Characterisation of expression of a gene encoding the asparagus intracellular PR protein AoPR1.

Thesis submitted for the degree of Ph.D.

at

The University of Leicester

by

Aideen Gill B.Sc. (N.U.I.)

Dept. of Botany

University of Leicester.

January 1995.

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Acknowledgements

I wish to express my gratitude to the following for their help and support: Drs. John Draper (my supervisor), Steve Smith (University of Edinburgh), Simon Warner and Luis Mur, also Edward Laverty, Rob Darby and Karl Deacon in particular, and all those whom I have worked with while at Leicester.

This work was funded by a Sainsbury studentship from the Gatsby Charitable Foundation whose encouragement and support I gratefully acknowledge.

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Abbreviations

(w/v)	weight:volume ratio
(v/v)	volume:volume ratio
^{o}C	degrees Celsius
Abs	absorbance
bp	base pair
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
Bq	bequerel
CoA	coenzyme A
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylenediaminetetraacetic acid (disodium salt)
g	gramme
g	relative centrifugal force
GUS	β-glucuronidase, encoded by the *uidA* gene from *E. coli* (Jefferson *et al.*, 1987)
gus	*uidA* coding sequence
h	hour
kb	kilobase pair
kDa	kilodalton
l	litre
p.s.i.	pounds per square inch
M	molar
mM	millimolar
min.	minute
MW	molecular weight

mRNA	messenger RNA
MU	methyl umbelliferone
MUG	methyl umbelliferone glucuronide
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
vol.	volume

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Chapter 1:
General Introduction.

1.1 Introduction.

The constraints imposed by a rooted existence have resulted in the evolution of a highly sensitive and coordinated defence response mechanism among higher plants. Throughout the duration of their life cycle plants are continuously subjected to a vast array of stresses, both environmental - temperature fluctuations, drought and wind damage for example, and biological - primarily pathogen attack. The distinction between both types of stress may be made on the premise that environmental stresses are imposed by abiotic agents while biological stresses are imposed by living organisms or by agents derived therefrom, such as fungal elicitors. This chapter is primarily concerned with providing a review of recent literature concerning that aspect of the plant defence response invoked by biological stresses. Analogies and comparisons between the two types of induction will, where appropriate, be made. Since, however, the plants' response to physical damage shares many features in common with the biologically-induced response, for the purposes of this review a discussion of the wound response will be incorporated into discussions on pathogen-induced defence responses.

1.2 The Plant Defence Mechanism:

The ability of a plant to perceive and respond to stress stimuli is dependent on the presence of both a constitutive and an inducible defence mechanism. Some form of physical barrier must be in place to prevent or, at least, reduce the extent of physical damage sustained by the plant, limiting pathogen invasion. In plants, as in animals, this physical barrier is provided by modifications of the exposed surface of the organism. Plant morphology itself may be modified to minimise the risk of damage, while structural modifications of exposed surfaces

e.g. the formation of the cuticle on exposed leaf surfaces, provide a physical constraint to pathogen penetration or mechanical damage.

Inducible plant defence mechanisms comprise those concerning structural alterations in cell and tissue morphology and those concerning modulation of defence-related gene expression with a resulting alteration in the protein complement of affected cells. Common to both is the induction of the phenylpropanoid pathway, resulting in the accumulation of compounds facilitating structural modification of cell architecture and of chemicals directly involved in the biological aspect of the defence response, acting directly on the invading organism.

1.2.1 Structural alterations in cell and tissue morphology.

Lignin, defined as "a complex polymer formed by the random condensation of phenylpropanoid units" (Collinge and Slusarenko, 1987), is an integral component of secondary cell walls of vascular plants. Its accumulation occurs in an enhanced manner following challenge by various plant pathogenic fungi, viruses, nematodes and elicitors (Bell, 1981; Vance *et al.*, 1980), and is a component of the wound response (Lipetz, 1970; Vance *et al.*, 1980). Similarly, callose, a ß-1,3-glucan, accumulates in cell walls in response to a number of chemical, physical and biological stresses including pathogen attack (Bell, 1981; Jahnen and Hahlbrock, 1988). Its formation is brought about by a reaction catalysed by the membrane-bound enzyme callose synthase, otherwise referred to as 1,3-ß-glucan synthase or glucan synthase II (GS-II). This enzyme has been isolated and characterised from a number of species (Dhugga and Ray, 1994; Pedersen *et al.*, 1993), and is thought to consist of between six and nine subunits. Attempts to purify callose synthase from pea tissue resulted in the copurification of two polypeptides of 50 and 70 kDa, which likely form a catalytic complex upon activation (Dhugga and Ray, 1994). Other forms of cell wall modification include oxidative cross-linking of cell wall polymers brought about by the action of cell wall peroxidases (section 1.3.4), and the accumulation of a number of cell wall proteins referred to as proline-, glycine- and hydroxyproline-rich proteins (section 1.3.4). Taken together, these

forms of cell wall modification provide a means of physically reinforcing the cells' integrity, providing an enhanced barrier to further damage or pathogen invasion.

1.2.2 The phenomenon of dedifferentiation.

The wound stimulus is itself a potent initiator of mitotic activity and most of the cells in the vicinity of the wound, excluding, of course, those sufficiently injured to die, respond by dividing one or more times. This programme of cell division gives rise, in a number of cases, to a wound periderm, a visible manifestation of the phenomenon of dedifferentiation. All cellular processes which result in the repression of cell type-specific gene expression may be considered to contribute to the process refered to as cellular dedifferentiation. This phenomenon pertains to all cases wherein division cycle arrested cell types undergo a 'disorganisation' phase and become 're-organised' with a consequential reactivation of the cell cycle. Essentially, therefore, dedifferentiation may be considered as the process *via* which cells become re-programmed in their committment to differentiation; one set of cell type-specific genes becomes repressed with a concomitant expression of genes driving cell differentiation towards a different tissue specificity. The undifferentiated state is therefore that state which all dedifferentiating cells go towards. Since in the majority of cases the point at which a cell is fully undifferentiated is difficult to determine, dedifferentiation can be seen as the complex of cellular events by which a cell arrives at such a state and the maintenance of this state may also be deemed as an aspect of the dedifferentiation process.

1.2.3 Modifications in plant biochemistry.

1.2.3.1 Phenylpropanoid metabolism.

The phenylpropanoid pathway is that sequence of biological reactions leading from L-phenylalanine to 4-coumaroyl CoA (the 'core' reactions of the pathway), and includes all branch pathways derived from these core steps, leading to the production of lignin, suberin and other wall-bound phenolics, flavonoids and UV protectants, phytoalexins and a number of other compounds involved both in plant defence and development (Hahlbrock and Scheel,

1989). Studies of phenylpropanoid metabolism have been primarily concerned with analysis and characterisation of enzymes involved in two of the three core reactions of the pathway, the first and key enzyme being phenylalanine ammonia lyase (PAL) catalysing the conversion of L-phenylalanine to cinnamic acid, and the enzyme catalysing the final step in this core sequence, the conversion of 4-coumaric acid to 4-coumaroyl CoA, 4-coumaroyl CoA ligase (4CL). Substantial information has also been accumulated concerning the enzyme chalcone synthase (CHS), the enzyme catalysing the first committed step in flavonoid production. Further discussion regarding these enzymes and their regulation will be presented in section 1.3.2.

1.2.3.2 Pathogenesis-related (PR) proteins.

Defined by van Loon (1990) as "proteins encoded by the host plant but induced by various pathogens as well as under stress situations similar to those provoked by pathogens", PR proteins were initially described in 1970 by Gianinazzi and co-workers, and van Loon and van Kammen in tobacco cultivars reacting hypersensitively to TMV. These proteins were characterised by their low molecular weight, solubility at low pH and resistance to proteolytic degradation. Subsequently basic counterparts of these acidic proteins were isolated, and PR proteins are now generally assumed to be ubiquitous (van Loon, 1985).

Initial characterisation of the tobacco PR proteins was made on the basis of electrophoretic and immunological properties, and resulted in the determination of five PR groups (van Loon *et al.*, 1987). Recent developments, however, have revealed shortcomings in this system of nomenclature and necessitated a review of PR protein classification criteria (Linthorst, 1991; Somssich, 1994). PR protein groups 1-5 remain classified on the basis of size, sequence homology and function. Proteins of the PR-1 group possess an, as yet, unidentified function, while members of PR-2 possess ß-1,3-glucanase activity and PR-3 proteins chitinase activity (Linthorst, 1991). Members of the PR-5 group, including that PR-5 protein referred to as osmotin, demonstrate DNA sequence homology to the sweet-tasting thaumatin proteins of the african shrub *Thaumatococcus daniellii*. The classification of PR-6 has recently been

allocated to the well-characterised group of defence-related proteins showing proteinase inhibitor activity, PR-7 to proteases similar to the P69 protein of tomato (Vera and Conjero, 1988), and PR-10 to the intracellular PR (IPR) protein family described initially by Walter *et al.* (1990). The *wun*I (Logemann *et al.*, 1988) and *win*1 and 2 (Stanford *et al.*, 1990) proteins have also now been incorporated into the PR 'super group' of proteins (Somssich, 1994). A more detailed description of PR protein nomenclature, characterisation and function may be found in Linthorst (1991), while the role of PR proteins in plant defence is discussed by Bowles (1990), Dixon and Harrison (1990), Stintzi *et al.* (1993) and Collinge and Slusarenko (1987).

1.3 Regulation of defence-related gene expression.

1.3.1 Changes in gene expression as a consequence of the dedifferentiation process.

Having defined the phenomenon of dedifferentiation in terms of cell type-specific gene expression (section 1.2.2) it becomes apparent that this phenomenon is manifest in virtually all aspects of plant development. Any process which involves the committment of cells to one particular cell or tissue type i.e. the unidirectional process of differentiation has intrinsic within its nature an aspect of dedifferentiation. Thus studies of these cellular processes may provide an insight into those aspects of the process pertaining to dedifferentiation.

While the processes of cell and tissue differentiation are fundamental to our understanding of plant growth and development, molecular studies in this area appear to be rare. Structural studies, however, have generated quite large amounts of information regarding the study of development with results indicating that during development each cell determines its position relative to others and then differentiates accordingly. This therefore implies that cells have the ability to communicate with each other, and that this communication is fundamental to plant development (Verbecke, 1992).

The dedifferentiation process itself is manifest at all cellular levels and may be seen to introduce an inherent genetic instability, especially in tissue culture material (Hirochika,

1993; Kikuchi *et al.*, 1987). A large number of physiological and cytological perturbations have been reported in cells and tissues undergoing dedifferentiation (Yeoman and Street, 1977). Changes have been observed in gross protein profile (Grosset *et al.*, 1990b; Wozniak and Partridge, 1988; Harikrishna *et al.*, 1991, 1992; Bonham-Smith *et al.*, 1988; Fleck *et al.*, 1979, 1980) and gene expression (Guerri *et al.*, 1982; Grosset *et al.*, 1990a; Kelly *et al.*, 1990; Marty *et al.*, 1993; Neale *et al.*, 1990; Fujita *et al.*, 1994). Plastids revert to proplastids and a number of other ultrastructural alterations have also been reported (Goldberg *et al.*, 1986; Harikrishna *et al.*, 1991, 1992; Lipetz, 1970) the culmination of which is the loss of photosynthetic capacity and the reversion to heterotrophic growth (Harikrishna *et al.*, 1992).

Dedifferentiation occurs in explants placed into culture as the first step in the expression of totipotency, a property of plant cells which allows the regeneration of whole plants from cultured cells (Davidson *et al.*, 1976). Thus such cell populations provide a potentially enriched source of dedifferentiation-related gene expression. During protoplast isolation and in culture cells undergo fundamental changes in protein synthesis (Fleck *et al.*, 1979). Indeed it has been estimated that 50-70% of total RNA sequence complexity in soybean suspension cultured cells is contained as poly $(A)^+$ (Silflow *et al.*, 1979), a dramatic increase over that of cells *in planta*. Genes expressed in such systems may, broadly speaking, be grouped into three main groups: (i) those induced rapidly during culture initiation prior to the reactivation of DNA replication and cell division, (ii) those involved in mitotic division, and (iii) those whose induction appears to occur independently of cell division. Most work to date on these systems has concentrated on analysis of gene expression during mid- to late-log stages of suspension culture growth. Little work has centred on the characterisation of genes induced early during culture initiation and on those aspects of culture initiation which are sufficient to cause induction of these genes. Investigations into induced changes in gene expression during and immediately following protoplast isolation have provided a limited amount of information concerning this. The isolation of protoplasts (essentially wall-less cells), by its nature ie. the separation of cells from an organised tissue into a population of wall-less independent cells, results in a spectrum of cellular modifications similar to those observed

during early dedifferentiation. Indeed protoplasts may themselves be considered as undergoing a dedifferentiation response, the stimulus being the isolation procedure.

Fleck *et al.* (1979) showed that changes in protein synthesis were detectable as soon as protoplasts were incubated in culture medium. These alterations in protein pattern were subsequently observed to occur not as a result of changes at the level of transcription, but probably due to a change in transcript population (Fleck *et al.*, 1980). Such alterations in protein synthesis correlate with a shift in the transcript population (Grosset *et al.*, 1990a). Comparing transcript populations from freshly isolated protoplasts and mechanically damaged leaf material revealed that it is the wound stimulus that appears to be the essential event in the induction of dedifferentiation in this system (Grosset *et al.*, 1990a).

Characterisation of genes induced during this early induction phase in cell suspension culture specifically has been limited. However, Criqui and colleagues (1992) report the isolation and characterisation of three cDNAs from transcripts that accumulate to very high levels within the first 6 h of protoplast culture, prior to the onset of cell division. These encode a novel type I trypsin inhibitor, a novel anionic peroxidase and a clone of, as yet, unidentified function. The level of expression of these three genes was found to decrease at the onset of cell division and led the authors to suppose that the activation of these genes corresponds not only to a specific adaptation to the culture environment but also to a sequence of events connected with the 're-programming' of gene expression in the dividing cell. Similarly, Marty *et al.* (1993) reported the isolation of two 'early genes' expressed in freshly isolated protoplasts - a cytoplasmic thioredoxin (Marty and Meyer, 1991) and a clone encoding a tumor-related protein. After 48 h in culture transcripts encoding both these proteins was still detectable at relatively high levels. Summarising their results the authors state that (i) dedifferentiation begins during protoplast isolation and (ii) wounding alone is sufficient to induce dedifferentiation.

Those genes concerned with cell division and the reactivation of DNA replication isolated from cell suspension systems comprise, among others, a number of cyclins (Hirth *et al.*, 1992)

including the proliferating cell nuclear antigen (PCNA) (Kodama *et al.*, 1994) and kinases (Jonak *et al.*, 1993; Wilson *et al.*, 1993). A novel S-phase-specific clone, *cyc*07, has also been isolated from *Catharanthus roseus* (periwinkle) (Ito *et al.*, 1991).

A number of cDNA clones have been isolated, the expression of whose corresponding genes appears to occur independently of DNA replication in both protoplast and callus-derived suspension cultures. Included among these types of genes are a number involved in plant defence. A number of PR proteins have been detected in tobacco protoplast-derived cell suspensions (Grosset *et al.*, 1990b). Immunodetection revealed the presence of chitinases, a ß-1,3-glucanase, and osmotin after four days in culture. Having illustrated this increase in PR protein accumulation with time in culture, further investigation at the mRNA level indicated that transcripts encoding similar PR proteins were detected following only 6 h in culture (Grosset *et al.*, 1990a). Following a further 6 h the transcript population was observed to be similar to that observed in callus material.

Coinciding with the induction of defence-related protein gene expression, a marked increase in enzyme activities associated with the phenylpropanoid pathway may also be observed (Funk and Brodelius, 1992; Pletsch *et al.*, 1993; Sudibyo and Anderson, 1993). Suspension cultures have been found to be an enriched source of, for example, enzymes involved in lignification (Eberhardt *et al.*, 1993) and flavonoid biosynthesis (Bokern *et al.*, 1991).

1.3.2 Modulation of expression of genes encoding enzymes of the phenylpropanoid pathway.

1.3.2.1 Phenylalanine ammonia lyase (PAL).

PAL is a tetrameric enzyme (Bolwell *et al.*, 1985) catalysing the removal of an amino group from the amino acid L-phenylalanine. Although present constitutively, in varying amounts, throughout development, PAL activity increases rapidly in response to a number of stress stimuli, correlated with increased transcription of the corresponding genes (Chappell and Hahlbrock, 1984). In response to pathogen attack leading to a hypersensitive response, PAL

transcript accumulates rapidly in the tissue surrounding hypersensitive cell death at fungal infection sites in parsley (Schmelzer *et al.*, 1989), in response to pathogen invasion in soybean (Esnault *et al.*, 1987), and in response to bacterial infection of french bean (Jakobeck and Lindgren, 1993). PAL transcript also accumulates in response to elicitor stimulation of suspension cultures of parsley (Hahlbrock *et al.*, 1991), alfalfa (Gowri *et al.*, 1991) and bean (Cramer *et al.*, 1985; Ellis *et al.*, 1989), being cultivar-specific in the latter case (Ellis *et al.*, 1989; Shufflebottom *et al.*, 1989). Wounding and mechanical damage have reported to cause an induction in PAL transcript levels at and close to the wound site (Lawton and Lamb, 1987), as has *uv* irradiation (Chappell and Hahlbrock, 1984; Hahlbrock and Scheel, 1989).

PAL has been shown to be encoded by multi-gene families in a number of species (parsley: Lois *et al.*, 1989; bean: Cramer *et al.*, 1989; rice: Minami *et al.*, 1989; *Arabidopsis*: Ohl *et al.*, 1990; potato: Joos and Hahlbrock, 1992), data correlating with the isolation of three different PAL isoforms from bean cell suspension cultures, each differing in pI and Km values from the others (Bolwell *et al.*, 1985) and with data demonstrating that the bean PAL-2 and PAL-3 genes encode different isoforms of the protein (Liang *et al.*, 1989). Members of the PAL gene family have been shown to exhibit differential tissue-specific expression (Shufflebottom *et al.*, 1993; Lois and Hahlbrock, 1992; Liang *et al.*, 1989) and to accumulate rapidly in response to uv irradiation, wounding, elicitation and pathogen challenge (Hahlbrock and Scheel, 1989). In parsley, where at least four members of the PAL gene family have been identified (Lois *et al.*, 1989) the expression of three of these PAL genes (PAL-1, PAL-2 and PAL-3) have been studied in detail in response to a number of stimulation events (Lois and Hahlbrock, 1992). All were found to be highly expressed in root tissues and during certain stages of leaf development, while PAL-3 was preferentially activated in wounded leaves. PAL-2 was shown to be primarily responsible for the high constitutive levels of expression detected in roots. Each gene was found to respond to *uv* irradiation and elicitor-treatment. There are three classes of PAL genes within the bean genome that exhibit differential regulation in response to environmental stimuli and during development (Cramer *et al.*, 1989). All are induced by wounding; however only PAL-1 and PAL-2 are induced in

response to fungal elicitor. PAL-2 transcript was found to accumulate in shoots, petals and light-treated hypocotyls, while PAL-3 transcript was detected predominantly in fungal-infected hypocotyls. The tissue- and cell-specific nature of expression of the bean PAL genes has recently been investigated in transgenic tobacco (Shuflebottom *et al.*, 1993). The PAL-2 promoter was found to direct reporter gene (*gus*) expression in developing xylem and in a narrow zone of cells immediately surrounding necrotic lesions produced during the hypersensitive reaction of tobacco to TMV. GUS activity as a consequence of PAL-3 promoter activity was detected in pigmented petal tissue, specifically in the epidermal layer. Expression of the *gus* gene under the control of this promoter during the hypersensitive response was localised to large halos surrounding the necrotic tissue, expanding to cover the whole explant by 24 h post-elicitation. Both promoters were active in pollen, root tips and stem.

1.3.2.2 4-coumarate:CoA ligase (4CL).

The enzyme 4CL plays a key role in linking general phenylpropanoid metabolism to the end products of specific branch biosynthetic pathways, as it is the enzyme catalysing the final step in the 'core' phenylpropanoid pathway (Hahlbrock and Scheel, 1989). 4CL exists as a monomeric enzyme in parsley, occurring as two isoforms, each encoded by a single-copy gene (Douglas *et al.*, 1987). Both genes are expressed at similar relative rates in unstimulated, uv-irradiated or elicitor-treated parsley cells (Douglas *et al.*, 1987), and transcript accumulates rapidly at sites of infection (Schmelzer *et al.*, 1988; Esnalt *et al.*, 1987).

1.3.2.3 Chalcone synthase (CHS).

CHS, a key enzyme of flavonoid biosynthesis (Hahlbrock and Grisebach, 1989), catalyses the stepwise condensation of three acetate residues from malonyl CoA with coumaroyl CoA. Most non-legume species contain one or two CHS genes per haploid genome (Herrmann *et al.*, 1988) while CHS exists as multi-gene families in bean (Ryder *et al.*, 1987), soybean (Wingender *et al.*, 1989) and petunia (Koes *et al.*, 1989). The induction of CHS gene

expression in parsley is light-dependent (Bruns *et al.*, 1986; Schmelzer *et al.*, 1988), being stimulated by *uv* irradiation, although a slight modulatory effect by blue light has been observed, with red light also exerting a small, but measurable, effect *via* phytochrome (Bruns *et al.*, 1986). This *uv*-induced increase in CHS enzyme activity arises as a consequence of *de novo* transcription of the CHS gene (Chappell and Hahlbrock, 1984). *uv* irradiation has also been demonstrated to induce expression of CHS genes in *Arabidopsis* (Feinbaum and Ausubel, 1988), where transcript accumulation preceedes anthocyanin accumulation, and in soybean (Wiegender *et al.*, 1989), petunia (van Tunen *et al.*, 1988), bean (Ryder *et al.*, 1987), *Antirrhinum* (Lipphardt *et al.*, 1988) and tobacco (Kaulen *et al.*, 1986). In the case of parsley this light-induction of transcript accumulation has been localised to the epidermal cells of leaves (Schmelzer *et al.*, 1988). In mustard seedlings, however, CHS gene induction is not dependent on *uv* irradiation, although this response is developmentally regulated with uv-dependent CHS induction being observed during later stages of development (Hahlbrock and Sceheel, 1989). Developmental regulation of expression of this gene is also reported for petunia (Koes *et al.*, 1989) and bean (Schmid *et al.*, 1990). Pathogen- and elicitor-mediated induction of CHS gene expression has been reported (Schmelzer *et al.*, 1989; Ryder *et al.*, 1987; Wingender *et al.*, 1989; Ellis *et al.*, 1989), and is not dependent on the development of the hypersensitive response (Jakobek and Lindgren, 1993). Petunia and parsley CHS genes, however, do not display this response (Koes *et al.*, 1989). Induction in response to both elicitation and developmental stimuli leads to the differential expression of members of the CHS gene family (Koes *et al.*, 1989; Wiegender *et al.*, 1909; Ellis *et al.*, 1989; Ryder *et al.*, 1987).

1.3.4 Induction of PR protein gene expression.

In addition to displaying developmental regulation (Eyal and Fluhr, 1991; Cutt and Klessig, 1992; Lotan and Fluhr, 1989), expression of genes encoding PR proteins is also modulated in response to a number of environmental stimuli. PR proteins accumulate in pathogen-infected tissue (Casacuberta *et al.*, 1992; Brederode and Linthorst, 1991; van Loon, 1985; reviewed in Collinge and Slusarenko, 1987) and in response to exogenously supplied elicitor (Somssich *et*

al., 1986; Marineau *et al.*, 1987; Roby *et al.*, 1991). Chemical agents, including jasmonic acid, its methyl derivative and octadecanoid precursors (Farmer *et al.*, 1992; Farmer and Ryan, 1992), ß-amino-butyric acid (Cohen *et al.*, 1994), and salicylic acid and its acetyl derivative, aspirin (Antoniw and White, 1980; Hennig *et al.*, 1993; Malamy, 1990) also induce PR protein accumulation to relatively high levels. Ethylene and abscissic acid both induce PR gene expression (Eyal *et al.*, 1993). Auxin and cytokinin together have been reported to repress both chitinase and glucanase gene expression (Shinshi *et al.*, 1987; Mohnen *et al.*, 1985). Physical stimulation by wounding also results in the induction of PR gene expression (Warner *et al.*, 1992). Thus induction of PR gene expression occurs as a consequence of both exogenous e.g. wounding, pathogen attack and elicitation stimulation, and endogenous stimulation, phytohormones, salicylic acid, jasmonic acid and its derivatives for example. Also included in these endogenous PR protein-inducing factors is the 18 amino acid peptide, systemin (Pearce *et al.*, 1991; McGurl *et al.*, 1992) which has been found to induce PI synthesis in tomato and potato leaves, and has been proposed as a long distance wound signal (McGurl *et al.*, 1994). These endogenous agents comprise components of signal transduction pathways transforming an external stimulation event into a response characterised by changes in gene expression. Investigations into the means by which these stimuli modulate PR gene expression have concentrated on explorations of the effect of salicylic acid, ethylene and jasmonic acid on PR gene expression primarily and thus shall be discussed further below.

Exposure of plants to the above stimuli may not only give rise to a local accumulation of PR proteins, but may also cause the accumulation of such proteins at sites distant from the site of initial stimulation. This systemic accumulation of PR proteins is correlated with a systemic induction of transcription of the corresponding PR genes (Ward *et al.*, 1991) and acts to protect the plant from subsequent exposure to the same or a different pathogenic stimulus. In this way the unstimulated regions of the plant become 'primed', possessing a basal level of PR proteins which further enhances the plants defence mechanism. This phenomenon is referred to as systemic acquired resistance (SAR) (Ryals *et al.*, 1994).

1.3.4.1 Salicylic acid and the regulation of the plant defence response.

Salicylic acid has been implicated in systemic signal transduction in the plant defence response to pathogen attack (Malamy, 1990; Métraux, 1990) and its induction of 'classical' (Antoniw and White, 1986) and intracellular (Crowell *et al.*, 1992) PR protein gene expression is well documented, with particular emphasis being given to its induction of expression of the tobacco PR1a gene (Ohshima *et al.*, 1990; van de Rhee *et al.*, 1990; Uknes *et al.*, 1993). In contrast, accumulation of tomato PR proteins (proteinase inhibitors I and II) is repressed in the presence of salicylic acid (Doherty *et al.*, 1988).

Having been identified as 'calorigen', an endogenous regulator of heat production in the *Arum* lily (Raskin *et al.*, 1987), salicylic acid has also been widely implicated in the development of systemic acquired resistance (SAR) (Ward *et al.*, 1991; Raskin *et al.*, 1992; Malamy and Klessig, 1992). Exposure of plants to salicylic acid, or indeed to its derivative aspirin, has been shown to result in the rapid expression of PR genes considered to be molecular markers of SAR (Ward *et al.*, 1991). Indeed work by Gaffney and colleagues (1993) clearly demonstrated that salicylic acid is indeed essential for the development of SAR. In an experiment utilising transgenic tobacco plants into which the *Pseudmonas putida nah*G gene, encoding the enzyme salicylic acid hydroxylase, had been introduced under the control of the CaMV 35S promoter, Gaffney *et al.* (1993) were able to demonstrate that plants depleted in salicylic acid were defective in their ability to respond to TMV infection through the development of an SAR response. Salicylic acid hydroxylase converts salicylic acid into catechol which is unable to induce PR protein gene expression. It was proposed (Ward *et al.*, 1991) that salicylic acid accumulated during the plant defence response as a consequence of phenylpropanoid metabolism. Subsequent work by Yalpani and colleagues (1993) has shown that this is indeed at least one of the sources of salicylic acid biosynthesis employed by the plant in response to pathogen attack. While not excluding the possibility that salicylic acid may be synthesised *via* some other route(s) Yalpani *et al.* (1993) demonstrate the synthesis of salicylic acid directly from *trans*-cinnamic acid, an intermediate of the phenylpropanoid pathway *via* benzoic acid, a reaction catalysed by the putative cytochrome P450 benzoic acid

2-hydroxylase. This latter enzyme has itself been found to accumulate in response to pathogen infection (Leon *et al.*, 1993). Once synthesised, salicylic acid is rapidly conjugated into its glucoside (Eneydi *et al.*, 1992; Hennig *et al.*, 1993) in which state it becomes unable to cause induction of PR protein gene expression. Ryals (1994) observes the likelihood that this salicylic acid conjugate may function as a storage form which may be hydrolysed as needed within the cell in response to various stimuli, or that this modification of the bioactive agent such that is rendered inactive is a prerequisite for the appropriate targeting of the conjugate for catabolism. Either way it is clear that salicylic acid turnover and the regulation of endogenous salicylic acid levels are important aspects of the regulation of the plant defence response by this chemical. The role of salicylic acid in SAR and the consequences of exposure to this chemical are, as previously discussed, well documented, illustrating the ability of salicylic acid to stimulate the accumulation of a range of defence-related proteins. The exact nature of those mechanisms involved in bringing about this response remains, as yet, unclear.

Salicylic acid, it has been proposed, operates *via* an ethylene-dependent pathway (Eyal *et al.*, 1992; Raz and Fluhr, 1992), unlike the fungal elicitor xylanase which operates *via* an ethylene-independent mechanism (Raz and Fluhr, 1992). Calcium has recently been found to be a component of the transduction pathway leading to this ethylene-dependent stimulation of PR protein gene expression (Raz and Fluhr, 1992). Ethylene responsiveness has, in this case, been shown to be dependent on and mediated by phosphorylation events (Raz and Fluhr, 1993). Thus the signal transduction pathway leading from the salicylic acid stimulus to PR protein gene expression may be seen to be, potentially, comprised, at least in part, of phosphorylation cascades and ion fluxes, events previously documented as being important in the regulation of plant gene expression in response to physiological stimuli (Bennett, 1991; Carter *et al.*, 1991) and elicitation (Dietrich *et al.*, 1990; Felix *et al.*, 1991).

Another hypothesis that exists concerning salicylic acid stimulation of plant defence responses has been proposed by Klessigs group. Chen and co-workers (1993a) reported the isolation of a 280 kDa salicylic acid binding protein (SABP) from tobacco which appears to

be a complex containing a 57 kDa subunit. It was found that the inducibility of PR1 genes by salicylic acid was directly proportional to the abundance of SABP in different organs. These observations were consistent with a role for SABP in perceiving and transducing the salicylic acid stimulus in plant defence. A further report from the same group (Chen *et al.*, 1993b) documented the isolation and characterisation of the cDNA encoding SABP. Based on predicted amino acid similarity the SABP was identified as a catalase, an identity borne out by protein sequencing and catalase assay experiments on SABP itself. Further, it was shown that salicylic acid binding to SABP inhibited the catalase activity of that protein. In the context of the plant defence response it is therefore postulated that the salicylic acid signal is propagated *via* the active oxygen species H_2O_2 which may act as a secondary messenger to activate defence-related gene expression (Chen *et al.*, 1993).

1.3.4.2 Octadecanoid-derived signals.

A number of C_{18} unsaturated fatty acid-derived C_{12} compounds have been shown to possess potent biological activities in plants in regulating developmental and environmental responses. These C12 compounds include jasmonic acid and its methyl derivative, cucurbic acid and traumatic acid. Of these the most extensively studied is jasmonic acid, and the role of this compound and its derivatives in modulating the plant defence response will be briefly discussed here.

Jasmonic acid is a naturally occurring compound identified in a diverse range of plant species (Meyer *et al.*, 1984) and its methyl ester has been shown to cause marked alterations in protein profiles of treated material, causing the accumulation of a number of novel proteins in excised barley leaves while simultaneously repressing synthesis of most pre-existing, control proteins (Reinbothe *et al.*, 1993). Control protein synthesis was found to be repressed as a consequence of translational impairment, while novel proteins accumulated as a result, not of changes in rates of transcription, but of some form of implied post-translational modification. Expression of three genes encoding pathogen-inducible lipid transfer proteins is repressed upon exposure to 10 μm methyl jasmonate (Molina and Garcia-Olmedo, 1993) while *de novo*

induction of genes encoding phenylpropanoid pathway enzymes is stimulated by jasmonic acid, with a resultant accumulation of flavonoids and other pathway end products (Gundlach *et al.*, 1992). Both jasmonic acid and methyl jasmonate induce PI gene expression (Farmer and Ryan, 1990; Farmer *et al.*, 1992; Xu *et al.*, 1993), as do a number of octadecanoid precursors of jasmonic acid - linolenic acid, 13(S)-hydroperoxylinolenic acid and phytodienoic acid (Farmer and Ryan, 1992). These results led the authors to speculate that jasmonic acid and its octadecanoid precursors may participate in a lipid-based signalling system activating PI gene expression in response to external stimuli, and to propose a model by which this may occur (Farmer and Ryan, 1992). This model distinguishes between signals arising as a consequence of wounding and herbivory i.e. systemin and those likely to be produced by the plant as a result of signals arising from a wound stimulus are systemic in nature, while those arising as a consequence of pathogen attack will be of a local nature.

The jasmonic acid biosynthetic pathway (Vick and Zimmerman, 1984) includes a lipoxygenase-catalysed reaction converting linolenic acid into 13(S)-hydroperoxylinolenic acid. This enzyme is itself induced following wounding (Hildebrand *et al.*, 1988) and it has been suggested that lipoxygenase may play a role in a general stress response (Bell and Mullet, 1991). Inhibition of lipoxygenase activity results in a loss of wound-inducible *pin*II gene expression (Peña-Cortés *et al.*, 1993). Aspirin also inhibits proteinase inhibitor gene expression in tomato (Doherty *et al.*, 1988), and has been found to mediate this *via* the inhibition of jasmonic acid accumulation normally observed after wounding (Albrecht *et al.*, 1993) through blocking jasmonic acid biosynthesis (Peña-Cortés *et al.*, 1993). Levels of endogenous jasmonic acid also increase in response to elicitation of cell suspension material of a number of plant species in a rapid and transient manner (Gundlach *et al.*, 1992). Exposure of such material to methyl jasmonate has been found to enhance the response of conditioned cells to low concentrations of fungal elicitor, enhancing elicitation of active oxygen species (Kauss *et al.*, 1994). A similar conditioning response had previously been reported following preincubation of suspension cultured cells in the presence of either salicylic acid or its derivative dichloroisonicotinic acid (Kauss *et al.*, 1992).

1.3.4.3 Ethylene and other phytohormones.

An important mediator of the plant defence response is the phytohormone ethylene which accumulates in response to wounding, pathogen attack and elicitation (Paradies *et al.*, 1980; Yang and Hoffman, 1984; Lotan and Fluhr, 1990). It has also been shown to induce a number of defence-related and PR proteins (Ecker and Davis, 1987; Weiss *et al.*, 1991; Roby *et al.*, 1991; Brederode *et al.*, 1991; Eyal *et al.*, 1992). While capable of inducing PR gene expression directly, ethylene also serves to mediate perception of other stress stimuli e.g. elicitors such as α-aminobutyric acid and salicylic acid (Eyal *et al.*, 1992). Not all pathogenesis-related responses are dependent on ethylene however, and thus ethylene-independent and -dependent pathways exist mediating PR gene expression (Raz and Fluhr, 1993).

Abscissic acid is involved in the wound-induced expression of proteinase inhibitor genes in potato and tomato (Peña-Cortés *et al.*, 1989; Xu *et al.*, 1993). Experiments have shown that wounding results in increased levels of this phytohormone both in wounded and non-wounded systemically induced leaves (Peña-Cortés *et al.*, 1989). However, the role of ABA in mediating PI gene expression is apparently limited to the wound response. Tissue specific expression remains unaffected in ABA-deficient mutants (Peña-Cortés *et al.*, 1991), suggesting the action of two separate pathways regulating developmental and environmental induction of PI gene expression. Two members of the PR-10 family are classified as ABA-responsive proteins. ABR17 and ABR18 (Barratt *et al.*, 1989) were initially isolated as novel proteins accumulating in pea embryos in response to osmotic stress together with exogenous 10 μM ABA.

Further evidence for the involvement of plant hormones in modulating defence-related gene expression comes again from observations based on expression of genes encoding members of the PR-10 protein family. Phytohormone depletion results in the accumulation of SAM22 transcript (Crowell *et al.*, 1992), while this is reversed upon addition of cytokinin to cytokinin-starved or auxin to auxin-starved cells with SAM22 message levels decreasing

dramatically within 4 h under these conditions. Other PR proteins whose accumulation is modulated by the presence/absence of auxin and cytokinin are members of the PR-2 and PR-3 families. ß-1,3-glucanase transcript levels accumulate in cultured tobacco tissues treated with either auxin or cytokinin, while simultaneous exposure to both hormones results in transcriptional inhibition of the corresponding gene (Mohnen *et al.*, 1985). Similarly, chitinase activity is blocked and transcription of the corresponding gene inhibited in the presence of auxin and cytokinin (Shinshi *et al.*, 1987).

1.3.4.4 Signal transduction leading to PR gene expression.

The response of a number of plant species to wounding, pathogen attack and elicitation involves the activation of PR genes and the accumulation of the respective proteins at the local site of injury and systemically throughout the plant. At least two different pathways are thought to be involved (Lotan and Fluhr, 1990). In the case of PI induction in response to wounding the nature of the systemically propagated signal(s) mediating this response has been the subject of some debate. Candidates include systemin, an 18 amino acid endogenous polypeptide from tomato found to induce PI activity when supplied to young tomato leaves, and to be transported away from the wound site following stimulation (Pearce *et al.*, 1991), a signal based on electrical activity (Wildon *et al.*, 1992), and a hydraulic signal (Malone *et al.*, 1991). Evidence for the involvement of an electrical signal comes from observations that mechanical damage to cotyledonary leaf tissue leads to electrical activity which is propagated throughout the subject explant correlating with the systemic induction of *pin* activity (Wildon *et al.*, 1992). The involvement of some other phloem-mobile signal(s) appears to be excluded by the observation that phloem translocation can be completely blocked, through the use of cooling blocks, without effect on *pin* transcript accumulation or propagation of electrical signals. Hydraulic signals, propagating changes in water pressure, are also transmitted from wound sites in tomato (Malone, 1993) but cannot themselves induce PI synthesis (Malone *et al.*, 1994b). Recent work, however, has shown that this hydraulic signal is an essential requirement for the systemic induction of PIs by localised treatments (Malone *et al.*, 1994a). It was found that the presence of an endogenous Proteinase Inhibitor Inducing Factor (PIIF)

was required locally for induction of PIs. Systemic induction of PIs occurred only when both PIIF and a hydraulic signal are triggered at the treatment site, a criterion fulfilled by excision of the leaf explant under leaf extract. Thus, the authors conclude, systemic wound signalling involves a synergistic interaction between hydraulic signals and PIIF, and propose that PIIF is distributed rapidly from wound sites by the mass flows associated with wound-induced hydraulic signals. It thus appears from this study that hydraulic dispersal of elicitor-like agents is one possible mechanism of systemic signalling in response to wounding in tomato.

As previously discussed salicylic acid has frequently been proposed to function as the systemically transported signal causing induction of PR gene expression, especially PR1, gene expression in response to pathogen attack. It plays an essential role in the induction and maintenance of SAR (Ryals *et al.*, 1994; Gaffney *et al.*, 1993), although until recently the exact nature of this role was unclear. Accumulation of SA under conditions of SAR led many to propose that SA was itself being systemically transported and directly inducing PR gene expression. Recent data has revealed, however, that SA is not the SAR-inducing systemically translocated signal (Vernooij *et al.*, 1994). In a series of grafting experiments using both wild-type and transgenic (expressing *nah*G, a gene encoding salicylic acid hydroxylase) *N. tabacum* cv Xanthi plants the authors found that, when wild type scions were grafted onto transgenic root-stocks incapable of accumulating salicylic acid, non-transgenic scions were rendered resistant to further infection following infection of the root stock by TMV. It thus appears that the signal originating within the root stock in response to TMV infection and propagated throughout the plant thereby inducing SAR is not salicylic acid. Scions of reciprocal grafts, i.e. using a non-transgenic root stock and *nah*G-expressing scion, failed to display SAR following similar treatment. Therefore, the authors conclude, the signal requires the presence of salicylic acid in tissues distant from the infection site to induce SAR.

Analysis of cellular events occurring as a consequence of a defence-related stimulation event have revealed changes in protein synthesis, kinase activity, ion fluxes and an oxidative burst (Choi and Bostock, 1994; Viard *et al.*, 1994; Raz and Fluhr, 1993; Nürnberger *et al.*, 1994; Vera-Estrella *et al.*, 1994). Changes in the distribution of membrane-bound calcium,

activated calmodulin and callose visualised by Thonat *et al.* (1993) as a consequence of thigmomorphogenesis in *Bryonica dioica* suggest a rapid influx of calcium into the cytosol and the involvement of this ion in signal transduction, data supported by observations made by other workers (Choi and Bostock, 1994; Raz and Fluhr, 1992). Ca^{2+}/H^{+} influxes have also been reported to occur in response to the binding of the fungal oligopeptide elicitor Pep-13 to a specific, novel membrane-bound receptor in parsley (Nürnberger *et al.*, 1994), as have K^{+}/Cl^{-} effluxes, an oxidative burst, and activation of defence-related genes. In this study the authors propose a model summarising current data concerning elicitor signal perception and transduction in the parsley system (Nürnberger *et al.*, 1994).

1.3.5 Factors affecting expression of genes encoding other defence-related proteins.

Cinnamyl acid dehydrogenase (CAD) which catalyses the formation of cinnamyl alcohols, the direct precursors of lignin biosynthesis is encoded by a single gene (Walter *et al.*, 1988) and is rapidly (within 1.5 h) and transiently activated in response to elicitation (Walter *et al.* 1988), with a consequential increase in enzyme activity (Grand *et al.*, 1987; Mitchell e*t al.*, 1994). It has been suggested that this rapid elicitor stimulation of CAD may play a role in the generation of secondary signals involved in the induction of the plant defense response (Walter *et al.*, 1988), since certain lignin precursors (dihydrodiconiferyl glucosides) have been found to exhibit cytokinin-like activity in plant cells (Binns *et al.*, 1987).Antisense inhibition of CAD has been found to result in the production of a modified lignin in otherwise normal plants (Halpin *et al.* 1994). The lignin complement of such plants was found to be altered in chemical structure and composition.

Genes encoding a number of other enzymes involved in the lignin biosynthetic pathway including S-adenosyl-L-methionine:caffeic acid 3-O-methyltransferase have been cloned and characterised (Gowri *et al.*, 1991; Jaeck *et al.*, 1992). Expression of these genes is developmentally regulated and elicitor-inducible, increases in enzyme activity correlating with increased transcription.

During the plant defence response peroxidases are involved in the scavenging of hydroxyl radicals produced as a consequence of the superoxide dismutase (SOD)-catalysed reaction converting superoxide radicals into oxygen (Bowler *et al.*, 1989) and accumulate rapidly and transiently following elicitation (Wingate *et al.*, 1988). They are also thought to play a role in the polymerisation of phenolic compounds to generate suberin, in the formation of lignin precursors, and in the cross-linking of cell wall components such as HRGPs (Bowles, 1990). While extracellular peroxidases are inactivated in response to elicitation, cellular forms of the proteins are induced (Messner and Bell, 1994; Mitchell *et al.*, 1994) with enzyme activity also being induced following pathogen attack (Nickerson *et al.*, 1993; Ludwig-Mueller *et al.*, 1994) and wounding (Mohan *et al.*, 1993a). Some developmental regulation of gene expression has also been observed (Mohan *et al.*, 1993b, Sherf and Kolattukudy, 1993).

HRGPs are encoded by a small multi-gene family (Hood *et al.*, 1993) and referred to as extensins (reviewed in Cassab and Varner, 1988; Kieliszewski and Lamport, 1994) in dicotyledonous species. Accumulation of HRGP mRNA depends, to a large extent, on tissue-specific factors, as well as on the physiological state of the plant. In general, HRGP mRNA accumulates in meristematic tissue (José and Puigdomenach, 1993). Genes encoding such proteins show elevated levels of expression in elicitor-treated material (Roby *et al.*, 1985; Bolwell *et al.*, 1985; Wingate *et al.*, 1988; Showalter *et al.*, 1985). Transcripts encoding HRGPs accumulate in response to wounding (Ludevid *et al.*, 1990), ethylene (Showalter *et al.*, 1992; Tagu *et al.*, 1992), and developmental stimuli (Ruiz-Avila *et al.*, 1992; Stiefel *et al.*, 1990) with induction arising as a consequence of transcriptional activation of the appropriate genes (Lawton and Lamb, 1987).

Proline-rich proteins (PRPs) differ from extensins both in the sequence of the characteristic repetitive motifs and in features of the expression of their genes. Such proteins have been classified into a number of groups based on their amino acid sequences (José and Puigdomenech, 1993). They have been immunolocalised to the cell wall (Marcus *et al.*, 1991; Ye and Varner, 1991) and found to show profiles of developmental regulation of gene expression specific for the different groups of PRPs. Some PRPs do show an induction of

gene expression in response to wounding; in general, however, no induction has been observed in response to ethylene, elicitation or fungal attack (José and Puigdomenach, 1993).

Despite being obviously distinct from PRPs, GRPs share many features in common with this class of proteins. Both are localised to the cell wall, share many gene expression features, and exhibit some amino acid sequence similarities. Like both HRGPs and PRPs, synthesis of GRPs is also developmentally regulated. Interestingly, GRPs may be either repressed or induced following wounding, depending on the specific gene being observed, and do not appear to be strongly responsive to ethylene or ABA (José and Puigdomenach, 1991). Expression of genes encoding GRPs has been correlated with the lignification process. In both bean and soybean GRP-encoding genes were found to be expressed in all cells that were, or were going to become, lignified (Ye and Varner, 1991). Both processes were subsequently found to occur independently (Ryser and Keller, 1992; Keller *et al.*, 1989).

