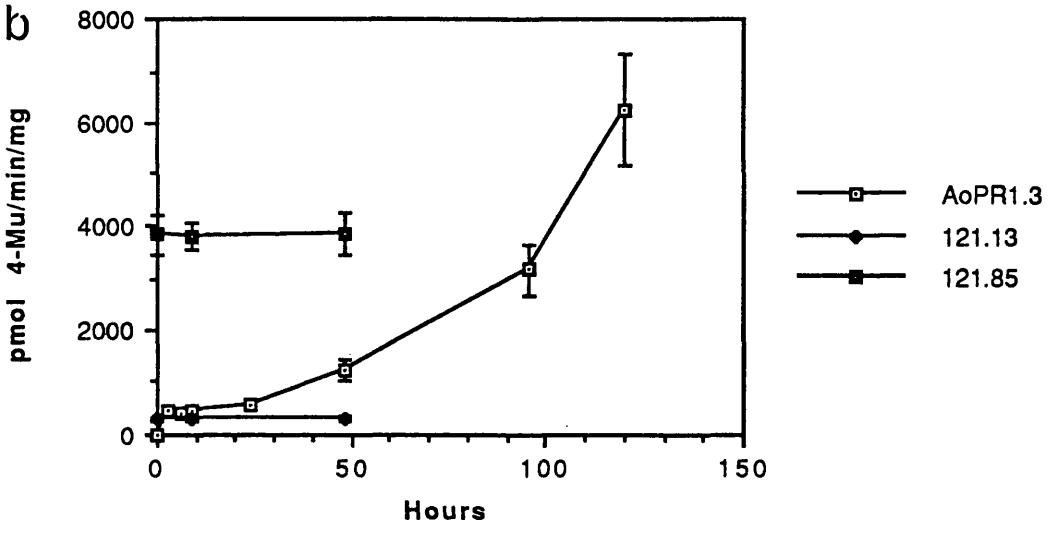
The general results showed that 9 of the kanamycin resistant plants harbouring the AoPR1-*gus* 1.1 kb Full construct gave GUS activity in wounded leaf tissue. None of the AoPR1-*gus* 3'D 0.7 kb or AoPR1-*gus* 5'D 0.4 kb kanamycin resistant plants

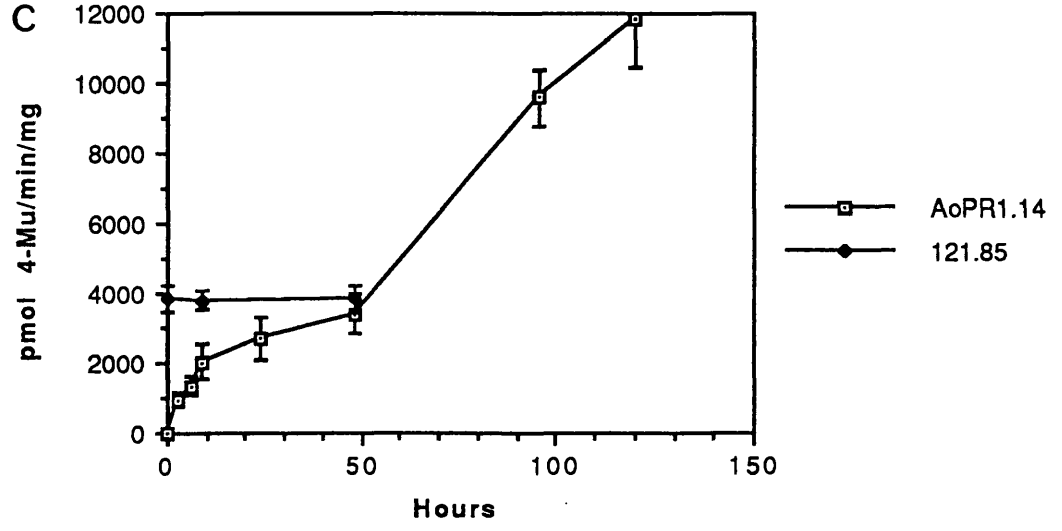
Figure 6.3. GUS activity measured for 3 independent transformants harbouring the AoPR1-*gus* 1.1 kb Full transgene at various time points following wounding. Two CaMV 35S-*gus* transformed plants (121.13 and 121.85) are included as controls.

Graphs a, b and c Wound-induction of GUS activity observed for AoPR1-*gus* in pmol 4-Mu/ min/mg total pr
1.1 kb Full transformed tobacco lines 1, 3 and 14.

(Points represent the mean and the vertical bars represent the standard deviation$_{n-1}$ of data obtained from three independent measurements).


a
2000
1000
0
pmol 4-Mu/min/mg
0
50
100
150
Hours
AoPR1.1
121.13


b
8000
6000
4000
2000
0
pmol 4-Mu/min/mg
0
50
100
150
Hours
AoPR1.3
121.13
121.85


c
12000
10000
8000
6000
4000
2000
0
pmol 4-Mu/min/mg
0
50
100
150
Hours
AoPR1.14
121.85

gave any measurable activity.

This data suggested that plants containing the deleted versions of the promoter either were not transformed, contained truncated T-DNA inserts, did not exhibit wound inducible expression or alternatively could have wound inducible expression levels that were too low to be detectable.

AoPR1-*gus* Full 1.1 kb plants 1, 3 and 14 were chosen as plants that exhibited low, medium and high relative expression levels of GUS respectively. Individuals from the T_2 population of self fertilized plants from these lines that were both kanamycin resistant and expressed *gus* were chosen for fluorometric study. T_2 plants harbouring CaMV 35S-*gus* constructs were chosen as comparative controls (kindly provided by Jen Topping in the Leicester Biocentre). Untransformed plants were used as negative controls.

Leaf sections were wounded uniformly by stabbing 15 holes/cm with fine forceps and incubated on sterile Whatmann 3MM disks dampened with water for varying lengths of time. Sections were then harvested, protein extracted in GUS extraction buffer, quantified and a fraction of the extract used for fluorometric assays. The GUS activity was calculated and expressed in terms of pmoles of 4-MU produced/min/mg of total protein. The experiment was repeated in triplicate using different sections of the same leaf for each plant. The results are shown in graphical form (fig. 6.3). The data shows that an increase in GUS activity is detectable from a few hours after wounding and increases with time up to 5 days in all three plants containing the full promoter fusions. The fluorometric data confirmed that line 1 was the weakest expressing plant and 14 was the strongest being approximately an order of magnitude stronger than line 1. Differences in copy number of the AoPR1 promoter *gus* fusions in each of these plants is insufficient to explain the observed differences in expression levels between plant line 1 and 14. A more likely explanation is that the surrounding context of sequences flanking the inserted constructs influences the level of expression. The two CaMV 35S promoter-*gus* fusion controls also show expression levels that are a magnitude different from one another, but exhibit no apparent significant increase in GUS activity over the two day time period from

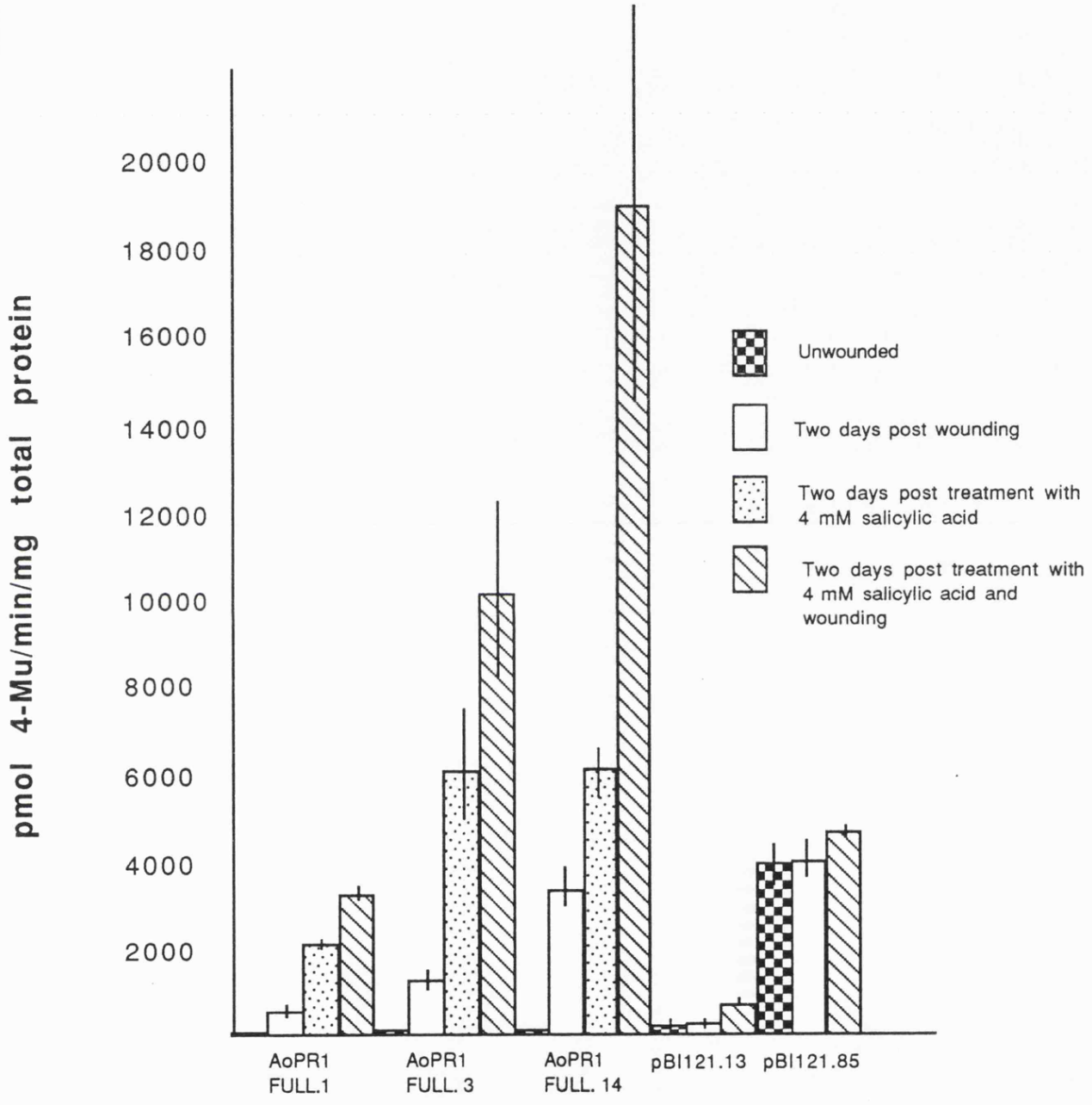
which data were obtained. However the possibility of wound induction of GUS activity, directed by the CaMV 35S promoter, post the two day period can not be discounted.

Localization of the *gus* expression during wounding was achieved by histochemical staining with X-gluc. Fig. 6.7 contains some pictures showing a general summary of staining post wounding on line 14 and line 3 plants. Fig. 6.7g shows expression around a lesion in a line 3 leaf demonstrating that *gus* expression correlates with the localized wound response. Fig. 6.7h shows expression of two whole neighbouring leaves from plant line 3 treated in different ways. The left leaf was removed from the plant, stabbed and left to age for one day. The leaf on the right was removed the following day, stabbed and both leaves were then incubated in the presence of X-gluc overnight. Only the leaf that had been stabbed and aged showed visible staining around the wound sites whereas the leaf that was stabbed and not aged showed no visible staining, implying that GUS fusion protein has accumulated sufficiently to give rise to staining surrounding the damaged area in a wound-inducible manner only after aging. The veins in each leaf both show faint GUS staining that appears more intense in areas very close to wound sites. The observation that stronger staining in the vascular tissue is associated with proximity to wound sites suggests either better penetration of X-gluc in these regions or a genuinely higher activity that may be associated with a 'wound'-messenger travelling through this tissue.

To address the possibility that the localized staining was restricted to the wound site due to ease of penetration through wound sites by X-gluc a CaMV 35S promoter-*gus* fusion plant was stained and showed more or less uniform staining across the leaf section, suggesting that X-gluc penetration was efficient, uniform and accurately reflects the strong and 'constitutive' nature of this promoter (fig. 6.7l). Fig. 6.7k shows staining in young line 3 plants that have been sliced with a scalpel again showing expression around the wound sites and vascular tissue. Expression is also observable in the roots and this will be discussed later in this chapter.

The transcriptional activity of other wound-induced genes, notably PAL, CHS and other important members of the phenylpropanoid biosynthesis pathway show simi-

Figure 6.4. GUS activity measured for 3 independent transformants harbouring the AoPR1-*gus* 1.1 kb Full transgene two days following wounding and treatment with salicylic acid. transformed plants are included as controls. (Filled columns represent the mean and the vertical bars represent the standard deviation$_{n-1}$ of data obtained from three independent measurements.)


pmol 4-Mu/min/mg total protein
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
Unwounded
Two days post wounding
Two days post treatment with 4 mM salicylic acid
Two days post treatment with 4 mM salicylic acid and wounding
AoPR1 FULL.1
AoPR1 FULL. 3
AoPR1 FULL. 14
pBI121.13
pBI121.85

larities to the temporal expression profiles exhibited by the AoPR1-*gus* fusion. When transcript levels are measured in bean PAL2-*gus* transformed tobacco plant using S1 mapping the *gus* transcript appears to be induced in sliced transgenic tobacco leaf tissue within 12 hours of wounding with an associated GUS activity in the range of 200-600 pmol 4-MU/min/mg (Liang *at al.*, 1989 [115]). At the same time point after wounding AoPR1-*gus* transgenic plants have similar GUS activities in lines 3 and 14. Similarly, bean CHS8-*gus* fusions in transgenic tobacco show weak X-gluc staining around wound sites within sixteen hours of wounding and strong staining within 48 to 72 hours. In contrast to the *win 2-gus* potato transformants (Stanford *et al.*, 1990 [186]), the AoPR1-*gus* tobacco transformants are induced more rapidly post wounding. Unfortunately there is little data relating to the expression profiles of the parsley PR proteins but following fungal invasion of parsley *in situ* analysis of transcript localization revealed strong up-regulation of PR1 transcript within 4-6 hours post invasion (Schmelzer *et al.*, 1989 [171] and Somssich *et al.*, 1988 [183]) which is a similar time scale to that observed for the initial induction of the GUS protein in AoPR1-*gus* plants following wounding.

These data clearly demonstrated that the monocotyledon inverse PCR-generated upstream regions conferred strong wound-inducibility of GUS in the AoPR1-*gus* transformed tobacco and suggested that the IPCR approach may be a useful technique for the isolation and study of gene regulatory sequences. The temporal and spatial expression profiles of AoPR1-*gus* wound-inducibility resembled those obtained from phenylpropanoid promoter-*gus* fusion analysis also carried out in transgenic tobacco.

6.4.1 Salicylic acid induction of GUS activity in AoPR1-*gus* fusion plants

Salicylic acid causes the induction of many defence-related genes in tobacco including the classical PR proteins and has been implicated as a general 'wounding' messenger (Yalpini *et al.*, 1991 [205]) as discussed in the introduction to this section. For this reason a series of experiments were carried out to discover if this was the case for the AoPR1-*gus* fusions. For this experiment leaf disks were either wounded by stabbing

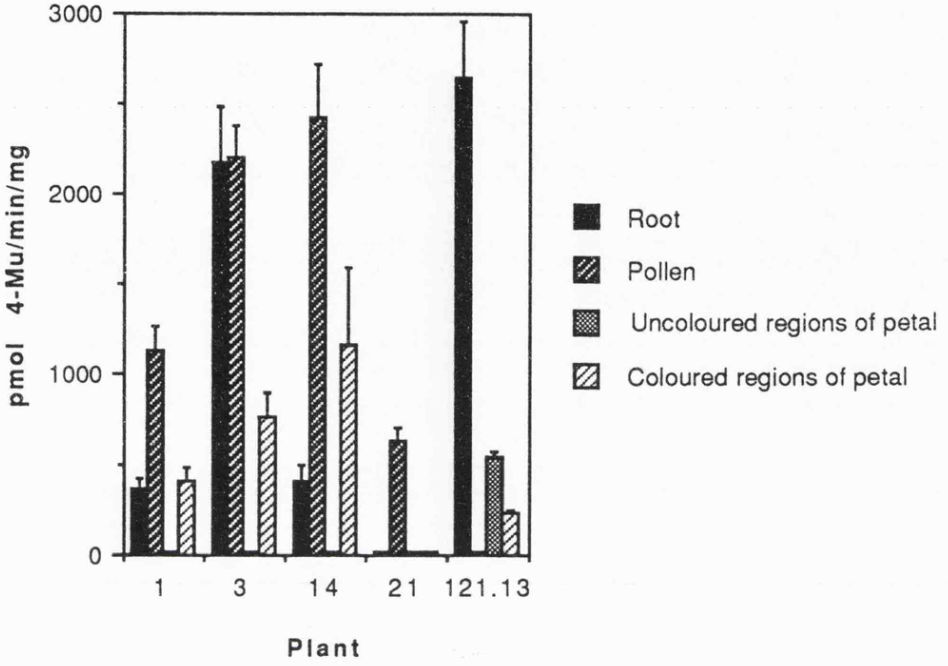
or untreated and incubated for two days on filter paper dampened with either distilled water or 4 mM salicylate. The results are shown in histogram form (fig 6.4). The relative levels of expression observed for each of the plant lines are in accordance with those seen previously in wounding experiments. The CaMV 35S promoter-*gus* fusion plants are apparently not significantly effected by treatment with salicylic acid or wounding. However treatment with 4 mM salicylic acid alone induces the measured GUS activity in the AoPR1 promoter-*gus* fusion plants. Treatment with 4 mM salicylic acid and stabbing further induces measurable GUS activity, presumably as either a synergistic effect of the two treatments or by facilitating penetration of the leaf tissue by salicylic acid. Fig. 6.7j shows the effect of wounding alone and wounding plus salicylic acid treatment as discussed earlier this section. The leaf on the left has been stabbed and the leaf on the right has been stabbed and treated with salicylic acid showing salicylic acid is able to induce GUS activity across the leaf area it is in contact with. Leaf tissue that had been treated with salicylic acid following stabbing were stained with X-gluc. It is interesting to observe that of the leaf tissue that has been in contact with the salicylic acid solution showed strong staining which was further intensified around the wound site, (fig. 6.7j right leaf section). Staining is mainly observed around the wound site in the control which had been stabbed and not treated with salicylic acid (fig. 6.7j left leaf section).

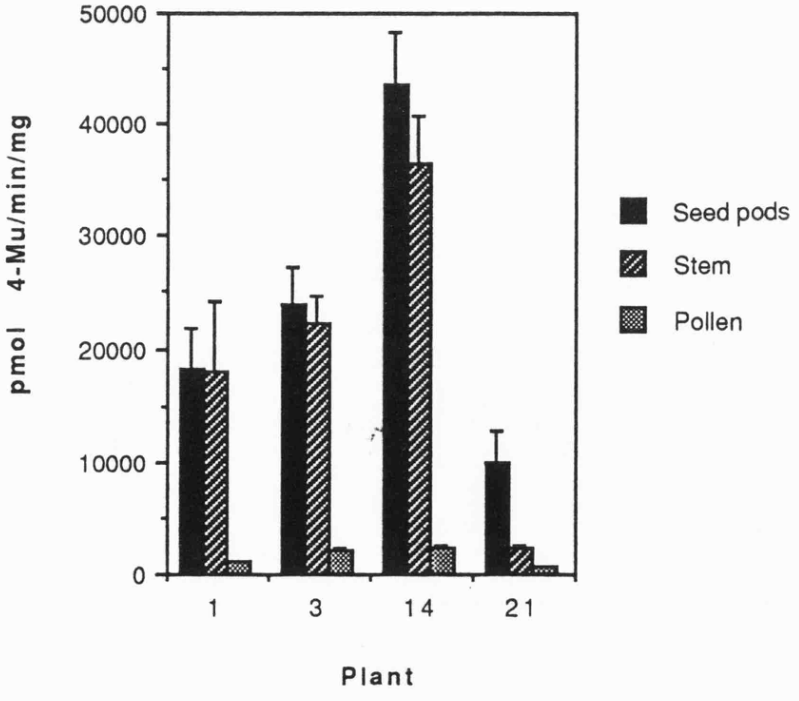
Although no time-course or dosage experiments were carried out, these data show that the AoPR1 promoter is responsive to the levels of salicylate that were applied to the transgenic tobacco leaf disks. The levels of induction measured two days after wounding and salicylic acid treatment and wounding alone for the tobacco PR1a-*gus* construct (Ohshima *et al.*, 1990 [140]) resemble those observed for AoPR1-*gus* expression. The activity of GUS measured for the PR1a-*gus* plants was expressed as μmol 4-Mu/gram fresh weight. However, comparisons of the localization of expression following X-gluc staining between the PR1a-*gus* and AoPR1-*gus* tobacco plants suggest that the AoPR1 promoter may be more active than the PR1a promoter in response to 4 mM salicylate as blue staining in the AoPR1-*gus* tobacco was much more intense than in plants containing PR1a promoter-*gus* fusions. Similarly, the 1129 bp arabidopsis PR- acidic chitinase promoter-*gus* fusion also gives inducible

Figure 6.5. GUS activity measured in different unwounded tissues for three independent transformants harbouring the AoPR1-*gus* 1.1 kb Full transgene, one transformant harbouring the AoPR1-*gus* 0.4 kb 5'D construct and two CaMV 35S-*gus* transformed plants.

Plant 1	AoPR1-*gus* 1.1 kb Full line 1
Plant 3	AoPR1-*gus* 1.1 kb Full line 3
Plant 14	AoPR1-*gus* 1.1 kb Full line 14
Plant 21	AoPR1-*gus* 0.4 kb 5'D line 21
Plant 121	CaMV 35S-*gus* line 13

(Filled columns represent the mean and the vertical bars represent the standard deviation$_{n-1}$ of data obtained from three independent measurements.)


3000
2000
1000
0
pmol 4-Mu/min/mg
1
3
14
21
121.13
Plant
Root
Pollen
Uncoloured regions of petal
Coloured regions of petal

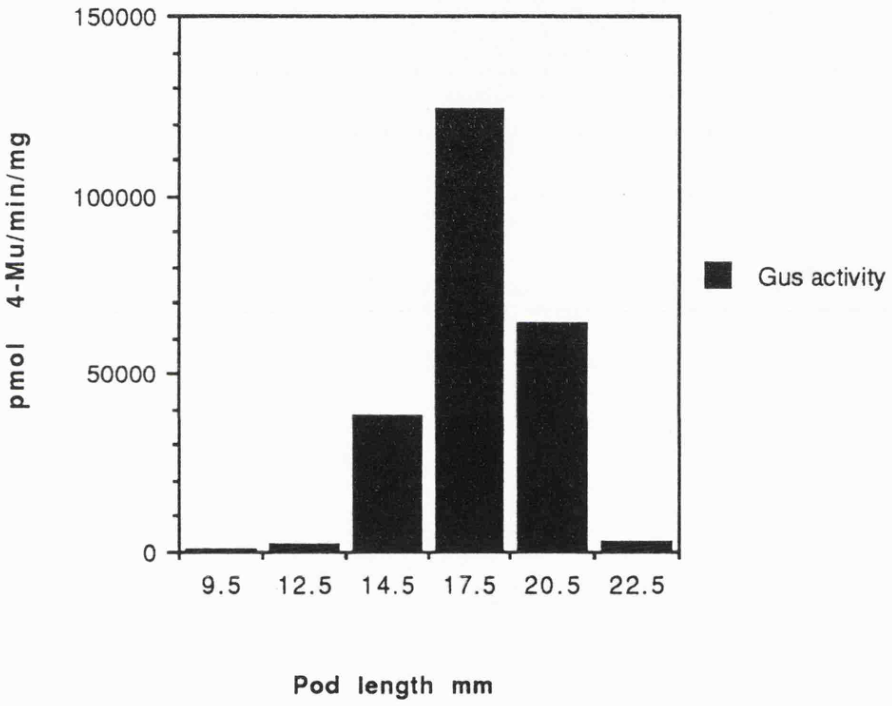

50000
40000
30000
20000
10000
0
pmol 4-Mu/min/mg
1
3
14
21
Plant
Seed pods
Stem
Pollen

GUS expression in transgenic *arabidopsis* when treated with 0.1 - 1.0 mM salicylate for 2 days as well as being responsive to pathogenic attack (Samac and Shah, 1991 [164]). If there are correlations in gene expression patterns between the AoPR1 gene and other defence related genes, these data suggest that the AoPR1 promoter may too be inducible by a range of pathogenic organisms, either as a direct or an indirect consequence (ie via a 'wound' messenger) of pathogen attack.

6.5 Developmental expression in non-wounded AoPR1-*gus* plants

Several plant defense genes also have roles in plant development and are often expressed in a stage specific manner in specific tissues during different stages of a plants life cycle. Particularly good examples are the genes involved in the phenylpropanoid biosynthesis pathway. Bean chalcone synthase promoter-*gus* fusions in transgenic tobacco show strong GUS activity in the coloured regions of the petal where naturally chalcone synthase would be involved in the biosynthesis of pigments as well as in etiolated cotyledons that have been exposed to light, presumably where phenylpropanoid-derived UV protectants are being synthesized (Schmid *et al.*, 1990 [172]). PAL promoters from *Arabidopsis* and bean show expression of *gus* fusions in pollen where the phenylpropanoid tetrahydroxychalcone is found (de Vlaming and Kho, 1976 [45]), in the secondary xylem parenchymal rays, where PAL is a marker for lignin biosynthesis, in regions of the vasculature showing secondary thickening, in the coloured regions of the petal, in the ovary and stigma (Bevan *et al.*, 1989, Liang *et al.*, 1989 and Ohl *et al.*, 1990 [3,115,139]). More recently the parsley 4CL-1 promoter-*gus* fusion has been shown to be highly active in the parenchymal rays, mature pollen and the epidermis of developing seeds and is also active in coloured regions of the petal and receptive stigma surface (Hauffe *et al.*, 1991 [82]). As noted previously in chapter 5, the AoPR1 promoter had sequence motifs displaying some homology to sequence boxes that are associated with the transcriptional regulation of genes encoding enzymes of the phenylpropanoid pathway. The apparent similarities

Figure 6.6. GUS activity measured in developing seed pods of AoPR1-*gus* 1.1 kb Full transformed plant line 14.


150000
100000
50000
0
pmol 4-Mu/min/mg
9.5
12.5
14.5
17.5
20.5
22.5
Pod length mm
Gus activity

between AoPR1 and phenylpropanoid gene expression suggested it was important to learn about the temporal, spatial and developmental expression patterns of the AoPR1 promoter in transgenic plants.

Although due to time constraints, very few experiments could be carried out regarding the quantitation of the interesting aspects of expression in various organs and tissues some preliminary data was obtained (fig. 6.5). Generally large differences in GUS activity was observed in various tissue and organ types throughout the plant lines. Wound-induction could be discounted since this expression was observed when freshly harvested material was used in GUS assays. Fig. 6.5a shows expression levels in roots, mature pollen from dehiscent anthers, noncoloured petal tissue and pigmented tissue for plant lines 1, 3, 14, 21 (a plant containing the AoPR1-*gus* 5'D 0.4 kb construct) and a CaMV 35S promoter-*gus* fusion plant. For the plant lines 1, 3 and 14 containing the AoPR1-*gus* full promoter constructs, GUS expression was observed in roots, mature pollen and the coloured regions of the petal. The plant containing the AoPR1-*gus* deleted promoter showed weak expression in pollen, whilst the CaMV 35S promoter-*gus* plant showed high expression in roots, leaves, stems and floral tissues except for the mature pollen. Plant line 3, containing the full AoPR1 promoter construct has higher expression levels of GUS activity observable in root tissue than predicted from the general trend of expression in other plant lines tested histochemically for GUS. This effect may be explained in terms of positional effects within the genome where it is possible that the construct in plant line 3 is under the effect of enhancer sequences that direct strong expression in roots.

Strong activity was measured in developing seed pods 15 mm in length and in mature, woody stem tissue from a just above soil level. The fluorometric data for these tissues showed activity that was more than ten fold higher than the levels observed in mature pollen grains (fig. 6.5b). The plant harbouring the 5' deleted AoPR1 promoter-*gus* construct revealed significant expression in developing seed pods but poorer expression in stem tissue (fig. 6.5b).

A complete analysis of promoter activity in each tissue type throughout the developmental process would have occupied a great deal of time and was not undertaken.

Figure 6.7. Histochemical localisation of GUS expression in AoPR1-*gus* 1.1 kb Full transgenic tobacco plants.

6.7a staining of stem xylem paranchymal rays in line 3.

6.7b autofluorescence of lignified stem tissue in line 3.

6.7c staining of developing seeds from line 14.

6.7d staining of mature pollen from line 14.

6.7e an unstained flower (left) and stained flower (right) from line 14.

6.7f staining of the surface of a receptive stigma from line 14.

6.7g staining surrounding a lesion in a leaf from line 3.

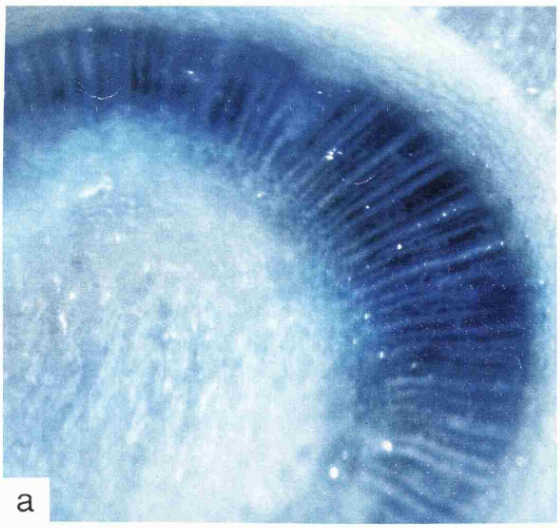
6.7h staining observed in a wounded and aged leaf (left) and a wounded and not aged neighbouring leaf (right) from line 3.