1.3.6 Analysis of regulatory elements determining specificity of defence-related gene expression.

The transcription of genes by RNA polymerase II is regulated by *cis*-acting DNA sequences which exert their effects on transcription through interactions with specific *trans*-acting proteins. Specificity and inducibility of gene expression is therefore tightly regulated at the level of transcription, although subsequent regulatory mechanisms may also operate for any particular gene. The location of these *cis*-elements within the regulatory regions (for the purposes of this discussion these regions will be referred to as 'the promoter') may be defined on the basis of the ability of truncated promoter fragments to confer transcriptional activation upon a reporter gene and through analysis of protein-DNA interactions. In the former case, the promoter fragment is successively shortened by either 5' or 3' deletion, fused to a reporter gene coding sequence and the construct assayed for reporter gene activity. In this way, specific regions within the promoter may be defined that potentially harbour regulatory elements important in the determination of specificity of gene expression. Analytical approaches aimed at exploring protein-DNA interactions directly centre primarily on

observations that protein-DNA complexes exhibit altered mobility through a polyacrylamide matrix when compared to non-complexed DNA. Such gel shift or electrophoretic mobility shift assays (EMSA's) are, however most effective when employed in conjunction with deletion analysis, the DNA fragment being utilised in the EMSA's having been implicated in transcription regulation. A further technique, DNA footprinting, also investigates protein-DNA interactions through electrophoretic analysis. In this case, the experimental procedure is based on the observation that DNA that is complexed with a protein will be 'protected' from chemical or enzymatic cleavage. Thus, comparison of complexed and naked i.e. non-complexed forms of the same DNA fragment following exposure to the cleavage agent - generally DNaseI - allows determination of protein-DNA interaction events since the ladder of DNA fragments generated upon cleavage of the promoter will differ only at those regions where protection has been afforded the DNA through DNA-protein interactions. A number of modifications of this technique are also commonly used, including dimethyl sulphate (DMS) protection assays - a technique based on the ability of a transcription factor to protect against the methylation of guanine resudues within a DNA sequence by contacting those nucleotides during DNA binding. Thus, in addition to delineating the DNA binding site of a protein factor, this technique also allows the determination of the precise guanine residues within the DNA sequence that interact with the protein.

Such methodology has been widely applied in the analysis of regulatory regions of genes involved in the plant defence response. Deletion analysis has been employed in the analysis of the PAL (Hashimoto *et al.*, 1992; Levya *et al.*, 1992; Shufflebottom *et al.*, 1993), 4-CL (Hauffe *et al.*, 1993) and CHS (Wingender *et al.*, 1990) promoters, for example, allowing the delineation of sequence elements implicated in light- and developmental regulation of these genes. DNA footprinting revealed a number of footprints, both constitutive and inducible, within the bean PAL1 promoter (Lois *et al.*, 1989). The inducible footprints corresponded to DNA sequence designated boxes P (centered around position -181), A (-163) and L (-110) (da Costa e Silva *et al.*, 1993), and a cDNA encoding a Box P Binding Factor (BPF-1) has been isolated and characterised. Box P is conserved at similar positions relative to the start of

transcription in a number of other defence-related genes (de Costa e Silva *et al.*, 1993), and binding of BPF-1 to this sequence indicates a probable role in the regulation of expression of other defence-related genes. Similar approaches have been employed in the analysis of promoter regions of a growing number of PR- and defence-related genes, the results of which are comprehensively reviewed by Somssich (1994).

1.3.7 Regulatory features common to defence-related genes.

Through the use of approaches described above, a number of regulatory elements common to defence-related gene promoters have been identified. The H-box (CCTACC) (Loake *et al.*, 1992; Yu *et al.*, 1993) is related to a consensus sequence, (T/A)CT(C/A)ACCTA(C/A)C(C/A), present in the promoter sequence of a number of genes encoding enzymes of the phenylpropanoid pathway (Lois *et al.*, 1989). Sequence motifs exhibiting homology with this consensus have been found to play a role in mediating uv light-induced gene expression (Schulze-Lefert *et al.*, 1989; Wingender *et al.*, 1990), to be involved in regulating gene expression in response to environmental stimuli (Yu *et al.*, 1993; Dron *et al.*, 1988), and, in conjunction with a second regulatory element, to function in 4-CA-induction of the parsley CHS15 gene (Loake *et al.*, 1992). Similar sequences have also been found in the bean PAL2 and PAL3 promoters (Shufflebottom *et al.*, 1993), and defined as an *in vivo* footprint in the 4CL-1 promoter in parsley protoplasts (Hauffe *et al.*, 1991). In the case of 4CL, this motif has been implicated in the repression of phloem-specific gene expression (Hauffe *et al.*, 1993), while Levya *et al.* (1992) infer the ability of this sequence element to mediate xylem-specific PAL gene expression.

The activity of this regulatory motif is modulated by its ability to interact in a sequence-specific manner with certain nuclear proteins. Two such proteins, KAP-1 and KAP-2, have been found to recognise conserved features in the H-box motif of the CHS15 gene (Yu *et al.*, 1993). The binding activity of both these proteins has been shown to increase markedly in bean in response to elicitor-treatment of bean cell suspension cultures (Yu *et al.*, 1993). Similarly, the appearance of a specific footprint mapping to the same sequence following uv

irradiation or elicitor-treatment of parsley suspension cultured cells (Schulze-Lefert *et al.*, 1989) indicates a inducible DNA-protein interaction at this motif in response to specific stimuli. Recently, a further H-box-binding protein has been isolated and characterised (Sablowski *et al.*, 1994). The authors demonstrate that tobacco and *Antirrhinum* petal-specific proteins bind to H-box-like sequences within the promoters of a number of phenylpropanoid pathway genes. These petal proteins were found to be serologically related to *Myb*305, a flower-specific *Myb* protein (a *trans*-acting factor). Indeed, Grotewold *et al.* (1994) have shown that these H-box-like seqences conform to the consensus CCT/AACC, the target site for binding of plant *Myb* homologues and classifies all sequence motifs containing this consensus as *Myb* binding sites.

The consensus sequence CCA(A/C)C(A/T)AAC(A/T)CC, designated Box P by da Costa e Silva *et al.* (1993) and PAL Box 1 by Hauffe *et al.* (1993), bears some similarity to the H-box consensus sequence, and may, indeed, be considered to be a degenerate form of H-box sequence. This sequence is conserved in a number of PAL promoters from bean, parsley and *Arabidopsis*, and also in a number of other stress-inducible promoter sequences (Lois *et al.*, 1989; Ohl *et al.*, 1990; da Costa e Silva *et al.*, 1993; Dron *et al.*, 1988). The nuclear protein BPF-1 interacts specifically with this sequence (da Costa e Silva *et al.*, 1993), and transcript encoding this protein shown to accumulate rapidly in elicitor-treated parsley cells and around sites of fungal infection on parsley leaves (da Costa e Silva *et al.*, 1993).

The G-box motif CACGTG (Williams *et al.*, 1992) first defined in the promoter of the light-regulated ribulose bisphosphate carboxylase promoter (*rbc*S), has been implicated in the regulation of defence-related gene expression in response to ethylene (Somssich, 1994), intermediates of the phenylpropanoid pathway, namely CA and 4-CA (Loake *et al.*, 1992), and methyl jasmonate (Kim *et al.*, 1992). This motif is also found within the parsley CHS box II sequence (Schulze-Lefert *et al.*, 1989), where it is found to interact with at least one nuclear protein in response to uv irradiation. A similar role for this sequence, i.e.. in the mediation of uv-induced gene expression, has also been demonstrated for a number of other CHS genes (Wingender *et al.*, 1990; Lipphardt *et al.*, 1988).

Both parsley and *Antirrhinum* G-box sequences have been shown to be recognised by the *trans*-acting factor CG-1, while no interaction is observed between this protein and soybean G-box sequence (Staiger *et al.*, 1989). A number of other bZIP-like CPRFs (Common Plant Regulatory Factors) also bind this sequence motif (Armstrong *et al.*, 1992), as well as binding other ACGT-containing promoter elements, although with markedly different affinities.

A 10 bp motif, TCATCTTCTT, designated the TCA motif, found to be repeated several times in the barley glucanase promoter is also found in the promoter sequences of over 30 different stress-inducible genes including glucanases, chitinases, PR1a and b, osmotin, CHS, 4-CL, *wun*1, *win*1 and *pin*II (Goldsbrough *et al.*, 1993). This sequence has been implicated in the quantitative determination of basal levels of gene expression observed, and possibly in elicitor-mediated induction of gene expression (van de Löcht *et al.*, 1990), while co-operation with other promoter elements is required for tissue-specific expression (Hauffe *et al.*, 1992). The TCA motif is recognised by the 40 kDa nuclear protein TCA-1 whose binding activity increases dramatically following exposure to SA (Goldsbrough *et al.*, 1993).

The AGC-box sequence, AGCCGCC, is conserved in virtually all chitinase and glucanase promoters studied (Ohme-Takagi and Shinshi, 1990; Hart *et al.*, 1993), and has been found to bind specific leaf nuclear proteins (Hart *et al.*, 1993). A 61 bp fragment from the basic tobacco glucanase gene containing two copies of this sequence and one sequence identical at 6 out of 7 bp is capable of functioning as an enhancer in *N. plumbagnifolia* protoplasts (Hart *et al.*, 1993); point mutations of all three boxes abolished activity. The AGC-box does not appear to function as an enhancer in the bean system, however, since deletion of a region containing this element has no effect on gene expression (Roby *et al.*, 1991).

An 11 bp motif, CTAATTGTTTA, has been shown to be functionally relevant within the context of the parsley PR2 promoter (Korfhage *et al.*, 1994). This sequence element is specifically bound by factors in nuclear extracts of both elicitor-treated parsley and *Arabidopsis* material. For both plant species full-length cDNAs have been isolated, the corresponding transcripts of which encode proteins that interact with this target sequence.

Sequence analysis of these cDNAs has identified the encoded proteins as homeodomain proteins and transient assay studies suggest a functional role of the homeodomain protein target sequence interaction in the regulation of PcPR2 gene expression.

1.4 Scope of this thesis.

It may thus be seen that the plant defence mechanism represents a highly sensitive, coordinated and plastic response, evolved as a direct consequence of the very nature of the plants existence itself. The function(s) of many of the involved gene products *in planta* has been defined, either by functional assays or by inference based on DNA or amino acid sequence homology. Analysis of the expression profiles of these genes reveals many striking similarities, primarily arising as a consequence of the involvement of the respective gene products in the defence response. These similarities are reflected on consideration of the 5' regulatory regions of these genes, where the presence of common sequence motifs within promoter sequences of genes displaying similar induction profiles suggests the operation of a common induction mechanism, leading, in turn, to a coordinate induction of gene expression.. The exact nature of this induction process and events mediating the transduction of a perceived stimulation event is currently the subject of much research.

In quite a large number of cases, however, despite observed similarities in expression profiles and regulatory elements, no conclusions regarding the possible function of respective gene products have been drawn. In such cases, the absence of significant homology to genes or gene products of known function has confounded attempts to characterise these proteins in detail. This thesis is concerned with the analysis of one such protein, AoPR1, an intracellular PR protein isolated from the monocot *Asparagus officinalis* here at Leicester (described further in section 3.1), and hence a member of the PR-10 group. In order to investigate the role(s) of this protein *in planta* a comprehensive analysis of the expression profile of this gene was undertaken, both in its native host, *Asparagus*, and, as an AoPR1 promoter-*gus* fusion, in transgenic tobacco. Characterisation of when, where and how this gene is induced will, it is

hoped, provide an insight into the nature of its involvement in the plant defence response, and, perhaps, facilitate determination of the function of this protein *in planta*.

Chapter 2:

Materials and Methods:

2.1 Glassware and Plasticware:

All glassware and plasticware used in experiments involving nucleic acids were siliconised and autoclaved at 121^{o}C, 15 p.s.i. for 20 min. Siliconisation was carried out by rinsing inner surfaces of glassware with 'Repelcote' water repellent, inverting thus treated glassware on a few layers of paper towel and leaving to dry in a fume hood. After siliconising, glassware was thoroughly rinsed with distilled water before autoclaving.

For work involving RNA samples glassware was treated with 0.01% diethyl pyrocarbonate (DEPC) after siliconisation, followed by autoclaving.

2.2 Chemicals and Biological Reagents:

Unless otherwise indicated, all chemicals and reagents used were obtained from BDH Chemicals Ltd. and were of analytical grade (AnalaR). Other materials were obtained from the designated sources as listed below:

Bovine serine albumin (BSA), polyvinyl pyrolidone (PVP), herring sperm DNA, xylene cyanol, bromophenol blue, ethidium bromide (EtBr) and ampicillin were obtained from Sigma Chemical Company. Augmentin was from Beecham Research. Caesium chloride was from Rose Chemicals Ltd. Ficoll 400 was from Pharmacia. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-ß-D-thiogalactopyranoside (IPTG) were purchased from Boehringer Mannheim GmbH.

Restriction endonucleases and DNA modifying enzymes were purchased from Northumbrian Biologicals Ltd., Boehringer Manheim GmbH, and New England Biolabs. *Taq* DNA polymerase with 10x reaction buffer for PCR reactions and dNTP stocks were purchased from Promega.

3MM paper, filter papers and GF/C glass fibre discs were from Whatman Ltd. 'Hybond-N' nylon filters, radioactive ^{32}P-dCTP (370 MBq/ml, specific activity = 100 TBq/mol) and ^{35}S-dATP (370 MBq/ml, specific activity>22 TBq/mol) were from Amersham International plc. 'Immobilon-P' was obtained from Millipore Corporation. X-ray films (Fuji RX100) were from Fujimex. High gelling temperature-, and low melting point- agarose were from Sigma.

Yeast extract and Bacto-tryptone were purchased from Oxoid. MS (Murashige and Skoog) salts were from Imperial Laboratories (Europe) Ltd.

2.3 Bacterial strains and cloning vectors:

2.3.1 Bacterial strains:

E. coli **strains:**

- XL1-blue: (Stratagene, La Jolla, California, U.S.A.)

*end*A1, *hsd*R17, (r_K^-, m_K^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, (*lac*-), [F', *pro*AB, *lac*I^{q}ZΔ M15, Tn10, (*tet*^{r})].

- HB101::pRK2013: (Ditta *et al.*, 1980)

Agrobacterium tumefaciens **strains:**

- LBA4404: Binary vector host strain, *rif*^{r}. (Hoekema *et al.*, 1983)
- PGV2260: Binary vector host strain, *rif*^{r}. (Deblaere *et al.*, 1985)

2.3.2 Cloning vectors:

Plasmids:

- pBluescript II SK$^+$: (Stratagene) A multi-purpose vector derived by replacing the pUC19 polylinker with a synthetic polylinker containing twenty-one unique restriction sites. This plasmid carries sequences encoding the N-terminal fragment of ß-galactosidase derived from *E.coli* which flank the polylinker. The synthesis of this fragment is induced by the presence of IPTG and is capable of complementation with a defective form of ß-galactosidase encoded by the host strain, e.g. XL1-Blue. On exposure to IPTG both fragments of the

enzyme are synthesised and blue colonies are formed when plated on medium containing the chromogenic substrate X-gal. Interruption of the polylinker site by the introduction of foreign DNA inactivates the N-terminal fragment of the enzyme giving rise to white colonies. Possession of the f1 origin of replication from the f1 filamentous phage allows for ss DNA rescue upon co-infection with helper phage. pBluescript II also carries an inducible *lac* promoter upstream from a *lacZ* coding sequence thus allowing for the production of fusion proteins upon induction with IPTG. The presence of an ampicillin resistance marker enables selection of bacteria carrying this plasmid when grown in medium containing 100 μg/ml ampicillin.

◦ pBI101.1: (Clontech) A general purpose binary vector for constructing gene fusions to the ß-glucuronidase (GUS) coding sequence. pBI101.1 was made by the ligation of the *gus* coding sequence to the 5' end of the *nos*(A)$^+$ site in the polylinker of pBin 19 (Jefferson *et al.*, 1987).

2.4 Growth, maintenance and storage of bacterial cultures:

2.4.1 Growth of bacterial strains:

Aliquots of bacterial media containing the required concentration of antibiotic were inoculated with a single colony of bacteria and incubated overnight at the appropriate temperature with constant agitation.

2.4.2 Storage of bacterial cultures in solid media:

Bacteria were stored for periods of up to three weeks at 4^{o}C on sealed agar plates containing the appropriate antibiotic. For storage of up to two years cultures were preserved as stabs prepared as follows:

Glass vials (2-3 ml) were prepared by filling with LA (2.22.2) until 2/3 full and autoclaved. Having allowed the media to cool a single, well-isolated colony of bacteria was inoculated

into the agar using a sterile inoculating needle, the vial sealed and stored in the dark at room temperature.

2.4.3 Long-term storage of bacterial cultures:

Storage of bacteria for indefinite periods of time was achieved by mixing 750 µl of an overnight bacterial culture grown in LB (2.22.1) with an equal volume of 40% (v/v) glycerol in LB in a cryotube and flash-freezing in liquid nitrogen. Cultures thus treated were stored indefinitely at -80°C; when such cultures were required for use, an inoculum was obtained by scraping a sterile cocktail stick over the surface of the culture and grown up overnight in 5 ml 2x YT broth (2.22.6) with antibiotic selection. This reactivated culture was subsequently used as a starter innoculum for larger culture volumes.

2.4.4 Antibiotics used for bacterial selection:

Antibiotic	Selective concentrations for		Stock (mg/ml)	Solvent
	E. coli (µg/ml)	*A. tumefaciens* (µg/ml)		
Ampicillin	50.0	-	100.0	70% ethanol
Kanamycin	100.0	100.0	50.0	water
Rifampicin	100.0	50.0	10.0	methanol
Tetracycline	12.5	-	12.5	ethanol

All water-soluble antibiotics were sterilised by filtration (0.22 µm pore size) and stored frozen at -20°C.

2.4.5 Preparation of competent cells:

A single colony of the *E. coli* strain XL1-Blue was inoculated into 10 ml of LB containing the antibiotic tetracycline at a concentration of 12.5 µg/ml. and grown overnight to stationary phase. The following day an aliquot of this culture was diluted 1:250 with LB and incubated

at 37°C with constant agitation for 3-4 hours until an OD_{600} of 0.5 was attained. Bacteria were recovered by centrifugation in a Sorvall RC-5B centrifuge at 4,000 rpm for 10 min. at 4°C having first pre-cooled the rotor. The resultant pellet was gently resuspended in a 1/2 volume of ice-cold 50 mM $CaCl_2$, and incubated on ice for 1 hour. Following this cells were spun down as before, resuspended in 1/10th volume ice-cold 50 mM $CaCl_2$, 20% glycerol (v/v) and dispensed into 500 µl aliquots. Cells were flash-frozen in liquid nitrogen and stored at -80°C.

2.5 Plant material:

2.5.1 Growth and maintenance of *Nicotiana tabacum* :

For the purposes of plant transformation the tobacco *N. tabacum* cv. SR1 was used. Plants were germinated on compost and maintained under standard glasshouse conditions.

2.5.2 Aseptic germination of tobacco seedlings:

Seed harvested from transgenic tobacco was surface sterilised in 1.5 ml eppendorf tubes using a 10% hypochlorite solution (Domestos). After a 10 min. incubation seed was washed thoroughly with sterile water and placed on germination medium (section 2.23) containing the selection antibiotic kanamycin at a concentration of 100 µg/ml. Plates were sealed with Nescofilm and incubated at 25°C.

2.5.3 Growth and maintenance of *Asparagus officinalis* :

A. officinalis seed (cv. Conovers' Colossal) was imbibed overnight at 4°C before planting in compost, and grown under controlled conditions. For the purposes of obtaining etiolated seedlings, seed was scattered on vermiculute, germinated in the dark at 25°C, and maintained under these conditions until they had reached the required age for experimental use.

2.5.4 Isolation of asparagus mesophyll cells:

Mesophyll cells of the monocot *Asparagus officinalis* were isolated from cladode material according to the method of Paul *et al.*, 1989.

A. officinalis seedlings at approximately six weeks post germination were harvested and cladode material stripped from the fronds. After sterilising in 10% bleach (Domestos) for approximately 15 min. and washing with copious amounts of sterile tap water, the cladode material was placed in a mortar and ground gently in a small volume of sterile tap water to release the individual mesophyll cells. The resultant cell suspension was then filtered through a 64 μm mesh filter to remove any debris. Isolated mesophyll cells were recovered by centrifugation at 800 rpm for 2 min. in a Sorvall RT6000B centrifuge, resuspended in 100 ml of sterile tap water, and the centrifugation repeated. After carefully removing the supernatant by aspiration, the pellet of cells was resuspended gently in 80 ml of asparagus medium and subjected to a further centrifugation step. The resultant pellet was resuspended in asparagus medium (section 2.23.1) to give a final cell density of 2.5×10^5 viable cells per ml. Cell counts were estimated using a haemocytometer, and viable cells visually identified as those having intact cytoplasmic contents. Sterile glutamine was then added to the cell suspension to give a final concentration of 1 mg/ml. The final cell suspension was then aliquoted in 10 ml volumes into sterile petri dishes which were then sealed with Whatman film, and incubated in the dark at 25°C on a gently shaking platform so as to maintain constant aeration. Visual screening of plates on a microscopic level allowed detection of infection and contamination. Such plates were rejected, while healthy cell populations were harvested by centrifugation at 1,000 rpm for 10 min. Harvested cells were either used immediately in experimental analysis or flash-frozen in liquid nitrogen and stored at -80°C.

2.6 Extraction of nucleic acids from bacteria:

2.6.1 Small-scale alkaline-lysis plasmid preparation:

Recovery of plasmid DNA from 1-1.5 ml cultures of bacteria was carried out essentially as described in Sambrook *et al.* (1989).

1.5 ml of an overnight culture of *E.coli* was centrifuged at top speed for 5 min. in a mocrocentrifuge (MSE) to pellet the bacterial cells. After removing the supernatant by aspiration cells were thoroughly resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0). 200 µl of solution II (0.2 M NaOH, 1% SDS) were then added and the contents of the tube gently mixed by inversion to lyse the cells. Following addition of 150 µl of solution III (3 M potassium acetate, 2 M glacial acetic acid) the tube was gently shaken to ensure adequate mixing and subjected to centrifugation at top speed in a bench top centrifuge for 5 min. After phenol-chloroform extraction of the supernatant, nucleic acids were precipitated from the upper aqueous phase by the addition of 2.5 volumes of ice-cold ethanol, recovered, dried and resuspended in 20-30 µl sterile distilled water. 2-3 µl of this final suspension were routinely used in restriction digests.

Since this method does not remove RNA digests were routinely carried out in the presence of 0.1 vol. 0.5 mg/ml RNase.

2.6.3 Large-scale alkaline lysis preparation of bacterial nucleic acids:

Large-scale plasmid preparations were routinely carried out in the extraction of nucleic acids from bacterial cultures of 500 ml volume according to the method of Sambrook *et al.* (1989). For the extraction of plasmid from cultures of smaller volume sizes this procedure was scaled down to the appropriate volumes but otherwise remained essentially as described below:

500 ml of an overnight culture (stationary phase) was spun down in a Sorval RC-5B centrifuge at 10,000 rpm for 10 min. at room temperature. After pouring off the supernatant the bacterial pellet was resuspended in 20 ml of solution I, 40 ml of solution II added to lyse

the cells, and genomic DNA and protein precipitated by the addition of 30 ml of solution III followed by a gentle yet thorough agitation of the mixture. A crude nucleic acid preparation was obtained by centrifuging this mixture at 10,000 rpm for 5 min. and filtration of the supernatant through polyallomer wool. Nucleic acids were precipitated by the addition of an equal volume of propan-2-ol to the filtrate and a further centrifugation at 10,000 rpm for 5 min.. After resuspension of the nucleic acid pellet in 3 ml sterile distilled water, 3 ml of 10 M LiCl were added in order to precipitate contaminating RNA which was then removed by centrifugation at 10,000 rpm for 10 s. The supernatant was transferred to a fresh tube and plasmid precipitated using 1 volume of propan-2-ol. Following incubation at room temperature for 5 min., the plasmid was recovered by centrifuging at 10,000 rpm for 5 min., dried thoroughly in a vacuum dessicator, resuspended in 500 µl sterile distilled water and transferred to a 1.5 ml eppendorf tube. After treating with RNase to remove any remaining contaminating RNA molecules, high molecular weight nucleic acids were precipitated with an equal volume of 1.6 M NaCl, 13% (w/v) PEG 6,000, and recovered by centrifugation at top speed in a microcentrifuge for 10 min. at 4°C. The resulting pellet was resuspended in 200 µl sterile distilled water and phenol / chloroform extracted twice. Plasmid DNA was finally precipitated with 10 µl 10M ammonium acetate and 2.5 volumes ethanol, pelleted by centrifugation, washed in 70% ethanol, dried and resuspended in 200 µl water. Nucleic acid concentration was estimated by spectrophotometric analysis (section 2.8.1).

2.6.4 Preparation of caesium chloride-purified plasmid:

Cells from a 500 ml overnight culture of the appropriate bacterium were harvested and lysed as in section 2.6.3. Subsequent caesium chloride-purification of recovered nucleic acids was carried out as described in Sambrook *et al.* (1989).

Following precipitation of the crude nucleic acid extract and resuspension of recovered DNA and RNA in 10 ml of sterile, distilled water solid CsCl was added at a concentration of 1 g CsCl per 1 ml of DNA solution. The solution was gently heated to 30°C to facilitate dissolution of the CsCl. In order to allow visualisation of nucleic acids, 0.8 ml of a 10 mg/ml

stock of ethidium bromide was added per 10 ml of DNA/CsCl solution and the solution mixed immediately the final density being adjusted to 1.55 mg/ml. The above solution was then centrifuged at 8,000 rpm for 5 min. at 4°C. A furry scum was observed to appear at the top of the tube, which comprised ethidium bromide/bacterial protein complexes. The underlying clear, red solution was transferred to a 15 ml Beckman Quickseal tube, balanced with light paraffin oil, and heat sealed. Centrifugation was carried out for 24 h at 20°C in a Beckman ultracentrifuge using a VTi 75 rotor at 55K. Following centrifugation, visualisation of the tubes using an ultra-violet lamp revealed an upper band corresponding to chromosomal and nicked circular plasmid DNA and a lower band corresponding to closed circular plasmid. Any residual protein was observed to aggregate at the CsCl solution/oil interface, while ethidium bromide/RNA complexes formed the pellet. The plasmid band was carefully removed using a wide gauge syringe, having first pierced the top of the tube to release any vacuum which may have built up during centrifugation. The ethidium bromide was removed from the sample by extraction with an equal volume of water-saturated 1-butanol. After vortexing and centrifugation, the lower, aqueous phase was re-extracted several times until the pink colour had completely disappeared. Dilution of the resulting DNA/CsCl solution 3-fold with distilled water allowed the nucleic acids to be precipitated using 2 volumes of ice-cold ethanol. Plasmid DNA was recovered by centrifugation following incubation for 15 min. at 4°C. The final plasmid pellet was vacuum dried, resuspended in 200 µl of water and spectrophotometrically analysed to determine concentration.

2.7 Extraction of plant nucleic acids:

2.7.1 Isolation of plant genomic DNA:

Genomic DNA was isolated from plant material (approximately 5 g) according to the method of Shirsat (1984).

Leaf material was frozen in liquid nitrogen and ground thoroughly into a fine powder using a pre-cooled mortar and pestle. The following were then added in quick succession: 5 ml homogenising buffer (0.1 M NaCl, 0.025 M EDTA, 2% SDS), 2.5 ml 5 M sodium

perchlorate, 5 ml Tris-equilibrated phenol and 5 ml chloroform. The mixture was then transferred to a 100 ml conical flask and incubated at 4°C for 1 h with constant, gentle agitation. After transferring the mixture to a 30 ml Corex centrifuge tube, centrifugation was carried out at 10 K, 4°C for 30 min. The upper aqueous phase was then extracted with an equal volume of chloroform:octanol 24:1 (v/v). Nucleic acids were recovered by spooling following ethanol precipitation of the aqueous phase, and transferred to a 15 ml Corex tube before washing once with 70% ethanol. Resultant pellets were dried and resuspended in 2-5 ml of TE. Contaminating proteins were removed by treating with 25 µl per ml of nucleic acid suspension of a 20 mg/ml stock of pronase and incubating at 37°C for 1 h. Treating with 5 µl of 20 mg/ml RNase at 37°C for 15 min. ensured a significant reduction in the amounts of contaminating RNA. Following extraction with chloroform:octanol nucleic acids were ethanol precipitated using 1/10th volume of 5 M ammonium acetate/100 mM magnesium acetate pH 5.6 and 2.5 volumes of ice-cold ethanol. The final nucleic acid pellet was dried and resuspended in 50 µl TE.

2.7.2 Extraction of plant DNA for PCR analysis:

A small piece of leaf material was removed, placed in an eppendorf tube and frozen on dry ice. Having ground this material thoroughly using a micro-homogeniser 400 µl of extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added and the tube vortexed for 30 s. After centrifugation for 5 min. at top speed in a microcentrifuge 300 µl of the supernatant were removed to a fresh tube and nucleic acids precipitated by the addition of an equal volume of isopropanol. Following incubation at room temperature for 2 min., the nucleic acids were recovered by centrifugation for 10 min., dried in a vacuum dessicator and resuspended in 100 µl of sterile, distilled water. 5-7 µl were routinely used in PCR analysis.

2.7.3 Extraction of total RNA from plants:

Total RNA was isolated from plant material using the method of Covey and Hull (1981).

Plant material was ground into a fine powder with liquid nitrogen plus an equal volume of acid-washed sand in a pre-cooled mortar and pestle. RNA extraction buffer (6% 4-amino-salicylic acid, 1% Tri-isopropyl napthalene (TNS) , 6% phenol, 50 mM Tris-Cl pH 8.4) was added (2 ml per gram fresh weight of tissue) and the material ground further. The resultant powder was transferred into 50 ml Sarstedt polypropylene tubes and allowed to thaw. An equal volume of phenol-chloroform was added and the tubes shaken well before centrifugation at 3,600 rpm for 10 min. The upper, aqueous phase was removed to a fresh tube and re-extracted until all precipitated protein at the interface was removed. At this stage the aqueous phase was transferred to a 30 ml siliconised corex tube and ethanol precipitated by the addition of 1/20 th volume 4 M sodium acetate, 2.5 volumes of ethanol and incubating at -20^{o}C for 2 h. Total nucleic acids were recovered by centrifugation at 10,000 rpm for 20 min. at 4^{o}C in a Sorvall RC-5B centrifuge. The resultant pellet was dried and dissolved in 0.5 ml water per gram of tissue.

RNA was differentially precipitated by incubation on ice for 1 h following the addition of 3 volumes of 4 M sodium acetate. Recovered RNA was dissolved in sterile, distilled water and ethanol precipitated at -70^{o}C after which the pellet was washed with 70% ethanol, air-dried and resuspended in a final volume of 200 µl.

2.8 Nucleic acid quantitation:

2.8.1 Spectrophotometric analysis of nucleic acids:

Concentrations of DNA (genomic and plasmid) and RNA were determined using the absorbance properties of nucleic acids. Both molecules absorb light at a wavelength of approximately 260 nm, while any contaminants will shift the absorbance peak from this wavelength. For the purposes of spectrophotometric analysis a Perkin-Elmer dual beam diode-array spectrophotometer (Perkin-Elmer Lambda 5 UV/VIS) was routinely used.

Samples were scanned over a wavelength range from 200-300 nm, and nucleic acid concentration determined by measurement of the absorbance value at 260 nm. The ratio of $Abs._{260nm}$:Abs_{280nm} gave an estimate of the purity of the sample.

2.9 DNA manipulation and modification:

2.9.1 Restriction digests:

Endonuclease digestion of plasmid and genomic DNA was routinely carried out using a 10-fold excess of enzyme. Reaction mixes containing the DNA to be digested, the relevant endonuclease at a dilution of no more than 1/10th the final reaction volume and 1/10th volume of the appropriate buffer supplied with the enzyme were incubated according to the manufacturers instructions for, generally, 1-2 hours.

When carrying out a double digest i.e. digesting with two different restriction endonucleases, was desired the enzymes were diluted in the reaction mix such that the combined enzyme volume did not exceed 1/10th the final reaction volume. The choice of buffer was such that each enzyme was capable of at least 50% activity. Where this was not possible the DNA was cleaved using one enzyme first, extracting this digest with phenol-chloroform followed by an ethanol precipitation of the upper aqueous phase. The resultant pellet was resuspended in an appropriate volume of sterile distilled water, and treated with the second restriction enzyme.

2.9.2 End-filling of restriction enzyme-generated 5' overhangs:

The cloning of fragments containing 5' overhangs resulting from restriction digestion into blunt-ended sites within the chosen vector necessitated the end-filling of these overhangs. This was achieved by the addition of 1/10th volume of 2 mM dNTP mix and 10 units of Klenow to the restriction mix immediately following digestion. The mixture was then incubated at room temperature for approximately 30 minutes.

2.9.3 Removal of 3' restriction enzyme-generated overhangs:

Removal of 3' restriction enzyme-generated overhangs was routinely achieved using T4 DNA polymerase which possesses both 3'-5' exonuclease and 5'-3' polymerising activity while lacking 5'-3' exonuclease activity. T4 DNA polymerase has been found to exhibit approximately 50% of maximal activity in all buffers routinely used with restriction enzymes, and hence it is possible to carry out the reaction by the addition of T4 DNA polymerase directly to the digestion mixture together with high concentrations of the four dNTPs.

DNA digestion was generally carried out in a reaction volume of 20 µl. Following digestion, 1 µl of a solution of dNTPs each at a concentration of 2 mM and 1-2 units of T4 DNA polymerase per µg DNA was added and the mixture incubated at 12°C for 15 min. The T4 DNA polymerase was them inactivated either by incubation at 75°C for 10 min. or by phenol-chloroform extraction of the reaction mix after the addition of 1 volume of sterile, distilled water.

2.9.4 Dephosphorylation of vector DNA:

The probability that a single-cut vector would re-ligate due to the presence of compatible ends was significantly reduced by the removal of the phosphate groups present on the 5' termini of the cut DNA. This was achieved by the use of calf intestinal alkaline phosphatase.

Following digestion of the vector with the appropriate restriction enzyme, the digest mix was phenol-chloroform extracted, ethanol precipitated, dried, and resuspended in a small volume of sterile, distilled water. 10x CIP buffer (100 mM Tris-Cl pH 8.5, 10 mM $MgCl_2$, 10 mM $ZnCl_2$) was added to a final concentration of 1x. One unit of the phosphatase enzyme was added and the mix incubated for 30 min. at 37°C. The dephosphorylated vector was then re-extracted with phenol-chloroform, ethanol precipitated, dried and resuspended to a final concentration of approximately 100 ng/µl in distilled water.

2.9.5 Generation of nested sets of deletions with Exonuclease III:

The generation of a 5' deletion series of the AoPR1 promoter using exonuclease III was achieved using the method of Sambrook *et al.* (1989).

Plasmid DNA (10 μg) containing the target sequence was digested with two restriction enzymes that cleave uniquely in the polylinker towards the 5' terminus of the target sequence. That enzyme cleaving nearer the target sequence generates a blunt end or a 5' overhang, while the other enzyme generates a 3' overhang. Digestion was first carried out using the enzyme that generates the blunt end or 5' overhang, the buffer adjusted, and the second enzyme added. Following restriction the DNA was purified by phenol/chloroform extraction, ethanol precipitated and resuspended in 60 μl exonuclease III buffer (0.66 M Tris-Cl pH 8.0, 66 mM $MgCl_2$). Exonuclease III digestion was carried out by the addition of 150 units of enzyme per picomole of recessed 3' termini , followed by incubation at 37°C for increasing lengths of time. At 30 s intervals a 2.5 μl aliquot was removed, added to a tube containing S1 reaction mix (172 μl H2O, 27 μl 10x S1 buffer, 60 units Nuclease S1) and incubated for 30 min. at 30°C to remove exposed single stranded DNAt. S1 buffer contains 2.25 M NaCl, 0.3 M potassium acetate pH 4.5, 50% glycerol, 1.8 mM $ZnSO_4$. Following this 1 μl S1 stop mixture (0.3 M Trizma base, 50 mM EDTA pH 8.0) was added and tubes transferred to ice prior to electrophoretic analysis of a sample aliquot from each time point. Samples containing DNA fragments of the desired size were pooled, 1 μl Klenow mixture (20 μl H2O, 6 μl 1 M $MgCl_2$, 3 μl 0.1 M Tris-Cl pH 7.6, 3 units Klenow fragment of *E. coli* DNA polymerase I) added per 10 μl pooled samples, and incubation carried out for 5 min. at 37°C in order to remove any protruding 3' termini from the digested DNA. A solution (1 μl) of dNTPs, each at a concentration of 0.5 mM, was then added and incubation continued for a further 15 min. Plasmids were then re-ligated by the addition of ligase buffer and enzyme as described in section 2.9.9 and introduced into *E. coli via* calcium chloride-mediated transformation methods (section 2.9.10).

Optimisation of incubation conditions for exonuclease III digestion were determined by incubation of 2-5 μg target DNA plasmid in the presence of varying concentrations of exonuclease III at 37°C for increasing lengths of time, followed by electrophoretic analysis of digestion products.

2.9.5 PCR-mediated amplification of DNA:

PCR reactions were routinely carried out in a 20 μl volume, with a mineral oil overlay, as follows:

Template	
11x PCR buffer	1.8 μl
Primer 1 (10 μM stock)	2.0 μl
Primer 2 (10 μM stock)	2.0 μl
Taq polymerase (5U/μl)	0.3 μl
H_2O	to 20 μl

- 11x PCR Buffer:

2 M Tris-Cl pH 8.8	165 μl
1 M Ammonium sulphate	85 μl
1 M Magnesium chloride	33.5 μl
ß-mercaptoethanol	3.6 μl
10 mM EDTA pH 8.0	3.4 μl
each dNTP (100 mM stock)	75 μl
10 mg/ml BSA	85 μl
	676 μl final volume

Store at -20°C.

The PCR programme used was designed to allow optimum amplification of target sequences according to Innis *et al.* (1990).

Denaturation of template was achieved by heating to 95°C for 30 s.

The temperature and length of time required for primer annealing is dependent of the base composition, length, and concentration of the chosen primers. In general, an appropriate annealing temperature was considered to be 5°C below the true T_m of the primer. The T_m of each primer was determined as follows:

$$T_m \ (^{o}C) = [(A+T) \times 2] + [(G+C) \times 4]$$

In general, annealing will require only a short length of time; an annealing time of 30 s was routinely used.

Extension time depends on the length and concentration of the target sequence and upon temperature. Primer extensions were carried out at 72°C for varying lengths of time. An extension time of 1 min. at this temperature was considered sufficient for products of up to 2 kb in length.

2.9.6 Geneclean purification of DNA fragments:

Following restriction digestion of appropriate plasmids or PCR-mediated amplification of required sequences, fragments of interest were routinely purified from agarose gels using the Geneclean kit (BIO 101 Inc.). All the required solutions were provided by the manufacturers.

The desired band was excised from an ethidium bromide-stained gel and the gel slice placed in a 1.5 ml microcentrifuge tube. Having determined the weight of the gel slice three volumes of NaI solution (90.8 g NaI, 1.5 g Na_2SO_3 per 100 ml) were added and the gel slice incubated at 50°C until completely melted. To this was then added 10 µl of glassmilk. An additional 1 µl of glassmilk was added for each 0.5 µg of DNA above 5 µg in the gel slice. The tube was then mixed well and incubated on ice for approximately 30 min. to ensure adequate binding of the DNA to the silica matrix. The glass beads were pelleted by centrifuging the tube for 5 s at top speed in a microcentrifuge and the NaI supernatant discarded. The pellet was resuspended in 300 µl of ice-cold NEW wash (50% ethanol, 0.1 M NaCl, 10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0) and spun for 5 s as before. Discarding the supernatant, the wash procedure was repeated twice more. After the supernatant from the third wash was removed the tube

was spun again for a few seconds and all remaining traces of liquid carefully removed. In order to elute the DNA from the glass beads the pellet was resuspended in 10 µl of distilled water and incubated at 55°C for 5 min. After centrifuging for 30 s the supernatant containing the eluted DNA was carefully removed and placed in a fresh tube. The elution procedure was repeated once more to ensure that all the DNA had been recovered from the silica and this second eluant added to the first. An aliquot of eluant was electrophoresed on a TAE/agarose gel in order to estimate quantity and quality of the recovered DNA. The remaining aliquot was stored at -20°C until required.

2.9.7 Purification of DNA fragments by "freeze-squeeze":

An alternative to the Geneclean method of purification of DNA fragments from agarose gels is the "freeze-squeeze" approach. In this method the band of interest was excised from the gel and placed over some siliconised polyallomer wool in a 0.5 ml eppendorf prepared by piercing a hole in the bottom using a needle. This eppendorf was then placed into a 1.5 ml tube and incubated at -20°C for 15 min. Once frozen the tubes were centrifuged at top speed in a bench top centrifuge for 15 min. and the resulting eluant ethanol precipitated to recover the fragment of interest.

2.9.8 Ligations:

100 ng of digested, dephosphorylated vector was mixed with a sufficient concentration of insert to ensure a three-fold excess of insert ends. The concentration of ends was determined as follows:

$(2 \times 10^6) / (660 \times \text{number of bases}) = \text{pmole ends} / \mu g$ double-stranded linear DNA

To the vector/insert mix was added 1/5th final volume of ligase buffer (0.25 M Tris-Cl pH 7.6, 50 mM $MgCl_2$, 50 mM DTT, 250 µg/ml BSA) and 1-10 units of T4 DNA ligase. Sterile, distilled water was added to make a final volume of 10-50 µl, and the mix incubated either at 37°C for 1 h or overnight at 16°C.

Blunt-ended ligations were essentially carried out as above, but with the addition of 1/10th volume of 10 mM hexamine cobalt chloride to the reaction before adding the ligase. 1-2 minutes following addition of the enzyme 1/5th volume of 100 mM NaCl was added. These conditions were found to greatly enhance the ligation efficiency in blunt-ended ligation reactions.

2.9.9 *E. coli* transformation using calcium chloride-competent cells:

Approximately 10-100 ng plasmid was added to 100 µl of transformation-competent *E. coli* XL1-Blue cells and incubated on ice for 30 min. Cells were then heat-shocked at 42°C for 2 min. 1 ml of LB, pre-warmed to 37°C, was added and the cells incubated at 37°C for 1 h. Following recovery of the cells by centrifugation for 5 min., cells were gently resuspended in 100 µl LB and spread on LA plates containing the appropriate antibiotic for selection of transformed cells. Where selection on a colorigenic basis was appropriate 50 µl 250 mg/ml X-gal (made up in dimethyl formamide) and 50 µl 0.5 M IPTG was added to each plate by spreading on the surface prior to the application of the cell suspension. Plates were incubated overnight at 37°C.

2.10 DNA sequencing:

2.10.1 Preparation of single-stranded sequencing template for manual sequencing:

Single-stranded sequencing template was prepared over a period of two days as follows:

A single colony of XL1-Blue harbouring the pBluescript II plasmid containing the DNA fragment to be sequenced was inoculated into 5 ml 2x YT containing 50 µg/ml ampicillin and 5µl helper phage (VCS M13). The culture was incubated for 2 h at 37°C. After this time had elapsed kanamycin was added to a final concentration of 50 µg/ml and the culture incubated at 37°C overnight.. The following day the culture was subjected to 10 min. centrifugation at 3,600 rpm. 1 ml of supernatant was then collected in a 1.5 ml eppendorf tube and centrifuged for a further 10 min. to ensure removal of all cells. Following transfer of the supernatant to a fresh eppendorf tube, 200 µl 20% PEG 6,000 / 2.5 M NaCl was added, the tube inverted a few

times to ensure adequate mixing of the contents, and the mixture incubated at room temperature for 10 min. After centrifugation for 10 min. the supernatant was discarded and the pellet centrifuged once again to remove any remaining PEG solution. The pellet was then resuspended in 100 µl distilled water and phenol extracted. This extraction step was followed by a chloroform extraction of the aqueous phase and recovery of single-stranded DNA by precipitation using 1/10 th volume 10 M NH_4Ac, 2.5 volumes ethanol. After centrifugation the DNA pellet was air-dried completely and resuspended in 20 µl sterile, distilled water. A 5 µl aliquot was analysed on a TAE/agarose gel.

2.10.2 Preparation of double-stranded sequencing template for manual sequencing:

Plasmid DNA was routinely used as double-stranded sequencing template. 10 µg of plasmid DNA was diluted to a final volume of 10 µl and denatured for 5 min. at room temperature following the addition of 2 µl of 2 M NaOH. The NaOH was neutralised by adding 4 µl of 5 M ammonium acetate pH 4.8, and the DNA precipitated upon addition of 3 volumes of ethanol and incubation at -80°C for 10 min. After centrifugation, the pellet was washed in 70% ethanol air-dried, and resuspended in 14 µl of distilled water.

2.10.3 Preparation of double-stranded template for automated sequencing:

Double-stranded template was prepared for automated sequencing reactions using a modified alkaline-lysis procedure (Applied Biosystems; Taq DyeDeoxy Terminator Cycle Sequencing Kit).

A 1.5 ml aliquot of an overnight culture of bacteria harbouring a plasmid containing the sequence of interest was centrifuged at top speed in a microcentrifuge to pellet the cells. Having resuspended the cell pellet in 200 µl of solution I, 300 µl of solution II were added and the tube gently inverted to ensure adequate lysis of the cells. The solution was neutralised by the addition of 300 µl of solution III and incubating on ice for 5 min. Cell debris was removed by centrifuging for 10 min. at room temperature and the supernatant transferred to a fresh tube, treated with RNase A at a final concentration of 20 µg/ml at 37°C

for 20 min. After the RNase A treatment, the supernatant was extracted twice with 400 µl of chloroform. DNA was precipitated by adding an equal volume of 100% isopropanol and immediately centrifuging the tube for 10 min. at room temperature. The nucleic acid pellet was washed with 500 µl of 70% ethanol and vacuum dried. Following this, the pellet was resuspended in 32 µl deionised water, and plasmid DNA precipitated by the addition of 8.0 µl of 4 µ NaCl and 40 µl of 13% $PEG_{8,000}$. After mixing the sample was incubated on ice for 20 min. and plasmid DNA recovered by centrifugation for 15 min. at 4°C in a microcentrifuge. The supernatant was removed and the pellet washed with 70% ethanol, dried, and resuspended in 20 µl of deionised water.