6.7i stained callus material from line 14.

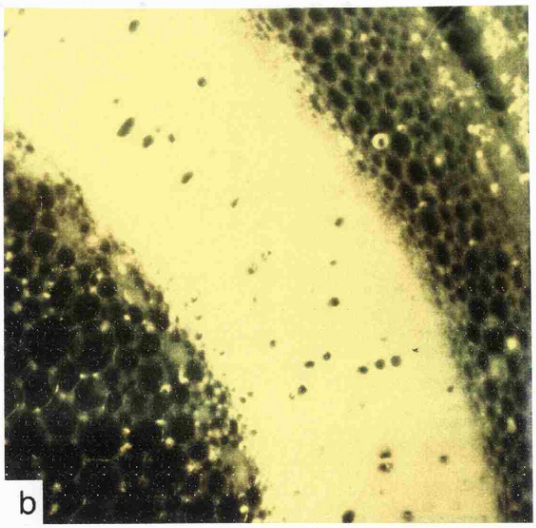
6.7j wounded leaf tissue treated with salicylic acid (right) and not treated with salicylic acid (left) from line 14.

6.7k sliced plant material aged two days before staining from line 3.

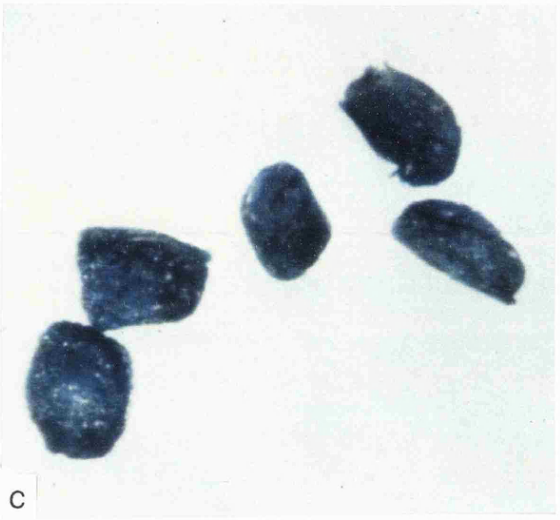
6.7l stained leaf from an untreated CaMV 35S-*gus* plant.



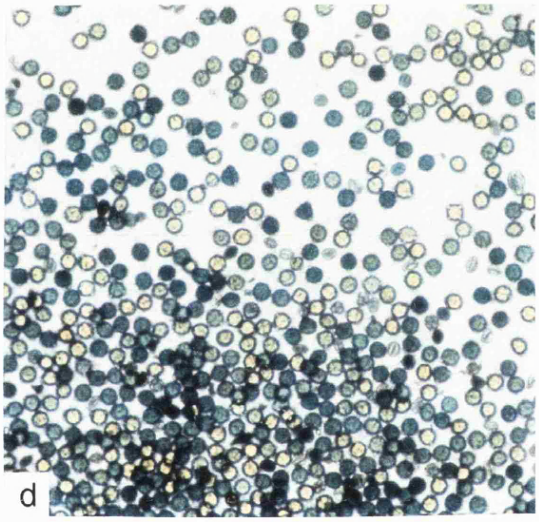
a



b



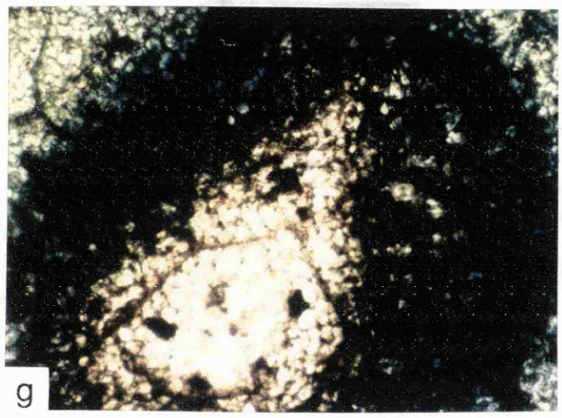
c



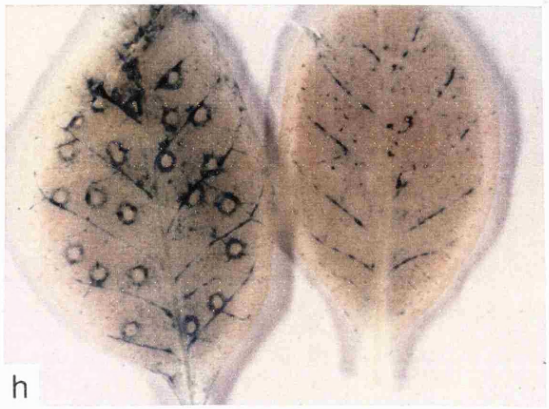
d

e

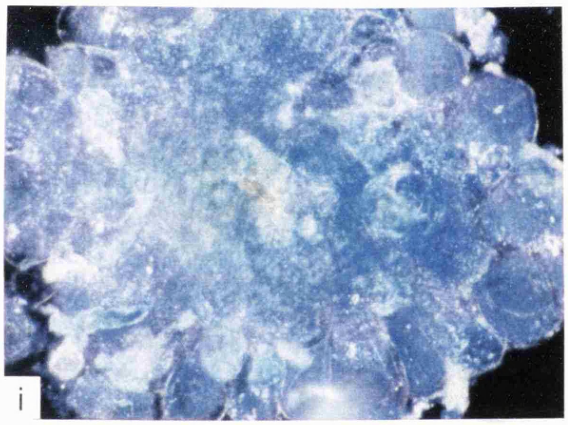
f



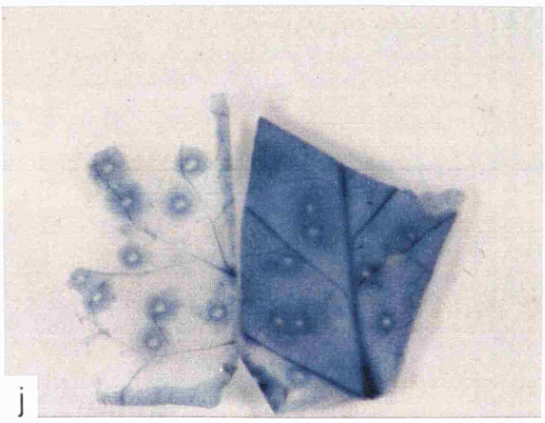
g



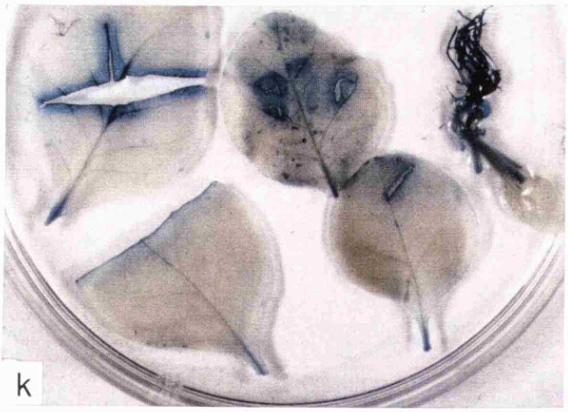
h



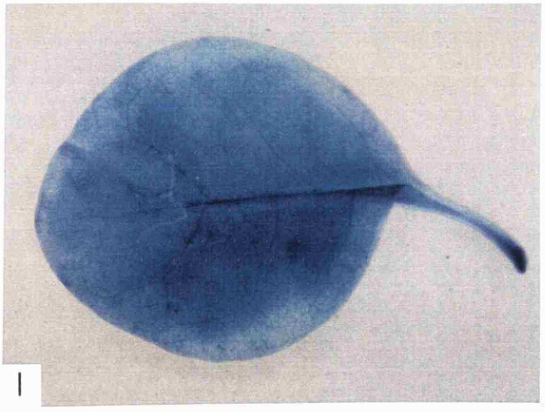
i



j



k



l

However, GUS activity in developing seed pods of different lengths were measured from plant line 14. (fig. 6.6) and plotted as a semi-log plot as there were large differences in GUS activity during seed pod development. GUS activity rises to a maximum at a pod length of around 17.5 mm which is approximately three orders of magnitude larger than GUS activity observable at a pod length of 9.5 mm. Following the maxima the pod starts to mature and desiccate and GUS activity declines.

GUS staining data correlates well with the numerical fluorometric data obtained for each of the tissues examined in this study. Fig. 6.7a shows stem tissue from plant line 3 where staining occurs in a time period of 20 minutes to two hours. The staining is mainly localized to the xylem parenchymal rays which are involved in the lignification and structural reinforcement of the secondary vasculature of the stem. This observation was seen in all the plant lines containing the AoPR1-*gus* Full 1.1 kb construct. When stem tissue of the AoPR1-*gus* 5'D 0.4 kb construct plants was stained little or no expression of GUS was observed in the ray tissue. Instead, activity was mainly restricted to the protoxylem and metaxylem (data not shown). Fig 6.7b shows autofluorescense of lignified tissue in the stem. The levels of autofluorescence observed from the lignified tissue correlate well with the localization of GUS activity in the stem (Compare fig 6.7a and 6.7b).

GUS staining of developing seed pods showed that most of the activity was mainly confined to the developing seeds. Further analysis of dissected seed showed that the activity was apparently restricted to the developing testa (data not shown). When seeds from different sized pods were stained in X-gluc, the activity was so great that staining was observable within a matter of 10 to 15 minutes and produced an intense blue colour after 30 mins. The rate of staining was developmentally regulated with nearly mature seeds with light brown seed coats showing no observable stain. Fig 6.7c shows stained seeds from a 15 mm pod from plant line 14. The seed staining activity was observable for both the full AoPR1 promoter and the 5' deleted AoPR1 promoter constructs although the rate of staining for the deleted promoter was less than for the full promoter. These data correlate well both with the high levels of expression measured fluorometrically for each of the plant lines and to the

developmental expression observed for different length seed pods.

When mature pollen from dehiscent anthers was stained in X-gluc and observed under the microscope it appeared blue. The rates of staining for different lines were variable but blue pollen was observable within a few hours for the AoPR1-*gus* full 1.1 kb plant lines and overnight for the AoPR1-*gus* 5'D 0.4 kb plants. This data again closely correlated with the data obtained fluorometrically. When immature pollen was squeezed from developing anthers no staining was observable for any of the plant lines suggesting no activity in immature pollen, although this was not checked fluorometrically. Fig. 6.7d shows the extent of staining for a heterozygous line 14 plant. If the plant was homozygous, 100% of the pollen would stain. Pollen staining experiments on initial transformants and the T_2 plants would be useful in determining the segregation pattern of the constructs and hence the number of loci carrying the *gus* constructs. Counting the number of blue and white pollen grains suggested that the individual used for Fig 6.7d is heterozygous for more than one locus containing the *gus* construct as greater than 50% of the pollen stained blue. It is also observable that out of the blue staining pollen grains there are mainly darker and lighter shades of staining. This could be attributed to chance differences in staining due settling of the pollen during incubation in X-gluc. Alternatively, the plant may have two un-linked loci containing inserts of the *gus* reporter construct, one with a higher activity than the other due to position effects. These sort of theories could easily be genetically confirmed or denied by out-crossing experiments.

Fig. 6.7e (right flower) demonstrates that GUS staining is observable in the coloured regions of the line 14 plant but is absent in the noncoloured regions of the petal. Only the plant lines that express GUS strongly (eg. line 14) show good staining of this tissue overnight, line 1 shows no staining and line 3 shows poor staining. This is reflected in the fluorometric data obtained for each line. GUS staining was also observable on receptive (wet) stigma surfaces (fig. 6.7f). No fluorometric data was obtained for this organ.

Fig. 6.7i shows a fragment of callus material derived from line 14. This culture and a line 3 culture was initiated by Neil Bate and Sebo Ozcan for their own studies and

was received as a gift for fluorometric measurements. Lines 3 and 14, when measured in triplicate showed an average activity of 18,814 (standard deviation of 2626) and 24,454 (standard deviation of 2626) pmol 4-Mu/min/mg respectively. This high activity in transgenic culture lines was not predicted since dividing asparagus cell suspension cultures were not tested for AoPR1 transcript.

The temporal and spatial expression patterns observed in AoPR1-*gus* plants were strikingly similar to those observed for PAL-*gus* or 4CL-1-*gus*. Each of these promoters is active the pigmented parts of the flower, developing seeds, xylem parenchymal rays and pollen. These phenylpropanoid genes are also light induced and this raises the question is the AoPR1 promoter light responsive? This question could be addressed using dark germinated AoPR1-*gus* tobacco plants treated with various light regimes.

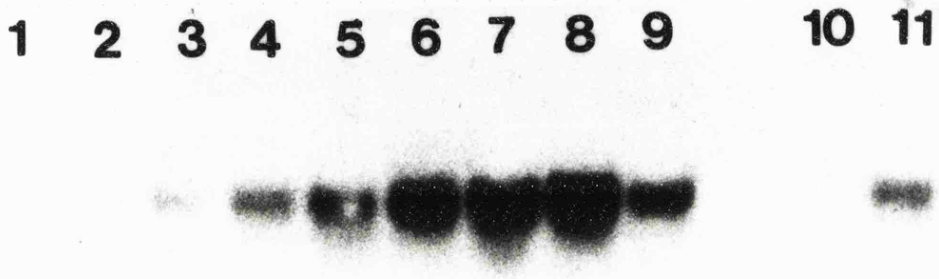
6.6 Experiments relating to developmental expression of the AoPR1 gene in asparagus

The data generated from histochemical staining of transgenic tobacco plants posed questions relating to the determination of whether the expression patterns observed in transgenic tobacco also occur in asparagus or whether they are aberrant. These questions could be answered quickly by northern hybridization experiments only in tissues that were available at Leicester. Unfortunately, there were no nearby fields of mature asparagus that were in flower or producing seed to provide enough material to carry out RNA preparations with. Instead a PCR approach was used to determine whether or not AoPR1 message was present or not in mature pollen from the few male flowers that were available. Other experiments could not be properly performed. For example, it was observed that GUS expression occurred in the coloured regions of the petal but not in the white regions in transgenic tobacco plants. Asparagus flowers are not coloured so gene expression in tobacco flowers could not be directly compared to gene expression in asparagus flowers. Ultimately *in situ* hybridization experiments may yield data revealing the spatial abundance of

Figure 6.8. Northern analysis of AoPR1 transcript induction following wounding. Total RNAs hybridized to the AoPR1 cDNA insert.