2.10.4 Manual sequencing of single- and double-stranded templates:

Sequencing was carried out using Sequenase 2.0 kits (USB Inc.) according to the manufacturers instructions. 7 µl of template (single- or double-stranded) prepared as in sections 2.10.1 and 2.10.2 were used in each reaction.

2.10.5 Automated sequencing of DNA template:

Automated sequencing was carried out using an Applied Biosystems 373A DNA Sequencer according to the manufacturers instructions.

2.10.6 Analysis of DNA sequences:

Computer-aided sequence analysis and database searches were carried out using the University of Wisconsin GCG programmes on an IRIX. (Genetics Computer Group, 1991).

2.11 Electrophoretic analysis of nucleic acids:

2.11.1 DNA electrophoresis:

Samples were routinely analysed by electrophoresis in agarose gels of 0.7-1.5% agarose concentration prepared in 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA, 0.5 µl per ml

ethidium bromide. The concentration of agarose chosen was dependent on the size of fragment being electrophoresed.

Gels were run at 100-150 V in 1x TAE buffer. Prior to electrophoresis, 1/5th volume of 5x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was added to the samples. DNA size standards were run alongside the samples in order to facilitate size determination of sample bands upon visualisation on a UV transilluminator (UVP Inc.). A photographic record of the gels was obtained using a video camera (UVP Inc.) and processor (Mitsubishi).

2.11.2 RNA formaldehyde gel electrophoresis:

RNA samples (6 µl final volume) were incubated at 65°C for 5 minutes in the following solution:

Deionised formamide	12.5 µl
10X MOPS buffer	2.5 µl
37% Formaldehyde	4.0 µl

After chilling on ice, 2.5 µl of RNA loading buffer (50% (v/v) glycerol containing 0.1 mg/ml bromophenol blue) were added and the sample electrophoresed on a 1.5% denaturing agarose gel made up by dissolving 1.5 g agarose in a solution containing 10 ml of 10x MOPS in 73 ml water. Once the agarose had been dissolved completely the mixture was allowed to cool to 50°C before adding 17 ml of a 37% (v/v) solution of formaldehyde and pouring the gel immediately. Electrophoresis was carried out in 1x MOPS. Visualisation of RNA samples was facilitated either by adding 0.5 µl of 1.0 mg/ml ethidium bromide solution to the sample prior to electrophoresis or, preferably, staining the gel with a dilute solution of ethidium bromide following electrophoresis.

2.11.3 Sequencing gels:

The following stock solutions were prepared and stored at 4°C until required:

- 40% acrylamide solution:

Acrylamide	380 g
N,N'-methylbisacrylamide	20g
Distilled water	to 600 ml.

- 10x TBE buffer:

Tris base	108 g
Boric acid	55 g
EDTA	5.8 g
Distilled water	to 1 litre

- 6% acrylamide/urea gel mix:

40% acrylamide mix	15 ml
10x TBE	10 ml
Urea	42 g
Distilled water	to 100 ml.

The above volume of gel mix was sufficient to pour one gel using the BioRad 20 cm x 40 cm x 0.5 mm gel kit.

The kit was assembled having first siliconised the top, glass plate, and sealed using 25 ml of the above gel mix polymerised by the addition of 200 µl of 10% ammonium persulphate solution made up in distilled water and 200 µl TEMED (N,N,N',N'-tetramethylethylenediamine). Once the plates had sealed adequately the remaining 75 ml of the gel mix was mixed with 750 µl 10% ammonium persulphate and 75 µl TEMED and poured carefully between the plates using a syringe. Care was taken to ensure that no air bubbles entered the gel. After inserting the comb the gel was allowed to polymerise at room

temperature for 1-2 h. Prior to loading samples the gel was pre-run at 2,500 V until a gel temperature of 50°C was attained. The running buffer used was 1x TBE and gels were routinely run at a voltage sufficient to maintain a constant temperature of 50°C throughout the gel.

Following electrophoresis, the gel kit was dismantled and the gel transferred to a piece of Whatman 3MM filter paper. This was then dried down at 80°C for 2 hours using a Bio-Rad gel drier, having first covered the gel in a sheet of ClingFilm.

2.12 Nucleic acid blotting:

2.12.1 Southern blotting:

The method employed in the transfer of electrophoresed DNA samples to nylon filters was modified after Southern (1975).

Following DNA electrophoresis samples were transferred to Hybond-N filters after being treated in the following manner:

Samples were depurinated by immersing the gel in 3 volumes of depurinating solution (0.25 M HCl) for 7 min. Denaturation was then carried out by transferring the gel to 3 volumes of denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min. with constant gentle agitation. After this time the gel was transferred to 3 volumes of neutralising solution (3 M NaCl, 0.5 M Tris-Cl pH 7.4) for 30 min. The gel was then transferred to a capillary blot system in order to effect transfer of the DNA to Hybond-N membrane.

2.12.2 Setting up a capillary blot:

A tray approximately 6" deep was filled with 20x SSC (3M NaCl, 0.3M tri-sodium citrate) and a layer of sponge placed in the dish. A layer of filter paper of the same dimensions as the sponge was placed on top of the sponge to act as a wick, drawing the buffer towards the gel. The gel was then placed onto the filter paper, ensuring adequate contact between the gel ad the filter paper and surrounded with cling film to prevent the buffer being absorbed directly

into the paper towels above. A sheet of Hybond-N membrane was cut to the exact size of the gel and placed on top of the gel, avoiding trapping any air bubbles beneath the membrane. Three sheets of 3MM paper cut to size and pre-wetted with buffer were then placed on top of the membrane, and a stack of paper towels placed on top of these. A glass plate was carefully placed on top of the paper towels and a weight balanced on this plate. Transfer was generally allowed to proceed overnight. After blotting the apparatus was carefully dismantled, taking care to mark the membrane with pencil so as to facilitate its orientation. Any adhering agarose was removed by briefly washing in 2x SSC before fixing the blot.

2.12.3 Northern blotting:

RNA gels did not require any further treatment following electrophoresis and so could be blotted directly using a capillary blot set up as described above (section 2.12.2).

2.12.4 DNA dot blots:

DNA samples were heated to 95°C and then chilled on ice before the addition of 1 volume of 20x SSC. Samples were spotted onto a Hybond-N membrane, pre-wetted with 10x SSC, in approximately 2 µl aliquot. The membrane was allowed to dry between each application. After all samples had been applied, the membrane was placed in denaturing solution for 5 min., transferred to neutralising solution for 1 min., and blotted dry with filter paper. Having allowed the membrane to air-dry, the DNA was fixed to the membrane as described in section 2.12.7..

2.12.5 RNA dot blots:

RNA samples were incubated at 65°C for 5 min. in three volumes of the following solution:

Formaldehyde (37%)	162 µl
10x MOPS buffer	100 µl

- 10x MOPS buffer:

  0.2M 3-[N-morpholino]-propane-sulphonic acid

  0.05M Na acetate pH 7.0

  0.01M Na_2 EDTA

One volume of cold 20x SSC was added after chilling the samples on ice. Samples were then spotted onto Hybond-N membrane which had been pre-wetted in 10x SSC, in 2 µl aliquots, allowing to dry between applications. The membrane was allowed to air-dry, and fixed as described above.

2.12.6 Colony blots:

Bacteria were grown overnight at 37°C on duplicate LA plates containing the appropriate antibiotic, which had been overlaid with a Hybond-N membrane. The filter was then carefully removed from the agar plate and floated on denaturing solution for 2 min. to lyse the cells. The filter was then gently transferred to neutralising solution for a further 2 min, after which it was allowed to air-dry for a few minutes before fixing the DNA to the membrane as described below

2.12.7 Fixing the blots:

Samples were fixed to Hybond-N filters by UV cross-linking in a Stratagene StrataLinker 2400 according to the manufacturers instructions.

2.13 Generation of radiolabelled probes:

2.13.1 Oligolabelling of double-stranded DNA fragments:

The method of Feinberg and Vogelstein (1984) was employed in the generation of labelled double-stranded DNA probes.

- 5X Oligolabelling buffer:

  250 mM Tris-Cl pH 8.0

  25 mM $MgCl_2$

  5 mM ß-mercaptoethanol

  2 mM each dATP, dGTP, dTTP

  1 M HEPES (pH 6.6 with 4M NaOH)

  1 mg/ml oligonucleotides.

  Store at -20^{o}C.

Oligolabelling of DNA probe stocks was carried out by mixing the following in a screw-cap microcentrifuge tube and incubating at 37^{o}C for 1 h.

5x oligolabeling buffer	3.0 µl
BSA (10 mg/ml)	0.6 µl
Klenow	0.6 µl
^{32}P-dCTP	1.0 µl
DNA	10 ng
Deionised water	to 15 µl.

The labelling reaction was terminated by the addition of 185 µl deionised water to the reaction mix.

2.13.2 Measuring nucleotide incorporation:

The efficiency of incorporation of ^{32}P during oligolabelling was determined as follows:

A 1 µl aliquot of labelling mix was precipitated along with 500 µl of carrier herring sperm DNA (10 mg/ml) by the addition of 125 µl of 50 % TCA and incubation on ice for 5 min. The nucleic acid precipitate was recovered by vacuum filtration through a GF/C filter and washed twice with 15 % TCA, once with IMS. After allowing the filter to air-dry the filter was subjected to scintillation counting and incorporation efficiency determined by comparison with a crude 1 µl aliquot of labelling mix spotted onto a similar filter.

2.14 Hybridisation and washing of filters:

2.14.1 Hybridisation of filters:

Filters prepared as described above were incubated for a minimum of 2 h in pre-hybridisation solution in order to block sites on the membrane to which the probe may potentially bind in a non-specific maner. DNA filters were incubated at 65°C, while RNA filters were incubated at 42°C. Generally sufficient prehybridisation solution was used to cover the filters to a depth of 2-3 mm.

- DNA prehybridisation solution:

20x SSPE	2.5 ml
10% SDS	0.5 ml
100x Denhardts	0.5 ml
Herring sperm DNA (10 mg/ml)	0.25 ml
Deionised water	to 10 ml.

- RNA prehybridisation solution:

Deionised formamide	50 ml
20x SSPE	30 ml
100x Denhardts	5 ml
10% SDS	4 ml
$PEG_{6,000}$	6 g
Herring sperm DNA (10 mg/ml)	2.5 ml

- 20x SSPE: 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA.
- 100x Denhardts: 2% BSA, 2% Ficoll 400, 2% PVP.

After sufficient time had elapsed, the labelled probe was added after first boiling for 5 min. and hybridisation allowed to progress overnight at the appropriate temperature with gentle, constant agitation.

2.14.2 Washing of hybridised filters:

Hybridised filters were routinely washed twice in wash A (3x SSC, 0.1% SDS) followed by at least one wash in wash B (0.5x SSC, 0.1% SDS). If a higher degree of stringency was required, then the above solutions were diluted appropriately.

2.14.3 Removal of bound probe from filters:

DNA probes were removed from filters by incubating the filters in approximately 500 ml of 0.1% SDS, which had been heated to boiling, until the solution had cooled to room temperature. This was generally sufficient to removal all bound probe. Removal of the probe was monitored by frequent assessment of the strip solution for radioactivity using a Geiger counter and filters judged to be sucessfully stripped when radioactive counts were no longer detectable.

2.15 Autoradiography:

2.15.1 Exposure of filters to X-ray film:

DNA and RNA filters were covered in saran wrap and exposed to Kodak X-ray film for the required length of time in sealed cassettes at -80^{o}C.

Sequencing gels were placed directly onto Kodak X-ray film in a sealed cassette and incubated for the required length of time at room temperature.

2.15.2 Development of autoradiographs:

X-ray film exposed as above was developed using a Cronex CX-130 automatic developer from DuPont.

2.16 *Agrobacterium* methods:

2.16.1 Conjugation of recombinant plasmids into *Agrobacterium*

Transfer of the recombinant plasmid of interest from *E. coli* to *Agrobacterium* was carried out using triparental mating essentially as described in Draper *et al.* (1988).

Single colonies of donor (i.e. containing the plasmid of interest) and helper (carrying the mobilisation and transfer functions) strains of *E. coli* were grown at 37°C overnight in 5 ml NB with selection. Similarly, a single colony of the recipient *Agrobacterium* strain was grown for 48 h at 27°C. Triparental mating was achieved by mixing 100 µl of each (donor, helper and recipient) and spreading on an NA plate with no selection This was then incubated at 27°C until a lawn of growth appeared. An inoculum was removed from this plate using a sterile inoculating loop and streaked out on an NA plate containing selective antibiotics both for *Agrobacterium* and for the presence of the introduced recombinant plasmid. After incubating for a further 48 h individual colonies of recombinant *Agrobacterium* were observed.

2.16.2 Extraction of nucleic acids from *Agrobacterium* :

Agrobacterium cells were pelleted by centrifugation and resuspended in 300 µl of distilled water. Cell lysis was achieved by the addition of 100 µl of 5% sarkosyl and 150 µl of Pronase E (5 mg/ml stock), followed by incubation at 37°C for 1 h. After phenol-chloroform extracting three times the supernatant was transferred to a fresh tube. 1/20th volume of 6 M sodium acetate and 3 volumes of ethanol were added to precipitate nucleic acids. After centrifuging for 10 min. at 4°C the resultant pellet was washed with 70% ethanol, air-dried and resuspended in 50 µl of sterile, distilled water. A 10 µl aliquot was generally used in restriction analysis.

2.17 *Agrobacterium* -mediated transformation of tobacco:

2.17.1 Tobacco transformation and regeneration:

Tobacco leaf disc transformation was essentially as described in Draper *et al.* (1988).

Tobacco leaves were surface-sterilised in a 10% hypochlorite solution (Domestos), followed by washing several times in large amounts of sterile tap water. Leaf explants of approximately 0.5 cm^2 were dissected from the leaf lamina taking care to avoid the mid-rib. These explants were then incubated in a 1/20 dilution of a mid- to late-log culture of the desired recombinant *Agrobacterium* strain in MSO (2.23.2) for 15-30 min. Thus inoculated explants were then transferred to antibiotic-minus MSD 4x2 (2.23.3) plates, the plates sealed with Nescofilm, and incubated at 25°C for two days. After this period of time had elapsed the explants were transferred to MSD 4x2 plates containing the bacteriostatic agent augmentin at a concentration of 400 µg/ml and kanamycin at 100 µl to select for transformed cells. Following a period of a few weeks in culture, explants begin to produce shoots as a result of the regenerative capacity of transformed cells on kanamycin-containing medium. Such shoots were aseptically excised from the explant taking care to avoid taking any callus material as well, and placed on solid MSO containing kanamycin and augmentin as above. This facilitates rooting of shoots, while maintaining selection. Once rooted, regenerated tobacco plantlets were planted in soil and grown under standard glasshouse conditions.

2.18 Screening transgenic plants:

2.18.1 PCR analysis of transgenic plants:

DNA extracted from transgenic tobacco (2.7.2) was analysed for the presence of the relevant transgene using PCR methodology. Aliquots of 5-10 µl of DNA extract were subjected to PCR analysis as in section 2.9.5, primer selection being dependent on the context of the sequence of interest within the binary vector. Annealing and extension times were selected appropriately.

2.18.2 Histochemical localisation of GUS activity:

GUS histochemical analysis of transgenic tobacco was carried out by incubating plant material in GUS histochemical buffer (50 mM sodium phosphate buffer pH 7.0, 0.1 mM potassium ferrocyanide, 0.1 mM potassium ferricyanide, 0.1 % Triton X-100, 0.1 % ß-mercaptoethanol) containing 10 µl of a 100mg/ml solution of the histochemical reagent 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) made up in DMF per ml of histochemical buffer at 37°C for the appropriate length of time. The length of time required for a blue colour to appear was dependent of the level of expression of the GUS gene in the transgenic. Generally staining was monitored regularly for up to four hours, after which staining was left to progress overnight. Material thus stained was cleared by transferring to ethanol an incubating at room temperature overnight. Samples were stored in ethanol indefinitely.

2.18.3 Fluorometric determination of GUS activity:

GUS activity was determined quantitatively according to a method modified from Draper *et al.* (1988).

Approximately 0.05 g of plant material, depending on the tissue type being analysed, was homogenised in 400 µl of GUS extraction buffer (GEB) (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10 mM ß-mercaptoethanol) and clarified by centrifugation at 13K for 10 min. Reactions were initiated by the addition of 20 µl of this extract to 180 µl of reaction mix (1 mM methyl umbelliferyl glucuronide (MUG) in GEB. Immediately after the addition of sample to reaction mix a 20 µl aliquot was removed and added to 180 µl of STOP (0.2 M Na_2CO_3 pH 11.2) in a microtitre plate (Dynatech) in order to stop the reaction at T=0 min. Similar aliquots were removed and thus treated at appropriate time points following reaction initiation, generally at T=20, 40 and 60 min. When all time points were taken fluorescence readings were obtained using a Dynatech MicroFLUOR plate reader and GUS activity determined by comparison with a standard curve constructed using increasing concentrations of 4-methyl umbelliferone (4-MU), the fluorogenic compound released upon the GUS-catalysed cleavage of MUG.

2.19 Protein extraction and quantitation:

2.19.1 Induction and recovery of fusion proteins from *E. coli* :

E. coli strains harbouring the plasmids pT7-7, containing a cDNA encoding the protein of interest, and pGP1-2 (section 3.2.1) were grown overnight at 30°C with constant agitation. The following day an aliquot of the culture was diluted and grown to $O.D._{600}$=0.6. Induction of cDNA expression was carried out by heat-shock treatment at 42°C for 30 min. followed by a recovery period of 30 min. at 30°C. 1 ml of cells thus treated were harvested by centrifugation and resuspended in 50 µl of 2x cracking buffer (100 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% glycerol). After boiling for 2-5 min. the cell suspension was spun to remove any cell debris and a 10 µl aliquot analysed on SDS-PAGE.

2.19.2 Extraction of protein from plant material:

Plant material was homogenised in approximately 1 volume of extraction buffer in a pre-cooled mortar and pestle at 4°C. The homogenate was then transferred to an appropriate volume centrifuge tube and clarified by centrifugation for 10 min. at 4°C. Following this the supernatant was transferred to a fresh tube and stored at 4°C. For extraction of protein from small amounts of material homogenisation was carried out in a 1.5 ml eppendorf tube using a micro-homogeniser.

2.19.3 Concentration of protein samples:

Dilute protein extracts were concentrated into an suitable working volume by freeze-drying. Samples were first dialysed overnight against a large volume of distilled water at 4°C. Following this samples were dispensed into appropriate aliquots and lyophilised overnight. Thus treated samples were resuspended in a small volume of distilled water and stored at 4°C for further use.

2.19.4 Protein quantitation:

Protein quantitation was carried out essentially as described by Bradford (1976).

A protein calibration curve was prepared using known concentrations of an appropriate protein over a range of 0 - 1.0 mg/ml as follows.

20 µl of protein solution was pipetted into each well of a flat-bottomed microtitre plate and 180 µl of Bradfords reagent (600 mg Coomassie R-250 (Serva Blue 250) in 1 l of 2% perchloric acid) added. Both solutions were mixed well. Protein samples prepared as previously described were treated in a similar manner; when assaying protein samples it was generally considered appropriate to assay a series of dilutions of each sample so as to obtain as accurate a measurement as possible. After all samples had been thus treated the microtitre plate was placed in a Dynatech MR5000 and absorbance measured at 595 nm. Protein concentration was determined by comparison of sample absorbance readings with those of the protein standards and expressed an mg/ml.

2.20 Electrophoretic analysis of protein samples:

2.20.1 SDS-PAGE analysis of protein samples:

The method used in electrophoretic analysis of protein samples is a modification of that described by Laemmli (1976).

One-dimensional electrophoresis of protein samples was carried out using the Bio-Rad Protean II xi kit for large-scale gels and the Bio-Rad mini-protean kit for small-scale gels. following the manufacturers guidelines.

Separation of proteins was routinely achieved by electrophoresis of samples through a 15% acrylamide, 0.375 M Tris pH 8.8 separating gel. In order to enhance the resolution obtained, the separating gel was overlain with a 4% acrylamide, 0.125 M Tris pH 6.8 stacking gel and samples applied directly to this.

• Denaturing gel:	7.5%	10%	12%	15%	20%
Dist. water	4.9 ml	4.1 ml	3.4 ml	2.4 ml	0.8 ml
Tris-Cl pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Acryl:bis (29:1)	2.5 ml	3.3 ml	4.0 ml	5.0 ml	6.7 ml
10% AMPS	37 µl	37 µl	37 µl	37 µl	37 µl
TEMED	3 µl	3 µl	3 µl	3 µl	3 µl
10% SDS	100 µl	100 µl	100 µl	100 µl	100 µl

- Stacking gel:

Distilled water	3.25 ml
1 M Tris-Cl pH 6.8	1.25 ml
Acryl:bis (29:1)	0.5 ml
10% SDS	50 µl
10% AMPS	15 µl
TEMED	5 µl

- Acryl:bis (29:1):

  30 g acrylamide, 0.8 g bis-acrylamide to 100 ml with distilled water.

Electrophoresis was generally carried out at 150 V for 3-4 hours for large-scale gels, and a similar voltage for 1-2 hours for smaller gels in 1x Tris-Glycine running buffer (25 mMTris, 250 mM glycine, 0.1% SDS).

2.20.2 Isoelectric focusing of protein samples:

The Bio-Rad Protean II xi kit, as above, was used in the IEF separation of proteins and the manufacturers guidelines followed. Generally a broad range of ampholytes (3-10) was used

in initial protein analysis. Once the approximate pI of the proteins of interest had been ascertained the ampholyte range was adjusted, the range chosen being dependent on the pI range of proteins to be separated. Electrophoresis was carried out for 2 h at 200 V, 2 h at 500 V and 16 h at 800 V.

2.20.3 2-dimensional analysis of protein samples:

A method derived from that of O'Farrell (1975) was employed in the two-dimensional electrophoretic analysis of protein samples.

Two-dimensional gel electrophoresis was carried by first subjecting protein samples to isoelectric focusing as in section 2.20.2. After extrusion, the rod gels were equilibrated in transfer buffer (0.07 M Tris-Cl pH 8.8, 3% SDS, 0.001% bromophenol blue,) for 2-5 min. Thus treated, rods were transferred to the second dimension, SDS-PAGE and electrophoresed as in section 2.20.1.

2.21 Staining and analysis of protein gels:

2.21.1 Coomassie-staining of protein gels:

Visualisation of protein samples on acrylamide gels was achieved by staining with Coomassie reagent (0.25% Coomassie R-250, 50% methanol, 10% acetic acid) for 3-4 h. Gels were then incubated in destain (50% methanol, 10% acetic acid) overnight with constant agitation in order to facilitate visualisation of discrete protein bands.

2.21.2 Blotting of protein gels:

Small-scale protein gels were generally transferred to Immobilon-P membranes using a Bio-Rad semi-dry blotter. Single gels were blotted at 30 mA for 1 h, while two gels could be transferred together at 130 mA for a similar length of time. The apparatus was assembled as follows:

Six pieces of Whatman 3MM filter paper were cut to the same dimensions as those of the gel to be blotted. Two of these pieces were soaked in anode 1 buffer (0.3 M Tris-Cl pH 10.4, 10% methanol), one in anode 2 buffer (25 mM Tris-Cl pH 10.4, 10% methanol), and three in cathode buffer (25 mM Tris-Cl pH 9.4, 40 mM 6-amino hexanoic acid, 20% methanol). The filter paper was layered onto the blotter anode by placing the two anode 1 pieces first followed by the single anode 2 piece. A piece of Immobilon-P membrane pre-wetted in methanol and rinsed in distilled water was then placed on top of the filter paper and the gel carefully placed onto this. Care was taken to ensure that no bubbles were trapped between the membrane and the gel. The three pieces of cathode buffer-soaked 3MM were then layered onto the gel and the apparatus completely assembled. Transfer was carried out as above.

For large-scale protein gels transfer was achieved using the Bio-Rad Transblot system as follows:

Having electrophoresed protein samples as in sections 2.20.1 or 2.20.3, gels were washed 2 x 10 min. in 0.1% SDS followed by one wash in transfer buffer (27 g sodium acetate in 4 l distilled water, pH 7.0 with acetic acid). The transfer apparatus was assembled by placing a sheet of 3 MM pre-wetted in transfer buffer on the cathode side of the cassette. The treated gel was then positioned on top of this, and a sheet of membrane (Immobilon-P pre-treated as above) layered on to the gel. One more sheet of pre-wetted 3 MM was finally placed on to the membrane and the cassette closed. Following assembly transfer was carried out in 3.5 l of transfer buffer at 30 mA overnight.

2.21.3 Ponceau staining of PVDF membranes:

Following transfer of protein samples to Immobilon-P membrane the efficiency of transfer was estimated by visualisation of transferred protein. Membranes were incubated with Ponceau stain (0.6% Ponceau-S, 1% acetic acid) for 5-10 min. before washing excess stain off with distilled water. Protein samples become visible as the stain is rinsed off. Remaining stain was removed by rinsing in TBS buffer.

2.21.4 *In situ* detection of protein using polyclonal antibodies:

Immunodetection of immobilised proteins was carried out as described in Worrall *et al.* (1993).

Membranes obtained as above were incubated in 4% Marvel made up in TBS-Tween (0.1% Tween-20 in TBS) for approximately 2 h. Binding of the primary antibody was carried out by the addition of the relevant amount of antibody to the membrane incubated in 1% Marvel in TBS-Tween and membranes incubated thus for a minimum of 2 h. Following this the membranes were washed twice in large volumes of TBS-Tween before being incubated with secondary antibody in a similar manner as with the primary. After approximately 2 h membranes were again washed in TBS-Tween. Blots were developed by incubating with 50 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate) (50 mg/ml in dimethyl formamide(DMF)) and 50 µl NBT (nitro-blue tetrazolium) (30 mg/ml in 70% DMF) in 5 ml of BCIP buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) until the desired development was achieved.

2.22 Bacteriological media:

- **Luria-Bertani (LB) medium (l^{-1}):** Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g. Adjust pH to 7.0 with 5 M NaOH prior to autoclaving.
- **LB agar (l^{-1}):** As for LB, but with the addition of 15 g/l agar prior to autoclaving.
- NZY **medium (l^{-1}):** NaCl 5 g, MgSO4.7H2O 2 g, yeast extract 5 g, caesin hydrolysate 10g. For solid NZY 15 g/l of agar were added to the above solution before autoclaving.
- NA **(nutrient agar):**NA was purchased from Difco and reconstituted according to the manufacturers instructions.
- NB **(nutrient broth):** NB was purchased from Difco and reconstituted according to the manufacturers instructions.

- **2x YT (l^{-1}):** Bacto-tryptone 16 g, Bacto-yeast extract 10 g, NaCl 5 g. Adjust pH to 7.0 with NaOH prior to autoclaving.

2.23 Plant media:

- **Asparagus medium:** The medium used in the culture of isolated asparagus mesophyll cells is modified from Nagata and Takebe (1971).

  Major salts (ml^{-1}): NH_4NO_3 825 mg, KNO_3 950 mg, $CaCl_2.2H_2O$ 220 mg, $MgSO_4,7H_2O$ 1233 mg, KH_2PO_4 680 mg, Na_2EDTA 37.3 mg, $FeSO_4.7H_2O$ 27.8 mg.

  Minor salts (ml^{-1}): H_3BO_4 6.2 mg, $MnSO_4.H_2O$ 22.3 mg, $ZnSO_4.H_2O$ 10.58 mg, KI 0.83 mg, $NaMoO_4$ 0.25 mg, $CuSO_4.5H_2O$ 0.025 mg.

  Other components: Thiamine 1.0 mg, NAA 1.0 mg, 6-BAP 0.3 mg, glutamine 1 g, myo-inositol 100 mg, sucrose 10 g/l. mannitol 30 g/l.

This medium was routinely made up by combining a number of stock solutions as follows:

A ($100\ ml^{-1}$): NH_4NO_3 8.25g, KNO_3 9.50 g. Add 10 ml/l medium.

B ($100\ ml^{-1}$): $CaCl_2.2H_2O$ 2.20 g. Add 10 ml/l medium.

C ($100\ ml^{-1}$): $MgSO_4.7H_2O$ 12.33 g, KH_2PO_4 6.0 g. Add 10 ml/l medium.

D ($100\ ml^{-1}$): Na_2EDTA 373 mg, $FeSO_4.7H2O$ 278 mg. Add 10 ml/l medium.

E ($100\ ml^{-1}$): H_3BO_4 62 g, $MnSO_4.H_2O$ 223 g, $ZnSO_4.H_2O$ 105.8 g, KI 8.3 g, $NaMoO_4$ 2.5 g, $CuSO_4.H_2O$ 0.25 g. Add 10 ml/l medium.

Thiamine: 20 mg/ml in water. Add 1 ml/l medium.

NAA: 20 mg/ml in 50% ethanol. Add 1 ml/l medium.

6-BAP: 30 mg/ml water + drop 1M HCl. Add 100 µl/l medium.

Glutamine: 2.35 g/100 ml water + drop 1 M HCl. Filter sterilise. Add 3.4 ml/80 ml media.

Sucrose: 10 g/l medium. Add dry.

myo-inositol: 100 mg/l medium..

Mannitol: 30 g/l medium.

All stocks were stored at -20°C.

Having combined the appropriate volumes of each stock, the pH of the solution was adjusted to 5.8 with KOH, the solution dispensed into 80 ml aliquots and autoclaved. Sterile glutamine was added immediately prior to use.

- **RMS (l^{-1}):** MS salts 4.71 g, B5 vitamins 5 ml, sucrose 30 g, NAA 1 mg, 2,4-D 0.1 mg, BAP 0.1 mg, MES to a final concentration of 3 mM. The above constituents were combined and dispensed into appropriate aliquot volumes before sterilising by autoclaving at 121°C for 15 min. at 15 p.s.i.

- **MSO (l-1):** MS salts 4.71 g, sucrose 30 g. pH 5.8 with KOH before autoclaving. For solid media agar was added at a concentration of 0.8 % prior to autoclaving.

- **MSD 4x2:** As MSO with the following additions: NAA 0.1 mg/ml, BAP 0.2 mg/ml

- **Germination medium (l-1):** MS salts 2.35 g, sucrose 5 g. These constituents were combined and agar added to a final concentration of 0.8% before autoclaving.

Chapter 3:

Characterisation of the DD1 family of proteins.

3.1 Introduction:

3.1.1 Use of *Asparagus* as a model system for the study of the dedifferentiation process:

As previously mentioned (section 1.3.1) limitations are imposed on the study of dedifferentiation due to the difficulty in obtaining sufficiently large populations of cells exhibiting synchrony of response to a particular stimulus inducing the dedifferentiation process, a phenomenon frequently associated with the manifestation of the wound response. Callus cultures, while providing such a population of cells, maintain some degree of cell-cell interaction due to the structure of the material. Callus cells are still, in effect, capable of transmitting signals to and receiving signals from their neighbours thereby reducing their potential to exist as fully undifferentiated cells. Protoplast cultures, already shown to be an enriched source of dedifferentiation-related gene expression, too are of limited potential. Their tendency to regenerate cell walls and re-enter the cell cycle after relatively short periods in culture means that they are restricted in the time span of gene expression one can investigate using this system. Further complications arise as a consequence of the cells response to osmotic stress imposed during isolation. Mechanically isolated whole cells on the other hand may provide suitable material for detailed studies of the dedifferentiation response in wounded tissue. Such cell populations comprise relatively large (often $>500x10^6$ cells) populations of physiologically and cytologically uniform wounded mesophyll cells (Rossini, 1972; Paul *et al.*, 1989) capable of dedifferentiation and cell division in a synchronous manner (Paul *et al.*, 1989). Mechanically isolated cell cultures of the monocot *Asparagus officinalis* (fig. 3.1) are easily obtainable by either scraping cladophylls with a plastic card (Foyer *et al.*, 1982) or by grinding with a mortar and pestle (Walton *et al.*, 1984). Such cell

populations have been shown to exhibit a photosynthetic capacity of approximately 50-70% that of intact cladophyll material, a reduction accounted for by the redirection of carbon sources from sucrose synthesis to starch and other carbon compounds (Hills, 1986). Work here at Leicester revealed this system to be a potentially rich source of wound-induced gene expression (Harikrishna *et al.*, 1991). The complete exclusion of the possibility of cell-cell communication through the mechanical severance of intercellular plasmodesmatal links necessitates a reversion to the 'default' state described by Verbecke (1992). Maintenance of cells in a suspended state also implies that all cells within a population are exposed to similar external factors. Thus large populations of synchronously dedifferentiating wounded cells are readily attainable.

3.1.2 The DD1 protein family:

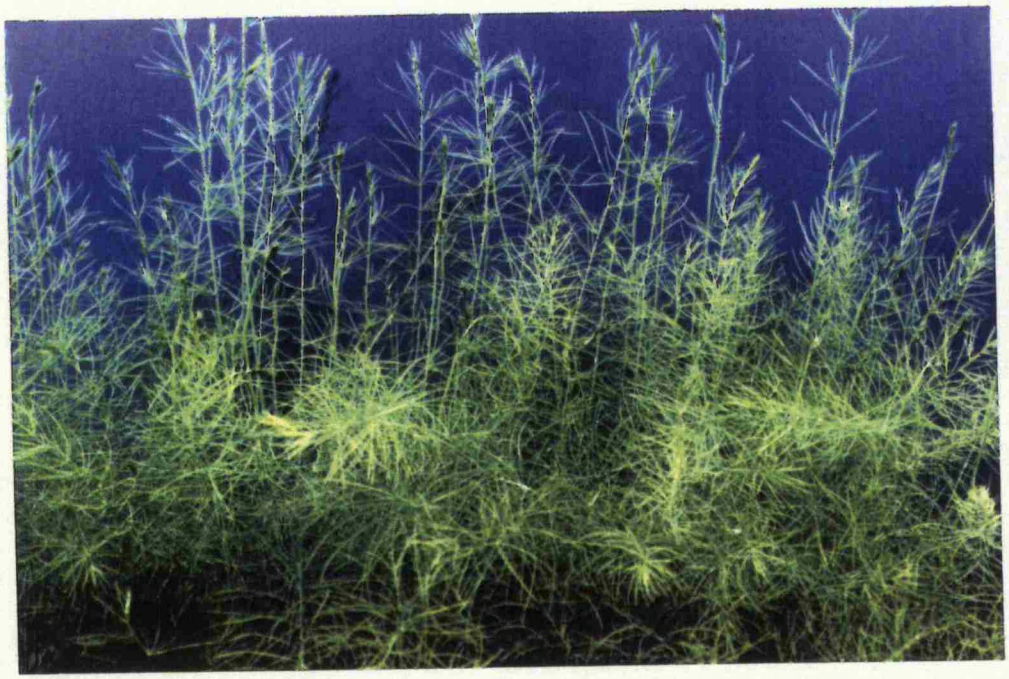
In previous studies at Leicester one-dimensional SDS-PAGE analysis of total cell protein extracted at various times following mechanical isolation of asparagus cells revealed apparent major alterations in protein synthesis following cell isolation (Harikrishna *et al.*, 1991). These changes were reflected at the transcript level, as determined by 2-D analysis of proteins translated *in vitro* from mechanically-isolated cell-derived message isolated at different times post-cell isolation The number of detectable polypeptides in 2-D gel analysis was found to increase with time in culture, to a maximum achieved between 2 and 5 days in culture, decrease after 6 days to a stable level, and maintained at this lower level between 7 days and 6 months in culture. This change in the complexity of protein profile was observed to comprise a down-regulation of transcription of large numbers of genes as determined by the reduction in intensity or disappearance of the corresponding spot on a 2-D gel, and the enhanced and *de novo* transcription of a relatively large number of genes (Harikrishna *et al.*, 1991). Among those proteins exhibiting an apparent specificity to mechanically isolated cell-derived material, one group of polypeptides of molecular weight approximately 16 kD was observed to be *de novo* induced under such conditions and was termed the DD1 family.

Figure 3.1:

(a) Six week old greenhouse-grown asparagus (*Asparagus officinalis* cv. Connovers' Colossal) seedlings.

(b) Mechanically isolated asparagus mesophyll cells derived from six week old seedlings three days post-cell isolation.

(a)



(b)

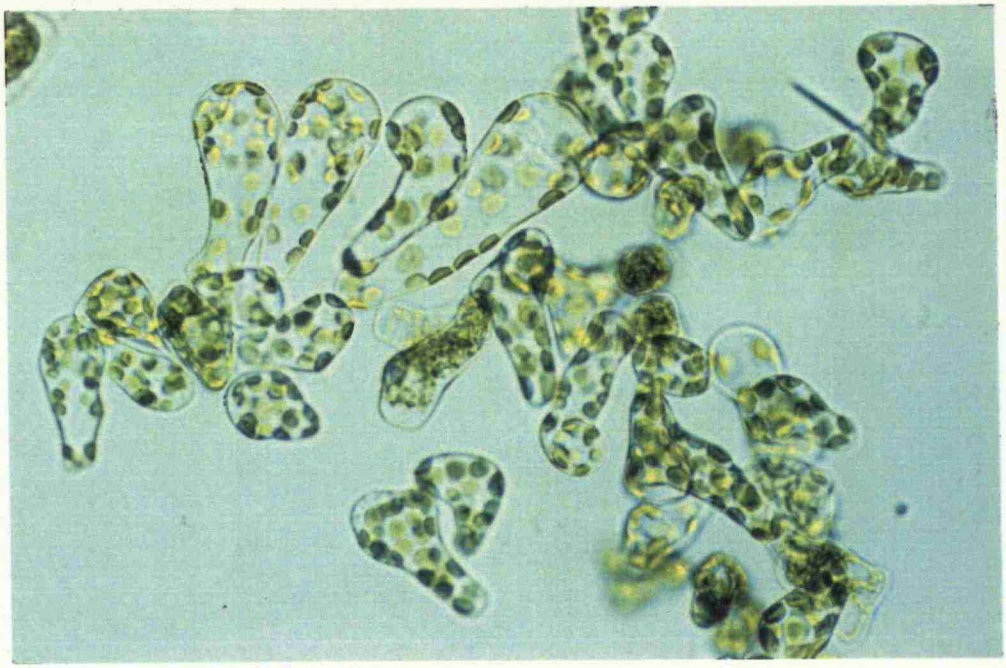


Figure 3.2:

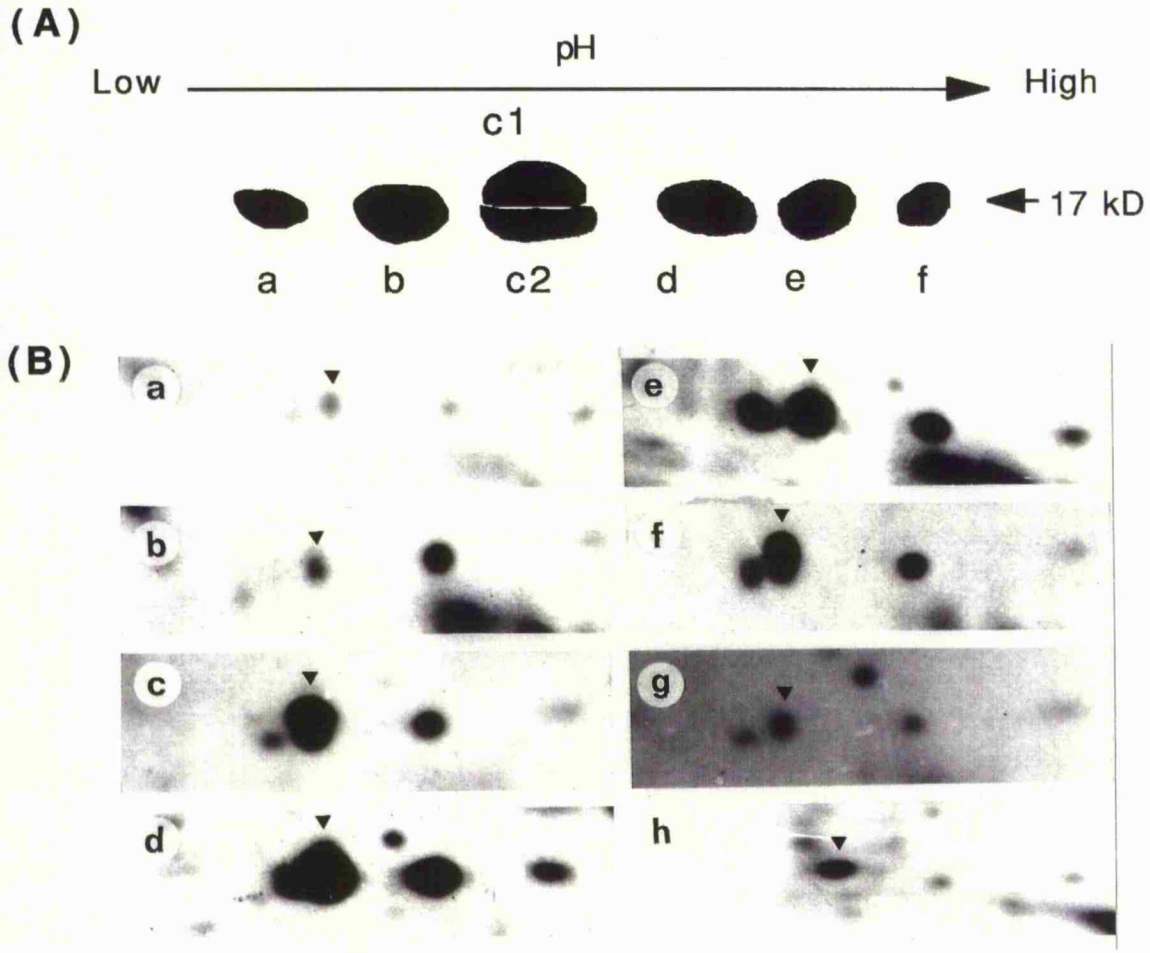
The DD1 protein family.

(A) Schematic representation of the DD1 protein family.

(B) Time-course of DD1 expression in *in vitro* cultured material.

(a) Cladode (b) 3 h post-cell isolation; (c) 1 d post-cell isolation; (d) 2 d post-cell isolation; (e) 5 d post-cell isolation; (f) 7 d post-cell isolation; (g) 2 months post-cell isolation; (h) 6 months post-cell isolation.

The arrow indicates the same spot on each gel, DD1c.


(A)
pH
Low
High
c1
17 kD
a
b
c2
d
e
f
(B)
a
b
c
d
e
f
g
h

3.1.3 Members of the DD1 family exhibit differential regulation during culture.

The DD1 family of proteins was found to comprise at least five separate polypeptides of similar molecular weight but differing in pI over the range 4-6 (Fioroni, 1989) (fig.3.2a). The DD1 proteins were not initially detected in translation products of mRNA isolated from non-wounded cladode material. At one day post-cell isolation at least two members of this family were detectable at relatively high levels, with the remaining polypeptides accumulating over the following three days (fig.3.2b). The intensity of all DD1 protein spots except DD1c, the most abundant, had diminished significantly by day 7. After 6 months in culture it was observed that a spot corresponding to DD1c on a 2-D gel was still detectable, although at significantly reduced levels.

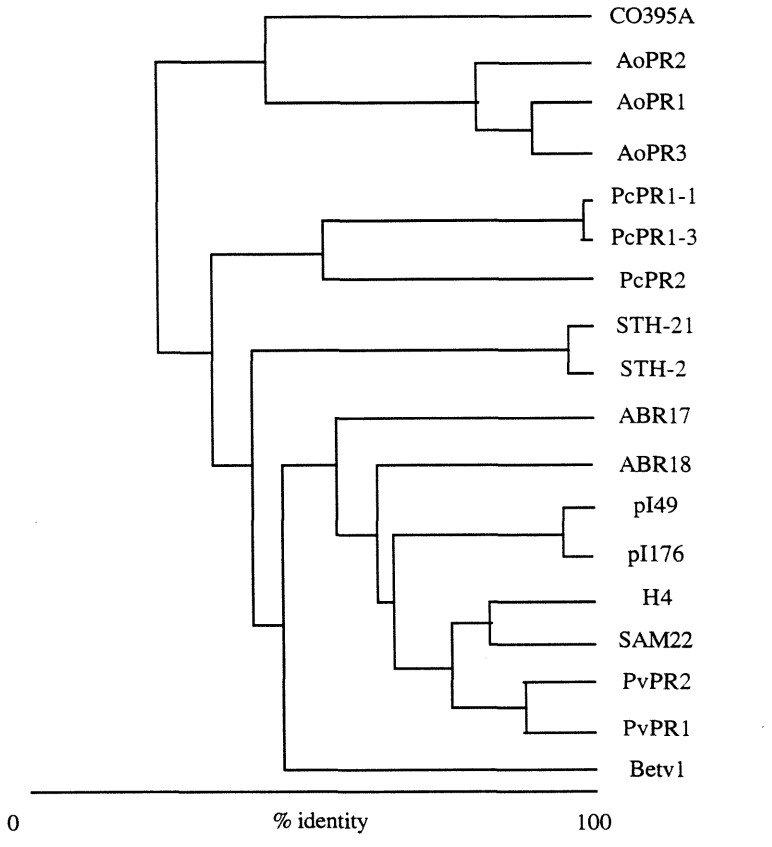
3.1.4 Production of an anti-DD1 antibody and immunological characterisation of the DD1 family:

In order to investigate the possibility that the DD1 family of proteins are subject to post-translational modification *in planta* a polyclonal antibody was raised to a protein sample obtained from 2-D gels of protein translated *in vitro* from transcript isolated from 8 week old asparagus culture material (Fioroni, 1989). Screening 2-D western blots of total cell protein extracts from 5 days and 6 months in culture revealed no apparent alterations in either molecular weight or pI of the relevant DD1 proteins. It was found that after five days in culture the antibody raised to DD1c cross-reacted with three other members of the DD1 family: DD1a,b and d. After 6 months in culture a strong signal was still detectable at a position corresponding to the polypeptide DD1c. Of those proteins initially assumed to belong to the DD1 family, one was found to lack an immunological relationship to the other DD1 proteins; it was also found that a further protein, not initially thought to belong to the DD1 family and exhibiting a slightly higher molecular weight than the other members of that family, did cross-react with the DD1c-derived antibody. This latter protein was referred to as DD1f.

Figure 3.3:

Dendrogram illustrating the relationship between members of the intracellular PR protein family.

CO395 (rice): Unpublished sequence
AoPR1 (asparagus): Warner *et al,* 1992
AoPR2, 3 (asparagus): This thesis
PcPR1-1, PcPR1,3 (parsley): Somssich *et al.*, 1988
PcPR1 (parsley): van de Löcht *et al.*, 1990
STH-2, STH-21 (potato): Marineau *et al.*, 1987
ABR17, ABR18 (pea): Barratt *et al.*, 1989
pI49. pI176 (pea): Fristensky *et al.*, 1988
H4, SAM22 (soybean): Crowell *et al.*, 1992
PvPR1, PvPR2 (bean): Walter *et al.*, 1990
Betv1 (birch): Breiteneder *et al.*, 1989.


CO395A
AoPR2
AoPR1
AoPR3
PcPR1-1
PcPR1-3
PcPR2
STH-21
STH-2
ABR17
ABR18
pI49
pI176
H4
SAM22
PvPR2
PvPR1
Betv1
0
% identity
100

3.1.5 Isolation of a wound-inducible cDNA from asparagus showing homology to a group of intracellar PR proteins :

Having previously shown that mechanically isolated asparagus mesophyll cells are potentially an enriched source of wound-induced transcripts a λzap cDNA library was constructed from such material in an attempt to isolate cDNAs corresponding to such message. Differential screening of this library against non-wounded cladode-derived cDNA resulted in the isolation of several wound-induced cDNAs, one of which, cWIP, was subjected to further analysis (Warner *et al.*, 1992). Transcript corresponding to this cDNA was found to accumulate to high levels following mechanical isolation of mesophyll cells and wounding of etiolated asparagus seedlings. Northern analysis localised this transcript to sites proximal to the initial site of wounding. Sequence analysis of the predicted 17 kD protein encoded by this cDNA revealed striking homology between this protein and members of a novel intracellular PR protein family (Walter *et al.*, 1990) (fig. 3.3).

3.1.6 Chapter aims.

Having available a relatively large amount of information concerning the behaviour of the DD1 protein family in dedifferentiating cells and preliminary data concerning the behaviour of the AoPR1 transcript in the same system it was decided to further investigate the characteristics of expression of both and to explore the relationship, if any, existing between them. It was hoped that by buiding up a comprehensive profile of the expression of both DD1 and AoPR1 it would be possible to speculate as to the potential role of these proteins (or encoded proteins) in the dedifferentiation process itself.

3.2 Results:

3.2.1 Construction of an expression vector encoding the AoPR1 protein:

In order to further investigate the nature and properties of the AoPR1 protein it was decided to express the protein in bacteria such that sufficient quantities of the protein would be available for use as an inoculum in the production of an anti-AoPR1 antibody. This approach would

eventually facilitate the characterisation of AoPR1 expression in asparagus and possibly also allow for an investigation into the ubiquity of the intracellular PR protein family.

The AoPR1 cDNA was cloned as an 800 bp *Eco*R1 fragment from pSKII$^+$-cWIP rescued from the λzap cDNA library (S. Warner) into the bacterial expression vector pT7-7 (B. Gill). This plasmid contains the T7 RNA polymerase promoter and the translation start site for the T7 gene 10 protein. Thus the use of this vector facilitates the production of translational fusions with the gene of interest. The donor plasmid pSKII$^+$-cWIP and the recipient plasmid pT7-7 both contain a unique *Eco*R1 restriction site thereby allowing easy manipulation of the AoPR1 cDNA as an *Eco*R1 fragment. Cloning of the cDNA in this manner resulted in its insertion into the expression vector such that the sequence was in frame and the AoPR1 protein could potentially be produced. The chimeric plasmid pT7-7-AoPR1 was then introduced into *E. coli* cells harbouring the plasmid pGP1-2 using a standard calcium chloride transformation protocol (section 2.9.10). This 7 kb plasmid encodes, among other proteins including the kanamycin resistance-conferring *npt*-II, the T7 gene 1 product RNA polymerase which is under the control of its native heat-inducible promoter. The interaction between this protein and the T7 RNA polymerase promoter in pT7-7 facilitates expression of the sequences inserted downstream of the RNA polymerase promoter. Thus only those bacterial cells containing both pT7-7-AoPR1 and pGP1-2 are capable of producing the AoPR1 protein under appropriate conditions.

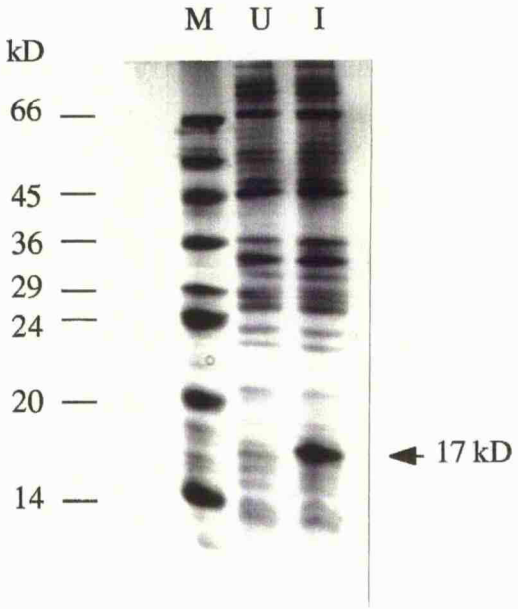
3.2.2 Expression of the AoPR1 protein in bacteria:

Transformed *E. coli* cells containing both pT7-7-AoPR1 and pGP1-2 as determined by kanamycin selection and restriction analysis were grown at 30^{o}C overnight and AoPR1 protein production induced as in section 2.19.1. Analysis of pre- and post-induced cells was carried out by one-dimensional SDS-PAGE through a 15% gel followed by Coomassie staining of the gel. As can be clearly seen from fig.3.4 a protein of approximately 17 kD appears to be synthesised *de novo* in those cells which have been subjected to heat stress. This band corresponds in size to that expected by predictions based on the AoPR1 cDNA, and

Figure 3.4:

Induction of AoPR1 expression in *E. coli* containing both the pT7-7-AoPR1 and pGP-1 plasmids.
M: Size standards; U: 10 µl total bacterial protein from pre-induction cells; I: 10 µl total bacterial protein from heat-induced cells.

The synthesis of the AoPR1 protein is indicated by the appearance of a highly abundant protein of approximately 17 kD in extract from induced cells.


M U I
kD
66
45
36
29
24
20
14
17 kD

its *de novo* appearance and abundance presuppose that this band does indeed correspond to the AoPR1 protein.

3.2.3 The AoPR1 protein is recognised by the anti-DD1 antibody.

Given that both DD1 and AoPR1 are stress-induced as both were isolated from the same source, namely mechanically isolated asparagus mesophyll cells, appear to be induced *de novo* in response to the stress imposed during the isolation of such cells, and their similarity in size, it was thought that there would, perhaps, be some degree of homology between these two proteins. Thus it was decided to explore this possibility further using western blot analysis (section 2.21.4).

It became apparent that the anti-DD1 antibody was capable of detecting the AoPR1 protein under the chosen conditions (fig.3.5; lane 1). A band of similar size, 17 kD, was also detected in protein extract from mechanically isolated asparagus cells isolated three days post-cell isolation (fig.3.5; lane 2). Hence, the possibility exists that DD1 and the AoPR1 gene product may in fact be similar, if not the same, proteins.

3.2.4 An antibody to the potato IPR STH-2 cross-reacts specifically with the AoPR1 protein.

As described previously (section 3.1.7), the predicted protein encoded by the AoPR1 cDNA exhibits significant homology to members of an intracellular PR protein family. The extent to which these proteins are related is evident only upon consideration of the corresponding protein sequences and is not apparent when DNA sequences are compared. Thus it is interesting to speculate that members of this family may show some degree of immunological relatedness.

Having obtained an antibody raised against the potato intracellular PR protein STH-2 from Dr. Normand Brisson the opportunity to explore this area presented itself. As with the anti-DD1 antibody, the anti-STH-2 antibody did indeed exhibit a striking degree of cross-reactivity with both the AoPR1 protein and with a 17 kD protein in extracts from day 3 cell

culture (fig.3.5). The protein-antibody interaction appeared to be specific. In contrast, an antibody raised to the similar sized tobacco PR1a protein did not cross-react under similar conditions (fig.3.5).

3.2.5 The anti-DD1 and anti-STH-2 antibodies recognise an identical group of proteins on 2-D gels of cell culture-derived material.

Having ascertained that both anti-DD1 and anti-STH-2 antibodies cross-react with apparently identical protein bands on one-dimensional gels, the extent to which both antibodies recognise similar proteins was investigated further using two-dimensional electrophoretic analysis.

Preliminary experiments subjected protein samples from day 3 cell culture material to an initial separation based on isoelectric point across an ampholyte range of pI 3-10. Following this, separation in the second dimension was on the basis of size. Results from these initial experiments suggested that both antibodies were capable of specifically recognising the same group of proteins on 2-D gels. The pI range over which these proteins were distributed as determined from these preliminary experiments was approximately 6.5-8.5. Further analysis was thus carried out using a narrower ampholyte range, pI 6-9.5, in order to reveal the true extent of this recognition. Both anti-DD1 and anti-STH-2 antibodies specifically recognised an identical group of proteins under these conditions (fig.3.6). Since both antibodies also cross-react specifically with the AoPR1 fusion protein it is feasible to conclude that DD1 and AoPR1 are in fact one and the same.

In both cases using the narrower ampholyte range those proteins identified were within the pI range 6.85 to 8.5. The anti-STH-2 antibody differentiated between at least six individual proteins, with possibly one further member. While differentiation of proteins using the anti-DD1 antibody was less discrete at least five spots were detectable, and the general pattern of spots appeared identical to that recognised by the anti-STH-2 antibody. This poor resolution of individual proteins generated upon using the anti-DD1 antibody is presumably attributable to the age of the antibody. The resolution of a single band as determined from 1-D gels into at least five individual immunologically related proteins of similar size but differing in

Figure 3.5:

Immunodetection of the AoPR1 protein using DD1, STH-2 and PR1a antisera.

(1) AoPR1 protein (5 µl total induced bacterial protein)
(2) Total protein extract (10 µg) from cell culture day 3 post-cell isolation.

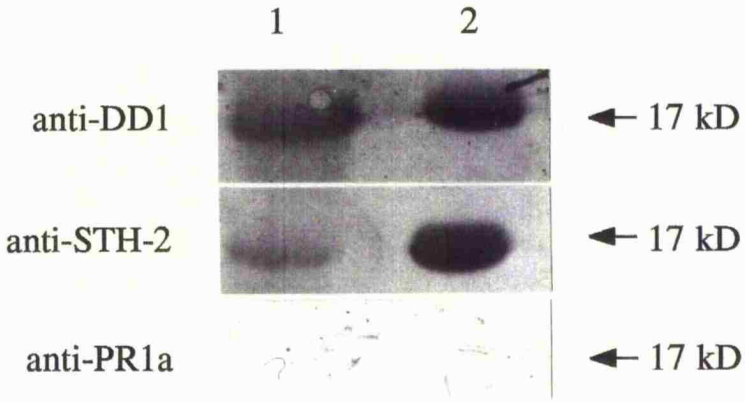
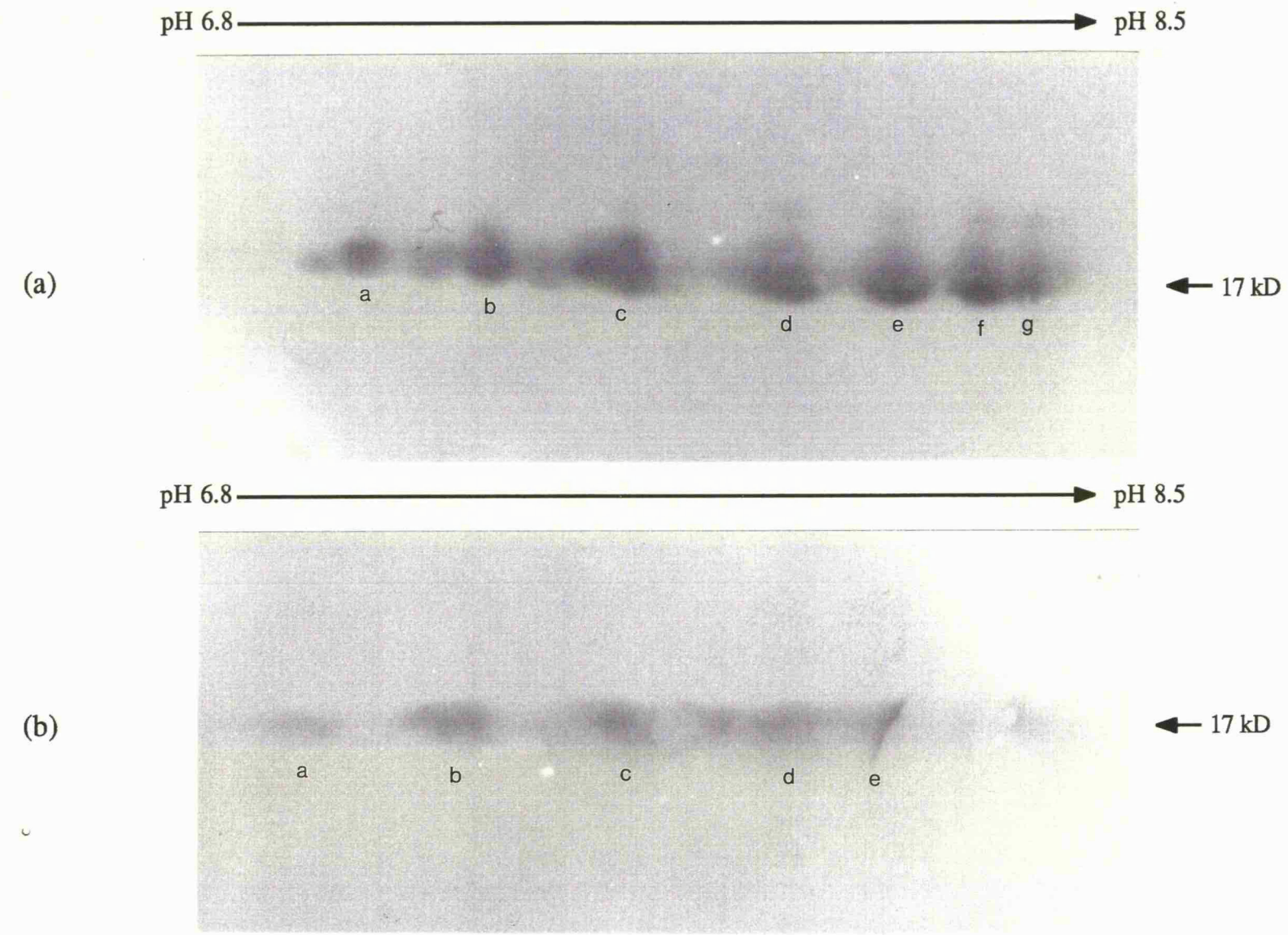

1
2
anti-DD1
17 kD
anti-STH-2
17 kD
anti-PR1a
17 kD

Figure 3.6:

2-D gels of total cell protein (20 μg) isolated from mechanically isolated asparagus mesophyll cells 3 d post-cell isolation.

(a) Total cell protein immunodetected with STH-2 antiserum.
(b) Total cell protein immunodetected with DD1 antiserum.


pH 6.8
pH 8.5
(a)
a
b
c
d
e
f
g
17 kD
pH 6.8
pH 8.5
(b)
a
b
c
d
e
17 kD

isoelectric point on 2D gels clearly illustrates the existence of DD1/AoPR1 as a protein family. Which member of the protein family corresponds to that encoded by the AoPR1 cDNA is difficult to ascertain on the basis of electrophoretic data alone since the predicted pI of that protein differs from that observed for any member of the AoPR1 family when analysed according to the above conditions. This apparent discrepancy may be due to modification of the encoded protein through e.g. phosphorylation, and/or to factors relating to the electrophoretic conditions employed e.g. ampholyte range and focusing. Similarly the presence of many members of the AoPR1 protein family may be the result of the post-translational modification of one or more 'core' proteins.

3.2.6 Computer analysis of the predicted sequence of the AoPR1 protein:

Translation of the AoPR1 cDNA using the GCG "Translate" program (Genetics Computer Group, 1991) installed on an IRIX system and subsequent analysis of the predicted protein sequence using the GCG "Plotstructure" program facilitated the compilation of a comprehensive analysis of structural features of the predicted AoPR1 protein. Results from this analysis are presented in fig. 3.7. The predicted AoPR1 protein may be seen to lack any significant structural features conferring notable properties on the protein. It appears not to contain any extensive regions of charged residues, not to contain regions of sequence predisposed towards antigenicity and, apart from a single potential glycosylation site towards the carboxy terminus, to lack any predicted sites of possible modification as determined within the parameters of the program used.

3.2.7 Members of the AoPR protein family accumulate in a similar manner in response to mechanical cell isolation and wounding.

Transcript encoding the AoPR1 protein has been shown to accumulate in response to wounding, in a wound site-specific manner (Warner *et al.*, 1991). To further explore this response a similar experiment was designed and AoPR1 protein accumulation monitored in response to the applied stimulus. It was found that, in agreement with previous data concerning the accumulation of AoPR1 transcript in response to wounding (Warner *et al.*,

Figure 3.7:

Computer-aided analysis of the predicted protein encoded by the AoPR1 cDNA.

DNA sequence was translated using the GCG package "Translate" and the resultant amino acid sequence analysed using the GCG "Plotstructure" package according to default parameters.

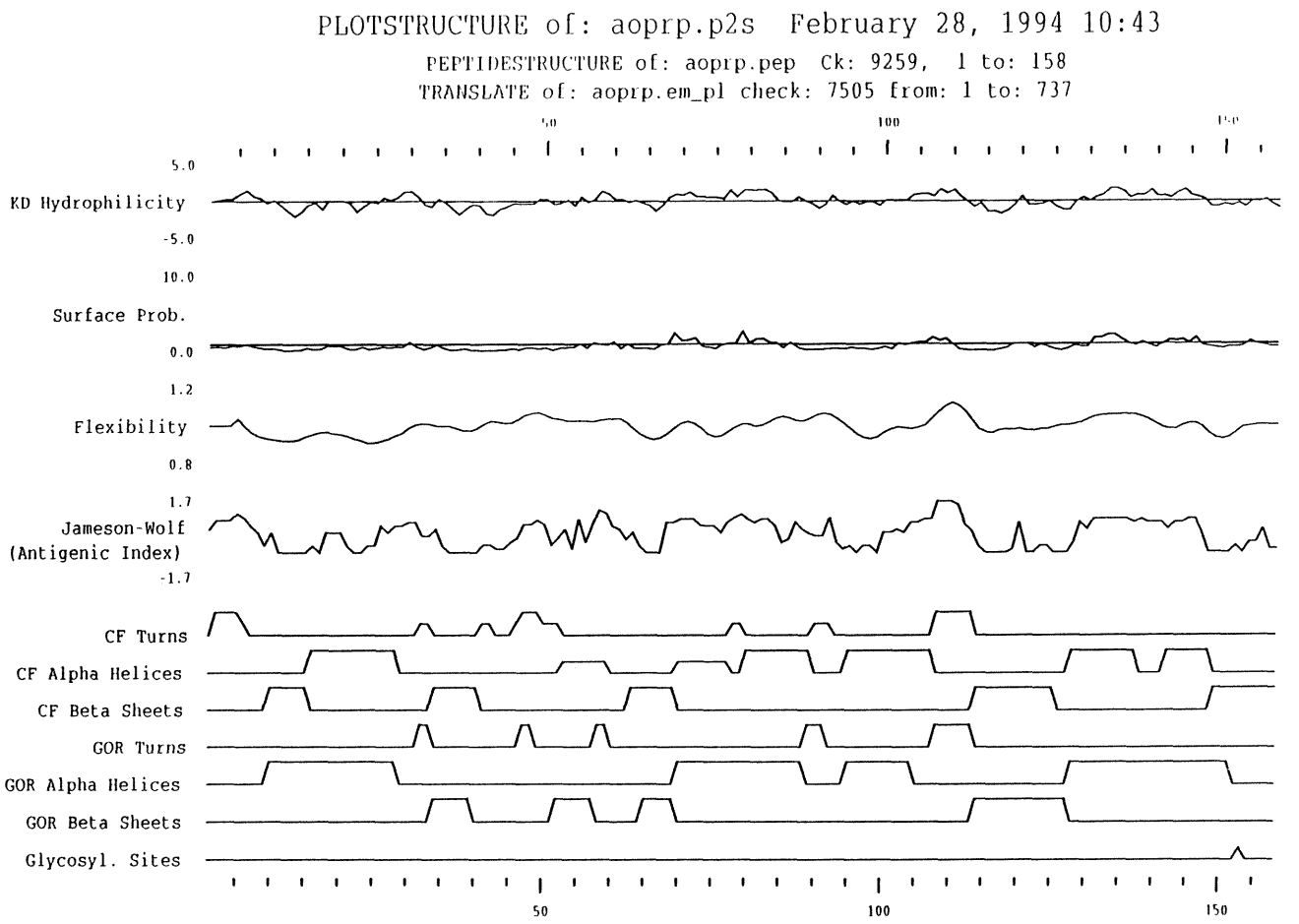

PLOTSTRUCTURE of: aoprp.p2s February 28, 1994 10:43
PEPTIDESTRUCTURE of: aoprp.pep Ck: 9259, 1 to: 158
TRANSLATE of: aoprp.em_pl check: 7505 from: 1 to: 737
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
50
100
150

Figure 3.8:

Western blot analysis of AoPR1 protein accumulation at the wound site.

Two week old etiolated asparagus seedlings were cut into 1.5 cm explants and allowed to age for 1 (A), 2 (B), and 3 (C) days. Sections were then cut and total cell protein extracted, electrophoresed through a 15% SDS-polyacrylamide gel, blotted and immunodetected with STH-2 antiserum.

Lane 1: 5 mm long sections 5 mm away from the wound site.
Lane 2: 2.5 mm long sections 2.5 mm away from the wound site.
Lane 3: 2.5 mm long sections that include the wound site.
fp: *E. coli*-expressed AoPR1 protein

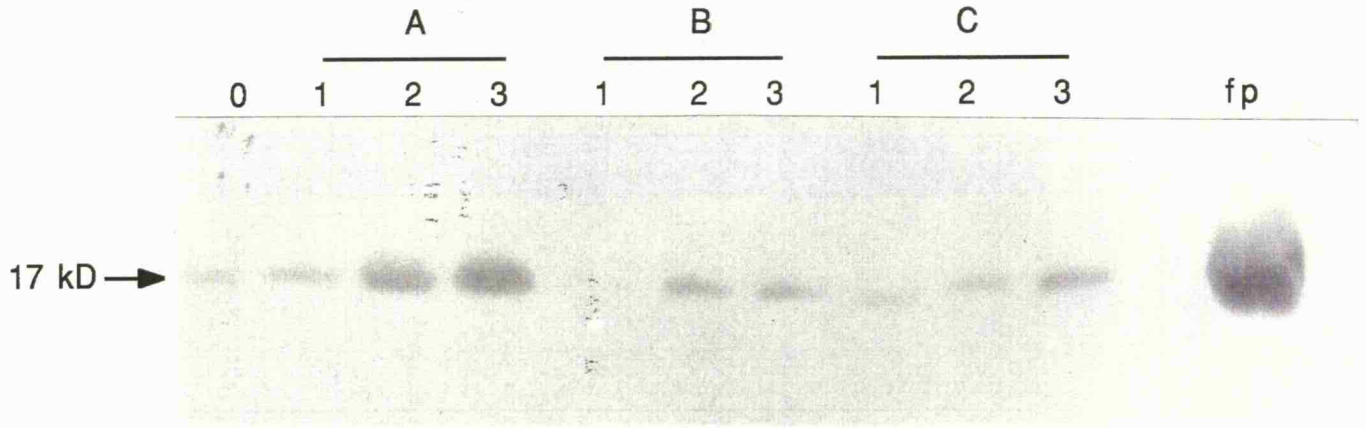
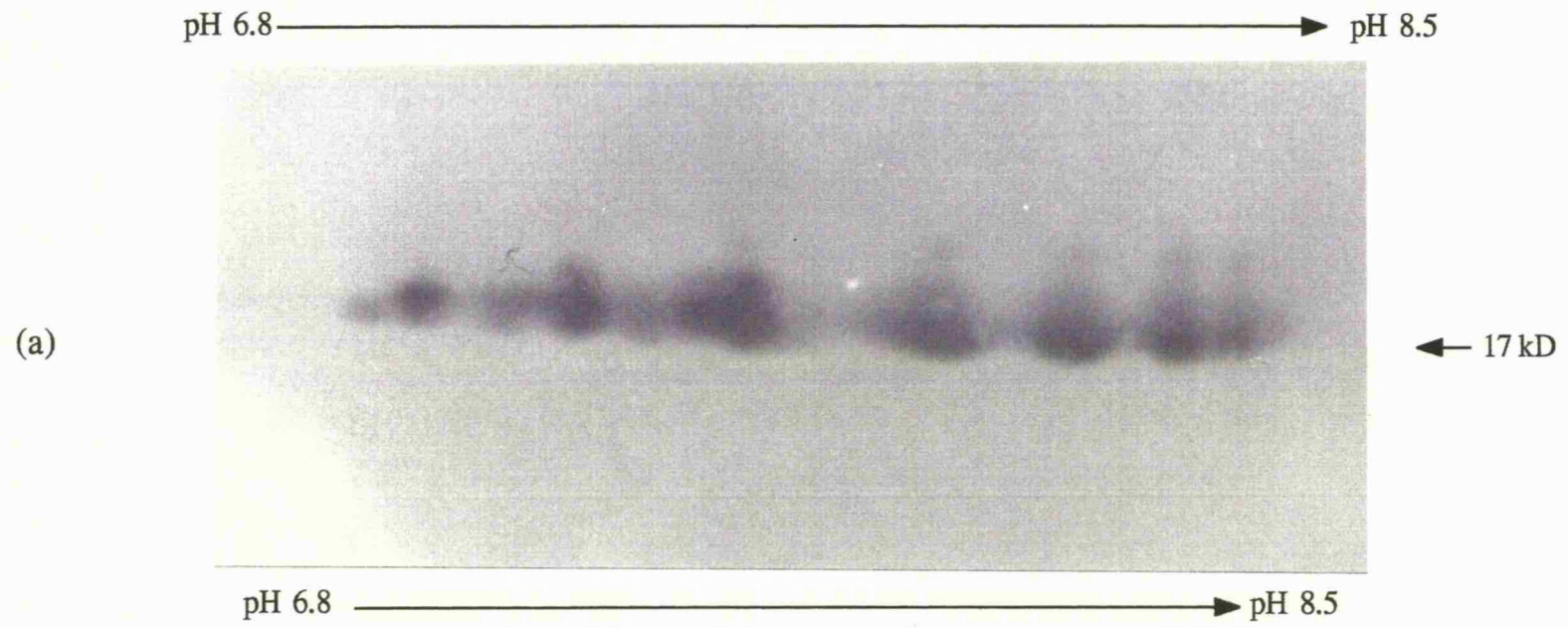

A
B
C
0
1
2
3
1
2
3
1
2
3
fp
17 kD

Figure 3.9:

2-D western analysis of total cell protein isolated from (a) mechanically isolated asparagus mesophyll cells 3 d post-cell isolation and (b) 5 mm explants of etiolated asparagus seedlings 3 d post-wounding using anti-STH-2 antiserum.


pH 6.8
pH 8.5
(a)
17 kD
pH 6.8
pH 8.5
(b)
17 kD

1992), induction of AoPR1 gene expression (measured, in this case, in terms of gene product accumulation) is restricted to the immediate vicinity of the wound site and does not appear to undergo any systemic induction as determined by the relatively low levels of AoPR1 protein detected in explant sections distant to the site of applied stimilus (fig. 3.8).

Further analysis of AoPR1 protein accumulation in response to wounding was carried out using 2-D gel and immunological analyses. Comparing the profile of isoforms accumulating in response to a wound stress (etiolated seedling material was chopped into 0.5 cm explant sections and incubated in the dark at 25^{o}C for 48 h prior to protein extraction) with that of dedifferentiating mechanically isolated cells 48 h post-cell isolation revealed some apparent differences in the pattern of spots detected on immunoreaction with anti-STH-2 antiserum (fig. 3.9). Although preliminary, these results appear to show the accumulation of similar isoforms in response to both stimuli, the characteristic a-g forms accumulating in cell culture material, while 5 spots, corresponding to spots a, b, c, d and e appear in extracts derived from wounded material. It is probable that proteins f and g are indeed synthesised in response to wounding, their apparent absence from the illustrated blot possibly being attributable to the experimental conditions employed. However, one must consider the possibility that these two proteins are not produced in response to the wound stimulus, their expression perhaps being regulated in response to developmental cues such as greening of tissues, stimuli to which the etiolated seedling material used in the wound assay was not exposed. This explanation would therefore account for the absence of these spots from 2-D gels of wounded seedling-derived protein.

3.2.8 Both transcription and translation of the AoPR1 gene occur following exposure to ABA.

The documented induction of intracellular PR proteins by the phytohormone abscissic acid (Barratt *et al.*, 1989) prompted the question 'would ABA induce expression of the AoPR1 gene in asparagus?'. In order to investigate this 6 week old asparagus seedlings were exposed to ABA by spraying with a 250 μM solution of this compound (prepared by the dilution, in

Figure 3.10:

AoPR1 accumulation in response to ABA.

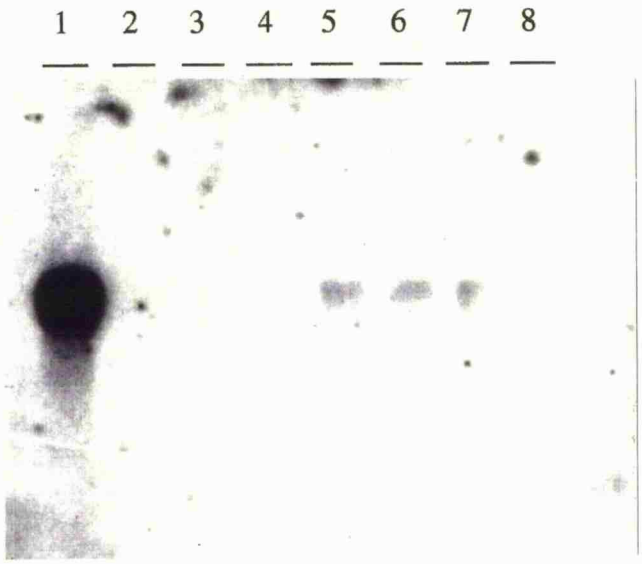
(a) Northern blot of total RNA isolated from 6 week old asparagus seedlings sprayed with 250 μM ABA and probed with labelled AoPR1 cDNA.

Lane 1: 10 μg total RNA isolated from mechanically isolated asparagus cells 3 d post-cell isolation.
Lane 2: Empty
Lane 3: 0 h post-treatment
Lane 4: 2 h post-treatment
Lane 5: 4 h post-treatment
Lane 6: 6 h post-treatment
Lane 7: 12 h post-treatment
Lane 8: 24 h post-spraying

(b) Western analysis of total cell protein extracted from seedlings treated as above (a) detected using STH-2 antiserum.

Lane 1: 24 h post-treatment
Lane 2: 12 h post-treatment
Lane 3: 6 h post-treatment
Lane 4: 4 h post-treatment
Lane 5: 2 h post-treatment
Lane 6: 0 h post-treatment.

(a)



(b)

water, of a 100 mM stock of ABA dissolved in ethanol). Following incubation periods of 2, 4, 6, 12 and 24 h cladophyll material was harvested and assayed for both AoPR1 transcript (Northern analysis using labelled AoPR1 cDNA as a probe) and protein (western analysis using STH-2 antiserum). Results obtained show the accumulation of both transcript and protein in response to the exogenous application of ABA (fig. 3.10). Both transcript and protein may be seen to accumulate 4 h post-treatment, with levels declining by 24 h. The apparent failure to correlate the presence of a 17 - 18 kDa immunoreacting band on western blots probed with anti-STH-2 antiserum at 0 h post-treatment with accumulation of the AoPR1 transcript on Northern blots of identically treated material presumably reflects the contributions of a number of experimental factors - differences in sensitivity between northern and western analysis or incomplete transfer of nucleic acids to membranes during blotting for example. It is also possible that the immunoreacting band detected at 0 h corresponds to a homologue of the potato 18 kDa protein immunologically related to STH-2 (Constabel and Brisson, 1992) although the failure to detect an immunoreacting band at this position in extracts from subsequent time points and in other westerns of asparagus protein extracts would tend to discount this hypothesis.

3.2.9 AoPR1 is not induced by arachidonic acid or jasmonic acid but does accumulate in response to salicylic acid.

The ability of the elicitor arachidonic acid to induce accumulation of the intracellular PR protein STH-2 and the serological relationship between the AoPR1 and STH-2 proteins led to an investigation into the effect, if any, exerted by this chemical on AoPR1 protein accumulation. Asparagus seedlings were sprayed with an emulsion of arachidonic acid (0.1 mg/ml in distilled water - a concentration 1/10th that used by Constabel and Brisson (1992), chosen in the light of observations of severe browning and disintegration of asparagus tissues when using higher concentrations) and incubated for increasing lengths of time. Western blot analysis of total cell protein extracted from this material at various times post treatment probed with the anti-STH-2 antiserum revealed no induction of AoPR1 protein accumulation whatsoever. Similarly, the proteinase inhibitor-inducing agent jasmonic acid, when

introduced at a concentration of 100 nl/1250 ml[3] (determined from Farmer and Ryan, 1990) into a sealed chamber containing asparagus seedlings, was not found to perturb levels of endogenous AoPR1 protein when assayed as described above, while levels of *pin*II transcript were seen to increase in leaves of tomato plants incubated in the same chamber. Unfortunately *pin*II mRNA levels also accumulated in untreated tomato control material thereby making accurate interpretation of this data impossible. Due to constraints imposed by limitations in time this experiment was not investigated further. In contrast to the above lack of induction in asparagus, spraying seedlings with a solution of 4 mM salicylic acid did result in the accumulation of AoPR1 protein (fig. 3.11a lanes 5 and 6 respectively).

3.2.10 Endogenous accumulation of the AoPR1 protein is regulated in a developmental manner.

Endogenous AoPR1 accumulation was studied by western blot analysis of protein extracts from different tissues and stages of asparagus development using the anti-STH-2 antiserum. Protein extracts from six week old, light grown, unwounded asparagus seedlings. six week old asparagus seedlings cut into 2 mm lengths 3 days post-wounding, asparagus roots and mature asparagus spears were assayed for AoPR1 expression as determined by the accumulation of the encoded protein. AoPR1 protein was detected by the STH-2 antiserum in wounded asparagus samples (fig. 3.11a; lane 5) and in root tissue and unwounded stem (mature asparagus spears) (fig. 3.11a; lanes 3 and 4 respectively). Attempts to characterise expression in seed during development were unsuccessful while analysis of AoPR1 expression in floral tissues was not undertaken due to a shortage of appropriate material.

3.2.11 Expression of endogenous AoPR1 protein in asparagus stems is associated with vascular tissue.

Having demonstrated the presence of detectable levels of the AoPR1 protein in stem tissue it was decided to investigate the localisation of this protein within this structure. Sections were cut accross a mature asparagus spear and pressed onto nitrocellulose, giving a tissue print. Membranes were then processed as for western blots and immunodetected using STH-2

antiserum. Results demonstrated that the STH-2 antiserum cross-reacted with protein associated with the vascular bundles (fig. 3.11c), regions of the stem found to produce lignin, as determined by staining with phloroglucinol (fig. 3.11d).

3.2.12 Petals of post-dehiscence flowers may be a source of the tobacco homologues of AoPR1.

Using the STH-2 antiserum to investigate the possible presence of proteins immunologically related to the AoPR1 and STH-2 IPR proteins failed to detect any such protein in extracts of wounded tobacco leaves and similarly treated *Arabidopsis* material. The material was thus treated on the assumption that the induction of a defense response would predispose the plant towards expression of IPR-related genes thereby enriching assay material for production of the desired proteins. Accumulation of PR proteins (and the mimicking of the defense response in this respect) in floral tissues (Lotan *et al.*, 1989; Neale *et al.*, 1990) coupled with the availability of such material prompted the analysis of tobacco petal material for IPR protein accumulation. It was observed that protein extracts from tobacco (SR1) petals of post-dehiscence flowers gave a probable signal on western blots. A 17 kD band was weakly detected in sample extracts of both white and pink regions of tobacco petals, corresponding in size to that detected upon analysis of the AoPR1 protein (fig.3.12). To examine this in more detail protein was extracted from large numbers of petals, both pigmented and unpigmented regions according to the method of Constabel and Brisson (1992). Ammonium sulphate fractionation was then carried out in order to selectively concentrate the desired protein fraction. Results from this experiment were, however, inconclusive (data not shown), although an immunoreacting doublet of MW 19-20 kD was observed at ammonium sulphate saturation of 40-60%. Some non-specific high molecular weight immunoreacting bands were also detected in lower fractions. Thus conclusive interpretation of these results in not feasible. It is possible, however, to speculate that this 20 kD immunoreacting doublet corresponds to the tobacco homologues of AoPR1. Further work is necessary to investigate this possibility further.

Figure 3.11:

Developmental accumulation of the AoPR1 protein in asparagus.

(a) Western analysis of total cell protein (10 μg) isolated from (2) Untreated cladodes from 6 week old asparagus seedlings; (3) roots from 6 week old asparagus seedlings; (4) asparagus spears from mature plants (5) etiolated 6 week old asparagus seedlings sliced into 2 mm lenghts and incubated on moist filter paper at 25°C for 3 d; (6) 6 week old asparagus seedlings 3 d post-spraying with 5 mM salicylic acid. Lane 1: 5 μl total bacterial protein from induced *E. coli* containing the pGP-1 and pT7-7-AoPR1 plasmids.

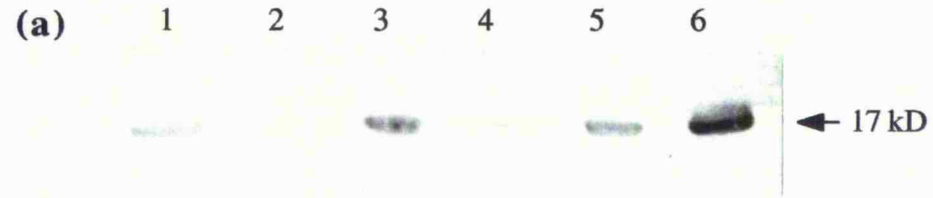
(b) Western blot of potato proteins detected with the STH-2 antiserum. 1 μg total protein from *E. coli* expressing the AoPR1 cDNA and 10 μg total plant protein were loaded per lane.

Lane 1: Sliced potato tubers.
Lane 2: Sliced potato tubers treated with arachidonic acid
Lane 3: *E. coli*-expressed AoPR1 protein
Lane 4: Untreated potato stem.

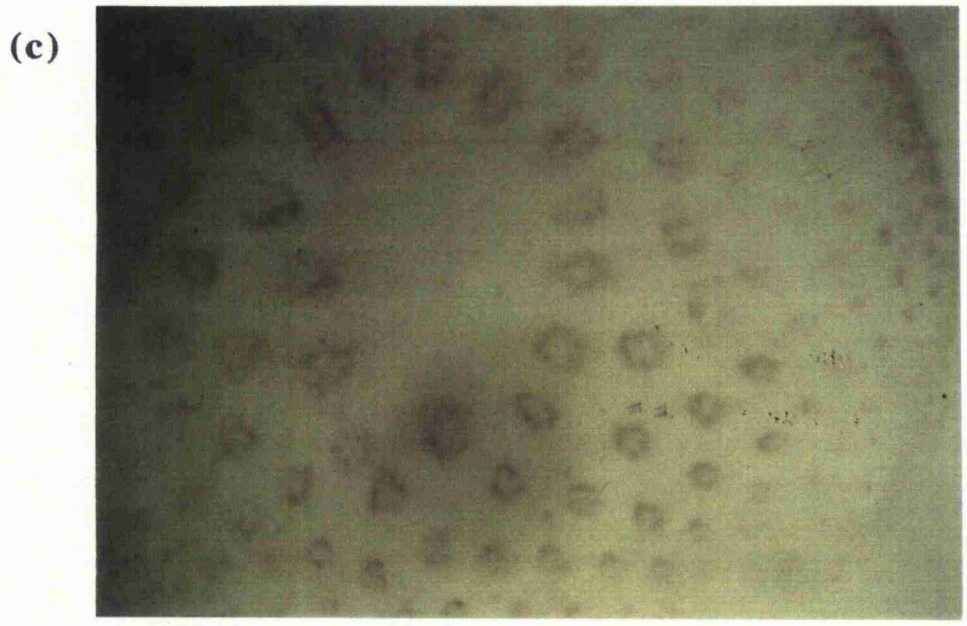
Treatment of potato tuber slices was as described in Marineau *et al.*, 1987.

(c) Tissue print of asparagus spear showing cross-reaction with the AoPR1 antiserum around the vascular bundles (x30).

(d) Phloroglucinol staining of T.S. of an asparagus spear showing the localisation of lignin surrounding the vascular bundles (x30).


(a)
1
2
3
4
5
6
17 kD


(b)
1
2
3
4
17 kD


(c)

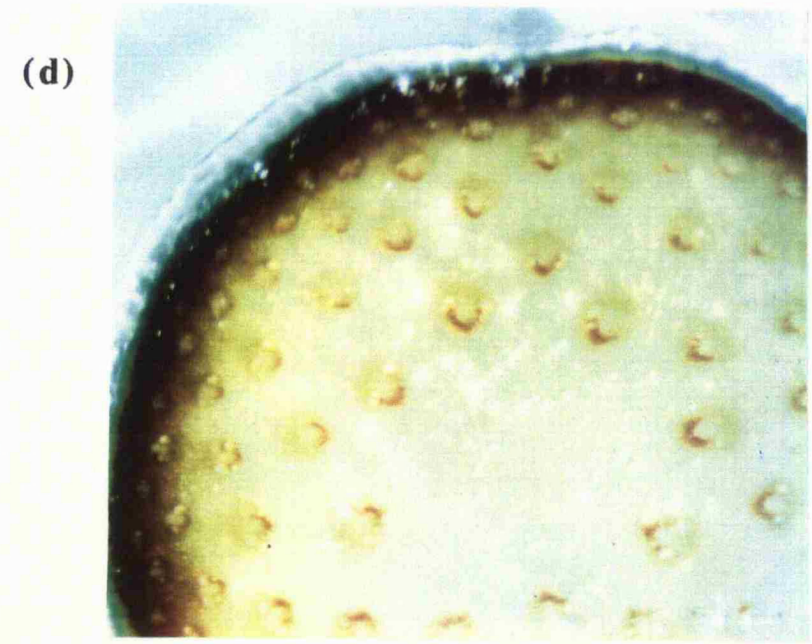
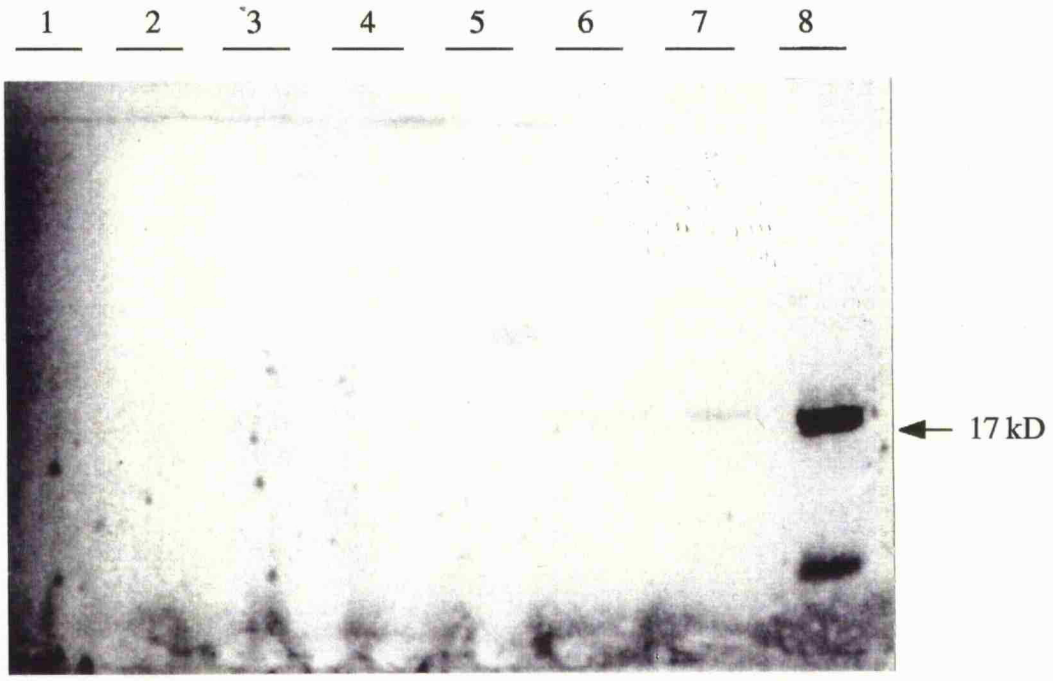

(d)

Figure 3.12:

Western blot analysis of AoPR1 protein accumulation in tobacco using the STH-2 antiseum. 10 μg total cell protein were loaded in each case.

Lane 1: Pollen from buds 0-1 cm long
Lane 2: Pollen from buds 1-2 cm long
Lane 3: Pollen from buds 2-4 cm long
Lane 4: Pollen from pre-dehiscence flowers > 4 cm long
Lane 5: Mature, dehisced pollen
Lane 6: Pigmented petal collar
Lane 7: White petal
Lane 8: Arachidonic acid-treated potato tuber slices.

The immunoreacting doublet at approximately 17-18 kD in lane 8 corresponds to the STH-2 protein, while the lower immunoreacting band is presumably a degradation product of this protein.