Lane 1	unwounded two week old dark grown asparagus seedlings.
Lanes 2 to 9	two week old dark grown asparagus seedlings cut into 5 mm sections and incubated in sterile conditions for 3, 6, 9, 12, 24, 48, 72 and 96 hours post slicing respectively.
Lanes 10 and 11	two week old dark grown asparagus roots cut into 5 mm sections and incubated in sterile conditions for 0 and 2 days post slicing respectively.

10 μg of total RNA was loaded per lane.


1 2 3 4 5 6 7 8 9 10 11

transcript in the native plant that corresponds to the spatial expression of the *gus* reporter observed in transgenic plants.

Following the fluorometric data obtained from transgenic tobacco it was apparent that GUS activity was induced rapidly following wounding and was present in root tissue, woody stem tissue, various floral organs and developing seeds. It was decided to test for AoPR1 transcript in roots, wounded roots and stems and to determine how rapidly the transcript was induced in chopped up asparagus seedlings. Dark grown, two week old etiolated seedlings were sliced into 5 mm sections and left for varying lengths of time. Root material was taken from unwounded seedlings, harvested and flash frozen as rapidly as possible to avoid wounding. Wounded root material was obtained from roots treated identically to chopped seedlings. Two year old asparagus plants were used to provide mature stem material for RNA. The vascular bundles of herbaceous monocotyledonous plants differ from those of woody dicotyledonous plants. The heavily lignified vascular cylinder, in tobacco, is replaced, in asparagus, by a number of collateral bundles. Autofluorescence of asparagus stem tissue showed very little lignified secondary thickening. This suggested that comparison of stem activity in asparagus and tobacco may be difficult. RNA was extracted from the different tissues, run on non-denaturing agarose gels, blotted and probed with the cAoPR1 insert. The autoradiograph showed that AoPR1 transcript was detectable from between 6 hours and a few days following the wounding event (fig. 6.8). Lower levels of transcript were present in wounded seedling roots; no detectable hybridization was detected in mature asparagus stem tissue (data not shown). This suggested that the rapidity of the wound inducibility of *gus* observed in tobacco correlated to inducible transcript detected in wounded asparagus stems and roots. There was no correlation between the lack of hybridizing transcript in seedling root and mature stem sections of asparagus as compared to the tobacco data. This may be interpreted as either aberrant expression in the transgenic plant or an inability to directly compare tobacco with asparagus. The numerous vascular collateral bundles in mature asparagus stems lack secondary thickening and show little autofluorescence indicating a lack of lignified tissue, whereas tobacco has a bicollateral stem structure with heavy secondary thickening in the vascular cylinder. The structural differences may

Figure 6.9. Amplification of AoPR1 transcript derived cDNA from wounded asparagus seedlings, mature pollen and unwounded asparagus seedlings.

Lane 1 1 kb marker (BRL)

Lane 2 PCR products from 0.4 ng of cDNA synthesized from RNA isolated from two week old dark grown asparagus seedlings cut into 2 mm sections and incubated in sterile conditions 3 days post slicing.

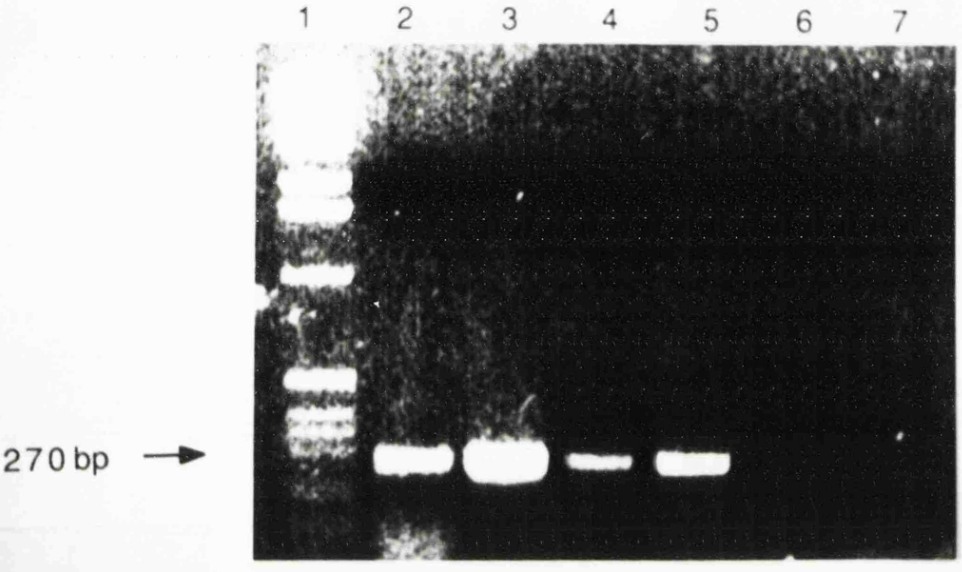
Lane 3 PCR products from 4.0 ng of cDNA synthesized from RNA isolated from two week old dark grown asparagus seedlings cut into 2 mm sections and incubated in sterile conditions 3 days post slicing.

Lane 4 PCR products from 0.4 ng of cDNA synthesized from RNA isolated from mature asparagus pollen.

Lane 5 PCR products from 4.0 ng of cDNA synthesized from RNA isolated from mature asparagus pollen.

Lane 6 PCR products from 0.4 ng of cDNA synthesized from RNA isolated from unwounded two week old dark grown asparagus seedlings.

Lane 7 PCR products from 4.0 ng of cDNA synthesized from RNA isolated from unwounded two week old dark grown asparagus seedlings.


1
2
3
4
5
6
7
270 bp

provide an explanation for the lack of correlation of expression observed in both plants. Similarly, it is not valid to compare transcript levels detectable in asparagus seedling roots with GUS expression observable in mature lignified transgenic tobacco roots.

6.6.1 The AoPR1 transcript can be detected using PCR in asparagus pollen

A few flowering asparagus plants were available at Leicester. These were collected and the anthers dissected from the male flowers. A small amount of pollen was washed from the anthers by pipetting water over the anthers and collecting the pollen by centrifugation in an Eppendorf tube. From this small amount of pollen a small scale RNA preparation was carried out. The resulting total RNA quantity was estimated at 1-5 μg from running a small quantity on an ethidium bromide containing agarose gel. This and similar quantities of total RNA from wounded and unwounded seedlings were then used in the synthesis of cDNA. A small amount of tracer radiolabelled dCTP was added to aid approximate quantitation of cDNA produced. This was used in conjunction with two primers in a direct PCR experiment. The IPCR 1. and POL. 1 primers described in the previous chapter were used (fig. 5.1). These primers were designed to span the intron containing region of the gene such that when transcript derived product was amplified a 272 bp fragment would be observed. If genic sequence was amplified then a 620 bp fragment would be observed such as was obtained in fig. 5.6. These primers were used to amplify sequences from the cDNA populations synthesized from RNA isolated from asparagus pollen, wounded seedlings and unwounded seedlings. Products were obtained only from the wounded seedling and pollen cDNA populations (fig. 6.9). The observed approximate size of these fragments was that expected if they were derived from transcript.

The size of the transcript derived product differs significantly from the 620 bp product derived from asparagus genic sequence (fig. 5.6) confirming that the products were derived from AoPR1 transcript and not the AoPR1 gene. These data suggest that AoPR1 is expressed in asparagus pollen and therefore AoPR1-*gus* expression is

authentic.

6.6.2 Transient assays in mature tobacco pollen

Transient assays may offer a rapid method of analysing reporter constructs without the need to generate and propagate transgenic plants. The assay is also free from positional effects that are associated with the integration of the construct into the plants genome where flanking sequences may influence the expression of the reporter construct. The assay is not quantitative as it is impossible to introduce an equal quantity of test reporter plasmid into each cell assayed. Instead, a non homogeneous population of cells is obtained with respect to the quantity of test reporter plasmid each cell receives. In order to make comparisons, a second reporter plasmid carrying a different reporter gene must be co-transformed into each batch of cells. This provides a reference. If the ratio of the quantity of test plasmid to the reference plasmid is constant between each experiment then the results obtained for different test constructs may be compared by expression level ratios of the test constructs to the reference constructs.

Reporter constructs provided by David Twell were used as both reference and test plasmids (Twell *et al.*, 1990 [191]). These plasmids were based on the LAT52 and LAT59 promoter sequences from tomato. LAT52 promoter-*gus* fusion expression is restricted to pollen from the onset of microspore mitosis to anthesis and results in very high activities of GUS. LAT59 promoter-*gus* fusions are co-ordinately regulated with LAT52 expression in the anther, but displays a comparatively lower activity and is also expressed in roots and seed.

The reference plasmid was provided by pLAT59-13 which consists of the LAT59 promoter mediating the transcription of the LUC gene (firefly luciferase) and a NOS 3' poly (A) signal. The pLAT52-7 plasmid contains the LAT52 promoter, the *gus* reporter gene and a NOS poly (A) signal. pCGUS, originally constructed by Jennifer Topping, was also used. This contains an 800 bp CaMV promoter-*gus*-NOS 3' reporter cassette.

A pUC based AoPR1-*gus* reporter construct was constructed using pGUS also from

J.Topping. This plasmid, named pAoPR1-*gus*, was constructed so that the AoPR1-*gus* fusion was as similar as possible to the translational fusion constructed in the binary vector.

The concentrations of each plasmid was assessed spectrophotometrically and then verified by agarose gel electrophoresis. Five micrograms of the relevant test plasmid and one microgram of the LAT59-LUC plasmid was then precipitated onto tungsten particles and three macro-projectiles prepared for each experiment. The pollen bombardment was carried out as described previously (Twell *et al.*, 1989 [190]) and the bombarded pollen incubated at 25°C for 6 hours. The pollen was then collected and protein extracted using 0.1 M phosphate buffer that contained 1 mM dithiothreitol (pH 7.5) in a mortar and pestle (as described in section 2.17). Luciferase and GUS assays were performed. The luciferase assays indicated the efficiency of the bombardments between experiments and the GUS activities reflected the transcriptional activity of the promoters. The results were expressed as a ratio of the GUS activity to luciferase activity. This allowed comparison between the activity of the different promoters. The LAT52 promoter was transcriptionally more active than the AoPR1 promoter which in turn was more active than the CaMV promoter in pollen. The LAT52 promoter was approximately 21 times as strong as the AoPR1 promoter in mature pollen as judged from transient assays (Table 6.1).

	Luciferase activity arbitrary units	δGUS activity/hour arbitrary units	δGus activity/hour/luc arbitrary units x 10^5
	238510	384	16.1
LAT52-*gus*	64549	107	16.5
	237290	376	15.8
			Ave. 16.1
			S.D. 0.35
	68670	4	0.58
AoPR1-*gus*	59900	6	1.00
	72931	5	0.68
			Ave. 0.75
			S.D. 0.23
	457850	0	0
pCGUS	494310	0	0
	416500	0	0
			Ave. 0

Table 6.1. Results of transient assays in bombarded mature tobacco pollen.

6.7 Conclusions and summary of the AoPR1-*gus* reporter fusion gene expression data obtained

Initially, the data that arose from the reporter construct analysis in transgenic tobacco seemed confusing when considered in the context of a wound-induced gene. Why should developmental expression be observed in the coloured parts of the petal, pollen, developing seed coats and vascular tissue during xylogenesis? There is no reported data on developmental expression patterns observed for other members of the 'intracellular' PR family as described by Walter *et al.*, 1990 [199] except for the birch pollen allergen which is clearly expressed in birch pollen. It is known that the parsley and bean homologues are expressed in established cultures after the addition of fungal elicitors and in parsley plants there is a localized induction of transcript at sites of attempted fungal invasion (Walter *et al.*, 1990 [199] and Schmelzer *et al.*, 1989 [171]). The relatively high expression of the AoPR1-*gus* fusion in tobacco culture suggests that expression of the AoPR1 gene in culture may be dissimilar to that observed for other members of this PR family. In the case of the parsley and bean homologues, transcript is not strongly expressed in established cultures prior to the addition of fungal elicitors (Walter *et al.*, 1990 [199] and Somssich *et al.*, 1989 [181]).

Reporter expression patterns are similar for the AoPR1 reporter constructs and reporter constructs synthesized using promoters derived from genes encoding enzymes of the phenylpropanoid biosynthesis pathway. For example, histochemical analysis of bean PAL-2 and the parsley 4CL-1 promoter-*gus* fusions in transgenic tobacco revealed developmental expression in coloured parts of the petal, secondary vasculature of the stem, pollen, developing seed coats, vasculature of roots and also showed wound-induction in leaf, stem and root tissue. (Liang *et al.*, 1989 [115], [116], Bevan *et al.*, 1989 [3] and Hauffe *et al.*, 1991 [82]).

Fluorometric data obtained for AoPR1-*gus* fusions also correlate well with GUS activities in tissues from tobacco plants transformed with phenylpropanoid gene promoter-*gus* fusions. AoPR1 promoter driven GUS induction in wounded tissues

can be detected rapidly (within 3 hours) and continues accumulating for a long period of time (up to 5 days as shown in fig. 6.3). This regulation of temporal expression is mirrored in chopped-up asparagus seedlings where AoPR1 transcript is easily detectable from 6 hours to 4 days after wounding with transcript levels reaching a maxima between 1 and 3 days post wounding (fig. 6.9). In the case of phenylpropanoid transcripts induced in wounded plants, for example excised bean hypocotyls, the picture is not so clear as bean PAL and CHS transcripts are detectable rapidly and transiently following wounding (PAL transcript being detectable between 2 and 30 hours, reaching a maxima between 8 and 12 hours post wounding and CHS transcript being detectable between 2 and 30 hours, reaching a maxima between 8 and 20 hours post wounding (Bell *et al.*, 1986, [1] and Mehdy *et al.*, 1987, [130])). In transgenic tobacco, the PAL2 and CHS8 promoter-*gus* fusions drive GUS expression following wounding such that GUS is detectable from 17 hours to 1 day post wounding (Liang *et al.*, 1989 [115] and Doerner *et al.*, 1990 [53]). This difference in timing between the onset of detectable transcript and GUS activity is explainable either: in terms of slightly different temporal regulation of transcription in transgenic plants compared to the native plant; or more probably, it is purely due to the differences associated with measuring steady state levels of transcript or an accumulation of GUS protein with time (GUS is a very stable protein with a half life *in vivo* of about 50 hours (Jefferson *et al.*, 1987 [92])) such that a sufficient quantity of GUS has accumulated to be detectable. In which case comparisons between the phenylpropanoid gene and AoPR1 gene promoters in transgenic tobacco possibly suggest that the AoPR1 promoter is transcriptionally more active immediately following wounding than the phenylpropanoid promoters. Northern analyses comparing PAL and CHS transcript levels, observed in excised bean hypocotyls, between AoPR1 transcript levels, in chopped up asparagus seedlings, also suggest the possibility that the AoPR1 promoter is transcriptionally active for a longer time period post-wounding compared to the bean phenylpropanoid gene promoters.

The induction of GUS by salicylic acid produces a 2-3 fold increase in the level of overall GUS activity compared to wounding. Wounding and simultaneous treatment with salicylic acid produces a greater induction. Whether this is due to a accumu-

lative synergistic effect of the two treatments on the transcriptional activity of the promoter or because salicylic acid enters the leaf tissue through the wound sites more efficiently has not been determined. Salicylic acid mediated induction of reporter expression has also been observed for the tobacco PR-1 promoter-*gus* fusions (Ohshima *et al.*, 1990 [140]). Since the asparagus AoPR1 gene and the tobacco PR-1 gene products are different with respect to their cellular location (tobacco PR-1 is extracellular and AoPR1 is probably intracellular) suggests that salicylic acid may not act by inducing certain families of wound-induced genes, but may have a more central role in inducing many defence-related genes. These observations are supported by those reported in recent literature (Yalpini *et al.*, 1991 [205], Ward *et al.*, 1991 [200] and Bol *et al.*, 1990 [10]).

Analysis of the predicted coding sequence indicated that the AoPR1 gene product was intracellular since the deduced protein lacked a lacked a signal sequence. The fact that the GUS translational fusion product is active suggests that the first 90 or so base pairs of the predicted coding sequence do not code for a signal peptide that is active in transgenic tobacco. This may be inferred because exported GUS fusion protein is glycosylated and inactive (Iturriaga *et al.*, 1989 [89]). This evidence supports the observations that the parsley PR proteins are not post-translationally processed as would be required for protein secretion or transport into the vacuole as is characteristic for classical PR proteins (Somssich *et al.*, 1986 [182] and Mauch and Staelein, 1989 [129]).

Techniques such as PCR were used to qualify the presence of transcript in asparagus pollen when data were obtained showing GUS activity in transgenic tobacco pollen. This approach was able to differentiate between genic and transcript derived PCR products on the basis of size and indicated that pollen and wounded seedlings from asparagus contained similar amounts of transcript and a higher level of AoPR1 transcript in comparison with non-wounded seedlings. The discovery that the AoPR1 gene was active in pollen did not come as a complete surprise since comparisons between the AoPR1 predicted protein sequence and other predicted protein sequences revealed homology to the birch pollen allergen (*BetvI* Brietender *et al.*, 1989 [26]).

Initially, due to lack of transgenic plant material, transient assays were performed by bombarding mature pollen with small plasmids containing reporter constructs. The results allowed relative data to be obtained for GUS reporter activity driven by the LAT52, AoPR1 and CaMV promoters, respectively. These showed that the LAT52 promoter was approximately 21 fold more active than the AoPR1 promoter which more active than the CaMV promoter. When more plant material was obtained the quantity of AoPR1 expression was measured in transgenic pollen. The previously reported GUS activity (Twell *et al.*, 1990 [191]) is 20 - 100 times stronger in transgenic tobacco pollen harbouring LAT52-*gus* constructs than in pollen carrying AoPR1-*gus* constructs (for example, 42,433 and 2428 pmol 4-Mu/min/mg protein respectively for LAT52 and AoPR1 mediated GUS expression for plant line AoPR1-*gus* 1.1 kb Full line 14). The differences observed for the expression levels in pollen from stably transformed tobacco plants are in good agreement with those seen in the transient assay. This suggests that the pollen bombardment experiment provides quantitative results that are consistent with data obtained through plant transformation experiments.

Although the AoPR1-reporter expression patterns are consistent with those observed for the genes involved in the first few steps of the phenylpropanoid pathway (the initial steps of the pathway are shown in fig. 1.1) it is evident that the AoPR1 gene shares no significant sequence homology to these genes. If the AoPR1 gene is a member of the uncloned phenylpropanoid biosynthesis pathway genes that codes for a protein with an enzymatic role that results in the biosynthesis of a molecule further down the phenylpropanoid pathway then one has to consider the branch-points of this pathway and the observed or expected expression patterns of the genes following these branch-points: For example, the bean CHS 8 gene is expressed in developing cotyledons and epidermis, presumably to produce light protectant molecules, around wound sites or elicitor treated site, presumably to produce phytoalexins and in floral tissue and root tips, presumably to make pigments and chemical attractants. Expression is not observed however, in tissue where extensive lignification is occurring such as the secondary xylem of mature stems as the CHS branch of the phenylpropanoid pathway does not produce metabolites with a major role in structural

molecule synthesis (Schmid *et al.*, 1990 [172]). This sort of data does not disagree with the assumption that gene expression is temporally and spatially controlled to be beneficial to the plant and when this is not the case the gene is not expressed. Given that expression of phenylpropanoid pathway genes coding for enzymes active on metabolites derived from 4-coumaraldehyde involved in the production of lignin and suberin are not expressed in the coloured parts of the flower, presumably because they have no beneficial role there, then it seems unlikely that AoPR1 gene is an unknown member of the phenylpropanoid pathway as it shows inappropriate expression. These assumptions have all been made on the basis of observable expression in transgenic tobacco. The possibility that these data are artifactual remains and direct comparative techniques such as *in situ* transcript hybridization or northern analysis may not necessarily provide evidence to base conclusions on regarding aberrant gene expression in transgenic plants. Ideally, in order to determine whether AoPR1 promoter activity is aberrant in tobacco, the gene fusion should be tested in transgenic asparagus. AoPR1-*gus* expression in transgenic asparagus should correlate with AoPR1 transcript abundance, showing the promoter was not driving aberrant expression. Unfortunately, although possible, asparagus transformation is a long and laborious process. Plants also take two years to mature and then there are the problems of sexual reproduction which would require either outcrossing or cross-fertilization in order to produce seed. For these reasons asparagus transformation was not attempted

The observations that the AoPR1 promoter contains sequence boxes that are important in the regulation of phenylpropanoid gene expression was briefly discussed in the concluding part of chapter 5 purely in terms of sequence similarity. The CC-TACC box of Loake *et al.*, 1991 [120] and the ACCTAACC sequence box of Lois *et al.*, 1989 [122] have been implicated as *cis*-acting elements that play roles in the regulation of phenylpropanoid gene expression by phenylpropanoid pathway intermediates and light. Although similar sequences are present in the AoPR1 gene's regulatory sequences these boxes are not present in the parsley PR genes or other genes of this class. If these sequences are important in regulating gene expression similarly to that observed for the phenylpropanoid genes this may explain why there

has been little data published relating to the developmental expression of this class of PR genes. Without experimental evidence obtained through promoter dissection it is possible that the presence of these boxes in the AoPR1 promoter may be coincidental and have no regulatory function in gene expression. However, as noted, there is a similarity between the expression patterns observed for the AoPR1-*gus* fusion gene and phenylpropanoid gene promoter-*gus* fusion genes in transgenic tobacco and these boxes may be candidates for future mutation analysis.

If the AoPR1 gene is expressed similarly to the first few genes of the phenylpropanoid pathway, but is not one of these genes, then what is its role? There are two possible answers to this question. Firstly, the AoPR1 gene product role is not part of the phenylpropanoid pathway and is simply expressed in a similar way to these genes Secondly, the gene product has a role in this pathway and that is why it is expressed similarly to PAL or 4CL. If AoPR1 has no role in the actual production of the metabolites of the pathway then the question of whether the AoPR1 protein has a regulatory role on one or more of the steps of the phenylpropanoid biosynthesis pathway arises. Regulation of gene expression can occur at the transcriptional, translational and post-translational stages. Transcriptional regulation refers to the chromatin structure, DNA methylation states and the efficiency of RNA polymerase to produce transcript which is generally governed by nuclear *trans*-acting factors that bind regulatory elements within a gene and interact through protein-protein interactions with RNA polymerase II to cause transcription of the gene to start at a specified point along the gene. Transcript stability in turn is governed by factors such as efficient processing of the 5'cap, the addition of poly(A) tails and removal of intervening sequences from the hnRNA. Translation rates may be altered by factors such as the absence or presence of translation factors, the secondary structure of the mRNA or the abundance of free ribosomes. Following the synthesis of the protein chain post-translational modifications such as proteolytic cleavage, phosphorylation, glycosylation or association with coenzymes, prosthetic groups or protein subunits may be required to generate an active protein. If the protein is extracellular or resides in the organelle, correct trafficking of the protein must be achieved to obtain appropriate expression. In times of physiological stress the protein may be modified

further, for example, by the association with a chaperone to ensure the correct protein structure and function is obtained (reviewed by Ellis, 1991 [63]).

The AoPR1 sequence and high expression levels provides some evidence that the AoPR1 gene does not encode a *trans*-acting factor. The abundance of message represented in the asparagus library is many fold higher than that expected for a *trans*-acting factor and the sequence of the cAoPR1 clone does not predict helix-turn-helix (Ludwig and Wessler, 1990 [123]), zinc finger (Lam *et al.*, 1990 [110]) or leucine-zipper (Hartings *et al.*, 1989 [80], Katagiri *et al.*, 1989 [98], and Singh *et al.*, 1990 [180]) DNA binding domains that are characteristic of DNA-binding proteins. The question of post-transcriptional and post-translational phenylpropanoid gene regulatory roles for the AoPR1 gene remain open.

If AoPR1 has a regulatory role in the phenylpropanoid biosynthesis pathway it would seem prossible that it may act on the expression of PAL as this is the first enzyme of the pathway and the expression of the AoPR1 gene is more analogous to the expression of a gene coding for an enzyme in the initial steps of the phenylpropanoid pathway rather than the latter steps (as discussed earlier this chapter). The observable patterns of expression do not provide data contrary to this speculation but the possibility of other points of action may not be ruled out.

Chapter 7

General conclusions, on-going and proposed future work

7.1 Summary of the work in this thesis

The ultimate aim of the work was to isolate and study a monocotyledon wound-induced gene. A target clone named DD1-34 had been isolated from a library constructed using poly (A^+) RNA isolated from a mechanically isolated asparagus cell suspension culture (Fioroni, 1989 [69]). Preliminary work on this clone suggested that it was derived from transcript that was wound-inducible. Genomic clones corresponding to the DD1-34 clone were isolated from an asparagus genomic library using the DD1-34 insert as a probe. The analysis of the genomic clones and DD1-34 showed that sequences that were almost identical to the complete DD1-34 clone were present in each genomic clone. Regions flanking the conserved sequences within the genomic clones displayed no similarity to each other and did not cross hybridize. Experiments using probes derived from separate parts of the DD1-34 clone demonstrated that the DD1-34 clone was probably not derived from a single transcript. The origin of this clone remains uncertain as comparisons with the genomic clones suggest direct identity of the DD1-34 clone which contains no full open reading frames.

Following the synthesis of a new cDNA library, cAoPR1 was obtained using a fragment of the DD1-34 clone as a probe. DNA sequence and northern analysis suggested that this near full length clone was derived from messenger RNA. The precise relationship between DD1-34, the genomic clones and cAoPR1 was never established but genomic Southern blots suggested that the genomic sequences encoding the DD1-34 clone and the AoPR1 gene were not closely linked on the asparagus genome.

RNA hybridizations demonstrated that AoPR1 transcript was up-regulated in a non-systemic manner following slicing of asparagus seedlings. The temporal abundance of detectable transcript in the chopped seedling sections compares with that observed in the asparagus cell suspensions suggesting that the asparagus cultures is an enriched source of wound-inducible transcript.

Sequence data base searches performed with the predicted protein sequence of the cAoPR1 clone showed that there were a number of homologous predicted proteins present in other plant species. Homologues from parsley and bean were isolated from cell suspension cultures treated with fungal elicitors (Somssich *et al.*, 1989 [181] and Walter *et al.*, 1990 [199]). A homologue in potato was identified following treatment of tuber slices with arachidonic acid or infection with fungus (Matton and Brisson, 1989 [128]). A pea disease resistance protein (Fristensky *et al.*, 1988 [70]) and a birch pollen allergen (Breitender *et al.*, 1989 [26]) are also homologous to the asparagus predicted protein. Transcript encoding the birch pollen allergen has also been shown to be up-regulated following wounding (A. Bito; Personal communication). Despite the name these clones are not homologous to the 'classical' tobacco PR1 proteins. There is no sequence homology and there is evidence that the parsley related family of PR proteins are intracellular whilst the tobacco PR1 protein is extracellular (Somssich *et al.*, 1989 [181]).

Initial attempts to obtain upstream regions containing the regulatory elements of the corresponding gene from a genomic library failed and led instead to the isolation of a number of clones that contained regions that were identical to the DD1-34 clone. These genomic clones did not contain sequences that would hybridize to the 5' coding region of the cAoPR1 clone. Inverse PCR allowed the amplification

of genomic sequences from the asparagus genome that hybridized to the cAoPR1 sequence. *Hind* III digestion of asparagus DNA led to the isolation of a 672 bp fragment. Sequence analysis of this fragment showed that it contained sequence that was highly homologous to the cAoPR1 clone interrupted by 347 bp of intervening sequence which contains donor and acceptor site consensus sequences (Breathnach and Chambon, 1981 [25]) and an AT rich region which have been shown to be important for correct intron removal (Goodall and Fillipowicz, 1989 [72]). Nuclease-S1 or R-loop mapping could be used to confirm this intervening sequence is an intron. The other amplified sequence obtained from inverse PCR was derived from *Eco* RI digested asparagus DNA. Sequence analysis suggested that this sequence contained the upstream regulatory region of the AoPR1 gene. Nuclease-S1 mapping was used to identify the putative transcriptional start site. Genomic Southern blot analysis using a probe derived from the upstream region of the PCR fragment suggested that hybridizing genomic sequence was present within the asparagus as one or two copies. Screening genomic libraries with this sequence was unsuccessful.

The IPCR putative promoter was tested using *gus* translational reporter constructs in transgenic tobacco. A simple deletion that contained only 309 bp and 982 bp (the full up-stream sequence) of sequence upstream from the predicted transcriptional start site both showed expression of the reporter in transgenic tobacco. A number of plants harbouring each construct were analysed. The analysis showed that there is developmentally regulated expression associated with the AoPR1 gene as well as a possible defence role. Tobacco plants harbouring the full up-stream sequences (982 bp) demonstrated wound-inducible expression in tissue immediately surrounding the wound site and 2 to 3 mm from the wound boundary. Developmental expression was observed in the vascular tissue where secondary thickening occurred. In floral tissues, expression was observed in the developing ovary, receptive stigma surface, the coloured regions of the petals and in mature pollen. Post fertilization, very strong expression is observed in the testa of developing seeds. The plants harbouring the 5' deleted promoter region did not demonstrate detectable wound inducible expression of the reporter, but there was detectable expression in the vascular tissue, mature pollen, developing seeds and in the coloured parts of the flower. The nature of the

fusions and the fact that active GUS expression was observed provided evidence that the AoPR1 protein is intracellular. This data was supported by analysis of the predicted protein sequence which did not appear to contain any signal consensus sequence (von Heijne, 1985 [198]).