1
2
3
4
5
6
7
8
17 kD

3.2.13 Isolation and characterisation of further AoPR1 cDNAs:

Two further cDNAs showing significant homology to the AoPR1 cDNA were isolated from an asparagus cell culture cDNA library by Pietro Piffanelli and termed AoPR2 and AoPR3 (fig. 3.13). Sequencing of both the 586 bp AoPR2 and 553 bp AoPR3 clones revealed an 89% (AoPR2) and 80% (AoPR3) overall identity to the AoPR1 nucleotide sequence. The levels of nucleotide identity in the 3' untranslated region fell to 52% between AoPR1 and AoPR2 and 75% between AoPR1 and AoPR3. Comparisons of the predicted protein sequences indicated a 79% identity between AoPR1 and AoPR2, and 91% identity between AoPR1 and AoPR3. EcoR1/Acc1 double digests were used to generate fragments of the cDNAs composed of coding and 3' untranslated regions which were subsequently used as probes in Southern and northern analysis of the three cDNAs AoPR1-3. The minimum overall sequence identity between any of these probes was estimated at 75% and was therefore insufficient to completely prevent cross-hybridisation. Results from Southern analysis revealed no distinguishable differences between banding patterns when all three probes were used to screen the asparagus genome. Similarly no detectable differences were observed upon comparison of transcript abundance in response to wounding for each cDNA (P. Piffanelli, pers. comm.).

Analysis of the predicted protein sequence of each of these individual cDNAs revealed differences sufficient to result in a shift in predicted pI of AoPR2 and AoPR3 relative to AoPR1. Using the GCG "Peptidesort" program (Genetics Computer Group, 1991) installed on an IRIX predicted pI values of 7.04, 7.72 and 7.07 were obtained for AoPR1, 2 and 3 respectively. All three predicted proteins were similar in size, being approximately 16.9 kD.

Figure 3.13:

Nucleotide and predicted amino acid sequences of the three asparagus PR cDNAs.

Asterisks and dashes represent bases or residues identical to those of AoPR1. The underlined sequence shows the *Acc*I site used to generate the coding and 3' untranslated probes.

```
AoPR1     1   ATGAGTTCAGGGAGCTGGAGCCACGAGGTCGCTGTCAATGTCGCCGCAGGACGGATGTTC  60
AoPR2         **************A****C*******C**T*C**G***C******
AoPR3       ***************A**T************************C******
AoPR1     1   M  S  S  G  S  W  S  H  E  V  A  V  N  V  A  A  G  R  M  F    20
AoPR2             -  -  -  -  -  -  -  A  -  -  P  S  -  K  L  -
AoPR3          -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  L  -

AoPR1    61   AAGGCGGCAATGCTCGACTGGCACAACCTCGGCCCTAAGATTGTGCCTGACTTTATTGCC  120
AoPR2         *****T**G*************************************G**T**C**C***
AoPR3         *********************************************G*****C***T**
AoPR1    21   K  A  A  M  L  D  W  H  N  L  G  P  K  I  V  P  D  F  I  A    40
AoPR2         -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
AoPR3         -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  S

AoPR1   121   GGTGGCTCAGTGGTGTCTGGAGATGGATCTGTAGGAACCATCCGAGAGATCAAGATCAAC  180
AoPR2         A*******T**TT****C****G****G****T****G*G**A************G*GT
AoPR3         A****T**T**A**************G*******G**********************
AoPR1    41   G  G  S  V  V  S  G  D  G  S  V  G  T  I  R  E  I  K  I  N    60
AoPR2         S  -  -  -  L  -  -  G  -  A  -  -  S  V  -  -  -  -  M  S
AoPR3         S  -  -  -  -  -  -  -  -  -  A  -  -  A  -  -  -  -  -  -  -
                                                                Acc I
AoPR1   181   AATCCTGCTATACCTTTCAGCTATGTGAAGGAACGCCTGGATTTCGTAGACCATGACAAG  240
AoPR2         **C****AAC*********A***CC*******G**********************G***
AoPR3         ********A*******************************A*********************
AoPR1    61   N  P  A  I  P  F  S  Y  V  K  E  R  L  D  F  V  D  H  D  K    80
AoPR2         -  -  E  -  -  -  N  -  L  -  -  -  -  -  -  -  -  -  E  -
AoPR3         -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

AoPR1   241   TTCGAGGTGAAGCAGACCCTCGTGGAAGGTGGAGGTTTAGGTAAGATGTTTGAATGTGCC  300
AoPR2         **********AA*C*****T**A*****C********G**A***CA********CG***
AoPR3         *******CT**********T********C*******************C****C****
AoPR1    81   F  E  V  K  Q  T  L  V  E  G  G  G  L  G  K  M  F  E  C  A   100
AoPR2         -  -  -  -  N  -  -  -  -  -  -  -  -  -  -  Q  -  -  S  -
AoPR3         -  -  A  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  S  -

AoPR1   301   ACCACTCACTTCAAGTTCGAGCCCTCGAGCAACGGTGGATGCCTCGTCAAGGTGACTGCA  360
AoPR2         T**********************T*T*******AA********A*T***********C**T
AoPR3         **T*****************************************A*T**T***********
AoPR1   101   T  T  H  F  K  F  E  P  S  S  N  G  G  C  L  V  K  V  T  A   120
AoPR2         S  -  -  -  -  -  -  -  -  -  -  N  -  -  I  -  -  -  -  -
AoPR3         -  -  -  -  -  -  -  -  -  -  -  -  -  -  I  -  -  -  -  -

AoPR1   361   TCCTACAAGATTCTCCCAGGTGTCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACC  420
AoPR2         **G******C********T******AA**T*********A*****C*********G*****
AoPR3         A****************T**GC**C*************A*************G******
AoPR1   121   S  Y  K  I  L  P  G  V  A  D  E  S  A  K  A  K  E  G  I  T   140
AoPR2         -  -  -  -  -  -  -  -  N  V  -  -  -  -  -  -  -  -  V  -
AoPR3         T  -  -  -  -  -  -  L  P  -  -  -  -  -  -  -  -  -  -  -

AoPR1   421   AACCACATGAAGGCAACCGAAGCTTACCTCCTAGCCAACCCAACTGCCTACGTTTAAATA  480
AoPR2         C*******C******G*T**************C**T***************C****G**
AoPR3         ****T**********G*T**************G**A***************C*******
AoPR1   141   N  H  M  K  A  T  E  A  Y  L  L  A  N  P  T  A  Y  V       158
AoPR2         H  -  I  -  -  A  -  -  -  -  -  -  -  -  -  -  -  A
AoPR3         N  L  -  -  -  A  -  -  -  -  -  -  -  -  -  -  -  A

AoPR1   481   TAGTGATTGTGTTTCTTTGCGTGAAGTGCTTGTGAGTTTGAATAAGGAGATTGGTTATGA  540
AoPR2         **TGCT*A**T*C*AA**TG***.....********C******A****G**CACC***
AoPR3         ***A*C**A*****.*C**TT***G**AAC*******************AC**A*T***

AoPR1   541   GGAAGCTTGATGGGGTCATACATAGTTAGTTTATGTTGAATGATCAGCCTTTTTTGTGTG  600
AoPR2         T*GT*A*G*C*AT*CATGGTT***C**TT*G*TGCC**GTATTAA*AGT*G*AG*CGTGT
AoPR3         *A*G*A**********TG**GT*TA*CA

AoPR1   601   AAGTACTTGGGAGTTTGAATAAGGAGACTGAATATGAGAAAGATTGATGGAGTTATCGTT  660
AoPR2         GTCGT

AoPR1   661   CATGTTGAATGATCAGCCTTATCAGTTTGTAACAGTGTCGAATGATCAGTCTTATCAGTT  720

AoPR1   721   TGTAATGGTGGCTTCAA
```

3.3 Discussion:

3.3.1 Mechanically isolated asparagus mesophyll cells are an enriched source of dedifferentiation-related transcripts:

Dedifferentiation, the cellular process whereby cell type-specificity is lost through alterations in gene expression, is widely reported *in planta* as a consequence of wounding. Its study has, however, been primarily confined to structural analysis of developing meristems (Verbecke, 1992). Results from this type of analysis clearly indicate the need for inter-cell communication in the direction of appropriate cell determination. This in turn implies that those cells being studied are, in effect, not in a fully dedifferentiated state. Thus investigations into the nature of the dedifferentiation response utilising this type of material are severely hindered by difficulties in obtaining sufficiently large populations of cells in the desired state of dedifferentiation. The presence of relatively large numbers of non-dedifferentiating cells effects a 'dilution' effect resulting in an apparently reduced bias towards dedifferentiation-related gene expression (Logemann *et al.,* 1988). Attempts to shift this bias towards a more favourable direction have resulted in the increased use of cell culture technology as opposed to *in planta* analysis.

As described in section 1.3.1 a large number of dedifferentiation-related transcripts have already been isolated from these systems . Comprising primarily cell cycle- and defense-related transcripts, the pattern of message obtained may be further enriched by cell culture elicitation. This approach has led to the isolation of a number of defense-related genes (Somssich *et al.*, 1990; van de Löcht *et al.*, 1988; Lois *et al.*, 1989; Gundlach *et al.*, 1992; Gowri *et al.*, 1991).

The use of non-elicitor treated material has, on the other hand, resulted in the isolation of a number of genes concerned with the cell division and wound response aspect of dedifferentiation. The isolation of a number of cyclins and kinases reflects the active mitotic state of these cells the induction of which may, in part, be attributed to the wound stimulus. Indeed it has been demonstrated that, in the initiation of cell cultures, it is the wound stimulus

itself that is sufficient for the induction of dedifferentiation (Marty *et al.*, 1993). Thus, it may be supposed that the majority of genes isolated from these non-elicited systems will exhibit a wound-inducible aspect to their expression profile.

The use of cultures of mechanically isolated mesophyll cells of the monocot *Asparagus officinalis* here at Leicester has resulted in the isolation of a number of such genes (R. Darby, pers. comm.) one of which was considered for further investigation. The cDNA corresponding to the gene encoding AoPR1, cWIP, was isolated by differential screening of a λzap cDNA library constructed using transcript derived from day 3 post-isolation asparagus mesophyll cells. The predicted protein derived from this cDNA sequence was found to exhibit homology to pathogenesis-related proteins from bean (PvPR1 and PvPR2) (Walter *et al.*, 1990), potato (STH-2 and STH-21) (Marineau *et al.*, 1987; Matton *et al.*, 1989) and parsley (PcPR1-1, PcPR1-2, PcPR1-3 and PcPR2) (Somssich *et al.*, 1988; van de Löcht *et al.*, 1990). Stress-induced proteins from soybean (SAM22 and H4) (Crowell *et al.*, 1992), the birch pollen allergen Betv1 (Breiteneder *et al.*, 1989) and the ABA-responsive proteins ABR17 and ABR18 (Barratt and Clark, 1991; Iturriaga *et al.*, 1994) also show similarity with the AoPR1 protein. The predicted protein encoded by the rice CO375A cDNA (unpublished sequence) shares 51% identity with the AoPR1 predicted protein and may also be considered as a member of the IPR protein family. Members of this protein family have been isolated as cDNA clones from libraries derived from a number of different systems including elicitor-treated tuber explants (Marineau *et al.*, 1987), mature pollen (Breiteneder *et al.*, 1989) and root tissue (Crowell *et al.*, 1992). Parsley and bean clones were derived from elicitor-treated cell suspensions (Somssich *et al.*, 1988; van de Löcht *et al.*, 1990; Walter *et al.*, 1990), while cytokinin-starved suspension cultures provided the source of the soybean SAM22 transcript (Crowell *et al.*, 1992). In addition the partial amino acid sequence of a recently isolated ginseng ribonuclease (Moiseyev *et al.*, 1994) has been reported to show high sequence similarity to the parsley IPR proteins. Further comparison of this sequence with that of other IPR ptoreins reveals that this similarity is shared among the IPR protein family as a whole.

Thus, the possibility that the members of the IPR family may exhibit ribonuclease activity exists. This idea will be discussed in greater detail in later chapters.

The AoPR1 cDNA was used as a probe to investigate the expression profile of the AoPR1 gene. Having isolated the cDNA from a mechanically isolated cell suspension the nature of the inductive stress was explored. During the isolation process the asparagus mesophyll cells are subjected primarily to a wound stress - the physical separation of cells from each other is achieved by grinding asparagus cladode material in a mortar and pestle (Paul *et al.*, 1989). This stress in itself has been reported to be sufficient for the induction of the dedifferentiation response (Marty *et al.*, 1993) Thus the effect of wounding on the accumulation of AoPR1 transcript was measured using northern analysis (Warner *et al.*, 1992). Results obtained clearly illustrated the ability of the wound stimulus to induce transcription of the AoPR1 gene. Since the dedifferentiation response is itself induced by the wound stimulus and is a fundamental aspect of the wound response it may be seen that AoPR1 is indeed an example of a dedifferentation-related gene.

3.3.2 Expression of AoPR1 is temporally regulated in culture.

The demonstration that the anti-DD1 antibody cross-reacted specifically with the *E.coli*-produced AoPR1 protein (section 3.2.3) brought together a large amount of data already held in this department. The expression of the DD1 family of proteins in culture had previously been characterised (Fioroni, 1989; Harikrishna *et al.*, 1991). Five members of this family were detected on two-dimensional gels of *in vitro*-translated mRNA from mechanically isolated asparagus mesophyll cells following culture initiation. Further analysis revealed a strong temporal aspect to the regulation of transcription of the genes encoding these proteins. It was observed that although a single band becomes apparent on one-dimensional gels following culture initiation this band may be resolved into at least five individual protein spots on 2-D gels the relative abundance of which alters with time in culture. The work carried out by Fioroni (1989) centred around the analysis of *in vitro*-translated protein and thus provides a reflection of induction at the transcript level. Some work was carried out using

total cell protein and did show that both systems were indeed comparable, the AoPR1/DD1 protein family appearing not to be subjected to any form of post-translational modification *in vivo*. Apparent discrepencies between 2-D gels obtained by Fioroni (1989) and in this study, which also employed total cell protein extracts in analysis, may possibly be accounted for by the use of a different ampholyte range (6-9.5) in this study as compared to that used by Fioroni (1989) (3-10), and differences in 2-D system used. Despite these differences, however, it is feasible to conclude that AoPR1 and DD1 are, potentially, one and the same.

3.3.3 Expression of AoPR1 is developmentally regulated *in planta* and induced by exogenously supplied salicylic acid and ABA.

Consistent with observations of developmental regulation of gene expression for a number of defence-related genes (section 1.2.4), the AoPR1 protein was itself seen to accumulate in a tissue-specific manner in its native host plant asparagus. The AoPR1 protein was detected in stem tissue in regions demonstrated to contain lignin, a product of the phenylpropanoid pathway, and in roots, although immunolocalisation of this protein in root tissue was not undertaken. These data correlate with data concerning the expression of SAM22, a soybean-derived IPR protein (Crowell *et al.*, 1992) transcript for which has been shown to accumulate to high levels in root tissue of soybean (*Glycine max* cv. Mandarin), of the related protein H4, whose corresponding cDNA was isolated from soybean (*G. max* cv. Williams) root tissue (Crowell *et al.*, 1992), and of PAL (Bevan *et al.*, 1989; Liang *et al.*, 1989, Shufflebottom *et al.*, 1993). The observed expression of AoPR1 in stem correlates well with observed expression profiles for the *gus* gene under the control of both the bean PAL3 promoter (Shufflebottom et al., 1993) and the rice CH10 (chitinase) promoter (Zhen *et al.*, 1993). Interestingly the STH-2 protein was itself detected in untreated potato stems (fig. 3.11b), an observation not previously documented (Constabel and Brisson, 1992). An immunoreacting band was also detected on western blots of tobacco petal material isolated from post-dehiscence flowers probed with STH-2 antiserum. Making the broad assumption that this band does indeed represent the tobacco homologue of STH-2, one may see that this petal-specific expression correlates with published observations on the behaviour of the PAL, 4-

coumarate:Co A ligase (4-CL) and CH10 promoters (Shufflebottom *et al.*, 1993; Bevan *et al.*, 1989; Liang *et al.*, 1989; Hauffe *et al,* 1991; Zhen *et al.*, 1993). All the above observations may be seen to point towards a striking correlation between accumulation of the AoPR1 protein and expression of genes involved in phenylpropanoid metabolism, a relationship which will be examined further in subsequent chapters.

Further correlations between AoPR1, PR and IPR protein induction profiles were obtained from investigation into the effect(s) exerted by a range of chemical agents - methyl jasmonate (an inducer of proteinase inhibitors; Farmer and Ryan, 1990), arachidonic acid (an inducer of the IPR protein STH-2 - Marineau *et al.*, 1987), salicylic acid (known to induce the 'classical' PR proteins; Hennig *et al.*, 1993) and abscissic acid (involved in the induction of the pea IPR proteins ABR17 and ABR18 - Barratt and Clark, 1991; also involved in the regulation of proteinase inhibitor gene expression - Peña-Cortés *et al.*, 1991). Neither methyl jasmonate nor arachidonic acid were found to cause any detectable alterations in levels of accumulated AoPR1 protein, although this observed lack of induction may, in part at least in the case of arachidonic acid, be attributable to the absence of wound sites limiting the intracellular concentration of active elicitor. This is in contrast to experiments by Brisson and colleagues (Marineau *et al.*, 1987) where the eliciting agent was applied directly to the cut surface of potato tuber slices thereby imposing the elicitation stress on wounded material. The AoPR1 protein was, however, found to accumulate in response to the exogenous application of 4 mM salicylic acid to levels comparable to those observed upon wounding of etiolated seedling material, and in response to 250 μM ABA, although to much lower levels. Comparisons of induction levels in response to salicylic acid with levels observed for other defence-related proteins are not possible in this case as data concerning PR gene induction by salicylic acid routinely utilises promoter-reporter gene constructs in contrast to the protein analysis carried out in this chapter. Further work to investigate the effect of salicylic acid on AoPR1 gene induction using promoter-reporter gene constructs will be detailed in a subsequent chapter. Little information is available regarding the effect of ABA on defence gene expression.

However, the pea IPR proteins ABR17 and ABR18 are responsive to levels of 100 μM ABA when supplied with 100 μM ABA *via* the transpiration stream (Barratt and Clark, 1993).

3.3.3 Some members of the IPR family are immunologically related.

Transcript encoding the 17 kD potato defence-related protein STH-2, a member of the IPR protein family, has been reported to accumulate in potato tuber slices treated with arachidonic acid (Marineau *et al.*, 1987). The isolation of the corresponding cDNA and subsequent production of a ß-galactosidase-STH-2 fusion protein allowed the generation of an anti-STH-2 polyclonal antibody (Constabel and Brisson, 1992). Having obtained this antibody from Dr. N. Brisson it was demonstrated that the AoPR1 protein was immunologically related to STH-2. The STH-2 antiserum was found to interact specifically wth the AoPR1 protein and was able to detect a 17 kD band in protein extracts from mechanically isolated asparagus cells. This serological relationship between members of the IPR family has not previously been reported. The ability of this STH-2 antibody to immunoreact with other IPR proteins has not been extensively investigated. Studies detailing the extent of relatedness between the STH-2 protein and the parsley IPR proteins have been carried out (I. Somssich, pers. comm.). No significant interaction was observed to occur. Thus it may be possible that AoPR1 and STH-2 represent an immunologically-related subgroup of IPR proteins. Further serological characterisation of other members of the IPR protein family is necessary to confirm/reject this hypothesis.

3.3.4 The AoPR1 protein family comprises at least five serologically related proteins showing differential induction in response to various stimuli.

Using the STH-2 antibody as an AoPR1 probe comparisons between 2-D westerns using both this antibody and the DD1 antibody revealed that the protein profile observed was identical accross the pI range 6.85-8.5 indicating that AoPR1 exists as a protein family in asparagus, data consistent with observations on the ABR protein family of pea (Barratt and Clark, 1993). Previous work by Fioroni (1989) indicated that these AoPR1(DD1) proteins arise as a result of the transcription of individual transcripts and not as a consequence of post-translational

modification. This data was confirmed by computer-aided analysis of the predicted protein sequence which failed to reveal possible sites of modification. Two further cDNAs were isolated by hybridisation to the AoPR1 cDNA (P. Piffanelli) and characterised. Sequence analysis indicated that all three cDNAs (AoPR1, AoPR2 and AoPR3) exhibited a high degree of identity within the coding region such that the development of gene-specific probes using these regions was not possible. This was reflected in northern and Southern analysis where results showed no significant differences between all three clones. All three cDNAs encode proteins of similar molecular weight, 17 kD, and therefore migrate the same distance on 1-D electrophoresis. It was observed, however, that nucleotide differences, where they did occur, were sufficient to result in changes in predicted amino acid sequence. This in turn resulted in alterations in pI of the predicted protein upon comparison with that of the predicted AoPR1 amino acid sequence. The two AoPR proteins encoded by the AoPR2 and AoPR3 cDNAs exhibit predicted pI values of 7.72 and 7.07 respectively and are immunologically related both to each other and to STH-2. The probability that further AoPR clones corresponding to other members of the AoPR1 protein family exist within the asparagus day 3 post-cell isolation cDNA library cannot be discounted and is considered as a proposal for future work in chapter 5.

It is interesting to observe that members of the AoPR1 protein family appear to show differential accumulation in response to the stresses imposed by wounding and mechanical cell isolation. Monitoring the behaviour of this protein family in culture indicated, as determined by 1-D gel electrophoresis and immunodetection, that AoPR1 is expressed in an hormone-independent manner, the absence of either auxin or cytokinin from the medium having no effect on levels of detectable AoPR1 protein (DD1: Fioroni, 1989). Also the observed changes in levels of detectable protein with time in culture show a marked reduction in AoPR1 (DD1) levels in established cultures. Taken together, these observations suggest that it is the wound stimulus imposed during cell isolation that is responsible for the induction of AoPR1 gene expression. This hypothesis is supported by observations by Marty *et al.* (1993) that the wound stimulus alone is sufficient to induce dedifferentiation and by

observations in this chapter indicating the accumulation of an apparently similar, if not identical, group of proteins in response to wounding and cell isolation.

3.3.5 Towards a function of the IPR proteins:

Having considered the above results it remains to be asked 'What do these intracellular PR proteins do *in planta*?'. Consideration of the sources of members of the IPR protein family would indicate some possible role in plant defence. However at the commencement of this research experiments concerned with ascertaining the nature of the involvement of IPR proteins in this response were limited. Having cloned cDNAs corresponding to genes encoding the majority of IPR proteins it is theoretically possible to over-express these proteins in transgenic plants and characterise any detectable alterations in the defence response. Such experiments are limited by the possibility that no measurable differences may be observed upon comparison of the response of plants over-expressing this gene with wild-type plants under the same conditions (Constabel *et al.*, 1993). Another possible approach would be the use of anti-sense technology to selectively "knock out" expression of the endogenous IPR protein-encoding gene. Again measurable alterations in the plants natural defence response could provide an insight into the involvement of IPR proteins in that response. Limitations are imposed upon this approach by the prerequisite of isolating IPR protein genes from readily transformable plant species such as tobacco and potato. Preliminary results presented in this chapter indicate the possibility of isolating the tobacco IPR protein homologue from petal material, while Brisson and co-workers have isolated the potato IPR protein. Similarly homologues from a number of other transformable species have recently been isolated. Thus the use of anti-sense technology may indeed prove a viable approach in investigating the role of the IPR proteins in plant defense.

The approach taken here at Leicester, however, centered around the characterisation of the expression profile of AoPR1 both in asparagus and in transgenic tobacco plants harbouring an AoPR1 promoter-*gus* construct. The AoPR1 promoter was isolated using inverse PCR (Warner *et al.*, 1993) and cloned into the binary plant transformation vector pBin19. Plants

transformed with this construct were subsequently analysed for GUS activity under a range of conditions and an extensive expression profile compiled detailing the ability of the AoPR1 promoter to drive expression of the *gus* reporter gene under these circumstances. Understanding the nature of the activity of this promoter under these conditions, it is hoped, will provide an understanding of the role played by IPR proteins, AoPR1 specifically, in the plant defence response.

Chapter 4:

Characterisation of AoPR1 promoter activity in transgenic tobacco.

4.1 Introduction:

4.1.1 Isolation of the AoPR1 promoter using inverse PCR:

Having previously isolated the AoPR1-encoding cDNA cWIP (Warner *et al.*, 1992) attempts were made to isolate the 5' untranslated region of the corresponding genomic sequence by screening an asparagus genomic library with this cDNA sequence. Following a number of unsuccessful attempts to achieve this Warner and co-workers turned to the technique of inverse PCR in an attempt to overcome difficulties which had arisen (Warner *et al.*, 1993). Designing primers towards sequence at the 5' end of the cDNA in such a manner that both primers faced in opposite directions when annealed to the target template and the use of these primers in the amplification of sequences from *Hin*dIII-digested asparagus DNA resulted in the isolation of a 1.1 kb fragment that was found to hybridise specifically with labelled AoPR1 cDNA sequence. Further analysis of this fragment revealed the presence of a 347 bp intron within the AoPR1 coding sequence containing an *Eco*RI site towards the 5' end. Amplification of putative 5' regulatory regions was thus again attempted using *Eco*RI-digested asparagus genomic DNA and resulted in the isolation of a 1.3 kb IPCR product hybridising specifically with labelled AoPR1 cDNA sequence. This fragment was subsequently cloned as two separate fragments, the larger of which was found to contain the longest contiguous stretch of upstream DNA and was chosen for further analysis. Results from S1 protection assays and sequence analysis provided strong evidence that the cloned fragment did indeed correspond to the AoPR1 5' regulatory regions. Further analysis involving the production of a gene fusion between this sequence and the coding sequence of the reporter gene *gus* were thus undertaken.

4.1.2 The AoPR1 promoter directs rapid, strong and persistent expression at sites of wounding and pathogen attack.

An AoPR1-*gus* translational fusion was created through the introduction of the AoPR1 5' sequence into the binary vector pBI101.1 (Jefferson *et al.*, 1987) as a *Bam*HI/*Sal*I fragment (Warner *et al.*, 1993). After introduction of this construct into tobacco and regeneration of plantlets, transgenic lines (as determined by Southern analysis) were assayed for GUS activity under certain conditions. Following wounding *gus* expression was elevated to high levels, as predicted from previous northern experiments where AoPR1 transcript accumulation in response to wounding was measured by detection with the AoPR1 cDNA (Warner *et al.*, 1992). Fluorometric analysis revealed a rapid and persistent induction of GUS activity, found by histochemical analysis to be localised to sites at or near to the wound site. Analysis of transgenic potato plants (*Solanum tuberosum* cv. Desirée) harbouring a similar construct revealed a similar pattern of expression in response to the wound stimulus (Wilkinson, 1993). In investigations into the effects of damage as a consequence of pathogen attack the AoPR1 promoter was found to direct expression at the edge of lesions formed upon infection of transgenics with *Botrytis cinerea* (tobacco; Warner *et al.*, 1993) and PVY (potato; Wilkinson, 1993). Infection of potato cv. Desireée with *Phytophthora infestans*, a pathogen towards which this potato cultivar is susceptible, resulted in positive GUS histochemical detection not in the halo of living cells immediately surrounding the lesion but in those cells adjacent to this halo (Wilkinson, 1993). The causal agent of soft rot in potato tubers, *Erwinia carotovora*, was also assayed for its ability to induce AoPR1-driven *gus* expression in transgenic potato (Wilkinson, 1993). In this case some slight positive staining was detected in areas around those affected by soft rot. Wilkinson concludes therefore that, since the AoPR1 promoter did not appear to respond to the presence of the pathogen itself but was activated once symptoms of disease (manifest as cell and tissue damage) appeared, the stimulus for the AoPR1 promoter appears to correlate with damaged tissue, whether that damage is caused by mechanical wounding or pathogen infection.

4.1.3 AoPR1-driven *gus* activity is detected in tissues enriched for phenylpropanoid metabolism.

Preliminary histochemical and fluorometric *gus* analysis of transformants harbouring the 'full' AoPR1 promoter-*gus* transgene revealed some developmental control aspects to the regulation of this promoter in transgenic tobacco. Interestingly those tissues where AoPR1-driven *gus* expression was detected appeared also to be those tissues where enzymes of the phenylpropanoid pathway have previously been reported to be active (section 1.3.2). GUS activity was observed in root, pollen, stigma, stem and petal tissue. Activity was virtually non-detectable in mature seed. It may be noticed that these regions of detectable GUS activity have, in a number of cases, also been shown to be sites of IPR protein localization (Barratt and Clark, 1991; Breiteneder *et al.*, 1989)

4.1.4 The response of the AoPR1 promoter to wounding is enhanced in the presence of salicylic acid.

The implication of salicylic acid (SA) as a general 'defence response' messenger (Yalpani *et al.*, 1991) eliciting the induction of a range of defence-related genes led Warner (1992) to a preliminary investigation into the possible effects exerted on the AoPR1 promoter by this elicitor. Assays were carried out by incubation of leaf discs, either wounded (leaf discs stabbed several times with a Gilson pipette tip) or unwounded (leaf discs which had not been so treated), on filter paper saturated with 4 mM SA for a period of two days at 25^{o}C followed by fluorometric estimation of GUS activity in chosen explants. Results indicated that treatment with 4 mM SA alone is sufficient to cause an increase in GUS activity in transgenic plants containing the 'full' AoPR1 promoter-*gus* transgene. Treatment of wounded explants with 4 mM SA further increased measurable GUS activity in a 'super induction'-type effect.

4.1.5 AoPR1-driven reporter gene expression as a marker for successful *Agrobacterium*-mediated transformation of leaf explants.

As a consequence of the source of the original cDNA corresponding to the AoPR1 transcript ie. mechanically isolated asparagus mesophyll cells, being a highly enriched source of dedifferentiation-related transcripts it was proposed that the AoPR1 promoter should be highly active in disorganised tissue such as callus material. Work by Warner (1992) and Ozcän (1993) confirmed this hypothesis. Thus further work aimed at exploring this expression further and the exploitation of this promoter sequence in plant transformation procedures as a component of transformation marker gene cassettes was undertaken (Firek *et al.*, 1993). Unlike other promoters commonly employed to drive expression of selectable marker genes during transformation (e.g. the CaMV35S promoter), the AoPR1 promoter drives strong expression at wound sites (Warner *et al.*, 1992) and in callus material while levels of expression are extremely low in leaf and root tissue (Firek *et al.*, 1993). Expression of an AoPR1-*gus* gene fusion during the callus stage of transformation was found to be maximal at approximately 4-5 days post inoculation, at which time wound-induced cell division has been initiated and cells are undergoing the dedifferentiation response (Firek *et al.*, 1993). The introduction of the *npt*II gene under the control of the AoPR1 promoter into tobacco using standard *Agrobacterium*-mediated transformation methods allowed selection of transformed material on medium containing 100 μg/ml kanamycin, while regenerated plantlet material showed very little NPT-II activity unlike extracts from CaMV35S-*npt*II-containing transgenics (Firek *et al.*, 1993). Thus the potential to exploit the AoPR1 promoter in plant transformation vector construction exists and has been shown to be a viable consideration as it provides a means of overcoming consumer problems and objections raised as a consequence of the expression of relatively high levels of selection gene products in transgenic plants.

4.1.6 Summary, discussion and chapter aims.

Having seen that the AoPR1 promoter is active in the heterologous hosts tobacco and potato in a manner similar, at least in relation to the expression profile observed, to that in its native

host asparagus (chapter 3), it may be speculated that the introduction of a monocot-derived promoter into a dicot species does not significantly perturb expression. This statement must, for the moment however, be restricted to observations made on the basis of the expression profile detected in both species - in asparagus as observations on the basis of northern and western analysis (chapter 3) and in tobacco on the basis of reporter gene expression under the control of the AoPR1 promoter (chapter 4). Conclusions relating to those aspects of gene expression concerning protein-DNA interactions and the conservation of these interactions or the potential to undergo these modifications between species and especially between monocots and dicots are not justifible on the basis of the above observations alone. For the purposes of this thesis it is prudent to bear in mind the possibility that although the expression profile conferred upon a gene by the AoPR1 promoter appears to be conserved accross species barriers those proteins whose interactions, either direct or indirect, with the promoter sequence confer this specificity of expression may differ. Thus any further reference to the AoPR1 expression profile in tobacco and inferences derived from its analysis must be restricted to, and not extrapolated from the characterisation of this monocot promoter in a dicot species (tobacco).

Having considered the above restrictions relating to interpretation of results obtained from an analysis of AoPR1 promoter activity in transgenic tobacco it was decided to further explore the many interesting aspects of the regulation of this promoter under these conditions such that a deeper understanding of what regulates transcription of genes under the control of this promoter and how this regulation occurs may be gained. A knowledge of those agents capable of causing the induction of any gene and of how these factors influence gene expression may be of considerable importance in postulating a role for the protein of unknown function encoded by that gene. Such is the case with AoPR1. The predicted protein encoded by the AoPR1 cDNA has been found to belong to a family of intracellular PR proteins (IPR proteins - PR10's). Unfortunately no function has as yet been ascribed to members of this family although among those proposed are that of an albumin-type storage protein (Barratt *et al.*, 1989) and, more recently, that of a ribonuclease (Moiseyev *et al.*,

1994). This latter function has been inferred by homology between members of the IPR protein family, in particular the parsley members PcPR1-1 and PcPR1-3 (Somssich *et al.*, 1988; van de Löcht *et al.*, 1990), and the recently characterised ginseng RNase reported by Moiseyev *et al.* (1994). RNase activity has not, however, been detected with *E. coli*-expressed PcPR1-1, PcPR1-3 (I. Somssich, pers. comm.) and similarly expressed AoPR1 (S. Warner, pers. comm.). Moiseyev *et al.* are quick to point out, however, that such activity, if found to be present in other members of the IPR protein family may not in fact represent the primary function of these proteins. Rather, they speculate, it may be the case that the IPR proteins play a much more significant role in plant defence and possibly development, possessing RNase activity only as a subsidiary function. Considering the expression profiles observed not only for AoPR1 but also for other members of this family and an apparent inability to detect ribonuclease activity in a number of IPR proteins this idea may be an attractive hypothesis. It must, however, be considered that the IPR proteins assayed - PcPR1-1, PcPR1-3 and AoPR1 - were derived from bacterial sources and not purified from the host plant, unlike the ginseng RNase which was initially purified from ginseng callus culture. It may be possible, therefore, that some *in planta* modification is necessary in order to confer RNase activity upon these proteins, and therefore the possibility that the IPR proteins (or at least some members of this protein family) do possess ribonuclease activity may not be conclusively discounted.

It is interesting to note that the ginseng RNase protein was isolated from callus culture, a highly dedifferentiated cell population. This type of material, as previously discussed (chapter 3) is highly enriched for the expression of defence-related and wound-inducible genes. Indeed a number of the IPR proteins have also been isolated from similar or related systems (Moiseyev *et al.*, 1994; Somssich *et al.*, 1988; Walter *et al.*, 1990) including AoPR1 (Warner *et al.*, 1993). The AoPR1 promoter has been shown to direct strong expression of the *gus* gene in transgenic tobacco persistent with the maintenance of the disorganised state (Ozcän, 1993). This phenomenon has been further explored with a view to employing this promoter to drive selection markers during plant transformation programmes (Ozcän, 1993;

Firek *et al.*, 1993). The rapidity and persistence of the response of this promoter to the wound stimulus which triggers the dedifferentiation process (section 3.1.1) leads one to speculate that the AoPR1 protein may have some involvement in the cell reorganisation aspect of this process ie. in those cellular events which allow cellular components to under go major alterations in morphology and biochemistry/physiology while simultaneously maintaining their fundamental integrity. AoPR1 may therefore play a role in sub-cellular stabilisation during the dedifferentiation phase. The exact nature of those elements of the wound stimulus responsible for the induction of expression of the AoPR1 gene appears somewhat ambiguous although it does appear from work carried out by Wilkinson (1993) that induction arises as a consequence of events dependent directly on the wound stimulus. With compatible plant-pathogen interactions, where mechanical damage is minimised during infection and HR is not induced, no significant increase in AoPR1 promoter activity is observed upon comparison with induction levels obtained following induction of the hypersensitive response. This would imply that the ability of the AoPR1 promoter to respond to the presence of a pathogenic organism is dependent on the ability of the plant to perceive the physical stimulus of mechanical damage caused either by the progress of the pathogen through the plant or by the hypersensitive response.

Interesting also is the observation that the AoPR1 promoter is responsive to salicylic acid at a concentration of 4 mM (Warner, 1992). Levels of induction obtained following prolonged exposure of leaf explants to this concentration of salicylic acid were found to be comparable with induction levels for the tobacco PR1a promoter under similar conditions (van de Rhee *et al.*, 1990; Ohshima *et al.*, 1990). One can clearly see from previous discussion (section 1.3.2.1) that salicylic acid induction of PR protein gene product accumulation is a complex and not yet fully understood process. As previously mentioned, the response of the AoPR1 promoter to 4 mM salicylic acid is comparable to the response of the tobacco PR1a promoter under similar conditions. It was therefore considered appropriate to further investigate the nature of this response in AoPR1-*gus* transgenics to gain a deeper insight into the effect of salicylic acid on intracellular PR protein gene expression and to provide a comparison

between salicylic acid induction of a member of the 'classical' PR proteins (PR1a) and a PR-10 protein (AoPR1).

Of interest also was the nature of the relationship between the phenylpropanoid pathway (section 1.2.3.1) and AoPR1 expression. Preliminary data available pointed towards a striking correlation between AoPR1-driven *gus* expression and known sites of phenylpropanoid pathway activity in transgenic tobacco. GUS activity was detected at sites previously shown to be sites of active phenylpropanoid metabolism - stems, pollen, petal collars, seed and stigma. These observations were made based primarily on visual data and thus a further, more detailed analysis of the relationship existing between AoPR1 activity and phenylpropanoid metabolism was deemed necessary in order to discern the extent of correlation, if any, existing between the two

In order, therefore, to answer the large number of questions posed by data already available concerning AoPR1 expression in transgenic tobacco a comprehensive and detailed analysis of the behaviour of the 'full' AoPR1 promoter in such a context was undertaken. Results from these investigations are presented below.

4.2 Investigating the behaviour of the 'full' AoPR1 promoter in transgenic tobacco.

4.2.1 Optimisation of assay techniques for the detection of the reporter enzyme ß-glucuronidase (GUS):

Fundamental to the analysis of the behaviour of the AoPR1-*gus* transgene in transgenic tobacco was the optimisation of analytical conditions employed in the assessment of GUS activity in extracts from various tissue types. Since the possibility exists that different tissues may exhibit differential tolerance to the accumulation of the GUS protein it was deemed necessary to investigate the effect, if any, exerted on *gus* expression by extracts of different tissues. Furthermore, while the tissue remains intact it is possible that the enzyme is unaffected by any inhibitors or 'promoters' of enzyme activity which may reside within the

cell as a consequence of compartmentalisation within the cell itself. Homogenisation of the tissue would therefore destroy this cellular integrity thereby releasing compounds into the immediate vicinity of the GUS protein which could potentially modify its activity in some way. In order to explore this possibility further the following series of experiments was designed and carried out.

Extracts from tissues which were to be subjected to GUS analysis in future experiments were obtained by homogenisation of the relevant material from an untransformed tobacco (SR1) plants in two volumes of GUS extraction buffer (GEB: section 2.18.3). Clarification was achieved by centrifugation at 13,000 r.p.m. for 10 min. in a bench top centrifuge. All extraction procedures were carried out at 4°C. The effect of increasing GUS concentration and incubation time on fluorescence was determined in order to define those assay conditions to be employed in tissue analysis experiments. Pure GUS enzyme (1 mg/ml in 0.1 M $NaPO_4$, pH 7.0) was diluted to appropriate concentrations (0-5 ng in 0.5 ng increments) with GEB to a final volume of 20 µl. This was then added to 180 µl 1 mM MUG in GEB and incubated at 37°C for 0-60 min. At times corresponding to 10, 30 and 60 min. incubation a 20 µl aliquot of the reaction mix was removed, added to 180 µl 0.2 M Na_2CO_3, pH 11.2 and stored at room temperature. Once all samples had been removed fluorescence was determined by analysis using a Dynatech MicroFluor plate reader. Results are presented in fig. 4.1. From these results it was determined that an incubation time of 30 min. was appropriate for future analysis of the effect of tissue-specific factors on GUS activity.

Experiments concerned with the determination of tissue-specific inhibition/promotion of GUS activity were conducted in a manner similar to that described above. The experimental set-up was essentially the same as previously utilised apart from the addition of specific concentrations of tissue extracts (1-20 µg total protein as determined by the method of Bradford (1976; section 2.19.4)). In all cases the final reaction volume was 200 µl, with substrate concentration being constant. The results of these investigations are presented in fig. 4.2. Table 4.1 summarises the results of statistical analysis of data obtained.

Figure 4.1:

Calibration curve illustrating the effect of increasing GUS concentration and incubation time on fluorescence as determined by measurement using a Dynatech MicroFluor plate reader.

Standard concentrations of GUS enzyme were prepared by dilution of a stock of 1 mM GUS (made up in sodium phosphate buffer pH 7.0) to the desired concentrations in a 20 µl volume with GUS extraction buffer (GEB). This was then added to 180 µl MUG (in GEB) and incubated at 37°C for the desired period of time. Samples were assayed at T=10, 30 and 60 min. by the removal of a 20 µl aliquot and addition of this to 180 µl 0.2 M sodium carbonate pH 11.2. Fluorescence was detected as described above.

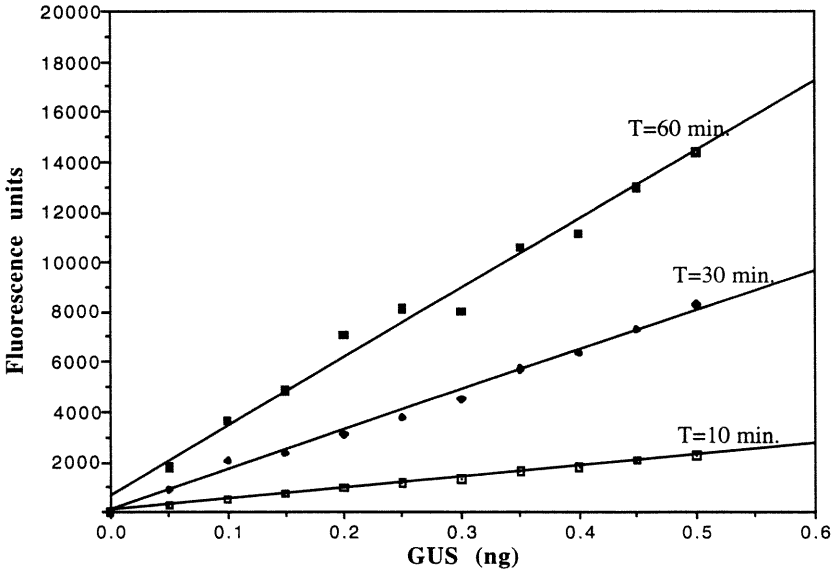
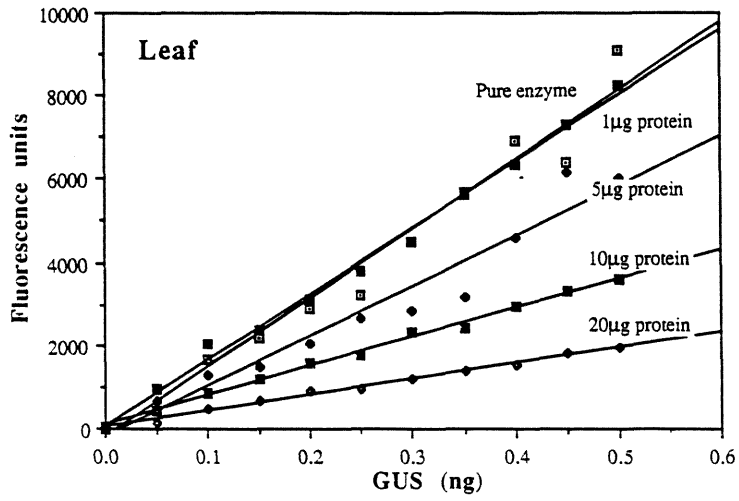

Fluorescence units
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0.0
0.1
0.2
0.3
0.4
0.5
0.6
GUS (ng)
T=60 min.
T=30 min.
T=10 min.