Transient expression analysis of particle bombarded tobacco pollen demonstrated that the AoPR1 promoter was able to drive the expression of the *gus* reporter in pollen. The ratio of *gus* reporter gene expression driven by the LAT56 and AoPR1 promoters was 20:1. PCR amplification allowed the detection of transcript derived sequence from asparagus pollen and the detection of genomic homologous sequence in another plant belonging to the Liliaceae.

7.2 Proposals for future work

There are many possible avenues of gene expression to explore but firstly consolidation and completion of existing work should be carried out. For example the IPCR fragment gave rise to sequence that probably contains errors. These errors could be resolved by either directly sequencing a PCR product or re-cloning the fragment from several PCR reactions and sequencing several clones until an indisputable consensus can be reached. The same constructs could be used to transform asparagus to show similar expression patterns as seen in tobacco. Although transgenic asparagus has been generated (Bytebier *et al.*, 1987 [29]) this was not done as the transformation, regeneration and the maturation time for asparagus would have taken a long time.

7.2.1 Towards a biochemical function of AoPR1

The major question as to the biochemical function of the AoPR1 gene product remains to be answered. Possible clues were gained from the reporter analysis. Why does the AoPR1 promoter drive expression in a similar way to promoters isolated from phenylpropanoid genes encoding the enzymes in the higher part of the pathway such as PAL2 and 4CL1? Sequence comparisons show that the AoPR1 gene is not one of these enzymes, so the following possibilities exist. The first is that the AoPR1

has nothing to do with the phenylpropanoid biosynthesis pathway and just happens to be regulated in a similar way to PAL and 4CL1. The second is that AoPR1 encodes a protein involved in this pathway that has not yet been identified and that has a role in several developmental processes as well as defence similarly to the first enzymes found in the pathway. The third explanation is that the AoPR1 gene product has a role in the phenylpropanoid biosynthesis pathway, but one of regulation rather than an enzymatic role. If this is the case then questions as to the regulation of the other members of this PR gene family are raised. There are no published data on promoter-reporter analysis in whole transgenic plants for the bean, parsley or other PR gene homologues similar to the AoPR1 gene, so it is unclear whether the expression of AoPR1 gene is significantly different to the expression of the other genes in this family. The fact that a birch pollen allergen is a member of this family suggests that the regulation of these genes is probably not restricted to a defence role but may also be important in aspects of development.

Studies on PAL gene expression have provided some very interesting data such as the role of *trans*-cinnamic acid on bean PAL expression. The synthesis of *trans*-cinnamic acid from L-phenylalanine is catalysed by PAL. Cinnamate suppresses elicitor inducible PAL activity and reduces the steady state level of PAL transcript (Bolwell *et al.*, 1988 and 1986 [14,13] and Billet and Smith, 1980 [5]). PAL is a tetrameric enzyme with two of the units possessing dihydroalanine which is involved in the catalysis at the active site (Hanson and Havir, 1970 [77]). There is an associated reduction of the dihydroalanine residue following treatment with cinnamate and an associated increase in a non dialysable PAL inactivating factor (Bolwell *et al.*, 1986 [13]). Tunicamycin treated potato tuber disks produce PAL that is reduced in catalytic efficiency but has unaffected substrate affinity suggesting that N-linked glycosylation is necessary for correct tertiary structure to carry out efficient catalysis (Shaw *et al.*, 1990 [177]). 2-D gel analysis of *in vitro* translated polysomal RNA showed that 1 mM cinnamate causes the *de novo* induction of at least nine different polypeptides ranging in size from 16 to 100 kDa (Bolwell *et al.*, 1988 [14]). Earlier work identified a macromolecular inhibitor of PAL in gherkin (*Cucumis sativum*) of approximately 19 kDa (Billet *et al.*, 1978 [6]) and a proteinaceous, cinnamate

inducible PAL inhibitor has been identified in bean (Bolwell *et al.*, 1988 [14]).

If AoPR1 has a regulatory role in the phenylpropanoid biosynthesis pathway it would seem probable that it may act on the expression of PAL as this is the first enzyme of the pathway. The observable patterns of expression do not provide data contrary to this speculation but the possibility of other functions may not be ruled out.

An approach to provide more evidence to the last of these theories would be to over-express the AoPR1 protein in tobacco using a construct incorporating a strong constitutive promoter. This work would rely on the creation of phenotypic mutants coupled with detectable over expression of the AoPR1 protein. The over expression of AoPR1 transcript could be detected by northern analysis. Antibodies to the AoPR1 protein would be required to obtain data relating to the expression of the AoPR1 protein. A possible way of generating these antibodies may be through the over-expression of a fusion protein using a bacterial expression vector in *E.coli* and immunizing a rabbit with purified fusion protein. The serum could then be used in western blotting experiments, immunohistochemical location or other experiments that lead to the determination of the proteins molecular characteristics in both asparagus and transgenic plants over expressing the protein. The type of mutant obtained may be similar to that observed by Elkind *et al.*, 1990, [60]. Tobacco over expressing a bean PAL 2 gene produced fluorescent lesions in the leaf, white petals and poor lignin biosynthesis in the stem and reduced pollen viability. The author argued that an accumulation of cinnamic acid resultant from the over expression of PAL may repress the endogenous phenylpropanoid pathway. This hypothesis is supported by the observation that cinnamate represses transcription of CHS-*gus* fusions by Loake *et al.*, 1991 [120]. This theory is speculative and it could be argued that this type of phenotype could not be easily explained, as a regulator of the phenylpropanoid pathway may act in a positive, or a negative way at different points in the pathway and that this in turn will be mediated by the phenylpropanoid pathway intermediates regulatory roles.

The observation that AoPR1 homologues have been obtained from several species including potato suggest that 'intracellular' PR proteins are ubiquitous in plants.

Bearing in mind that potato and tobacco are both members of the *Solanacae* the possibility of cloning a tobacco homologue using PCR primers that are designed on the basis of the known potato sequence exists. If a tobacco sequence homologous to the AoPR1 sequence was cloned then this may be used in anti-sense constructs that may reduce endogenous expression of the tobacco 'intracellular' PR1 gene resulting in an altered phenotype. Possibly the asparagus antibody may cross react with the tobacco homologue and be used to confirm that lower 'intracellular' PR1 protein levels might be associated with any altered phenotype. If the theory that AoPR1 is a protein associated with the regulation of phenylpropanoid metabolism is correct, then any altered phenotype may be correlated with the aberrant expression of the AoPR1 protein that results from changed activities of phenylpropanoid biosynthesis pathway enzymes as previously discussed.

Another approach would be to purify AoPR1 protein from asparagus or from a fusion construct expressed in bacteria (assuming that there are no major post-translational modifications required for activity) and try *in vitro* experiments on purified PAL, CA4H, 4CL *etc.* to test if changes in the enzyme activity could be observed following the addition of the partially purified AoPR1 protein

7.2.2 Analysis of the AoPR1 promoter and associated factors

Data obtained using the promoter-reporter fusions has hinted that there may be more than one *cis*-acting sequence box involved in the regulation of transcription in response to developmental or environmental stimuli. In comparison, for example, to the CHS promoter which is also transcriptionally active following wounding there have been several sequence boxes that have been shown to be important in transcriptional regulation following environmental and developmental cues for example light regulation and regulation by intermediates of the phenylpropanoid pathway (Lois *et al.*, 1989 [122] and Loake *et al.*, 1991 [120]). More data may be obtained by further analysis of the promoter. Current approaches to this goal include analysis of sets of 5' deleted promoter-reporter gene fusions either transiently, by directly introducing the constructs into amenable systems (such as protoplasts) or by the

stable transformation of plants. In many cases it is important to support any data from transient assays with experiments in stable plant transformants. This also allows data on the expression of the gene in complex organs to be collated. The 5' deletion analysis may be complemented by 3' deletion analysis. This technique relies on fusing upstream regions of the promoter which have their proximal regions missing to minimal sequences that can drive transcription. The -40 bp CaMV 35S promoter is such a candidate. This analysis complements the 5' deletion analysis since rather than looking for 'loss of activity' with the 3' deletion fusions the 'gain of activity' is sought. Taking this analysis to an extreme could be achieved by linker scanning type experiments were a small section of sequence is mutated but all other sequence surrounding the mutagenised region remains as cloned. This was previously achieved by matching 5' deletions with 3' deletions and joining these deletions with a synthetic linker such that only the base pairs that were in the position where the linker occupied are removed from the cloned sequence. Using PCR for site directed mutagenesis has provided an alternative to this laborious method.

Before trying such a time consuming technique other methods are available that may enable sensible targeting of sequence boxes important in gene regulation. *In vitro* and *in vivo* footprinting allows the detection of DNA sequence that binds to proteins. *In vivo* footprinting offers advantages that the results obtained are less likely to be artifactual in comparison to *in vitro* footprinting since the modification of the DNA is carried out with intact cells and with whatever nuclear factors that are bound to the DNA present. The disadvantage is that a large number of homogeneous cells are required to give a repeatable and detectable footprint. *In vitro* footprinting may provide results that are more easily obtainable as end labelled cloned DNA fragments are used in incubations with nuclear extracts. Similar *in vitro* experiments include gel retardation assays that allow regions of the promoter fragment that bind to proteins to be determined on the basis of increased observable molecular mass of a fragment that has bound to a protein, in comparison to the mass of a fragment that is not associated with a DNA binding protein. The *in vitro* experiments also allow determination of binding constants. Once footprinted regions are located a short DNA sequence that has the capacity to bind to proteins in the nucleus has been

defined.

Current research has often been aimed at the identification of these DNA binding proteins and cloning cDNAs and genes complementary to these proteins. Another line of interesting research is the construction of chimeric promoters based on the use of promoter 'building blocks' which consist of monomers or concatomers of sequence that has been shown to regulate transcription following certain stimuli, fused to sequence that allows transcription. In this way promoters with new functional properties may be obtained by using different arrangements of the 'building blocks'.

If the route to isolating genes encoding *trans*-acting factors is successful, experiments could be performed relating to the regulation of the trans-acting expression, perturbing the normal levels of expression of the factor may produce inappropriate regulation of a cascade of genes resulting in altered phenotypes. Other experiments investigating the role of post-transcriptional and post-translation modifications of the factor on its ability to bind to the target DNA sequences may allow partial elucidation of the signal transduction pathways involved in the response to be characterised. This may shed light on the more detailed questions of the regulation of gene expression.

7.3 Concluding remarks on the AoPR1 gene and possible applications

This work has concentrated on the initial cloning and characterization of both the coding and regulatory sequences of a novel monocotyledon gene. This is the first monocotyledon gene belonging to a new class of PR proteins that are probably intracellular. The function of this gene is unknown. Although there were initial concerns as to the transcriptional functionality of the AoPR1 promoter in a dicotyledon transgenic plant (as discussed in chapter 6), *gus* reporter data showed that the AoPR1-*gus* transgene was driving transcription in a temporally and spatially conserved manner in a foreign genetic background, as evidenced by efficient wound-inducible and developmentally regulated *gus* expression.

Insufficient data have been published to draw conclusions regarding differences in gene regulation between the asparagus gene and other members of the PR family. Therefore, it is not clear if the monocotyledon gene is significantly different from its dicotyledon homologues in terms of temporal and spatial expression around the plant.

There are differences in structure between the monocotyledons and dicotyledons which may be interpreted as resulting from differences in gene regulation or types of gene expressed within the organ that affect its biochemistry and consequently development. This may also be true of responses to environmental cues such as wounding where different genes may be up or down regulated at different times depending on whether they are in a monocotyledon or a dicotyledon. AoPR1-*gus* reporter data from transgenic tobacco may provide evidence supporting the hypothesis that there is a difference in gene regulation between monocotyledons and dicotyledons. This argument is based on the observation that the AoPR1-*gus* fusion gene is expressed similarly to genes of the phenylpropanoid biosynthesis pathway in transgenic tobacco and lignin biosynthesis is a product of the phenylpropanoid pathway. There is little lignin synthesis in the vascular bundles in the stele of asparagus but a great deal of lignin in the vascular cylinder of a tobacco stem. Consequently, it was difficult to detect AoPR1 transcript in asparagus steles, probably due to its low abundance, but GUS activity was high in the xylem parenchyma of transgenic tobacco stem. This suggests that the transgene is appropriately but differently expressed in both the monocotyledon and the dicotyledon in response to the appropriate developmental cues. Conversely, the other explanation is that the transgene is aberrantly expressed in the transgenic plant and is not responding correctly to the developmental cues either because of promoter recognition problems by host *trans*-acting factors or through a lack of *cis*-acting sequences.

Whatever the conclusions are regarding the expression of monocotyledon derived transgenes in dicotyledon hosts it would seem probable that a monocotyledon derived promoter that is responsive to wound stimulus may be commercially useful in the future and may be more so given some of the legal constraints enforced by patents

that may soon prevent unrestricted use of promoters such as the CaMV 35S. The observation made in chapter 6 that the AoPR1 promoter is highly active in culture systems implies that the promoter may be useful to drive the expression of genes in culture systems; for example, for the expression of selectable markers during transformation or for the production of plant cell products in large batch fermentors. Preliminary work to this end has been undertaken by Sebohattin Ozcan of Leicester University where AoPR1 promoter has been used to drive the NPT II selectable marker working towards an improved regeneration process in the development of transgenic plants that are generally recalcitrant to regeneration, such as pea. These transformed plants may have the added advantage of not expressing the selectable marker in a constitutive manner such as the CaMV 35S promoter. It could also be envisaged that the promoter would be useful to drive the expression of anti-microbial compounds at wound sites where most pathogens invade healthy plants thus offering protection to the plant. Potato plants transformed with the AoPR1-*gus* construct express *gus* similarly to transgenic tobacco plants following mechanical wounding (Joy Wikinson, Leicester University) and these transgenic potato plants are soon to be tested for GUS activity following viral, fungal and bacterial attack. It could be expected that the AoPR1-*gus* gene may be induced following pathogen invasion as the parsley PR homologue is induced around sites of attempted fungal invasion (Schmelzer *et al.*, 1989 [171]). The observation that salicylic acid induces AoPR1-*gus* expression in transgenic tobacco is also of interest and this may be exploited as the ability to control or increase the expression of any gene product by spraying with salicylic acid. Preliminary data suggest that the AoPR1 promoter induces transcription of the *gus* gene following the attack of transgenic tobacco by the non-specific fungal pathogen *Botritus cinnerea* (Karl Deacon-unpublished data). If the AoPR1 promoter is responsive to pathogenic attack as well as salicylic acid then this suggests the transgene may be expressed at a distance or systemically following wounding or pathogen invasion (this could be tested by wounding one leaf and measuring GUS activity in another leaf). As more is understood about the AoPR1 gene it is possible that more commercial applications may come to light.