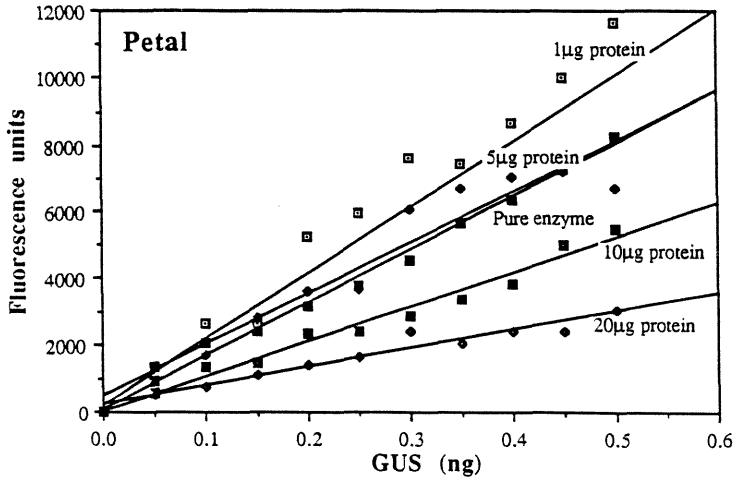
Figure 4.2:

Effect of increasing tissue extract concentrations on GUS activity:
(a) Leaf; (b) Petal; (c) Pollen; (d) Stigma; (e) Seed; (f) Stem

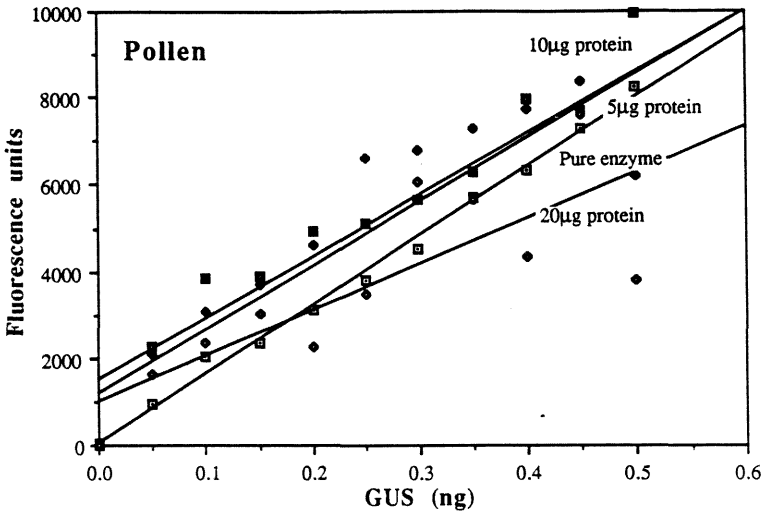
(A)


Leaf
Pure enzyme
1μg protein
5μg protein
10μg protein
20μg protein
Fluorescence units
GUS (ng)
0
2000
4000
6000
8000
10000
0.0
0.1
0.2
0.3
0.4
0.5
0.6

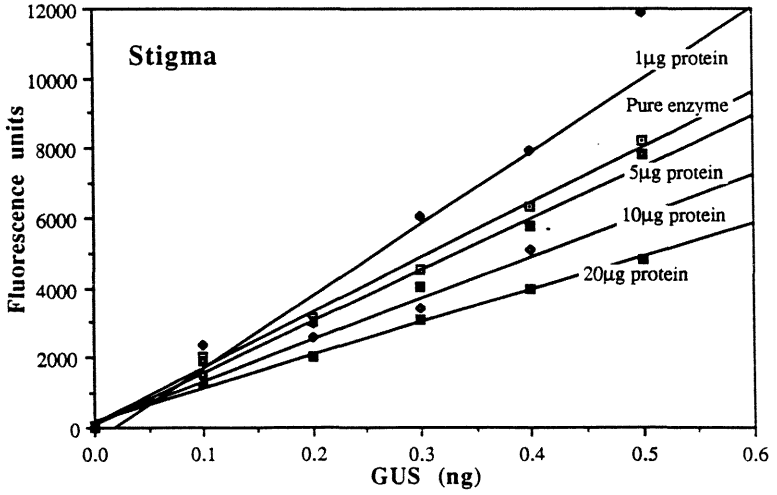
(B)


Petal
1μg protein
5μg protein
Pure enzyme
10μg protein
20μg protein
Fluorescence units
GUS (ng)
0
2000
4000
6000
8000
10000
12000
0.0
0.1
0.2
0.3
0.4
0.5
0.6

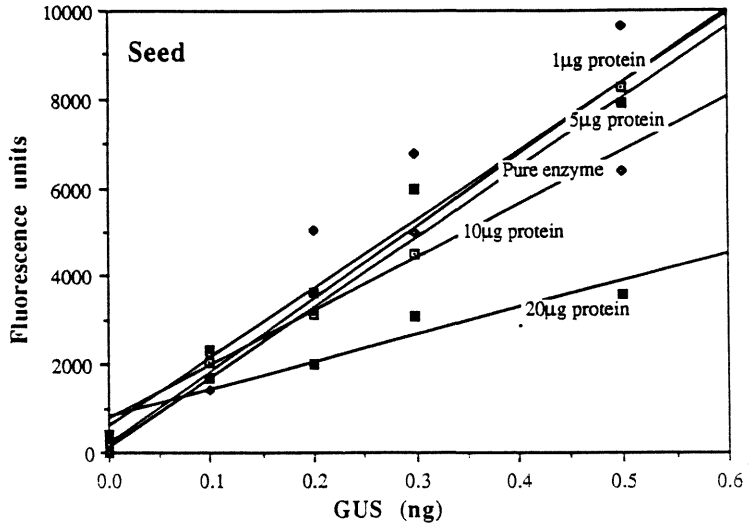
(C)


Pollen
10µg protein
5µg protein
Pure enzyme
20µg protein
Fluorescence units
GUS (ng)
0
2000
4000
6000
8000
10000
0.0
0.1
0.2
0.3
0.4
0.5
0.6

(D)


Stigma
1µg protein
Pure enzyme
5µg protein
10µg protein
20µg protein
Fluorescence units
GUS (ng)
0
2000
4000
6000
8000
10000
12000
0.0
0.1
0.2
0.3
0.4
0.5
0.6

(E)


Seed
1µg protein
5µg protein
Pure enzyme
10µg protein
20µg protein
Fluorescence units
GUS (ng)
0
2000
4000
6000
8000
10000
0.0
0.1
0.2
0.3
0.4
0.5
0.6

(F)

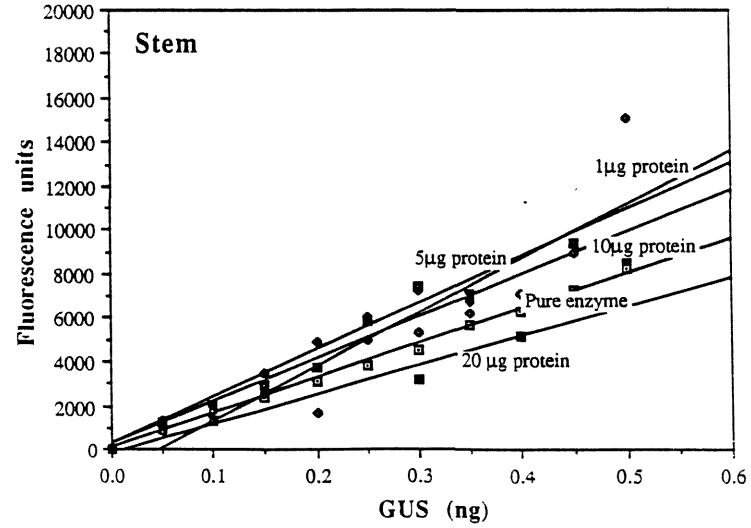

Stem
1µg protein
5µg protein
10µg protein
Pure enzyme
20 µg protein
Fluorescence units
GUS (ng)
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
0.0
0.1
0.2
0.3
0.4
0.5
0.6

Table 4.1:

Statistical analysis of the effects of increasing sample extract protein concentrations on GUS activity.

Results are represented by t-values indicating the significance of differences between enzyme activity in the presence of increasing concentrations of tissue extract when compared with that of pure enzyme.

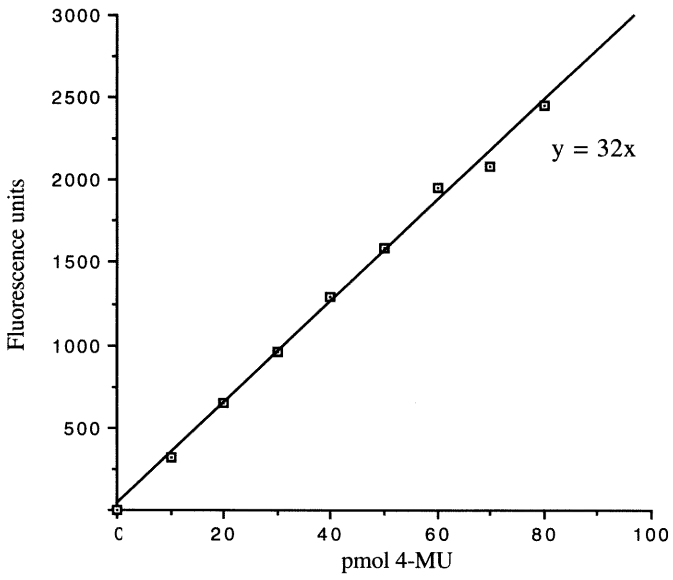
Key: NS: not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Protein (μg)	1	5	10	20
Leaf	t_{18}=2.26*	t_{18}=3.31**	t_{18}=4.63***	t_{18}=5.46***
Pollen	Not assayed	t_{18}=2.71*	t_{18}=2.24*	t_{18}=2.5*
Petal	t_{18}=3.08**	t_{18}=1.17 (NS)	t_{18}=3.76**	t_{18}=5.01***
Stem	t_{15}=3.30**	t_{17}=2.04 (NS)	t_{18}=5.41***	t_{12}=5.36***
Seed	t_6=4.56**	t_6=0.64 (NS)	t_6=6.16**	t_6=18.9***
Stigma	t_8=2.96*	t_8=0.12 (NS)	t_8=0.46 (NS)	t_8=0.77 (NS)

Figure 4.3:

Calibration curve illustrating the effect of increasing concentrations of 4-MU on fluorescence as determined by measurement using a Dynatech MicroFluor plate reader.

A standard curve of increasing concentrations (pmol/reaction volume) of 4-MU was prepared by dilution of a 1 mM stock of 4-MU (made up in GEB) to 20 µl with GEB. This was then added to 190 µl 0.2 M sodium carbonate pH 11.2 prior to fluorescence determination as described above.


3000
2500
2000
1500
1000
500
Fluorescence units
y = 32x
0
20
40
60
80
100
pmol 4-MU

Results obtained clearly indicate the tissue-dependent nature of GUS activity determination showing, for example in the case of leaf extract, assays carried out on 5 μg total cell protein would result in artificially low activity values as a direct consequence of the addition of significant amounts of chlorophyll concomittant with the addition of the GUS protein. The ability of chlorophyll to absorb light at those wavelengths emitted following excitation of 4-MU would thus decrease the levels of emitted fluorescence detected. The presence of pigments capable of absorbing emitted energy and the possible presence of agents capable of modulating GUS activity must therefore be acknowledged. It may be seen that the addition of increased concentrations of tissue extract does not necessarily lead to higher activity values; rather it appears that in general the addition of more extract biases the experiment in favour of significantly reduced levels of apparent detectable GUS activity. Subsequent GUS assays were carried out as described in section 2.18.3, fluorescence values being converted into units of product (4-MU) using the standard curve presented in fig. 4.3.

4.2.2 Detection of the AoPR1 promoter-*gus* transgene in transgenic tobacco.

Transgenic tobacco (SR1) plants into which an AoPR1 promoter-*gus* fusion transgene had been introduced were analysed for the presence of this transgene using a PCR-based approach. Seed from putative transformants was germinated on kanamycin (100 μg/ml)-containing media and kanamycin-resistant seedlings transferred to soil. Approximately 97% of seedlings plated out germinated, with 94% of these being kanamycin resistant as determined by the resistance of these seedlings to bleaching. This lack of apparent segregation of kanamycin resistance indicates the probable presence of multiple insertions of the introduced transgene into the SR1 genomic background. Genomic DNA was isolated from leaf discs of a number of chosen plants as previously described (section 2.7.2) and 5 μl used as template in PCR reactions. Using the primers WIP 1 (5' CCGGTACCTCAGGACTAGACC 3') and WIP 2 (5' GTAGCCACGATGTATTGGCGCCGACGTCGG 3') previously employed by Ozcan (1993) in the amplification of the AoPR1 promoter for use in the construction of a transcriptional gene fusion, a band of approximately 900 bp was generated under standard reaction

Figure 4.4:

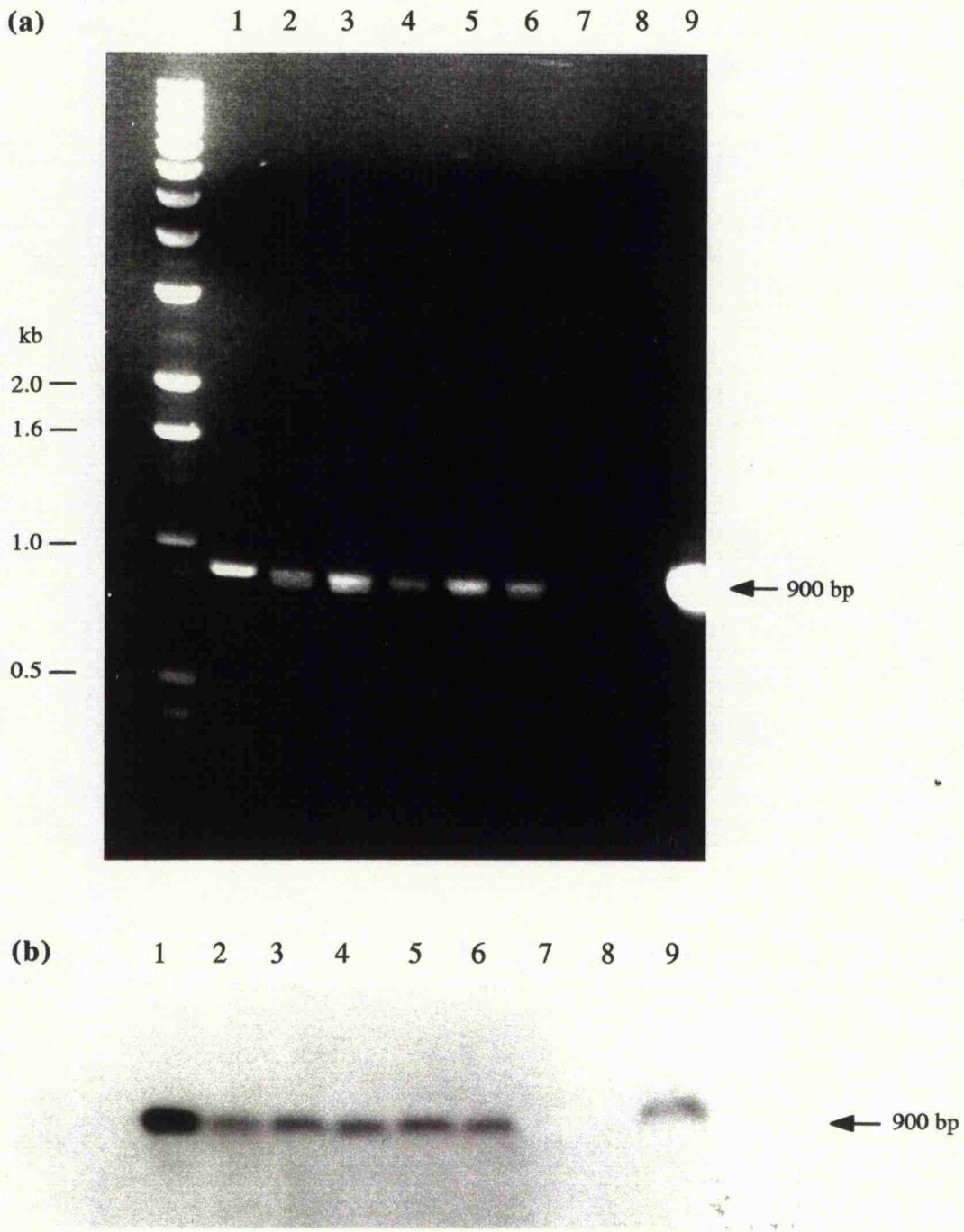
(a) PCR amplification of AoPR1 promoter sequence from DNA extracted from putative transformants harbouring the 'full' AoPR1-*gus* construct.

Lane 1: Positive control plant
Lane2: Line 1-S
Lane 3: Line 4-S
Lane 4: Line 5-S
Lane 5: Line 7-S
Lane 6: Line 14-S
Lane 7: Untransformed SR1
Lane 8: Empty
Lane 9: Plasmid control (pBI101.1-'full') (10 ng)

DNA extraction from plants was as in section 2.7.2. Primers used were WIP 1 and WIP 2 (section 4.2.2) under standard reaction conditions (95°C, 30 s; 55°C, 30 s; 72°C, 1 min. 30 s) for 25 cycles.

(b) Southern analysis of (a) probed with labelled AoPR1 promoter sequence.

Hybridisation was carried out overnight at 65°C followed by 1x wash A, 65°C, 15 min. and 2x wash B, 65°C, 15 min. Filters were autoradiographed overnight at -80°C.


(a)
1 2 3 4 5 6 7 8 9
kb
2.0
1.6
1.0
0.5
900 bp
(b)
1 2 3 4 5 6 7 8 9
900 bp

conditions (Denature: 95°C, 30 s; Anneal: 55°C, 30 s; Extend: 72°C, 1 min. 30 s) for 30 cycles from DNA into which the transgene had stably integrated. A control reaction using DNA extracted from untransformed SR1 failed to result in amplification of AoPR1 promoter sequence or of any non-specific sequences (fig.4.4a; lane 7). On the contrary, a plant previously shown to contain the AoPR1-*gus* transgene by histochemical methods (leaf discs were wounded several times, incubated overnight at 25°C and then histochemically analysed for GUS activity) yielded a single band of a size comparable with that generated using 10 ng of pSKII$^+$-WIP-*gus* as template (fig.4.4a; lanes 1 and 9). The presence of a correctly sized fragment in samples amplified from all chosen transgenics indicated successful integration of the AoPR1-*gus* transgene into the genomic DNA (fig.4.4a; lanes 2-6). Amplification of sequences from certain transgenics (lines 4-S, 7-S and 16-S) appeared to result in the generation of two similarily sized fragments (fig.4.4a; lanes 2,3 and 6) possibly indicating the occurrance of some form of genomic rearrangement of the introduced transgene. Southern blot analysis, however, revealed that only one of these fragments cross-hybridised with a radiolabelled AoPR1 promoter probe. This hybridising band corresponded in size to similarily cross-reacting bands in transgenic samples amplifying a single AoPR1 promoter fragment (fig.4.4b; lanes 1,4 and 5) as well as to the single fragment produced upon PCR amplification of AoPR1 promoter sequences from pSKII$^+$-WIP-*gus* (fig.4.4b; lane 9). Thus it is possible that the second band detected in some samples arises as a consequence of the non-specific interaction of the WIP 1 and WIP 2 primers with SR1 genomic DNA. The absence of this band in other samples including the untransformed SR1 sample is possibly attributable to differences in template concentration between PCR reactions. Since only a single band of approximately 900 bp is found to hybridise with the AoPR1 promoter in all cases except that of untransformed material and this band is of the same size as that detected in PCR amplified samples of a known positive plant and pSKII$^+$-WIP-*gus* a plasmid containing the full AoPR1 promoter sequence, it appears that all transgenics analysed do indeed contain the AoPR1-*gus* transgene in a stably integrated form within the genome. Confirmation of the transgenic nature of these transformants was obtained by histochemical analysis of a small piece of wounded leaf material as previously described. In all cases the presence of the AoPR1-*gus*

transgene was correlated with histochemically-detectable GUS activity following wounding of leaf material.

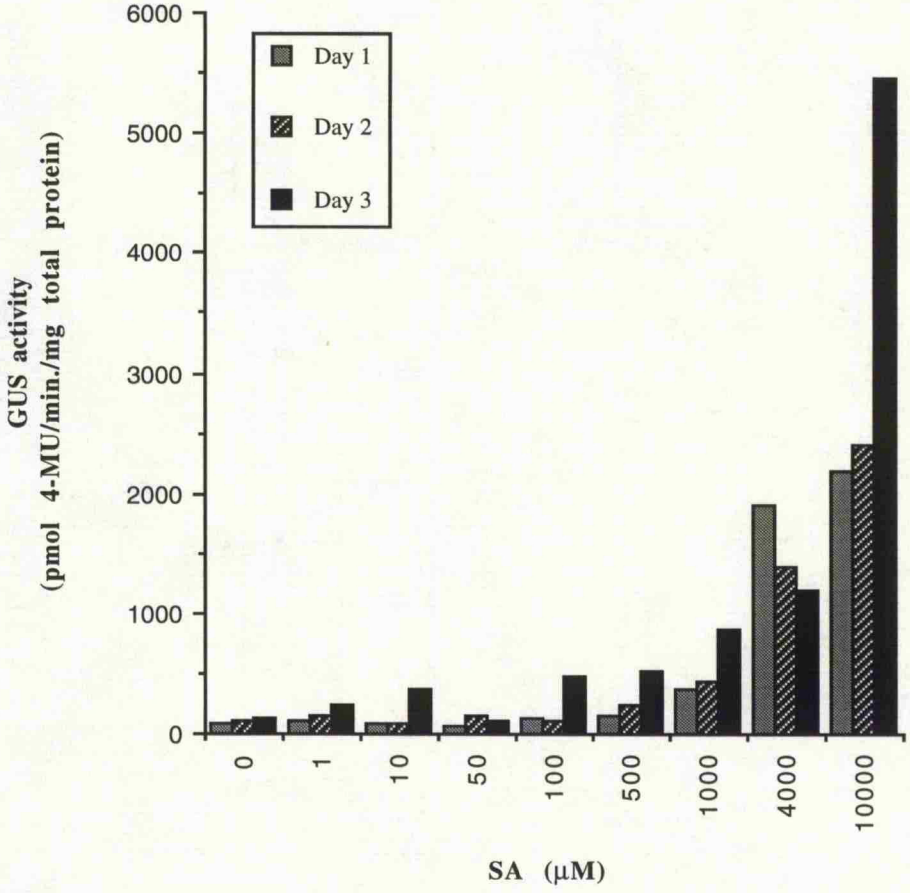
4.2.3 The AoPR1 promoter responds to exogenously supplied salicylic acid in a concentration-dependent manner.

Leaf discs (1 cm^2) were excised from transgenic tobacco plants previously shown to be stably transformed with the AoPR1-*gus* transgene and incubated at 25°C on filter paper soaked in increasing concentrations of SA for 1-3 days. At the appropriate time points (1, 2 and 3 days) samples were analysed for GUS activity using fluorometric assay methods. Leaf discs were rinsed several times with distilled water immediately prior to assay in order to remove any surface SA which could result in artifically high fluorescence readings as a result of the ability of SA to fluoresce at wavelengths similar to those used in the detection of 4-MU, the fluorescent product of the GUS-catalysed cleavage of MUG. Results from this analysis are presented in fig.4.5. and reveal that the AoPR1 promoter is responsive to exogenously applied SA at concentrations greater than 0.1 mM. At concentrations below this no marked induction of promoter activity was observed. After one day incubation this response appears to be saturated at concentrations greater than 4 mM as no significant induction was detected above that observed when leaf discs were incubated in the presence of this concentration of SA. However, at T=2 days AoPR1 promoter activity, as determined by levels of GUS activity, was seen to increase with increasing SA levels up to 10 mM. This would appear to indicate some form of dose-dependent induction of the AoPR1 promoter under the assay conditions employed. Incubation of explants for a further day maintained this apparent concentration dependency although the extreme increase observed between the effects of 4 and 10 mM SA and the non-linearity of the response now clearly observed indicates the possible involvement of some other factor(s) capable of inducing AoPR1 promoter activity.

Figure 4.5:

Graphical representation of the effect of increasing concentrations of salicylic acid on AoPR1-driven *gus* expression in transgenic tobacco.

Leaf discs (1 cm^2) were excised from leaves of tobacco plants previously shown to contain the 'full' AoPR1-*gus* construct and incubated in the presence of increasing concentrations of salicylic acid for 1, 2, and 3 days. Fluorometric GUS assays were carried out as in section 2.18.3. Each value represents the average of assays carried out on at least three independent explants.


GUS activity
(pmol 4-MU/min./mg total protein)
6000
5000
4000
3000
2000
1000
0
Day 1
Day 2
Day 3
0
1
10
50
100
500
1000
4000
10000
SA (μM)

4.2.4 The SA responsiveness of the AoPR1 promoter is further enhanced following wounding.

Having established to some extent that the AoPR1 promoter responds to exogenously applied SA in a concentration-dependent manner the question posed was 'Would the wound response of the promoter be able to override this SA induction or would both stimuli interact with a resulting 'super-induction' of AoPR1 activity?'. In order to investigate this further experimental analysis similar to that used above was employed. Parallel experiments in which explants were either wounded by stabbing a number of times with a Gilson pipette tip or left unwounded were set up, incubated at 25°C and samples assayed for GUS activity at 1, 2 and 3 days. Results (fig.4.6) showed that the dose-dependence of the SA response is retained although enhanced in the presence of the wound stimulus. After one day (fig.4.6a) the SA response appears to dominate with levels of super-induction as a consequence of wounding being apparent although not dramatic. Two days after application of the stimulus the effect of wounding seems to become more apparent resulting in a clearly visible super-induction of AoPR1 activity (fig.4.6b). Comparison of levels of measured GUS activity after one and two days shows some induction attributable to increasing incubation time but also some level of induction which may be ascribed to the manifestation of the wound responsive nature of the AoPR1 promoter. This effect is most clearly apparent when an SA concentration of 4 mM is used although 10 mM SA generates a similar but not quite as dramatic response. Incubation in the presence of SA for a period of three days causes further induction of GUS activity apportionable in the main to the superimposition of the wound stress upon SA exposure (fig.4.6c). Again this phenomenon is most visibly manifest in the presence of 4 mM SA. However, at a concentration of 10 mM SA the response appears to break down. As mentioned previously this may be the result of a number of secondary physiological effects of exposure to such a high level of SA for a prolonged period of time.

Investigations into the nature of this 'super-induction' effect showed that not only is the overall result to increase detectable levels of activity after a period of 1-2 days, but that differences in initial rates of AoPR1-*gus* induction of the response are detectable as early as 4

Figure 4.6:

Graphical representation of the effect of increasing concentrations of salicylic acid on AoPR1-driven *gus* expression in wounded and unwounded leaf explants derived from transgenic tobacco harbouring the 'full' AoPR1-*gus* construct.

Leaf explants were excised from AoPR1-*gus*-containing transgenic tobacco, eihter wounded by stabbing several times with a Gilson pipette tip or not, and incubated in the presence of increasing concentrations of salicylic acid for up to three days. At appropriate time points explant material was harvested and subjected to GUS analysis as in section 2.18.3. Values given represent the average of assays carried out on at least three independent explants and error bars represent the standard deviation for these values.

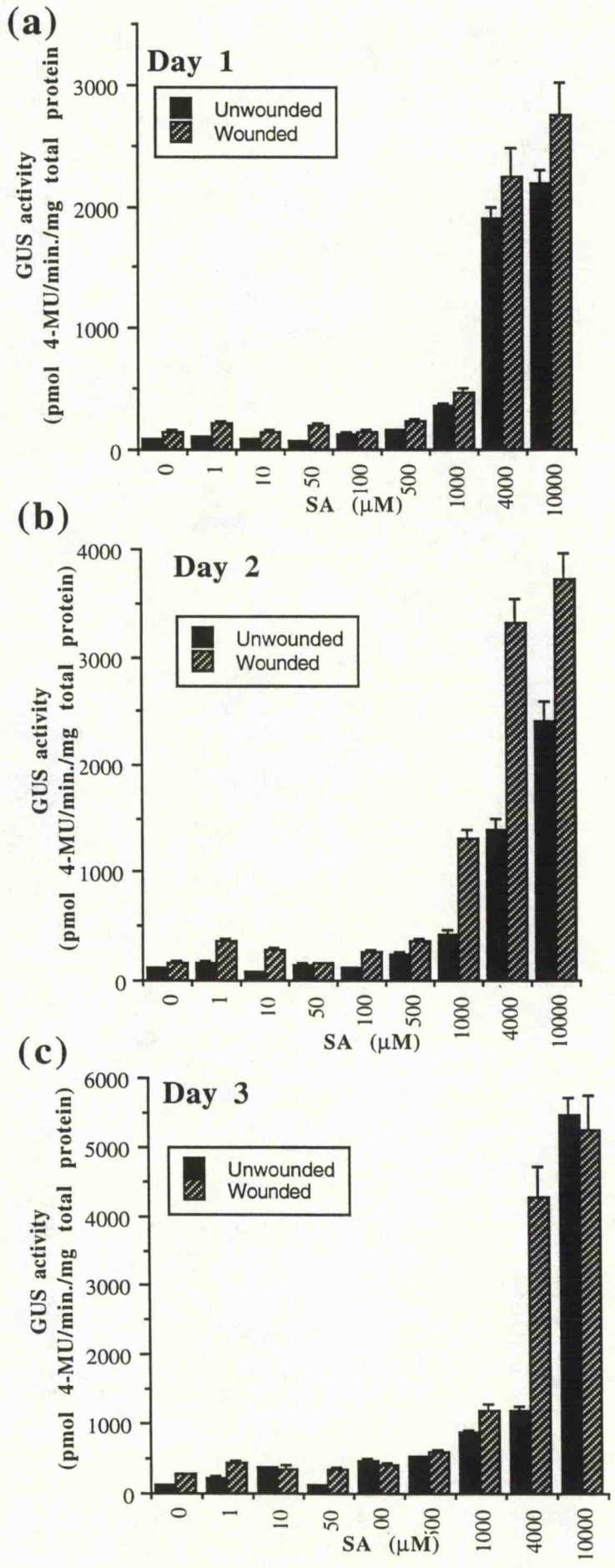
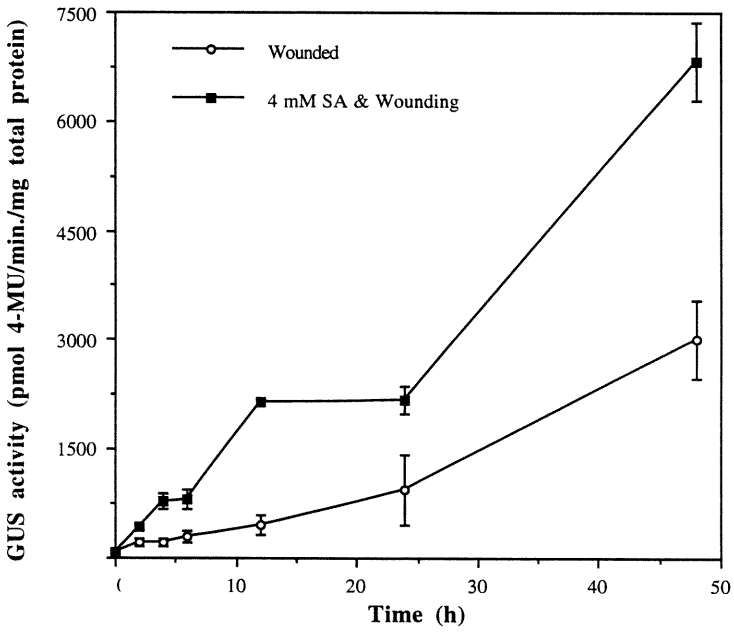

(a)
Day 1
Unwounded
Wounded
GUS activity
(pmol 4-MU/min./mg total protein
3000
2000
1000
0
0
1
10
50
100
500
1000
4000
10000
SA (μM)
(b)
Day 2
Unwounded
Wounded
GUS activity
(pmol 4-MU/min./mg total protein)
4000
3000
2000
1000
0
0
1
10
50
100
500
1000
4000
10000
SA (μM)
(c)
Day 3
Unwounded
Wounded
GUS activity
(pmol 4-MU/min./mg total protein)
6000
5000
4000
3000
2000
1000
0
0
1
10
50
00
00
1000
4000
10000
SA (μM)

Figure 4.7:

Graphical representation of GUS activity values obtained following incubation of wounded leaf discs in the presence or absence of 4 mM salicylic acid for increasing lengths of time.

Each point represents the average of assays carried out on five independent explants and error bars represent the standard deviation for these values.


GUS activity (pmol 4-MU/min./mg total protein)
7500
6000
4500
3000
1500
Wounded
4 mM SA & Wounding
10
20
30
40
50
Time (h)

h after application of the stimulus (fig.4.7). The wound response alone is detectable following 6 h incubation and activity gradually increases in a more or less linear fashion over the time course of the experiment (2 d). On the other hand, when SA exposure is super-imposed on the wound stimulus a marked change in initial rates of activity becomes apparent. This increase in induction rate is maintained throughout the 2 d period with a consequential super-induction effect.

4.2.5 Exogenous application of cinnamic and coumaric acids does not result in a significant alteration of detectable levels of AoPR1-driven *gus* expression.

Data available indicated the potential involvement of the AoPR1 gene product in the plant defense response. Central to this response is the phenylpropanoid pathway and expression of genes involved therein. Given the hypothesis that AoPR1 may, in some way, be regulated, either positively or negatively, by metabolites of this pathway or perhaps even that the gene product may be an as yet unidentified component of this pathway, the chemicals *trans*-cinnamic acid and *p*-coumaric acid, both intermediates of the phenylpropanoid pathway, were tested for their ability to influence AoPR1 expression. Under transient assay conditions it has been observed that at low concentrations (5 μM - 0.1 mM) *trans*-cinnamic acid slightly stimulated elicitor-induced expression of CAT under the control of the bean CHS promoter (Loake *et al.*, 1991). Concentrations higher than 0.1 mM severely reduced expression to below the levels observed in the absence of elicitor. In contrast, the addition of *p*-coumaric acid was found to stimulate CHS promoter activity up to 4-5 fold at a concentration of 0.5 mM. Bearing these data in mind, experimental conditions were chosen such that the dose response of the AoPR1 promoter over a range of concentrations that included those previously shown to exert an effect over the CHS promoter was analysed. Exposure to cinnamic and coumaric acids was imposed on wounded explants since it was necessary to ensure a certain level of induction in order to observe any repression effects that may arise as a consequence of the presence of exogenous chemicals. GUS activities obtained from treated explants i.e. wounded and incubated in the presence of cinnamic and coumaric acids, and untreated explants i.e. wounded only, were compared. It was found that in both cases no significant

effect was exerted on AoPR1 promoter activity by either cinnamic or coumaric acid (data not shown). Activities of explants incubated in the presence of cinnamic and coumaric acids were comparable with those of explants subjected to wounding alone. On one occassion, however, some apparent repression of activity in the presence of 1 mM cinnamic acid was detected, with repression being maintained over the duration of experimental sampling (3 d) (fig.4.8). Thus it is possible that some inhibition of AoPR1 promoter activity may occur in the presence of *trans*-cinnamic acid, although this result appears difficult to reproduce and therefore may not be an appropriate representation of the in planta effect of endogenous cinnamate levels. It is more than likely that neither *trans*-cinnamic acid nor *p*-coumaric acid exert any physiologically important effect on levels of AoPR1 expression *in vivo*.

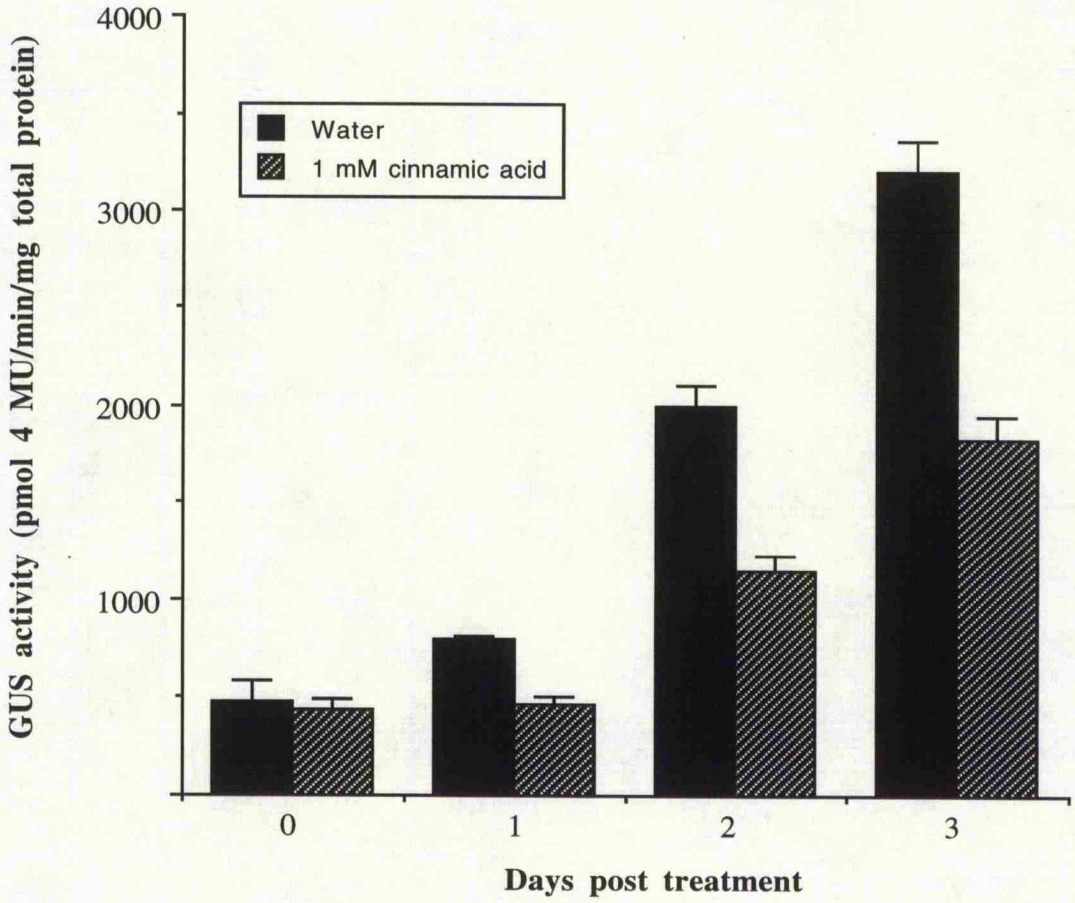
4.2.6 Strong expression of the AoPR1-*gus* transgene is not detected in established tobacco suspension cultures.

A number of the classical PR proteins and other defence-related genes studied to date have been isolated from established suspension culture-derived material (section 1.3.1). This includes the parsley cDNAs encoding the intracellular proteins PcPR1-1, PcPR1-3 (Somssich *et al.*, 1988) and PcPR2 (van de Löcht *et al.*, 1990) and other members of the IPR protein family (Moiseyev *et al.*, 1994; Walter *et al.*, 1990; Warner *et al.*, 1992). A number of suspension cultures generated from AoPR1-*gus* transgenic tobacco plants were available within the department here at Leicester. Thus it was possible to investigate the expression profile of the transgene in such a system. Cultures available were approximately six months old and were sub-cultured at weekly intervals. GUS activity was determined by fluorometric analysis at different times post-sub-culture. Results indicated that the amount of detectable AoPR1-driven *gus* expression was minimal in established suspensions. A basal level of expression was determined but no significant deviation from this was detected at any time during the sub-culture period. However, since those systems from which defence-related transcripts have previously been isolated comprise cell suspensions subjected to elicitation, one may speculate that AoPR1-driven *gus* expression may be modulated *in vitro* in a similar manner.

Figure 4.8:

Graph illustrating the effect of increasing incubation time in the presence of 1 mM cinnamic acid on AoPR1-driven *gus* expression in explants of transgenic tobacco.

Each value represents the average of assays carried out on at least three independent explants. Error bars represent the standard deviation of these values.


4000
3000
2000
1000
Water
1 mM cinnamic acid
GUS activity (pmol 4 MU/min/mg total protein)
0
1
2
3
Days post treatment

4.2.7 The AoPR1 promoter does not respond to enhanced sucrose levels in suspension culture.

The ability of sucrose to enhance the response of the potato proteinase inhibitor II to elicitation has been reported by Johnson and Ryan (1990), and the promoter element responsible for conferring this sucrose-inducibility delineated. Again, given the availability of material it was decided to undertake preliminary investigations into the possible effects of sucrose on AoPR1 promoter activity in suspension culture. Using conditions similar to those described in Johnson and Ryan (1990) GUS activity was determined for material cultured in the presence of increasing concentrations of sucrose for 48 h. The effect of addition of sucrose to the culture medium on levels of GUS activity detected was, in all cases, negligible.

4.2.8 The AoPR1 promoter exhibits developmental regulation in tissues active in phenylpropanoid metabolism.

4.2.8.1 The AoPR1-*gus* gene fusion is strongly expressed at sites of lignification in tobacco stems.

Preliminary GUS histochemical analysis revealed apparently high levels of *gus* expression in young, lignifying stem tissue (Warner, 1992) Histochemical work showed this to be localised to regions of active lignification, regions known to be highly active in phenylpropanoid metabolism (Bevan *et al.*, 1989). Staining was detected to quite high levels in young but lignifying stems and maintained through to mature stem material and correlates visibly with data obtained following phluoroglucinol staining of similar tissue for the presence of lignin (fig. 4.9). However, fluorometric data was found to be remarkably low in extracts from older material, material known to possess reduced levels of phenylpropanoid synthesis. For this reason subsequent analysis of the relationship between AoPR1 promoter activity and lignin content of stem tissue was undertaken only in material from plants immediately post-flowering but prior to seed maturity. Consideration of published methods for lignin extraction led to the decision not to directly compare lignin content with GUS activity.

Figure 4.9:

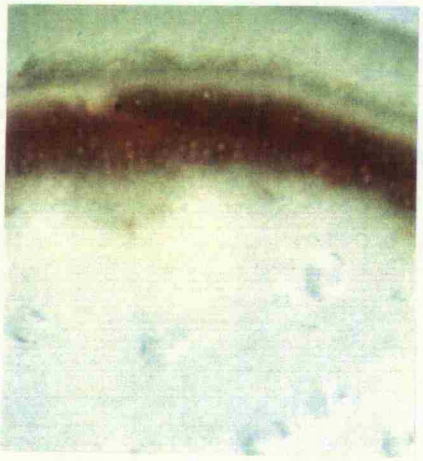
(a) T.S. of tobacco stem showing the location of lignin-containing regions in explants from transgenic tobacco plants ('full' AoPR1-*gus*).

Samples were treated with phloroglucinol (1% in water) for 2 min. Colour development was observed upon the addition of 2-3 drops conc. HCl to the section. Thus stained sections were covered in a layer of mineral oil immediately following the development of an intense red colour in those regions containing lignin to prevent crystallisation of HCl on the surface of sections prior to photography.

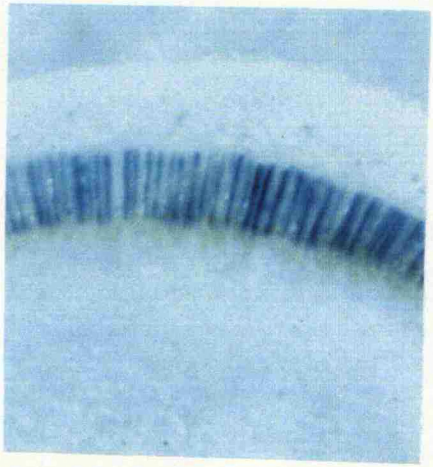
(b) Histochemical localisation of GUS activity (section 2.18.2) in stem tissue of transgenic tobacco analyses for lignin content as above.

(c) Alignment of sections treated as in (a) and (b) above. GUS acitivty and lignin content were determined for sections cut successively through the stem of one representative plant.

(a)



(b)



(c)

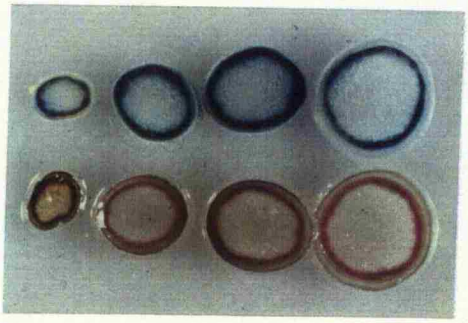


Figure 4.10:

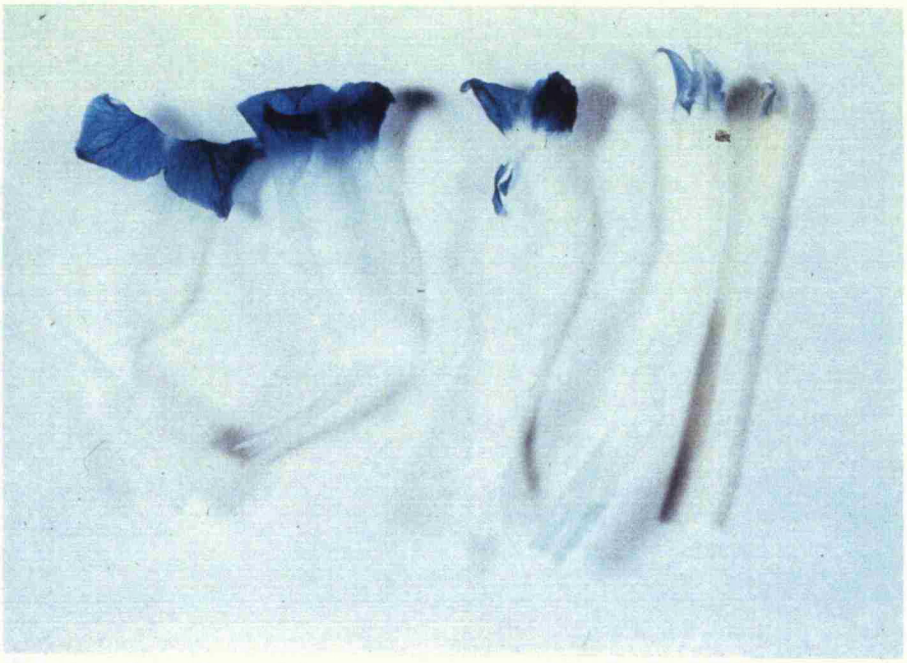
Histochemical analysis of AoPR1-driven *gus* expression in transgenic tobacco.

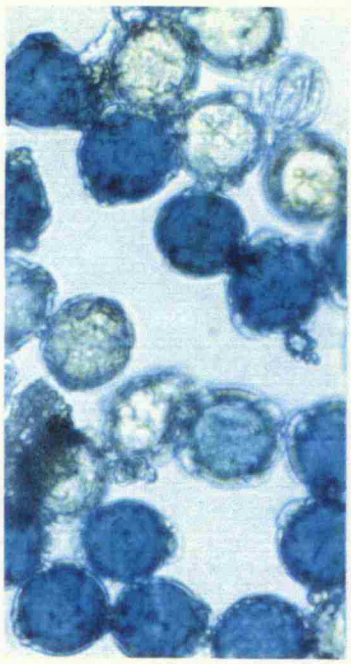
(a) Tobacco petals at various stages of flower development.

(b) Mature pollen post-dehiscence.

(c) Stigma.

(d) T.S. of a tobacco seed pod five days post-pollination.





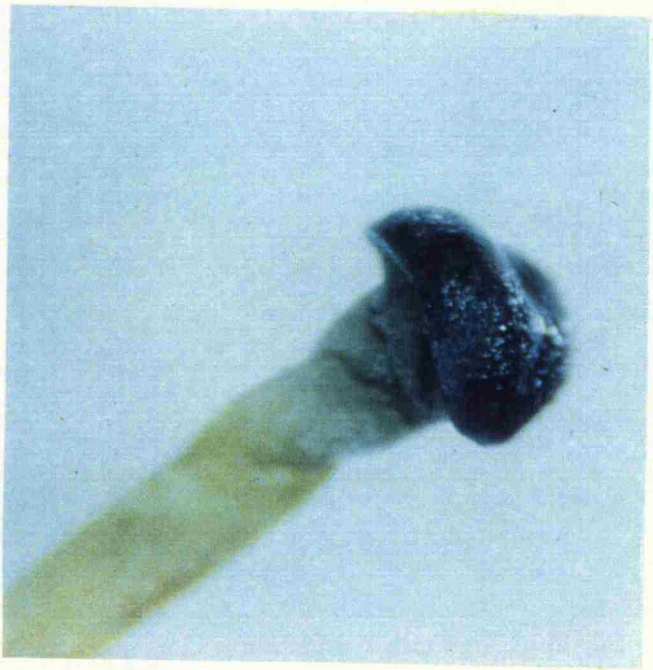
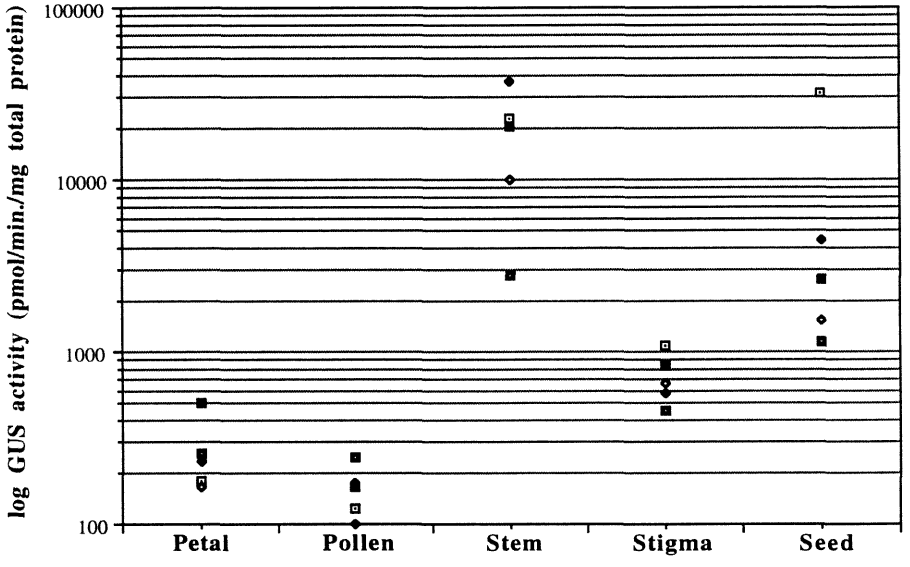


Figure 4.11:

Graphical representation of GUS activity in different tissues of transgenic tobacco plants containing the AoPR1 promoter-*gus* transgene.

Each point represents the mean GUS activity of at least three replica assays for one independent transformed line.


log GUS activity (pmol/min./mg total protein)
100000
10000
1000
100
Petal
Pollen
Stem
Stigma
Seed

Conclusions and speculations may therefore only be made based upon histochemical data obtained.

4.2.8.2 AoPR1 promoter activity is detected in pigmented regions of floral tissue.

Having previously observed the ability of the AoPR1 promoter to drive *gus* expression in pigmented regions of petals, the developmental aspect of this expression pattern was further investigated. It was found that detection of GUS activity correlated with the onset of anthocyanin synthesis (Warner *et al.*, 1994). Activity was observed only in those regions where anthocyanin accumulation had occurred and was detected at the onset of pigmentation, persisting through until senescence (fig. 4.10). A significant ($P<0.05$) correlation between GUS activity and anthocyanin content of petals was observed in three independent lines analysed (data not shown). Fluorometric values obtained on analysis of petal material showed levels of activity comparable to those observed in pollen extracts (fig. 4.11) but were markedly low in comparison to those of other tissue extracts.

4.2.8.3 Stigma-specific AoPR1-*gus* expression does not appear to be developmentally regulated.

One tissue which was found to yield consistently high GUS activity values independent of the developmental stage of the plant was the stigma (Histochemical: fig. 4.10; Fluorometric: fig. 4.11). Here histochemical investigations showed GUS activity to be detected at all stages of flower development. No significant alteration in levels of GUS activity were detected when the stigma became receptive for pollination, or at any other stage during flower development.

4.2.8.4 AoPR1-*gus* expression in pollen is limited to mature pollen grains.

Mature pollen released from dissected anthers immediately prior to dehiscence was found to exhibit positive GUS activity as determined from histochemical analysis (Warner *et al.*, 1993). While further investigations revealed the absence of detectable GUS activity in both developing microspores and immature pollen, activity observed in mature pollen grains was found to persist to dehiscence. Those levels of activity observed, where activity was

detectable, were, however, found to be rather low in comparison with expression levels observed in extracts from other tissues (fig. 4.10).

4.2.8.5 AoPR1-*gus* expression in seed occurs prior to the visible accumulation of pigments.

In agreement with Warner (1992), it was found that seed-specific GUS activity was maximal in immature pods at five days post-pollination. X-gluc staining showed strong activity in samples of immature seed that had not yet begun to accumulate visible phenylpropanoid pathway-derived pigments (fig. 4.10). Once such pigments had started to visibly accumlate, as determined by the appearance of a brown colour and changes in seed texture, detectable GUS activities were observed to decline. Under these conditions histochemical analysis proved unreliable as a consequence of the 'background' brown colour making detection of the GUS-positive state difficult to determine.

4.3 Sequence analysis of the AoPR1 promoter and generation of a 5' deletion series.

4.3.1 The AoPR1 promoter contains sequence motifs known to confer specificity of expression on other promoters.

While conclusions may not be drawn from the occurrence of certain sequence homologies between different promoters a comparison of those *cis*-acting elements previously shown to have possible roles in the specific regulation of one promoter with the promoter sequence of another gene can provide some clues and possible indications as to the potential involvement of these sequences in the regulation of the second gene. Although certain sequences may be present in both promoters their role, if any, in both may be different. Thus their mere presence does not infer a functional effect. In the case of defence-related genes a number of *cis*-acting elements have been defined as being essential for expression of the corresponding genes (for review see Somssich, 1994). Only in a limited number of these cases have further analyses been undertaken to delineate those specific sequences within the promoter region

Figure 4.12:

DNA sequence of the AoPR1 promoter as cloned in pSKII+-WIP-*gus*. The predicted TATA box is underlined. Letters in italics designate *gus* coding sequence.

1 CCTCAGGACT AGACCATCCG TGGTTAAATG ATCAAGTGCC TACTTGGCAG

51 AATTTCTTTC GAGCAGCCTC CTCCTACAAG TTGCATTTGT TGCGCTTACG

101 ATAATTGTCA AAGAAGTAGG TAAAATAAAG ACATGATCAC TAATATTAAG

151 GATAAGATTA AAAATAAGTC CAGGATTAAC CGGTCGGCCC ATCAATTACT

201 TGCTGACCTT TGTTGCCGTC CCACGACTTC CATTTTCTAA CCGTCCATTT

251 TTCATTTGTT TTTAGCTATA TTTAATATTA ATGGGATATA AATTATAAAC

301 ATTCCTCCTC CCAAAAAAAT AAGTTTAAGT AATACTGCAA TAGACAGTGT

351 TTTAAGCCAT GTAATTCAGT AAAAGTTCTT TTTTATTCTG AAGGTAGCCT

401 AAAAAGGCCA TGCGGGTAAT TAGTTCAGTC AACTGAATAT ACAACGTTTT

451 GAACCAAAGT TAACATGTAC AGGCCAATAG AAGTTATTTG ACCGTAAGCT

501 TAGTCTCTAC ATTCATTCAA CGTTCTTGAA TCAAAGTGAC CTGTACAGGC

551 CAATAGAAGT TACCTGACCG TAAGCTTAGT CTCTACATTC ATTCCTCTGA

601 GACGATATTC TAGAAGCCTG CTTTCAAGTC TAAAAGGCAC AATCTTTTTT

651 TCCTCACCAC TTGTTGAGGT ACTTATGATT TTAAAGATGA AACATTTTTT

701 TTACTTTTCC CCTTTAATTT CTTTGATTTT TTTTTTTCTT GGTAGTTGGA

751 AGTACTTTTC ATACCCTAGA AAATCCACTG TTGATCTTTG AAATATCAGC

801 AATCTTTGAA ATAATATCAG CAACCACGAC ACCTACCATT CTCAAATTCA

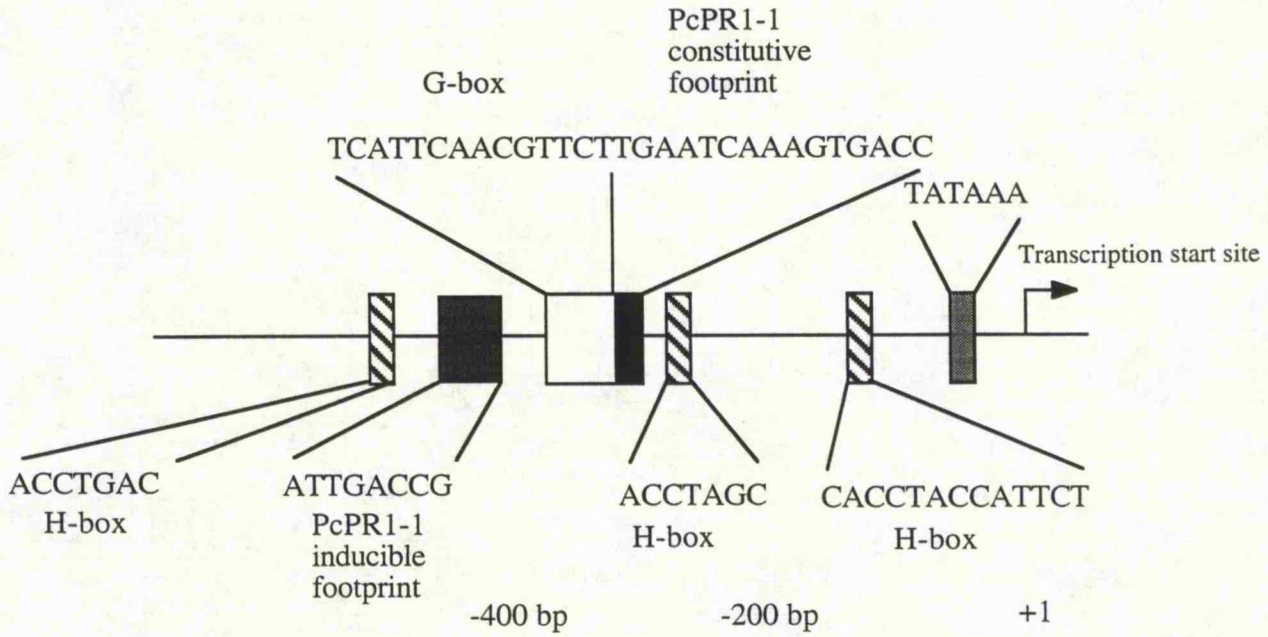
851 CTCTATAAAA GGGTAAACCT TTGCTTACCT CTATGCTCAC TCACAAGGAG

901 AACAAACACT CATCGGTGCT ACATAACCGC GGCTGCAGGT CGACGGATCC

CCGGGTAGGT CAGTCCCTT*ATGTTACGTCCTGTAGAAA*

Figure 4.13:

Schematic representation of regions of interest within the AoPR1 promoter.



Schematic representation of regions of interest within the AoPR1 promoter

H-box: Interacts with plant myb oncogene protein homologues and have been shown to be inportant in pigmented regions of petals and in other developmental processes.

G-box: Interacts with bZIP proteins. Found to have roles in many promoters that are responsive to light, ABA and other stimuli.

Others: Similar to regions found within the parsley IPR genes. Important for elicitor-mediated inducibility.

responsible for conferring expression in response to certain stimuli (examples include van de Rhee *et al.*, 1993; Lois *et al.*, 1989; Loake *et al.*, 1992; Matton *et al.*, 1993 and Uknes *et al.*, 1993). From such data it appears that in a number of cases specific expression profiles and expression in response to specific stimuli are conferred by the combinatorial interaction of *cis*-elements (van de Rhee *et al.*, 1993; Loake *et al.*, 1992). Comparisons of sequences found to play a role in the control of expression of such defence-related genes with the AoPR1 promoter sequence (fig. 4.12) revealed sequence similarity with a number of elements implicated in the regulation of defence-related gene expression. Homology was found with sequences within the parsley PcPR1-1 promoter (fig. 4.13). Meier and colleagues (1991) identified a region within this promoter which was found to interact specifically with a inducible DNA-binding protein upon elicitation of parsley suspension cultured cells. This region, designated iF (inducible footprint), contains the sequence ATTTGACCG which is also found in the AoPR1 promoter at position -244 to -235. Sequence homology has also been found with H-box sequences with implied involvement in the regulation of genes encoding enzymes of the 'core' phenylpropanoid pathway (Lois *et al.*, 1989; Loake *et al.*, 1992; Yu *et al.*, 1993). A region of similarity with a G-box element (Williams *et al.*, 1992; Weisshaar *et al.*, 1991) has also been found. While homology with known regulatory elements has been observed the presence of these sequences within the AoPR1 promoter does not necessarily imply a functional role in this context. To further investigate the role, if any, played by these sequences in the regulation of expression of the AoPR1 gene and to delineate those other regions responsible for determining the AoPR1 expression profile under certain conditions it was decided to construct a series of 5' deletions of the AoPR1 promoter and investigate their behaviour in transgenic tobacco.

4.3.2 Generation of a 5' deletion series of the AoPR1 promoter using exonuclease III.

Of those approaches available for the generation of a series of 5' deletions of the AoPR1 promoter that of exonuclease III digestion (Henikoff, 1987) was considered as the primary method to be investigated. Exonuclease III catalyses the stepwise removal of nucleotides

from ds DNA termini having either blunt ends or a 5' overhang. Termini having 3' overhangs are, however, resistant to digestion by this enzyme. As a result of this substrate specificity DNA that had been linearised in such a way as to generate a blunt terminus and one with a 3' overhang will be digested in a unidirectional manner. Furthermore, this exonucleolytic activity occurs at a relatively uniform and predictable rate which may be optimised such that the desired deletions are obtained. The procedure involves an initial restriction with two enzymes, one (A) which cleaves near the target sequence generating a 5' overhang, and the other (B) which cleaves further upstream of the target sequence generating a 3' overhang (fig. 4.14). Treatment of thus restricted ds DNA with exonuclease III results in the generation of a unidirectional deletion series based on the initial trarget sequence. Resulting ss DNA is then removed using S1 nuclease, plasmid DNA recircularised and self-ligated.

For the purposes of deleting the AoPR1 promoter (as found in the plasmid pSKII$^+$-WIP-*gus*) the enzymes chosen were *Not*I and *Sac*I such that conditions specified above were met. It was decided to generate a deletion series spanning the 'full' AoPR1 promoter (approximately 1 kb in length) in approximately 100-150 bp intervals thereby analysing the promoter sequence in depth while at the same time generating fragments sufficiently small enough to investigate further in isolation. Having optimised assay conditions such that an incubation period of 30 s was sufficient to result in the removal of approximately 100 nucleotides from the 5' end of the target sequence, a 5' deletion series of the AoPR1 promoter was generated as in section 2.9.5.. An example of the deletion series routinely obtained using this approach is shown in fig. 4.15a; fig. 4.15b illustrates Southern analysis of the gel in fig. 4.15a probed with labelled AoPR1 promoter sequence. A reduction in construct size from 6 kb to 5 kb was desired, reflecting the gradual removal of sequence from the 5' end of the AoPR1 promoter cloned into pSKII$^+$-WIP-*gus* (fig. 4.12). Hybridisation to the 1 kb ladder fragments was obtained as a result of the addition of a small amount of labelled 1 kb ladder to' the hybridisation reaction. It may be clearly seen that this approach is indeed appropriate to the generation of the desired deletion series. Unfortunately despite routine generation of such a series successful

Figure 4.14:

Schematic representation of the generation of a 5' deletion series using exonuclease III.

From Sambrook *et al.* 1989.

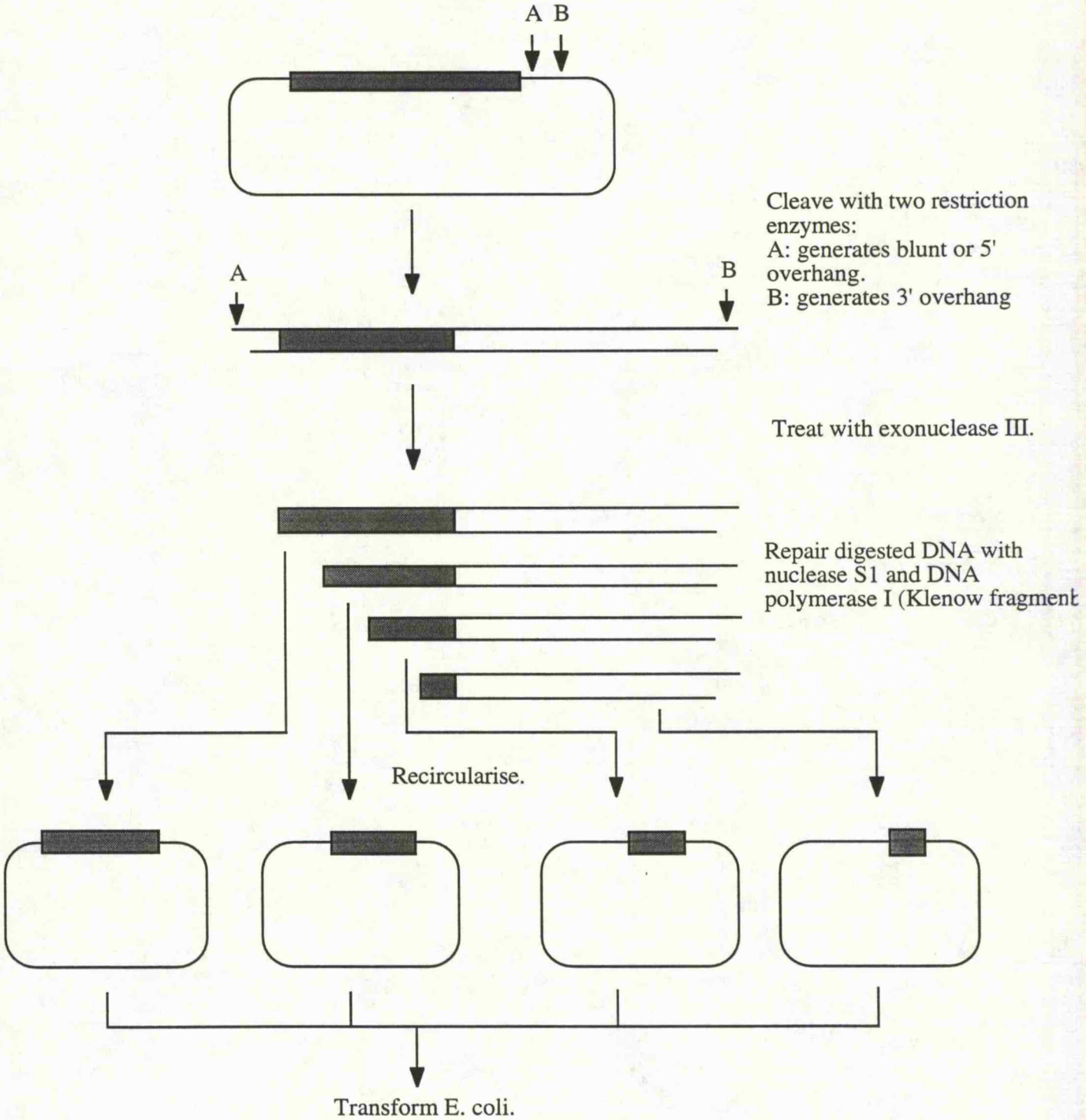

A B
A
B
Cleave with two restriction enzymes:
A: generates blunt or 5' overhang.
B: generates 3' overhang
Treat with exonuclease III.
Repair digested DNA with nuclease S1 and DNA polymerase I (Klenow fragment
Recircularise.
Transform E. coli.

Figure 4.15:

(a) Exonuclease III digestion of pSKII^{+}-WIP-*gus*. Samples were incubated in the presence of exonuclease III at 37^{o}C for increasing lengths of time. Aliquots were removed at 30 s intervals, treated with S1 nuclease and electrophoretically analysed.

(b) Southern analysis of (a) probed with labelled AoPR1 promoter sequence.

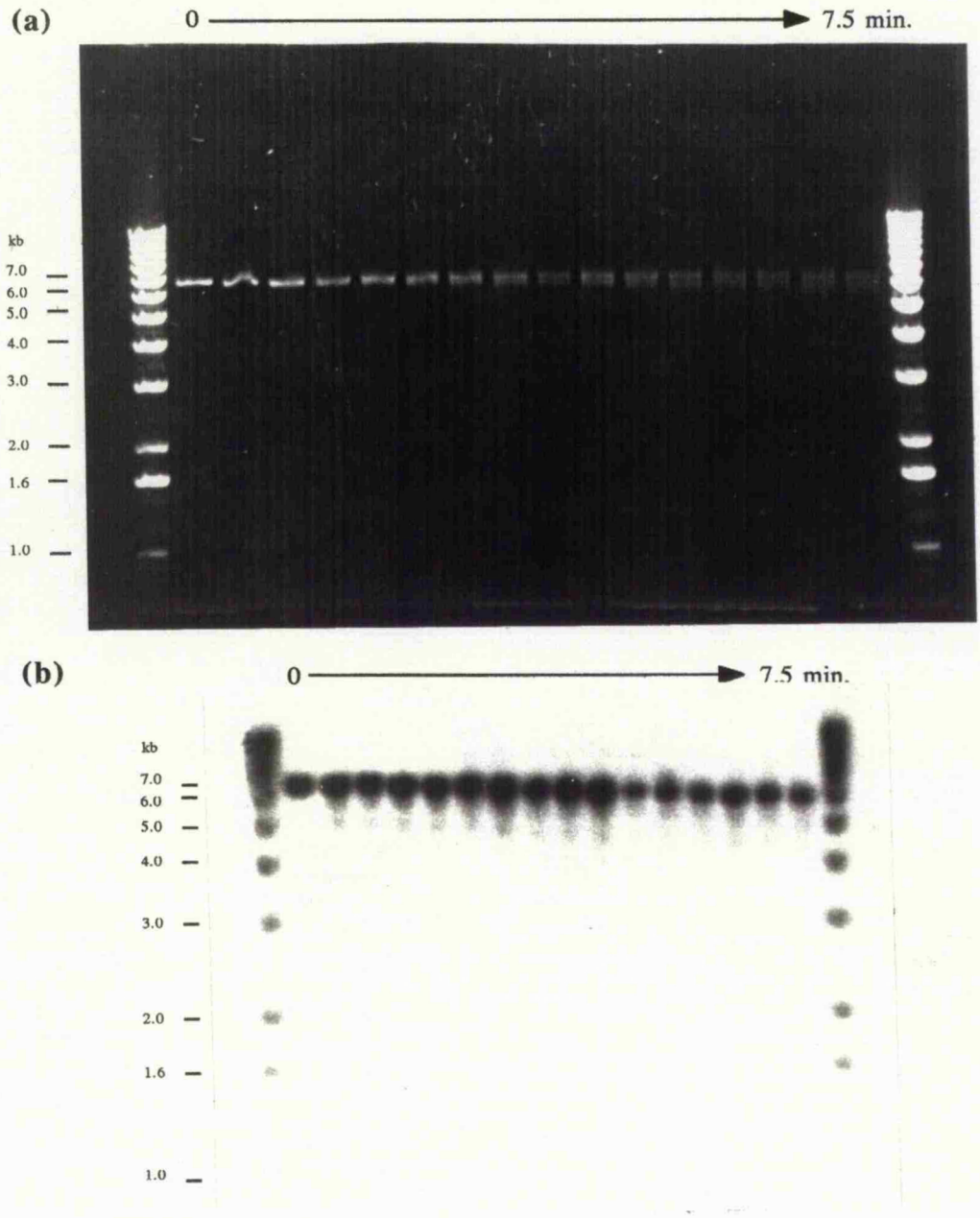
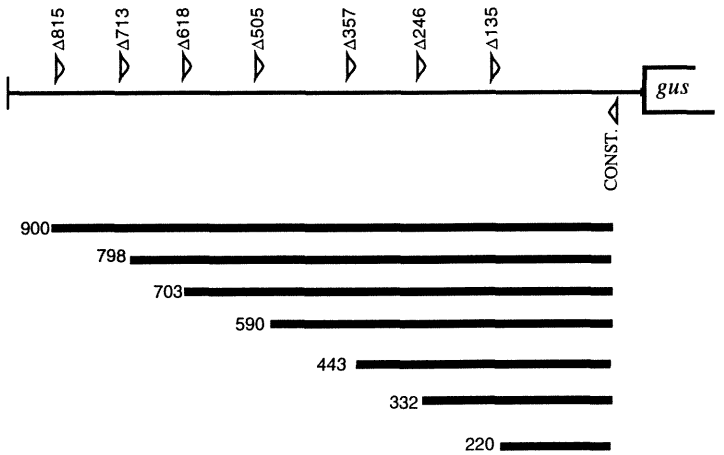

(a)
0
7.5 min.
kb
7.0
6.0
5.0
4.0
3.0
2.0
1.6
1.0
(b)
0
7.5 min.
kb
7.0
6.0
5.0
4.0
3.0
2.0
1.6
1.0

Figure 4.16:

Design of PCR primers for the generation of a 5' deletion series of the AoPR1 promoter. Δ815, Δ713, Δ618, Δ505, Δ357, Δ246, Δ135: numbers represent the approximate distance (in bp) upstream of the transcriptional start site of the published AoPR1 promoter sequence (Warner *et al.*, 1992); Δ indicates that AoPR1 promoter sequences amplified using this primer in conjunction with the 3' CONST. primer are deletions of the 'full' promoter sequence.



Primer name	Sequence (5' - 3')	Initial annealing temperature	Subsequent annealing temperature	Fragment size
Δ815	CTCCTCCTACAAGTTGC	52°C	84°C	900 bp
Δ713	CCAGGATTAACCGGTCG	54°C	86°C	798 bp
Δ618	GCTATATTTAATATTAATGG	48°C	80°C	703 bp
Δ505	CTTTTTTATTCTGAACCTAGC	56°C	88°C	590 bp
Δ357	CTTGAATCAAAGTGACC	48°C	80°C	443 bp
Δ246	GGCACAATCTTTTTTCC	48°C	80°C	332 bp
Δ135	GGAAGTACTTTTCATACCC	54°C	86°C	220 bp
CONST.	CCCGGGATCCGTCGGCCTG	68°C		

recircularisation of deleted molecules was not achieved thereby necessitating the consideration of an alternative approach, that of PCR.

4.3.3 Design of a 5' deletion series using PCR primers.

Using guidelines described in Innis *et al.* (1990) for the design of PCR primers a series of primers (designated Δ135, Δ246, Δ357, Δ505, Δ618, Δ713 and Δ815) were designed of approximately 20 nucleotides in length allowing sequential removal of 100-150 nucleotides from the 5' end of the AoPR1 promoter (fig. 4.16). The symbol Δ is used to designate a deletion, while numbers refer to the approximate distance in bp upstream of the transcriptional start site (+1) of the published AoPR1 promoter sequence (Warner *et al.*, 1993). In order to retain the 3' sequence of the promoter a constant primer (CONST.) was designed allowing, in conjunction with any specific Δ primer, amplification of the relevant promoter deletion. Computer-simulated PCR reactions were run using the 'Amplify' package for the Macintosh. Potential primer sequences were analysed against pSKII$^+$ and AoPR1 promoter target sequences in order to optimise both primer design and reaction conditions prior to fragment amplification. To facilitate subsequent cloning of deletions a *Sal*I site was engineered onto the 5'end of the Δ primers along with a GCG clamp. The CONST. primer contained a *Bam*HI site within its sequence. Deletions were thus subsequently manipulated as *Bam*HI/*Sal*I fragments.

4.3.4 PCR amplification and cloning of deleted promoter fragments.

PCR-based amplification of the relevant fragments of the AoPR1 promoter was achieved using combinations of the above primers targeted against sequences within the AoPR1 promoter sequence contained within the plasmid pSKII$^+$-WIP-*gus*. In order to conserve the 3' region of the fragment between deletions the CONST. primer was used in all reactions, with choice of 5' primer determining which fragment was specifically amplified. Reaction conditions were designed according to criteria discussed in section 2.9.6 and optimised by computer modelling of the relevant PCR reaction using 'Amplify' for the Macintosh. The amplification of a single fragment under chosen conditions (Denature: 95^{o}C, 30 s; Anneal:

43°C. 30 s; Extend: 72°C, 1 min.; 5 cycles. Denature: 95°C, 30 s; Anneal: 65°C; 30 s; Extend: 72°C, 1 min.; 15 cycles) reflected the optimal nature of these conditions. In all cases no non-specific fragments were observed to be amplified and primer-dimers were not generated thereby allowing for optimal amplification of appropriate DNA sequences. Fig. 4.17a shows the reaction products obtained following PCR reactions containing pSKII$^+$-WIP-*gus* (10 ng) template, CONST. primer (1 μM) and primers Δ135 - Δ815 (lanes 1 - 7) respectively. Southern blot analysis of this gel is illustrated in fig. 4.17b and shows the specific hybridisation of labelled AoPR1 promoter sequence to the amplified fragments under chosen conditions: Hybridisation was carried out overnight at 65°C; filters were washed 1 x 10 min. wash A, 65°C followed by 2 x 10 min. wash B, 65°C. Autoradiography was carried out overnight at -70°C.

Having optimised reaction conditions as above such that reaction products comprised, in all cases, a single band hybridising specifically to labelled AoPR1 promoter sequence, deletions were amplified according to these conditions and cloned into pBluescript SKII$^+$. Following PCR amplification the reaction mix was treated with chloroform to remove the mineral oil overlay and DNA precipitated with ethanol. Thus isolated DNA was then subjected to a *Bam*HI/*Sal*I double digest, phenol-chloroform extracted and precipitated. Resultant *Bam*HI/*Sal*I fragments were ligated into similarly cleaved pBluescript SKII$^+$ with the consequential generation of the plasmids pSKII$^+$Δ135 - pSKII$^+$Δ815. These plasmids were introduced into *E.coli via* calcium chloride-mediated transformation and transformed bacteria selected as ampicillin-resistant white colonies when grown on media containing ampicillin, X-gal and IPTG. The presence of AoPR1 promoter sequence within DNA sequences contained in chosen bacteria was confirmed by colony hybridisation experiments using labelled AoPR1 promoter sequence as a probe. Isolating plasmid DNA from positive transformants and subsequent *Bam*HI/*Sal*I digestion of this DNA revealed the presence of a single fragment corresponding in size to that observed upon PCR-mediated generation of AoPR1 promoter deletions which hybridises to linearised pSKII$^+$-WIP-*gus* (fig. 4.18). The upper hybridising fragment at approximately 2 kb presumably corresponds to linearised

pSKII$^+$ which, as a consequence of overloading the gel, exhibited slightly altered mobility through the gel.

4.3.5 Sequence analysis of PCR-generated deletions.

The introduction of PCR amplified fragments into pBluescriptII SK$^+$ allows for ease of sequencing of both strands using the T3 and T7 primers. Double stranded template was prepared and sequenced using a combination of manual and automatic approaches (section 2.10). Alignment of thus generated sequence with the published sequence of the AoPR1 promoter (Warner *et al.*, 1993) indicated that in all cases except that of pSKII$^+$Δ357 the sequence of the cloned fragment was identical to that predicted for that deletion on the basis of published sequence. In the case of pSKII$^+$Δ357, however, a rearrangement was observed to have occurred at the 5' end of the amplified fragment. Sequence analysis revealed the duplication of a region corresponding to sequence running 52 bp downstream of the primer site. It appears that during PCR the Δ357 primer bound correctly and initiated amplification of sequences immediately downstream of this site. However, at a position seven bp 3' of the primer site in the amplified sequence it appears that the sequence amplified corresponds to those 7 bp immediately 5' of the primer site in the 'full' AoPR1 promoter sequence. Immediately after the insertion of these 7 bp the sequence of the amplified fragment appears to return to the Δ357 primer site and correct amplification of sequence immediately downstream of this site is observed from this point on. This sequence rearrangement is schematically represented in fig. 4.19 and may possibly have arisen as a consequence of sub-optimal initial annealing conditions. Under the conditions utilised it is possible that, following correct annealing and a short extension, the extended primer may have dissociated from the template strand, annealing subsequently to the 'repeat' sequence upstream of the initial binding site. The Δ357 deletion may therefore be seen to actually represent a deletion to position Δ416 incorporating the base substitution T to C at positions -394 and -395. The nucleotide sequence of this deletion is given in fig. 4.20.

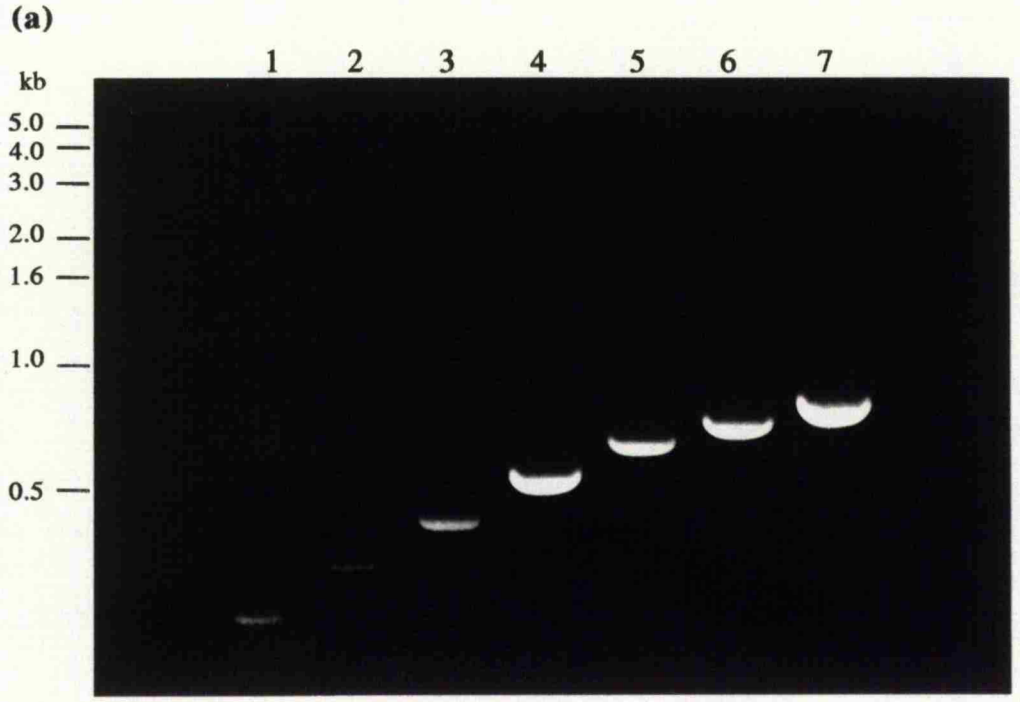
Figure 4.17:

(a) PCR amplification of a 5' deletion series of the AoPR1 promoter sequence.

Lane 1: Δ135
Lane 2: Δ246
Lane 3: Δ357
Lane 4: Δ505
Lane 5: Δ618
Lane 6: Δ713
Lane 7: Δ815

Each reaction was carried out using 100 ng template (pSKII^{+}-WIP-*gus*) and primers CONST. and Δ130-Δ815 (lanes 1-7 respectively). Reaction conditions are described in section 4.3.4.

(b) Southern analysis of (a) probed with labelled AoPR1 promoter sequence.


(a)
1 2 3 4 5 6 7
kb
5.0
4.0
3.0
2.0
1.6
1.0
0.5

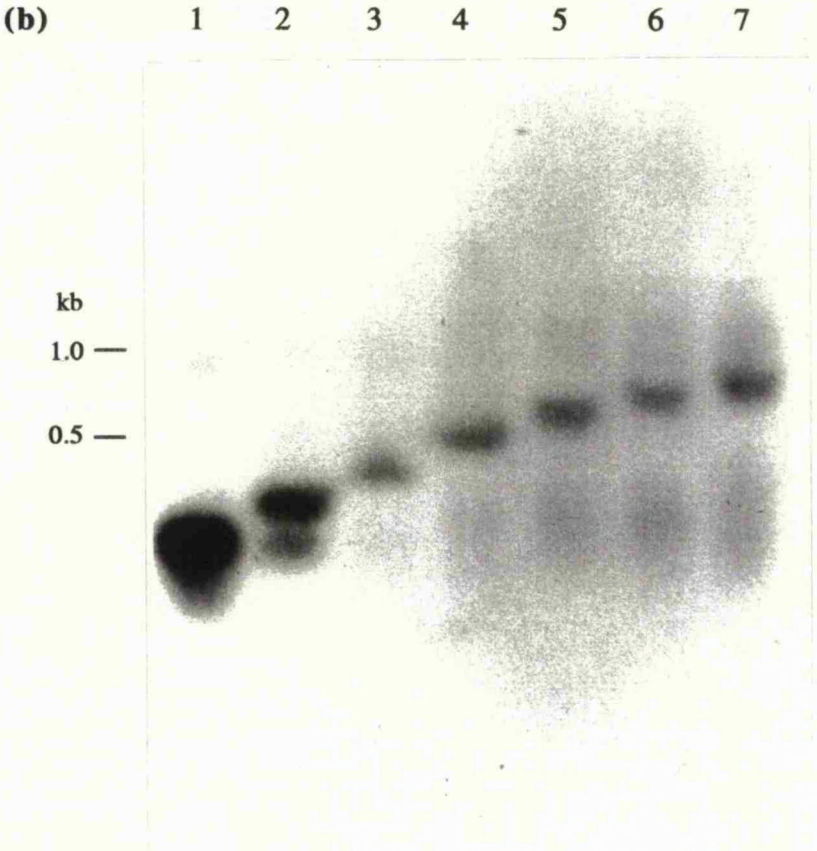

(b)
1 2 3 4 5 6 7
kb
1.0
0.5

Figure 4.18:

(a) Electrophoretic analysis of pSKII$^+$-Δ constructs following *Bam*HI/*Sal*I digestion.

Lane 1: pSKII$^+$-Δ135
Lane 2: pSKII$^+$-Δ246
Lane 3: pSKII$^+$-Δ357
Lane 4: pSKII$^+$-Δ505
Lane 5: pSKII$^+$-Δ618
Lane 6: pSKII$^+$-Δ713
Lane 7: pSKII$^+$-Δ815
Lane 8: pSKII$^+$-WIP-*gus*

(b) Southern analysis of (a) probed with labelled AoPR1 promoter.

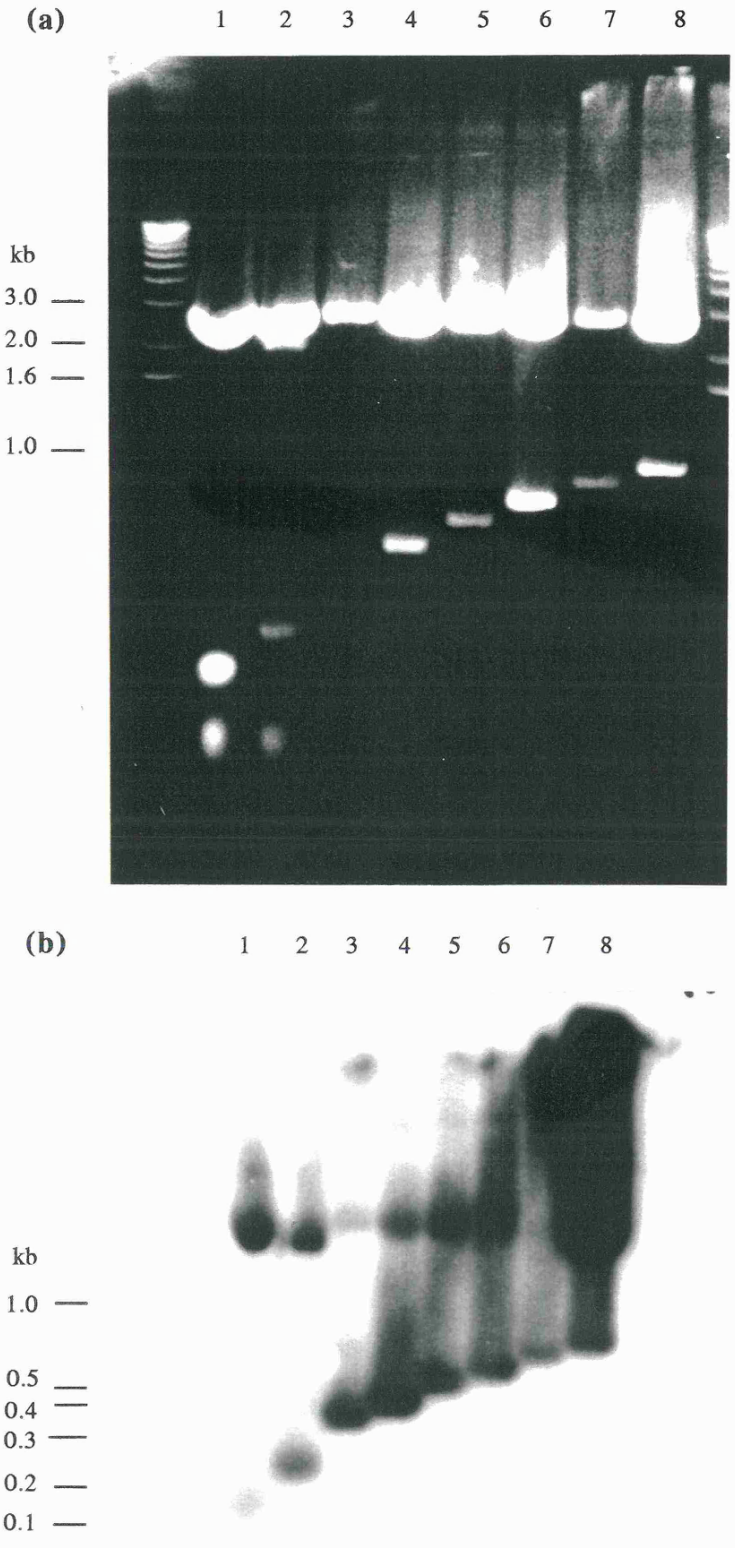

(a)
1 2 3 4 5 6 7 8
kb
3.0
2.0
1.6
1.0
(b)
1 2 3 4 5 6 7 8
kb
1.0
0.5
0.4
0.3
0.2
0.1

Figure 4.19:

Schematic representation of rearrangements occurring during PCR-mediated amplification of the Δ357 AoPR1 promoter deletion.

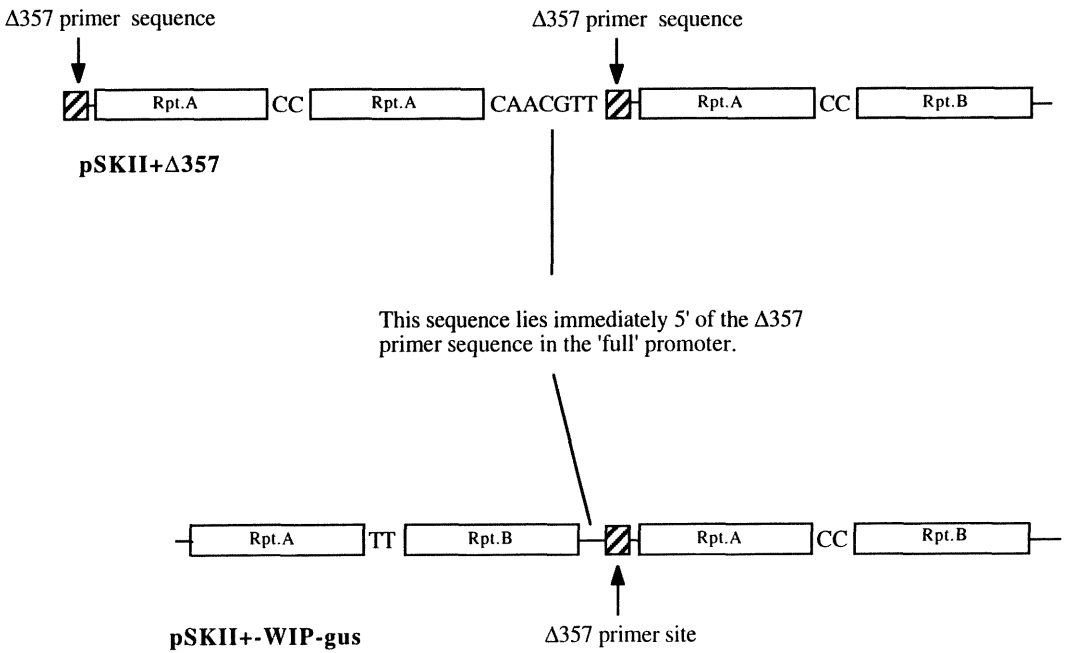

Δ357 primer sequence
Δ357 primer sequence
Rpt.A
CC
Rpt.A
CAACGTT
Rpt.A
CC
Rpt.B
pSKII+Δ357
This sequence lies immediately 5' of the Δ357 primer sequence in the 'full' promoter.
Rpt.A
TT
Rpt.B
Rpt.A
CC
Rpt.B
pSKII+-WIP-gus
Δ357 primer site

Figure 4.20:

DNA sequence of the Δ357 promoter deletion as cloned into pSKII^{+}. The Δ357 primer site is represented by underlined sequence.