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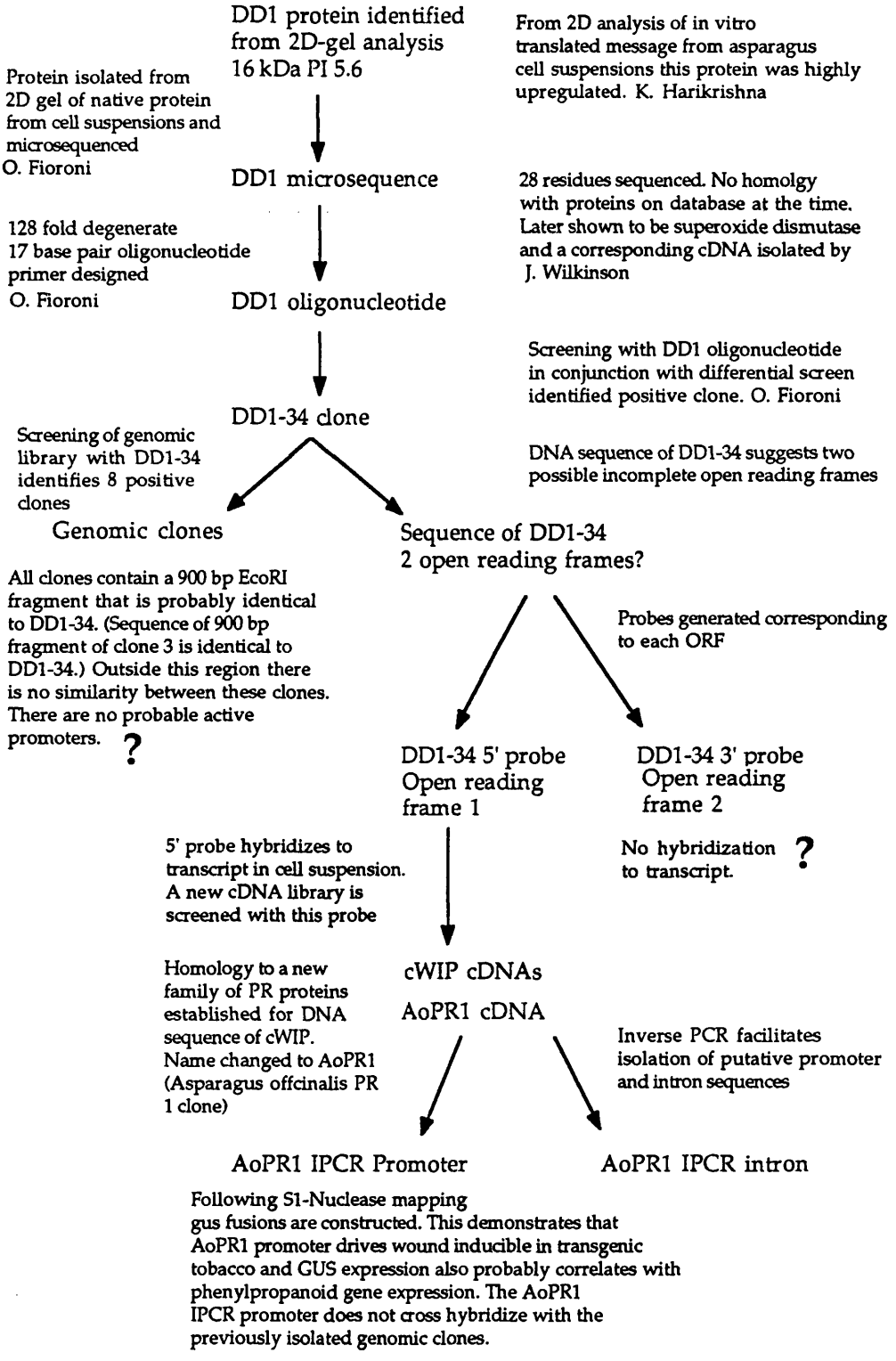
Appendix I. The derivation of clones described in this thesis.



Appendix II. Identity between DD1-34 and the 900 bp Eco RI fragment from genomic clone 3

```
       Gap Weight:  5.000       Average Match:  1.000
    Length Weight:  0.300    Average Mismatch: -0.900

           Quality:  943.0              Length:    943
             Ratio:  1.000                Gaps:      0
Percent Similarity: 100.000   Percent Identity: 100.000

DD1-34.1 x Clone 3 900 bp

                .         .         .         .         .
      1 GAATTCCAAGATGTTTGAATGTGCCACCACTCACTTCAAGTTCGAGCCCT 50
        ||||||||||||||||||||||||||||||||||||||||||||||||||
      1 GAATTCCAAGATGTTTGAATGTGCCACCACTCACTTCAAGTTCGAGCCCT 50
                .         .         .         .         .
     51 CGAGCAACGGTGGATGCCTCGTCAAGGTGACTGCATCCTACAAGATTCTC 100
        ||||||||||||||||||||||||||||||||||||||||||||||||||
     51 CGAGCAACGGTGGATGCCTCGTCAAGGTGACTGCATCCTACAAGATTCTC 100
                .         .         .         .         .
    101 CCAGGTGTCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACCCACCA 150
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    101 CCAGGTGTCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACCCACCA 150
                .         .         .         .         .
    151 CATCAAGGCAGCTGAAGCTTACCTCCTCGCTAACCCAACTGCCTACGCTT 200
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    151 CATCAAGGCAGCTGAAGCTTACCTCCTCGCTAACCCAACTGCCTACGCTT 200
                .         .         .         .         .
    201 AAGTATATGCTTAGGTTTCTAATTTGGTGTTGTGAGTTCGAATAAAGAGA 250
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    201 AAGTATATGCTTAGGTTTCTAATTTGGTGTTGTGAGTTCGAATAAAGAGA 250
                .         .         .         .         .
    251 GTGCTCCTGATGGTGATGCCTATGCATGGTTATACTTTTTGTTGCCTGAG 300
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    251 GTGCTCCTGATGGTGATGCCTATGCATGGTTATACTTTTTGTTGCCTGAG 300
                .         .         .         .         .
    301 TATTAAAAGTTTGTAGTCGTGTGTCGTGAAGTAATATTGTTGGTGGTGTG 350
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    301 TATTAAAAGTTTGTAGTCGTGTGTCGTGAAGTAATATTGTTGGTGGTGTG 350
                .         .         .         .         .
    351 CGTCTCGTCGTATGCGGAGATCGCGTGCTGGATGTTCACCGGAGAGAGGC 400
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    351 CGTCTCGTCGTATGCGGAGATCGCGTGCTGGATGTTCACCGGAGAGAGGC 400
                .         .         .         .         .
    401 AGGTTAGCGCGCTGCGGCGTCGGTATTTGGAGGCGGTGCTGAAGCAGGAC 450
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    401 AGGTTAGCGCGCTGCGGCGTCGGTATTTGGAGGCGGTGCTGAAGCAGGAC 450
                .         .         .         .         .
    451 GTCGGATTTTTCGACACCGATGCTCGGACCGGCGACATCGTCTTCAGTGT 500
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    451 GTCGGATTTTTCGACACCGATGCTCGGACCGGCGACATCGTCTTCAGTGT 500
                .         .         .         .         .
    501 TTCCACCGATACGCTGCTCGTACAGGACGCCATTAGTGAGAAGGTGGGCA 550
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    501 TTCCACCGATACGCTGCTCGTACAGGACGCCATTAGTGAGAAGGTGGGCA 550
                .         .         .         .         .
    551 ATTTCATTCACTACCTATCGACGTTCTTGGCGGGGCTTGTGGTGGGCTTC 600
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    551 ATTTCATTCACTACCTATCGACGTTCTTGGCGGGGCTTGTGGTGGGCTTC 600
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601 ATATCGGCATGAAACTGGCGCTTCTCAGTGTTGCGGTGATTCCCGGGATT 650
    ||||||||||||||||||||||||||||||||||||||||||||||||||
601 ATATCGGCATGAAACTGGCGCTTCTCAGTGTTGCGGTGATTCCCGGGATT 650

651 GCATTTGCTGGAGGTCTTTATGCTTATACTCTCACCGGGCTCACCTCAAA 700
    ||||||||||||||||||||||||||||||||||||||||||||||||||
651 GCATTTGCTGGAGGTCTTTATGCTTATACTCTCACCGGGCTCACCTCAAA 700

701 GAGCAGGAGTCCTATGCCAATGCTGGCATTGTAGCGGTTCAGGCAATTGC 750
    ||||||||||||||||||||||||||||||||||||||||||||||||||
701 GAGCAGGAGTCCTATGCCAATGCTGGCATTGTAGCGGTTCAGGCAATTGC 750

751 ACAAGTTCGTACAGAATATTCATTTGTGGGGGAGAGCAAAGCACTCAATG 800
    ||||||||||||||||||||||||||||||||||||||||||||||||||
751 ACAAGTTCGTACAGAATATTCATTTGTGGGGGAGAGCAAAGCACTCAATG 800

801 CCTACTCTGAAGCGATTCAAAATACATTGAAGCTTGGATACAAGGCTGGG 850
    ||||||||||||||||||||||||||||||||||||||||||||||||||
801 CCTACTCTGAAGCGATTCAAAATACATTGAAGCTTGGATACAAGGCTGGG 850

851 ATGGCTAAAGGTCTGGGCATTGGGTGCAGTTATGGAATTGCATGCATGTC 900
    ||||||||||||||||||||||||||||||||||||||||||||||||||
851 ATGGCTAAAGGTCTGGGCATTGGGTGCAGTTATGGAATTGCATGCATGTC 900

901 ATGGGCTTTGGTTTTCTGGTATGCCGGTGTATTCATGGAATTC 943
    |||||||||||||||||||||||||||||||||||||||||||
901 ATGGGCTTTGGTTTTCTGGTATGCCGGTGTATTCATGGAATTC 943
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haracterisation of a wound-induced transcript from the monocot sparagus that shares similarity with a class of intracellular athogenesis-related (PR) proteins

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y words: asparagus, mechanical cell separation, PR proteins, wound-induced gene expression

stract

e report the isolation and characterisation of a wound-induced cDNA designated AoPR1 from a spension of mesophyll cells that had been mechanically isolated from cladodes of light-grown *Asparagus ficinalis* seedlings by grinding in a mortar and pestle. The transcript abundance is up-regulated following ll separation and in chopped mesocotyl tissue from dark-grown seedlings. The expression of AoPR1 as shown by northern analysis to be located around the site of damage. Sequence analysis revealed milarity between the predicted AoPR1 polypeptide and bean PvPR1 and PvPR2 proteins, the potato STH2 protein, the pea PI49 protein, the parsley PcPR1-1 protein and a major pollen allergen from birch *Betv1*). These transcripts have been shown to be induced in response to microbial attack or fungal icitation. To our knowledge, this is the first example of a monocot cDNA belonging to this class of tracellular pathogenesis-related proteins (IPRs).

troduction

mongst some of the well characterised defence-lated proteins are the tobacco pathogenesis-lated (PR) proteins. Following infection of to-cco leaves by tobacco mosaic virus (TMV) PR oteins were shown to accumulate rapidly in the tracellular leaf spaces, with the most abundant ass being the small acidic PR1 group of proteins ,3, 6, 9, 26]. Basic isoforms of the PR1 proteins th a molecular mass of around 16 kDa have ce been identified that are targeted into the central vacuole in TMV-infected plants [for review see 1]. PR proteins also accumulate after treatment with fungal and bacterial pathogens, chemical elicitors (such as salicylic acid) and upon mechanical wounding [1]. A functional role has been assigned to some members of the classical PR protein groups, such as the β-1,3-glucanase [16] and the chitinase activity [17]; however, the PR1 subclass of protein-encoding genes, which have been studied intensively at the nucleic acid level, still have an unknown function in the defence response.

e nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under e accession number X62103 (AoPR1 cDNA).

The tobacco acidic PR1 subgroup of PR proins generally have a molecular mass range between 15.5 and 15.8 kDa [1, 6]. These polypepdes are all synthesised as precursor proteins with N-terminal extension of 30 amino acids, which cleaved during import into the endomembrane stem prior to secretion into extracellular spaces. haracterisation of fungal elicitor-induced proins and transcripts from cultured parsley cells entified proteins that were also acidic and nged in mass from 17.5 to 19.2 kDa [24]. These olypeptides were named parsley PR1 proteins, t sequence analysis of cDNAs coding for these oteins subsequently revealed no similarity beeen tobacco PR1 genes and three genes coding r parsley PR1-like proteins [25]. Evidence has so been presented suggesting that the parsley R1-like' proteins were not extracellular [25]. ecently, cDNAs homologous to the parsley PR1 DNA have been cloned from potato [18], funl elicitor-treated bean cell culture [27], birch llen [2] and pea [9]. As in the parsley clones, all cases there was no indication that the prected protein product of any of these similar nes contained and N-terminal extension charteristic of a signal sequence. It has been sugsted that these cDNAs represent a new catery of pathogenesis-related genes and hence the signation 'PR1' is perhaps misleading [27]. Alough there is no definitive proof, it is very likely at the proteins encoded by this new class of PR nes are intracellular [24, 25] and therefore, for e sake of clarity, perhaps it will be appropriate refer to them as intracellular PR proteins PRs).

In previous work we have shown that aspagus mesophyll cells mechanically separated grinding in a mortar and pestle are potentially rich source of wound-induced transcripts 3, 19]. Several wound-induced cDNAs were olated by differential screening of a cDNA lirary derived from message purified from meanically separated *Asparagus officinalis* cells. n sequencing, the predicted amino acid seence of one of these clones was found to be milar to the IPR group of proteins recognised eviously [27]. To our knowledge, this represents the first reported cloning of a monocot cDNA of this class.

Materials and methods

Plant growth and cell isolation

Asparagus officinalis (cv. Connover's Colossal) seed was purchased from Nickerson Seeds Ltd. Six-week-old greenhouse-grown plants were used for cell isolation by grinding in a mortar and pestle as described previously [19]. These freshly isolated, 'mechanically separated' cells were then incubated in a liquid nutrient as described previously [13, 19] and RNA isolated for cDNA library construction after 1, 2 and 3 days. For studies on the wound response in intact asparagus tissue, seeds were sown on sterile vermiculite and then grown in the dark at 26 °C. Etiolated seedlings were then harvested 2 weeks after gemination, and mesocotyls were then cut into various lengths and incubated on damp filter paper in Petri dishes at 26 °C in the dark until RNA isolation after 1, 2, or 3 days.

Construction and differential screening of cDNA library

Total RNA and poly $(A)^+$ RNA from cells and wounded seedlings were isolated as described previously [8, 13]. 2 μg of pooled poly$(A)^+$ RNA from cells 1–3 days after mechanical isolation (grinding) from asparagus seedling tissue were used to construct a λZAP II library. cDNA synthesis was carried out by a modification of the Gubler and Hoffman [12] method using a cDNA synthesis kit from Pharmacia. The cDNA was ligated into 'lambda ZAP' (Stratagene) and the phage packaged using Amersham's packaging extract. The resulting library contained 1.2×10^6 pfu. Ten thousand plaques were plated out at low density (2000 plaques per 15 cm plate) and duplicate filters lifted from the plates. One set of filters was probed with ^{32}P-labelled first-strand cDNA synthesised from the same mRNA used to

onstruct the library and the other set probed ith ^{32}P-labelled first-strand cDNA synthesised om unwounded seedlings. The labelling, and re-oval of unincorporated nucleotides, was carried ut as described [22]. Hybridisations were car-ed out essentially as described [22] in 6 × SSPE, enhardt's solution, 0.5% SDS and 100 μg/ml enatured salmon sperm DNA at 65 °C over-ght and the filters washed finally in 0.2 × SSC, 1% SDS at 65 °C. Autoradiography was car-ed out overnight using Amersham β-max 'Hy-rfilm'. Differentially hybridising plaques were cked and repurified by another round of differ-ntial screening. The clones of interest were sub-oned (according to Stratagene's protocol) by e *in vivo* excision of the Bluescript pSK(-) vec-r that follows super-infection of the λZAP-fected host cells with VCS-M13 helper phage.

NA gel blots

orthern blots were carried out essentially as de-ribed [8]. Total RNA was run on formaldehyde NA-denaturing gels. The gels were stained in hidium bromide and blotted. Pre-hybridisation d hybridisation were carried in the same hy-idisation solution as for the plaque lifts except e solution contained 50% deionised formamide. he probe was labelled by the oligo-labelling ethod as described [8].

NA sequencing

he dideoxy chain-termination method [21] was ed to sequence DNA from the recombinant K(-) clone with the Sequenase Version 2.0 kit SB). Single-stranded template was prepared phagemid rescue or by denaturing plasmid NA as recommended by the USB Sequenase ooklet. Computer-aided sequence analysis [5] d sequence database searches were carried out ing the University of Wisconsin-released pro-ams on a VAX. Protein sequence alignment as achieved using the CLUSTAL program on IBM personal computer [14].

Results

Isolation of the AoPR1 clone

cDNAs from a λZAP that differentially hybridised to a cDNA population from mechanically isolated asparagus cells versus a cDNA population from unwounded seedling cells were isolated, purified and used in northern dot blot analysis. Of several clones studied, clone 34 (200 bp in length) differentially detected large amounts of steady-state transcript in the RNA population from mechanically isolated cells compared to the RNA population from unwounded seedlings. This clone was then used to rescreen the cDNA library. From the 10000 plaques screened, there were 170

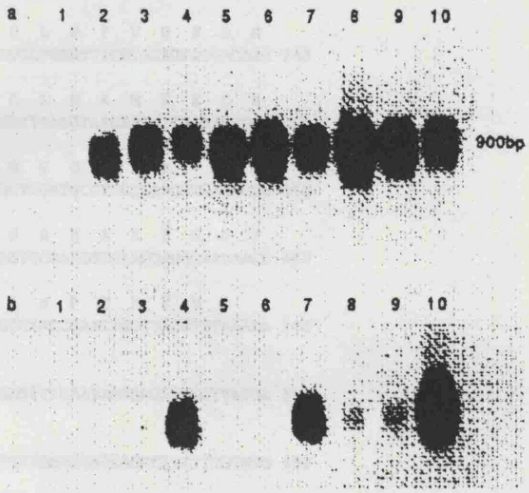

Fig. 1. Northern analysis of transcript following cell isolation and wounding. Total RNAs were extracted from wounded or unwounded etiolated asparagus seedling tissue or mechanically isolated cells and 10 μg of each sample was run out on denaturing gels, blotted and hybridised with probe made from the AoPR1 cDNA. The RNA samples are as follows: unwounded seedlings (lane 1), cell suspension one day after isolation (lane 2), seedlings cut into 2 mm length sections one day after wounding (lane 3), seedlings cut into 10 mm length sections one day after wounding (lane 4), cell suspension two days after isolation (lane 5), seedlings cut into 2 mm length sections two days after wounding (lane 6), seedlings cut into 10 mm length sections two days after wounding (lane 7), cell suspension three days after isolation (lane 8), seedlings cut into sections 2 mm long three days after wounding (lane 9), seedlings cut into sections 10 mm long three days after wounding (lane 10). The approximate transcript size is shown in base pairs.

ositive clones. Ten clones were chosen at ran-
om and the insert size determined. Three of these
ones contained inserts of ca. 750 bp. The size of
e transcript was estimated at 900 bp from
rthern gels (Fig. 1a).

ound induction of message

ne of the 750 bp cDNA inserts was used as
probe in northern analysis. As shown in Fig. 1a, the transcript is detectable in mechanically isolated asparagus cells reaching a maximum steady state one to three days after cell isolation. In chopped mesocotyl sections from etiolated seedlings, the transcript abundance is comparable to that in mechanically isolated cells and appears to follow similar time course kinetics (Fig. 1a). To determine the spatial distribution of message in relation to the wound site, total RNA was isolated from different areas of etiolated, wounded (chopped) mesocotyl and used in

```
     M  S  S  G  S  W  S  H  E  V  A  V  N  V  A  A  G  R  M  F
1    ATGAGTTCAGGGAGCTGGAGCCACGAGGTCGCTGTCAATGTCGCCGCAGGACGGATGTTC 60

     K  A  A  M  L  D  W  H  N  L  G  P  K  I  V  P  D  F  I  A
61   AAGGCGGCAATGCTCGACTGGCACAACCTCGGCCCTAAGATTGTGCCTGACTTTATTGCC 120

     G  G  S  V  V  S  G  D  G  S  V  G  T  I  R  E  I  K  I  N
121  GGTGGCTCAGTGGTGTCTGGAGATGGATCTGTAGGAACCATCCGAGAGATCAAGATCAAC 180

     N  P  A  I  P  F  S  Y  V  K  E  R  L  D  F  V  D  H  D  K
181  AATCCTGCTATACCTTTCAGCTATGTGAAGGAACGCCTGGATTTCGTAGACCATGACAAG 240

     F  E  V  K  Q  T  L  V  E  G  G  G  L  G  K  M  F  E  C  A
241  TTCGAGGTGAAGCAGACCCTCGTGGAAGGTGGAGGTTTAGGTAAGATGTTTGAATGTGCC 300

     T  T  H  F  K  F  E  P  S  S  N  G  G  C  L  V  K  V  T  A
301  ACCACTCACTTCAAGTTCGAGCCCTCGAGCAACGGTGGATGCCTCGTCAAGGTGACTGCA 360

     S  Y  K  I  L  P  G  V  A  D  E  S  A  K  A  K  E  G  I  T
361  TCCTACAAGATTCTCCCAGGTGTCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACC 420

     N  H  M  K  A  T  E  A  Y  L  L  A  N  P  T  A  Y  V  *
421  AACCACATGAAGGCAACCGAAGCTTACCTCCTAGCCAACCCAACTGCCTACGTTTAAATA 480

481  TAGTGATTGTGTTTCTTTGCGTGAAGTGCTTGTGAGTTTGAATAAGGAGATTGGTTATGA 540

541  GGAAGCTTGATGGGGTCATACATAGTTAGTTTATGTTGAATGATCAGCCTTTTTTGTGTG 600

601  AAGTACTTGGGAGTTTGAATAAGGAGACTGAATATGAGAAAGATTGATGGAGTTATCGTT 660

661  CATGTTGAATGATCAGCCTTATCAGTTTGTAACAGTGTCGAATGATCAGTCTTATCAGTT 720

721  TGTAATGGTGGCTTCAA 737
```

2. Northern analysis of transcript abundance at the wound site. Two week old etiolated asparagus seedling mesocotyls were into 1.5 cm explants and left to age as described in Materials and methods before taking the sections for analysis after 1, 2 3 days. Total RNA was extracted from asparagus tissue and 10 μg of each sample was run out on denaturing gels, blotted hybridised with probe made from the AoPR1 cDNA. The samples are as follows: unwounded seedlings (lane 1), 5 mm long tions 5 mm away from the wound site one day after wounding (lane 2), 2.5 mm long sections 2.5 mm away from the wound one day after wounding (lane 3), 2.5 mm long sections that include the wound site one day after wounding (lane 4), 5 mm long tions 5 mm away from the wound site two days after wounding (lane 5), 2.5 mm long sections 2.5 mm away from the wound two days after wounding (lane 6), 2.5 mm long sections that include the wound site two days after wounding (lane 7), 5 mm g sections 5 mm away from the wound site three days after wounding (lane 8), 2.5 mm long sections 2.5 mm away from the und site three days after wounding (lane 9), 2.5 mm long sections that include the wound site three days after wounding (lane

orthern analysis. This showed that after 1 or 2 ays the transcript was mainly located to the ound site. However, on the third day transcript as also detectable at much lower levels in tissue istant from the wound surface (Fig. 1b).

elationship of the cDNA to other gene sequences

s the transcript size was estimated to be 900 bp, was assumed that the approximately 750 bp DNA was not full-length. Following sequencing ig. 2), the 750 bp cDNA was found to contain major open reading frame of 474 bp with a 3 bp 3′ untranslated region containing a puta- e polyadenylation signal starting at position 618 t no apparent poly(A) tract. The predicted pep- le amino acid sequence shown in Fig. 2 was ed to screen the EMBL protein database. The quences that showed significant similarities are group of intracellular PR proteins found previ- sly [27]: a pea disease resistance protein, pI49 7% identity), a potato PR protein, pSTH2 4% identity), a parsley PR protein, PcPR1-1 2% identity), a birch pollen allergen *Betv1* (36% ntity), and the bean elicitor-induced transcripts PR1 and PvPR2 (29% identity). Because of is similarity we have named our cDNA clone PR1 (*Asparagus officinalis* pathogenesis-related clone 1). Sequence alignments are shown in Fig. 3.

Discussion

The initial differential screening of the cDNA library prepared from mechanically isolated asparagus cells confirmed our assertion [13] that this system is indeed a rich source of transcripts associated with the wound response. It was interesting to note that AoPR1, one of the most abundant cDNA clones in library, coded for a transcript with a predicted gene product that was similar to a group proteins known to be strongly unregulated *in planta* by pathogen attack or by treatment of cultured cells with microbial elicitors. AoPR1 and the IPR genes from parsley and bean were all derived from mRNA purified from suspension cultured cells. However, the major differences between the procedures used to isolate the AoPR1 and the parsley and bean IPR cDNAs is that the latter clones were obtained by treatment of long-established, dividing, heterotrophic suspension cell cultures with fungal elicitors, whereas AoPR1 was induced in freshly isolated mesophyll cells where the wound response is intense [25, 27]. In long-established suspension cultures, any wound signals would be diluted sub-

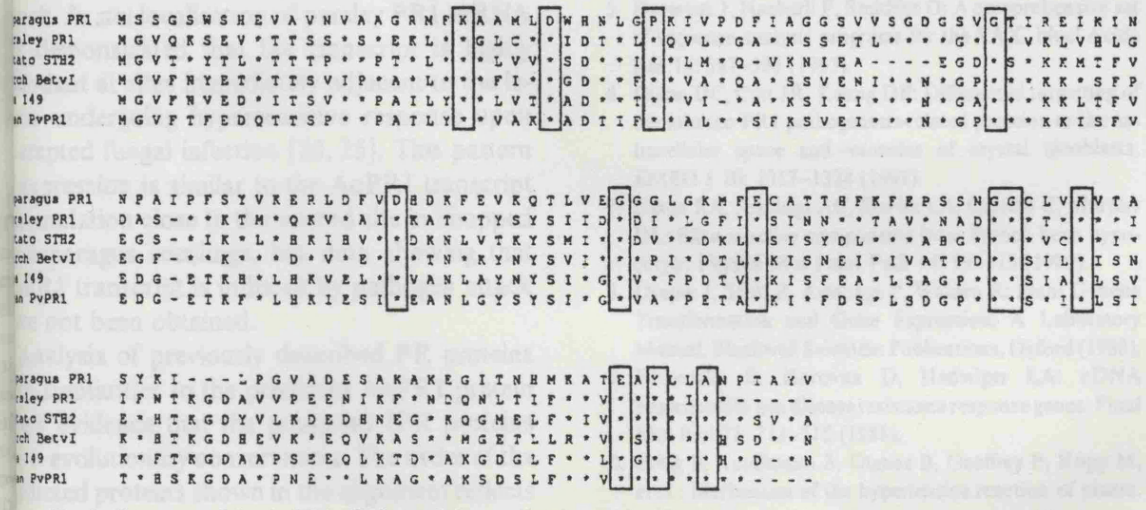

Fig. 3. Sequence of the AoPR1 cDNA and the predicted peptide. The stop codon is represented by an asterisk.

ılturing so that expression of PR1-like genes in ıese systems would predictably be very low until ıe addition of external elicitors. Because AoPR1 as derived from a cell system in the absence of ıternal elicitors, it is probable that the corre-ponding transcript is induced during mechanical ll isolation in which endogenous elicitors such plant cell wall fragments may be produced [4, 20]. Alternatively, the breaking of apoplastic nnections as a result of the shearing apart of ighbouring cells during grinding may be suffi-ent to induce the wound signal responsible for anscript induction. It has been shown that RNA coding for PR proteins accumulate in shly isolated tobacco protoplasts, which clearly ve lost all intercellular connections [11]. How-er, in these experiments the same PR genes ere induced by wounding only, so that it was fficult to determine any specific effect of cell paration on PR transcript induction.

The induction of PR protein expression is clas-cally associated with pathogen infection of ants, however, potato pSTH-2-related tran-ipts were found to be induced by slicing tubers stems as well as by the addition of arachidonic id to tuber discs or by treatment of leaves with *ytophthora infestans* mycelial homogenate [18]. hus, as with AoPR1, the pSTH-2 member of the R group of proteins is induced by wounding. It not known yet whether the birch, parsley or an transcripts are induced only following the tion of an elicitor or as a result of pathogen tack. *In situ* localisation of parsley PR1 mRNA s demonstrated that the transcript is highly undant at sites immediately adjacent to the le-ons undergoing hypersensitive response upon tempted fungal infection [23, 25]. This pattern expression is similar to the AoPR1 transcript cumulation close to the wound site in chopped asparagus seedlings, but data showing that oPR1 transcript is induced by pathogen attack ve not been obtained.

Analysis of previously described PR proteins ith similarities to the predicted AoPR1 protein fers evidence that the predicted IPR proteins ow evolutionary conservation. The order of the redicted proteins shown in the alignment reflects the degree of similarity among the AoPR1 protein and IPR proteins from other species, based on clustal analysis. The asparagus protein, which is derived from a monocot belonging to the family Liliacae, is most divergent from the protein sequence consensus. Although apparent sequence similarities are evident, there may be differences between the AoPR1 gene and those of other IPR genes, reflecting the differences between monocots and dicots.

In future experiments we hope to use this monocot clone to learn more about the role of the AoPR1 protein in the wound response and to utilise the asparagus wounded cell suspension as a model to investigate the extent of similarity of the wound response in monocots compared to dicots.

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