CTTGAATCAA AGTGGACCTG TACAGGCCAA TAGAAGTTAC CTGACCGTAA

GCTTAGTCTC TACATTCATT CAACGTTCTT GAATCAAAGT GACCTGTACA

GGCCAATAGA AGTTACCTGA CCGTAAGCTT AGTCTCTACA TTCATTCCTC

TGAGACGATA TTCTAGAAGC CTGCTTTCAA GTCTAAAAGG CACAATCTTT

TTTTCCTCAC CACTTGTTGA GGTACTTATG ATTTTAAAGA TGAAACATTT

TTTTTACTTT TCCCCTTTAA TTTCTTTGAT TTTTTTTTTT CTTGGTAGTT

GGAAGTACTT TTCATACCCT AGAAAATCCA CTGTTGATCT TTGAAATATC

AGCAATCTTT GAAATAATAT CAGCAACCAC GACACCTACC ATTCTCAAAT

TCACTCTATA AAAGGGTAAA CCTTTGCTTA CCTCTATGCT CACTCACAAG

GAGAACAAAC ACTCATCGGT GCTACATAAC CGCGGCTGCA GGTCGAC

4.3.6 Construction of a modified binary plant transformation vector.

In order to eliminate *Agrobacterium* read-through of the *gus* coding sequence which could result in bacterially-derived *gus* expression interfering with measurements of plant-derived expression levels in *Agrobacterium*-infected callus material, shown previously to be enriched for AoPR1 promoter activity (Firek *et al.*, 1993) it was decided to manipulate the binary transformation vector pBI101.1 (Jefferson *et al.*, 1987) which was to be used in the introduction of the AoPR1 promoter deletion series into plants. The introduction of a plant-derived intron into the *gus* coding sequence in such a way as to allow appropriate cleavage of the intron in plant systems while rendering any product produced on bacterial read-through inactive was achieved by Vancanneyt *et al.* (1990). Having obtained the p35s-GUS (intron) plasmid from Dr. Lothar Willmitzer it was decided to replace the pBI101.1 *gus* coding sequence, which lacks an intron and is therefore susceptible to read-through, with the intron-containing *gus* coding sequence from this plasmid. This was achieved by the *Bam*HI/*Sst*I digestion of both plasmids and subsequent ligation of digest products. Purification of the appropriate fragments from agarose gels and ligation of recovered products was not employed in this case. Instead total digest products from both digests was combined in the ligation reaction, the products of which were then introduced into *E.coli* (section 2.9.10). Positive transformants as determined by hybridisation with labelled gus sequence were then selected for further analysis in order to determine the presence or absence of the intron within the *gus* coding sequence. Five colonies were selected, plasmid DNA isolated (section 2.6.1) and restriction analysis carried out on this DNA. The enzymes used in this analysis (*Nco*I, *Rsa*I and *Nru*I) were chosen such that a restriction profile was obtained for the pBI101.1 plasmid with alterations in size of one or more fragments indicating the presence/absence of the intron. The results of this analysis are presented in fig.4.18a. Southern blot analysis of this gel probed with labelled linearised pBI101.1 (fig.4.21b) provides a clearer representation of the restriction profile obtained. Lanes 1 and 2 show the restriction patterns of p35s-GUS(intron) and pBI101.1 respectively. In the case of an *Nco*I digest it may be seen that the lower fragment corresponds to an intron-containing fragment, being larger in the p35s-GUS(intron)

Figure 4.21:

Generation of a modified binary plant transformation vector (pBI101.1(intron)).

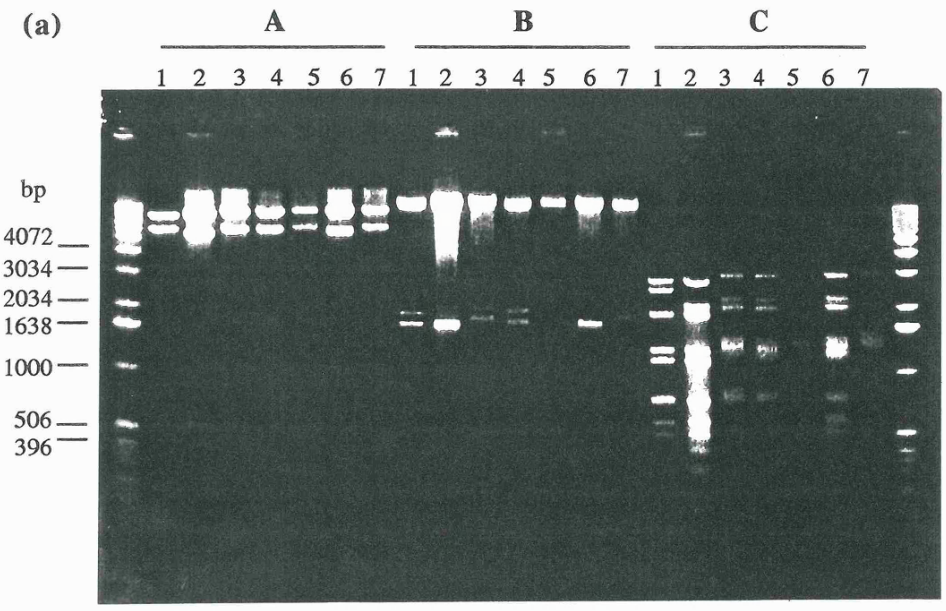
(a) Restriction analysis of recombinant plasmids.

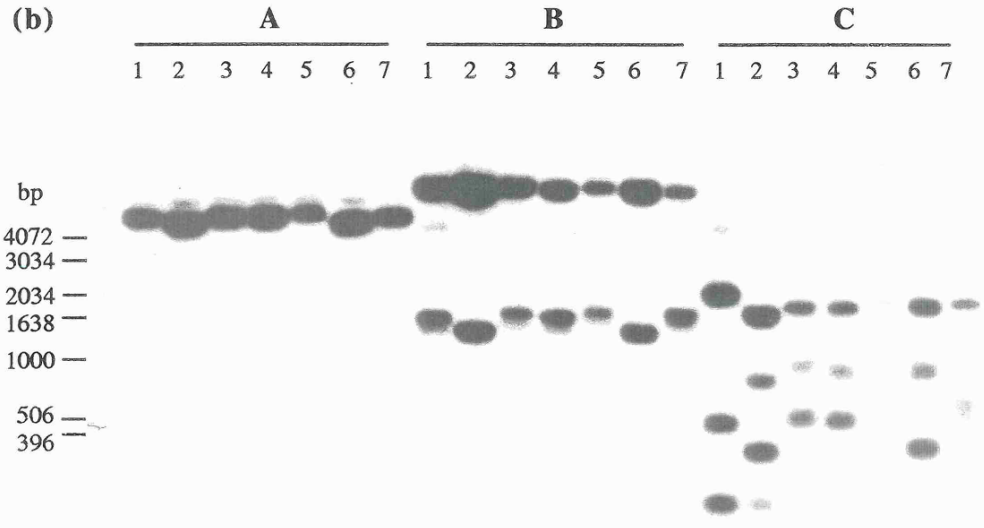
A:
Lane 1: pBI101.1 x *Nco*I
Lane 2: p35s-GUS(int.) x *Nco*I
Lane 3: Colony 1 x *Nco*I
Lane 4: Colony 2 x *Nco*I
Lane 5: Colony 3 x *Nco*I
Lane 6: Colony 4 x *Nco*I
Lane 7: Colony 5 x *Nco*I

B:
Lane 1: pBI101.1 x *Nru*I
Lane 2: p35s-GUS(int.) x *Nru*I
Lane 3: Colony 1 x *Nru*I
Lane 4: Colony 2 x *Nru*I
Lane 5: Colony 3 x *Nru*I
Lane 6: Colony 4 x *Nru*I
Lane 7: Colony 5 x *Nru*I

C:
Lane 1: pBI101.1 x *Rsa*I
Lane 2: p35s-GUS(int.) x *Rsa*I
Lane 3: Colony 1 x *Rsa*I
Lane 4: Colony 2 x *Rsa*I
Lane 5: Colony 3 x *Rsa*I
Lane 6: Colony 4 x *Rsa*I
Lane 7: Colony 5 x *Rsa*I

(b) Southern analysis of (a) probed with labelled pBI101.1.


(a)
A
B
C
1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7
bp
4072
3034
2034
1638
1000
506
396


(b)
A
B
C
1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7
bp
4072
3034
2034
1638
1000
506
396

sample than in pBI101.1. Comparing these samples with *Nco*I digests of colonies 1-5 (lanes 3-7 respectively) indicates the possible presence of the intron in plasmid DNA from colonies 1, 2, 3 and 5. Similar comparisons between samples digested with *Nru*I (B) in which the lower fragment (approximately 1.5-1.7 kb) is diagnostic agree with the above data obtained from *Nco*I digests. Digestion with *Rsa*I, however, results in the generation of a more complex restriction profile (C). Although appearing extremely similar on an agarose gel (fig.4.21a (C)) hybridising fragments reveal a number of marked differences between the p35s-GUS(intron) *Rsa*I digest and that of pBI101.1 (fig. 4.21b (C)). In this case a fragment of approximately 700 bp appears to indicate the presence of the intron, while the absence of this fragment and appearance of a 500 bp fragment indicates the absence of intron-derived sequence. Again colonies 1 and 2 may be seen to contain the intron as determined by the presence of the 700 bp fragment. Colony 4 again lacks the intron, while the profile obtained from *Rsa*I digests of DNA from colonies 3 and 5 is somewhat ambiguous. Bacteria from colony 1 were therefore used as future sources of the modified binary vector pBI101.1(intron).

4.3.7 Production of binary vectors containing the AoPR1 deletion series and subsequent introduction into *Agrobacterium.*

A series of recombinant binary vectors containing the AoPR1 promoter 5' deletion series was generated using standard molecular biology techniques. Deleted promoter fragments were removed from pSKII^{+}Δ135 - Δ815 plasmids as *Bam*HI/*Sal*I fragments and ligated into similarly digested pBI101.1(intron). As above, restriction fragments were not purified from gel slices prior to ligation. In this case it was possible to combine all the restriction products of both reactions in the ligation mix. Selection for transformed *E.coli* containing the desired plasmid was carried out by growth of possible transformants on kanamycin-containing media. Since the pBluescript-derived plasmids carry ampicillin resistance self-ligated plasmids of this type will not be favoured in the selection process and thus all surviving colonies contain pBI101.1(intron)-derived plasmids. The presence of deletion fragments within kanamycin-resistant transformants was determined by colony hybridisation methods (section 2.12.6) using labelled AoPR1 promoter sequence as a probe. Bacteria found to contain the

appropriate plasmids were further analysed by restriction digest to investigate the possibility of sequence rearrangment during cloning. Plasmids were restricted in a *Pst*I/*Bam*HI double digest, electrophoresed and analysed by Southern hybridisation to a labelled Δ135 AoPR1 promoter fragment (fig.4.22). From this it appears that no gross rearrangements have occurred that could have resulted in alterations in predicted fragment size.

The plasmids were then introduced into *Agrobacterium tumefaciens* LBA4404 *via* conjugative transfer. Following co-incubation of mating strains in the presence of the pRK2013-containing *E. coli* on non-selective media transformed bacteria were selected by streaking an inoculum from the lawn of non-selected growth on rifampicin and kanamycin-containing media. Single colonies selected on this basis were purified and analysed for the presence of the relevant binary plasmid. Total nucleic acid was extracted from *Agrobacterium* exhibiting both kanamycin and rifampicin resistance (2.16.1), digested using *Bam*HI and *Sal*I, electrophoresed, blotted and probed with the AoPR1 promoter Δ135 fragment (fig.4.23). In all cases a single hybridising band is observed at a size corresponding to that expected for the relevant deletion fragments.

4.3.8 *Agrobacterium*-mediated transformation of tobacco and regeneration of putative transgenics.

Agrobacterium-mediated tobacco leaf disc transformation was carried out according to standard techniques (section 2.17.1). Regenerated shoot explants (referred to as T0 ie. primary transformants) were transferred to kanamycin-containing media and rooting allowed to progress until a good root system had developed. Plantlets were then transferred to soil and maintained under standard growth conditions (25°C, 18h day length). Each individual plantlet was, theoretically, derived from an independent transformation event and will thus henceforth be referred to as an individual line. Leaf explants from each potentially transgenic line were removed, surface sterilised and placed on MSD4x2 medium to callus. Successful callus production was indicative of the kanamycin-resistant nature of regenerated plants. Only those plants retaining kanamycin resistance after regeneration were grown on to set

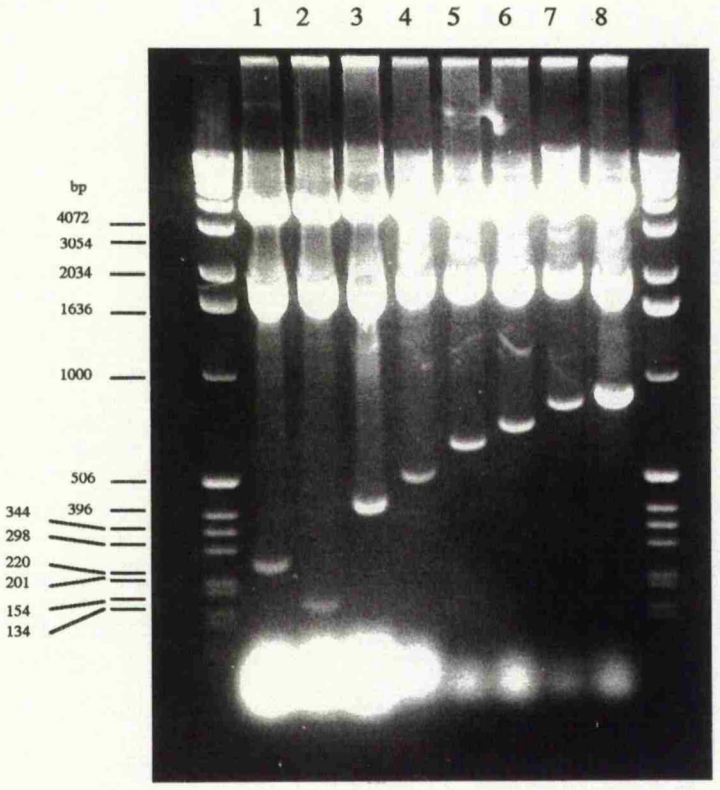
Figure 4.22:

(a) Electrophoretic analysis of pBI101.1(int.)-Δ constructs.

Lane 1: pBI101.1(int.)-Δ246 x *Pst*I/*Bam*HI
Lane 2: pBI101.1(int.)-Δ135 x *Pst*I/*Bam*HI
Lane 3: pBI101.1(int.)-Δ357 x *Pst*I/*Bam*HI
Lane 4: pBI101.1(int.)-Δ505 x *Pst*I/*Bam*HI
Lane 5: pBI101.1(int.)-Δ618 x *Pst*/*Bam*HI
Lane 6: pBI101.1(int.)-Δ713 x *Pst*I/*Bam*HI
Lane 7: pBI101.1(int.)-Δ815 x *Pst*I/*Bam*HI
Lane 8: pBI101.1(int)-'full' x *Pst*I/*Bam*HI

(b) Southern analysis of (a) probed with labelled Δ135 fragment.

(a)


1 2 3 4 5 6 7 8
bp
4072
3054
2034
1636
1000
506
344
396
298
220
201
154
134

(b)

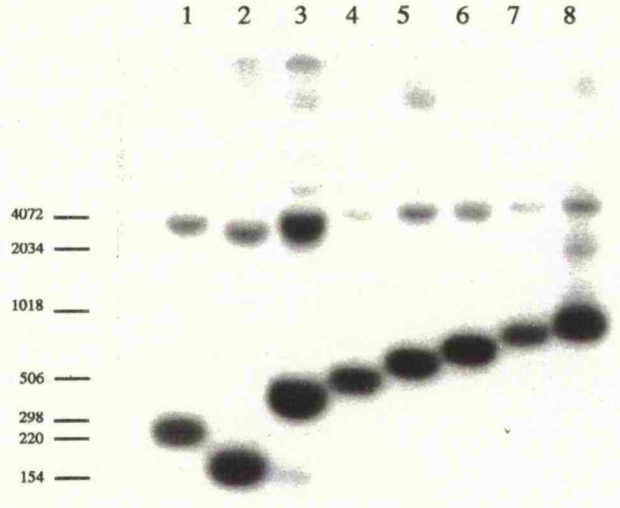
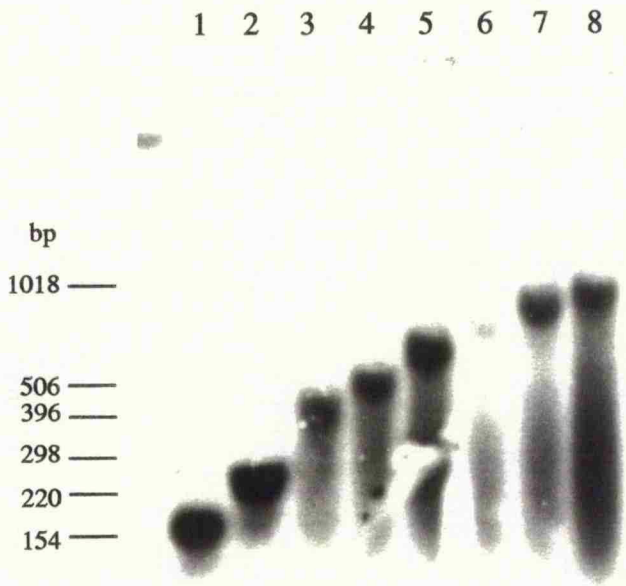

1 2 3 4 5 6 7 8
4072
2034
1018
506
298
220
154

Figure 4.23:

Southern analysis of *Bam*HI/*Sal*I-restricted total nucleic acid extracted from *Agrobacterium* containing the Δ-*gus* T-DNA probed with labelled Δ135 fragment.

Lane 1: Δ135-*gus*
Lane 2: Δ246-*gus*
Lane 3: Δ357-*gus*
Lane 4: Δ505-*gus*
Lane 5: Δ618-*gus*
Lane 6: Δ713-*gus*
Lane 7: Δ815-*gus*
Lane 8: 'full'-*gus*


1 2 3 4 5 6 7 8
bp
1018
506
396
298
220
154

seed. Since these plants contain deleted versions of the AoPR1 promoter fused to the *gus* coding sequence it was not possible to investigate the transgenic nature of regenerants using GUS-based assay methods. Hence the presence of the *nptII* gene, as determined by the expression of kanamycin resistance, was considered evidence for the probable transgenic nature of regenerants in respect of the AoPR1-*gus* transgene.

4.4 Investigating the effect of 5' deletions on AoPR1 promoter activity in transgenic tobacco.

4.4.1 Detection of the AoPR1-*gus* transgene in transgenic tobacco.

Having ascertained that regenerated transgenics (T0) were kanamycin resistant, seed from these plants was harvested, germinated on kanamycin-containing medium and the frequency with which kanamycin resistance was distributed within resultant populations determined. The number of seeds germinating after five days was determined and compared with the number of these seedlings exhibiting bleaching of the cotyledons (indicating kanamycin sensitivity) after a further sixteen days. As can be seen from table 4.2 in virtually all cases germination frequencies reflect the presence of multiple copies of the *nptII*-containing transgene stably integrated into the tobacco genome. Since, among other factors affecting expression of the transgene, the *nptII* portion of the transgene is carried at the right border side of the transferred DNA, the region from which DNA transfer into the plant cell starts, it is possible that the expression of kanamycin resistance does not necessarily correlate with transfer of those regions of the transgene further towards the left border, in this case the Δ AoPR1-*gus* constructs. As already stated, due to the nature of the transgene under investigation it is not possible to devise a method of screening transgenics for the presence of this transgene based on GUS assay approaches. It was therefore necessary to determine the presence of the transgene in a stably integrated form within the tobacco genome by DNA-based methodology. Two main approaches were to be considered in this area: genomic Southerns could provide information not only about the presence/absence of the transgene but also about the number of copies integrated into the genome and possible rearrangement events

Table 4.2:

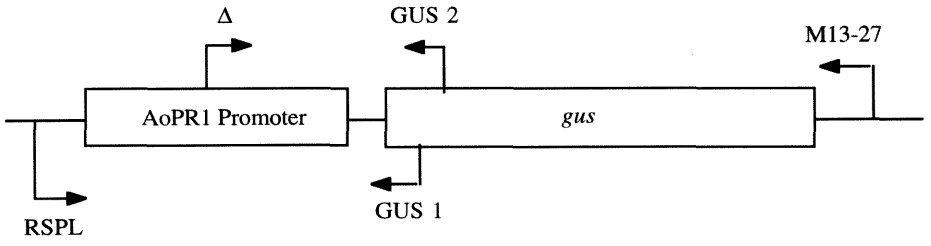
Frequency of kanamycin resistance in populations of T1 Δ-*gus* transformants.

Seed was germinated on kanamycin-containing (100 μg/ml) medium and sensitivity to kanamycin determined as bleaching of cotyledons at 21 d post-germination.

Line	% kan^r	Line	% kan^r	Line	% kan^r	Line	kan^r
135.1	82.8	357.1	82.0	618.1	96.0	815.1	00.4
135.2	74.7	357.2	78.3	618.2	0	815.2	4.4
135.3	86.4	357.3	80.6	618.3	78.4	815.3	100.
135.4	9.7	357.4	71.4	618.4	86.1	815..4	66.6
135.5	88.6	357.5	96.3	618.5	100.0	815.5	97.4
135.6	88.0	357.8	64.9	618.6	100.0	815.6	97.8
135.7	7.3	357.9	56.4	618.7	96.9	815.7	100
135.8	89.0	357.10	34.9	618.8	83.9	815.8	96.4
135.9	70.4	357.11	86.5	618.9	67.5	815.9	99.4
135.10	84.0			618.10	85.2	815.10	86.6
135.11	87.8	505.1	0	618.11	78.6	815.11	100
135.12	85.0	505.2	82.0	618.12	77.4	815.12	100
		505.3	100.0				
246.1	79.4	505.4	53.4	713.1	11.0		
246.2	0	505.5	93.5	713.2	90.8		
246.3	94.9	505.7	62.0	713.3	0		
246.4	89.3			713.4	2.3		
246.5	75.6			713.5	88.7		
246.6	32.3			713.7	100.0		
246.9	65.4			713.8	74.6		
246.10	89.4			713.9	66.1		

Figure 4.24:

Schematic representation of primers available for the amplification of AoPR1 promoter sequence from putative tobacco transformants. Arrows indicate the direction of amplification, while the position of primers above and below the line indicates which strand is used as template.



Primer	Sequence (5' - 3')	Tm (degree C)
RSPL	CACACAGGAAACAGATATGACC	46
Δ	see fig. 4.16	
GUS 1	GACTGAATGCCCACAGG	54
GUS 2	GCTAAAGCCGACTGCAGCAG	60
M13-27	CAGTCACGACGTTGTAAAACGACGGCC	74

that could potentially occur during the DNA transfer and/or integration process. The other approach considered was that of a PCR-based approach as previously employed in the analysis of transgenic plants containing the AoPR1 'full' promoter-*gus* construct (section 4.2.2). Considering the number of plants to be analysed and given limitations of facilities and time available this latter approach was deemed to be the more appropriate in this case. Information regarding copy number was not considered to be of vital importance in the context of this work as, having already shown the probable integration of multiple copies of the transgene by observations of the frequency of kanamycin resistance within ΔAoPR1-*gus* populations, it was hoped that the analysis of a number of lines for each construct would be sufficient to overcome any possible gene doseage-related effects on gene expression. In order to investigate the presence or absence of the ΔAoPR1-*gus* transgene within the tobacco genome while at the same time addressing the possibility of DNA rearrangement having occurred during the transformation process it was essential to optimise PCR reaction conditions such that the entire ΔAoPR1 region of the transgene was amplified. Thus any rearrangement resulting in a gross alteration in size of that sequence could be detected by its altered mobility through an agarose matrix. Primers available are illustrated in fig. 4.24. Using the Macintosh package 'Amplify' reaction conditions were devised for all possible primer combinations. Each combination was then assayed for its ability to amplify the desired fragment from DNA isolated from an AoPR1-*gus* plant shown previously to contain the 'full' AoPR1 promoter (section 4.2.2). Based on data obtained from these experiments it was found that the only primer combination capable of consistently amplifying the appropriate fragments was that of GUS 1 in conjunction with the relevant Δ primer. Conditions for amplification using these primers were determined as in section 2.9.6. Unlike the primary amplification of deletions where a portion of the Δ primer did not initially correspond to target sequence necessitating preliminary reaction conditions of low annealing temperature followed by an increase in this temperature in later cycles (section 4.3.4), the integrated deletions are homologous across almost the entire primer sequence except the GCG clamp at the 5' end. This case has arisen as a consequence of cloning steps retaining the *Sal*I site at this end of the Δ primers. Reflecting these considerations, reaction conditions chosen

for the amplification of AoPR1 promoter-derived sequences from transgenic tobacco DNA were a denaturing step of 95°C for 30 s, annealing at 49°C for 30 s and extension at 72°C for 1 min. 30 s. Computer modelling of these reactions predicted the amplification of a single band in all cases.

Having extracted nucleic acids from T1 explant material as in section 2.7.2 the integrity of the DNA as template for PCR amplification of transgene sequences was ascertained by the amplification of the endogenous PR1a promoter sequence using the primers PR1a 3' and PR1a 5'. In all cases amplification of a single band corresponding in size to that predicted for amplification of the correct sequences was observed indicating that DNA extracted in this manner was suitable for use as a reaction template.

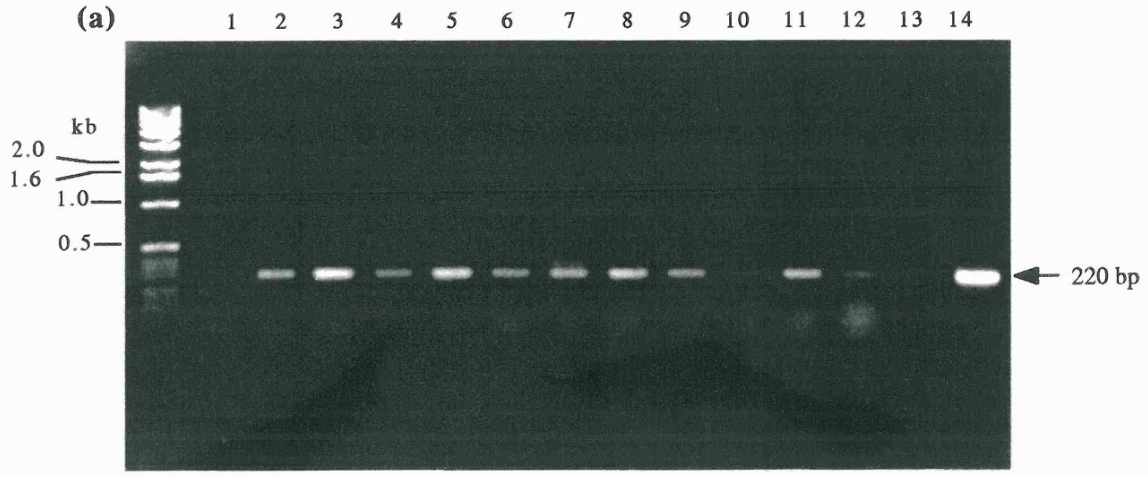
As can be seen from figs.4.25 - 4.31 all putative transformants, as determined by kanamycin-based selection methods, were found to contain the relevant transgene. Amplification of a single band corresponding in size to that predicted for amplification of the relevant AoPR1 promoter sequence was observed against a background of randomly amplified fragments generated as a consequence of the interaction of primers with sequences within the SR1 genomic background in all cases except that of plants containing the Δ357-*gus* transgene. In this case the presence of the AoPR1 promoter Δ357-deleted promoter was determined by the amplification of two fragments differing in size by approximately 100 bp. These fragments correspond in size to those generated as a consequence of the Δ357 primer binding at the original site within the promoter sequence and at the duplicated site which arose as a result of rearrangement during initial PCR-mediated generation of promoter deletions (section 4.3.4). As a control, PCR reactions were carried out using 10 ng of the plasmid pBI101.1(int.)-'full' as template. This plasmid contains the 'full' AoPR1 promoter sequence, and fragments amplified from this using GUS 1 and Δ primers are comparable in size to those amplified from DNA of transgenic tobacco harbouring the ΔAoPR1-*gus* transgene. Similarily, a second positive control reaction was carried out using DNA extracted from a transgenic tobacco plant previously shown to contain the 'full' AoPR1 promoter-*gus* transgene (section 4.2.2) as template. DNA extracted from an untransformed SR1 plant was used as a negative control,

Figure 4.25:

PCR amplification of Δ135 AoPR1 promoter sequence from kanamycin-resistant tobacco plants thought to contain the Δ135-*gus* transgene.

(a)
Lane 1: Untransformed SR1
Lane 2: Line 135.1
Lane 3: Line 135.2
Lane 4: Line 135.3
Lane 5: Line 135.5
Lane 6: Line 135.6
Lane 7: Line 135.8
Lane 8: Line 135.9
Lane 9: Line 135.10
Lane 10: Line 135.11
Lane 11: Line 135.12
Lane 12: Positive control plant
Lane 13: Empty
Lane 14: Plasmid (10 ng) control (pBI101.1(int.)-'full')

(b) Southern analysis of (a) probed with labelled Δ135 fragment.


(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
2.0
1.6
1.0
0.5
220 bp

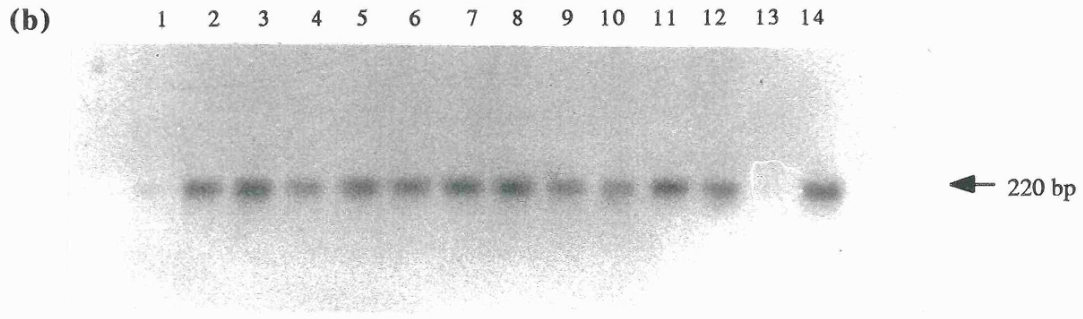

(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
220 bp

Figure 4.26:

PCR amplification of Δ246 AoPR1 promoter sequence from kanamycin-resistant tobacco plants thought to contain the Δ246-*gus* transgene.

(a)
Lane 1: Untransformed SR1
Lane 2: Line 246.1
Lane 3: Line 246.3
Lane 4: Line 246.4
Lane 5: Line 246.5
Lane 6: Line 246.6
Lane 7: Line 246.9
Lane 8: Line 246.10
Lane 9: Positive control plant.
Lane 10: Empty
Lane 11: Plasmid (10 ng) control (pBI101.1(int.)-full)

(b) Southern analysis of (a) probed with labelled Δ135 fragment.

(c) PCR amplification of Δ246 sequence from line 246.3 (10 μl nucleic acid extract).

Lane 1: Untransformed SR1
Lane 2: Empty
Lane 3: Line 246.3
Lane 4: Empty
Lane 5: Plasmid control (10 ng; pBI101.1(int.)-'full')

(d) Southern analysis of (c) probed with labelled Δ135 fragment.

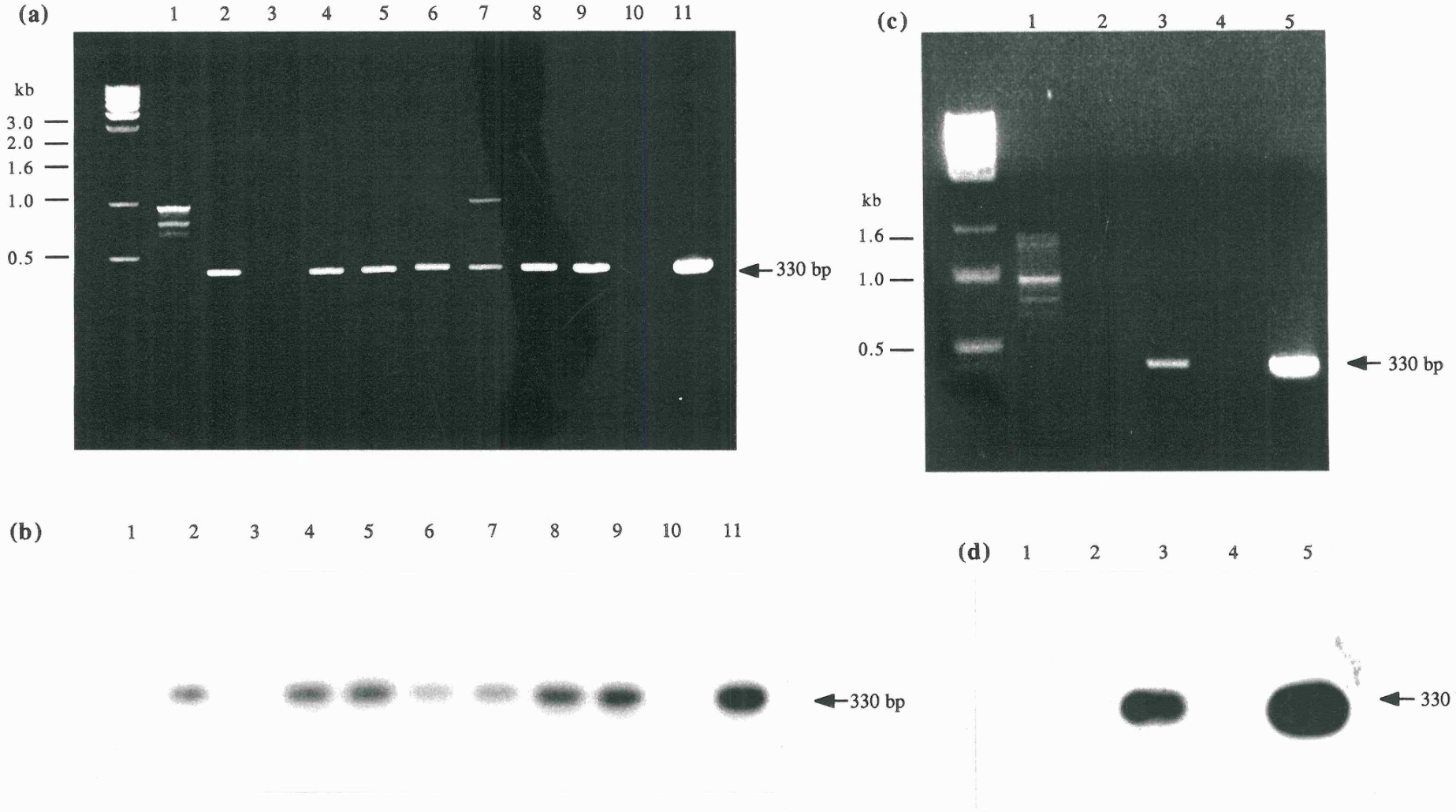

(a)
1 2 3 4 5 6 7 8 9 10 11
kb
3.0
2.0
1.6
1.0
0.5
330 bp
(c)
1 2 3 4 5
kb
1.6
1.0
0.5
330 bp
(b)
1 2 3 4 5 6 7 8 9 10 11
330 bp
(d)
1 2 3 4 5
330 bp

Figure 4.27:

PCR amplification of Δ357 AoPR1 promoter sequence from kanamycin-resistant tobacco plants thought to contain the Δ357-*gus* transgene.

(a)
Lane 1: Untransformed SR1
Lane 2: Line 357.1
Lane 3: Line 357.2
Lane 4: Line 357.3
Lane 5: Line 357.4
Lane 6: Line 357.5
Lane 7: Line 357.8
Lane 8: Line 357.9
Lane 9: Line 357.11
Lane 10: Positive control plant
Lane 11: Empty
Lane 12: Plasmid (10 ng) control (pBI101.1(int.)-'full')

(b) Southern analysis of (a) probed with labelled Δ135 fragment.

(c) PCR amplifiction of Δ357 sequence from line 357.2 (10 μl nucleic acid extract).

Lane 1: Untransformed SR1
Lane 2: Line 357.2
Lane 3: Positive control plant
Lane 4: Empty
Lane 5: Plasmid control (10 ng; pBI101.1(int.)-'full')

(d) Southerm analysis of (c) probed with labelled Δ135 fragment.

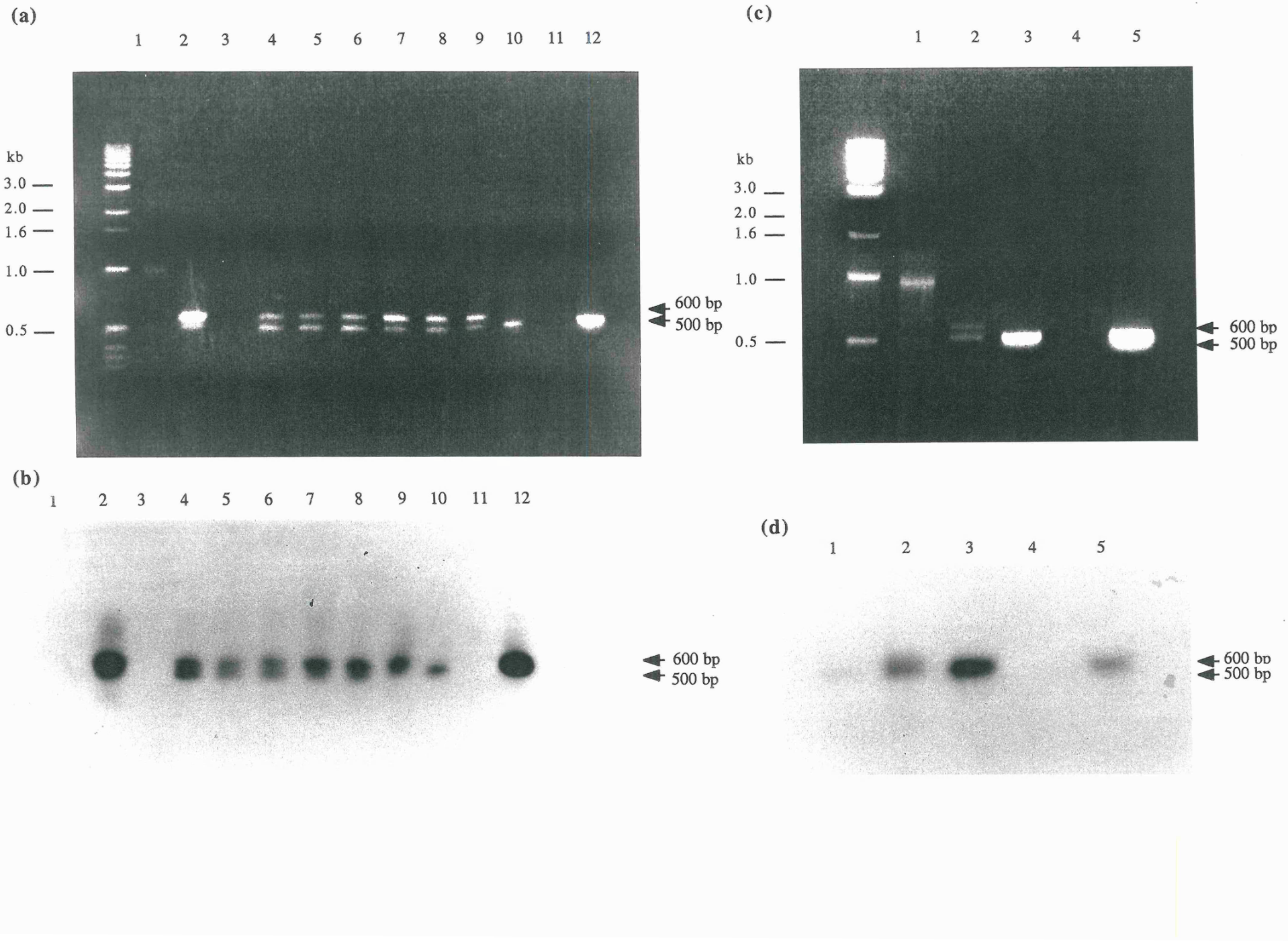

(a)
1 2 3 4 5 6 7 8 9 10 11 12
kb
3.0
2.0
1.6
1.0
0.5
600 bp
500 bp
(b)
1 2 3 4 5 6 7 8 9 10 11 12
600 bp
500 bp
(c)
1 2 3 4 5
kb
3.0
2.0
1.6
1.0
0.5
600 bp
500 bp
(d)
1 2 3 4 5
600 bp
500 bp

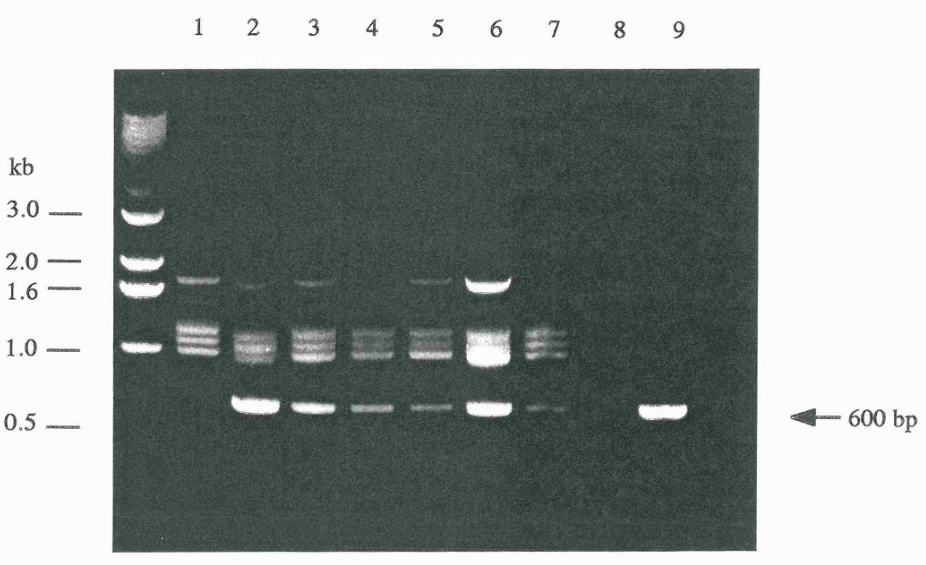
Figure 4.28:

PCR amplification of Δ505 AoPR1 promoter sequence from kanamycin-resistant tobacco plants thought to contain the Δ505-*gus* transgene.

(a)
Lane 1: Untransformed SR1
Lane 2: Line 505.2
Lane 3: Line 505.3
Lane 4: Line 505.4
Lane 5: Line 505.5
Lane 6: Line 505.7
Lane 7: Positive control plant
Lane 8: Empty
Lane 9: Plasmid (10 ng) control (pBI101.1(int.)-'full')

(b) Southern analysis of (a) probed with labelled Δ135 fragment.

(a)



(b)

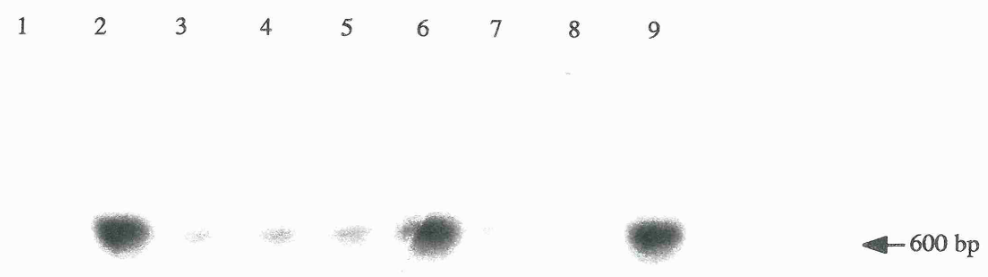
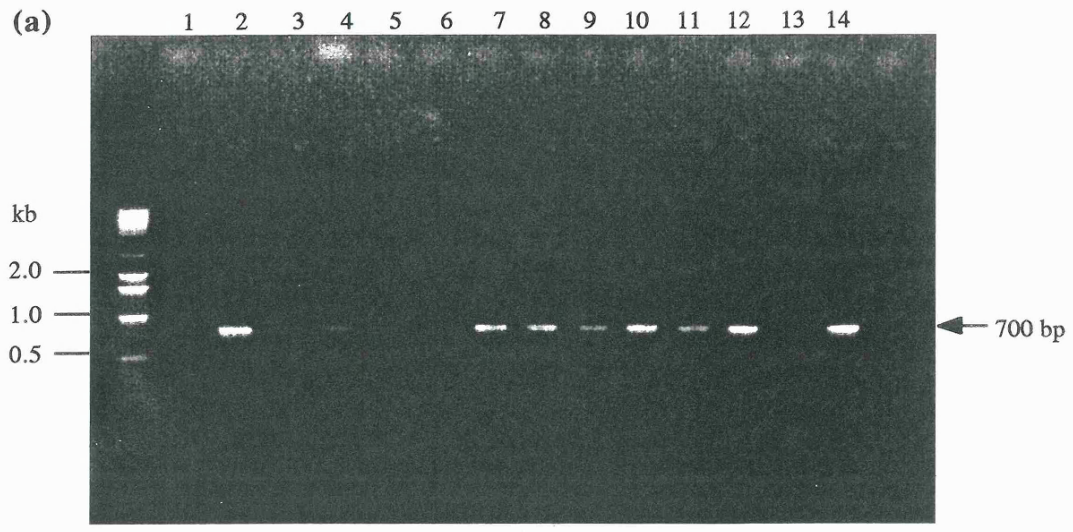


Figure 4.29:

PCR amplification of Δ618 AoPR1 promoter sequence from kanamycin-resistant tobacco plants thought to contain the Δ618-*gus* transgene.

(a)
Lane 1: Untransformed SR1
Lane 2: Line 618.1
Lane 3: Line 618.3
Lane 4: Line 618.4
Lane 5: Line 618.5
Lane 6: Line 618.6
Lane 7: Line 618.7
Lane 8: Line 618.8
Lane 9: Line 618.9
Lane 10: Line 618.10
Lane 11: Line 618.11
Lane 12: Positive control plant
Lane 13: Empty
Lane 14: Plasmid (10 ng) control (pBI101.1(int.)-'full')

(b) Southern analysis of (a) probed with labelled Δ135 fragment.


(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
2.0
1.0
0.5
700 bp

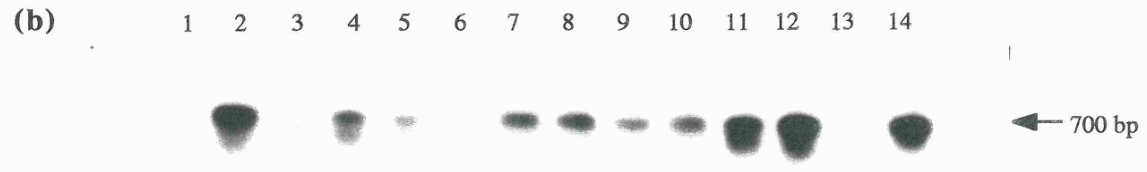

(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
700 bp

Figure 4.30:

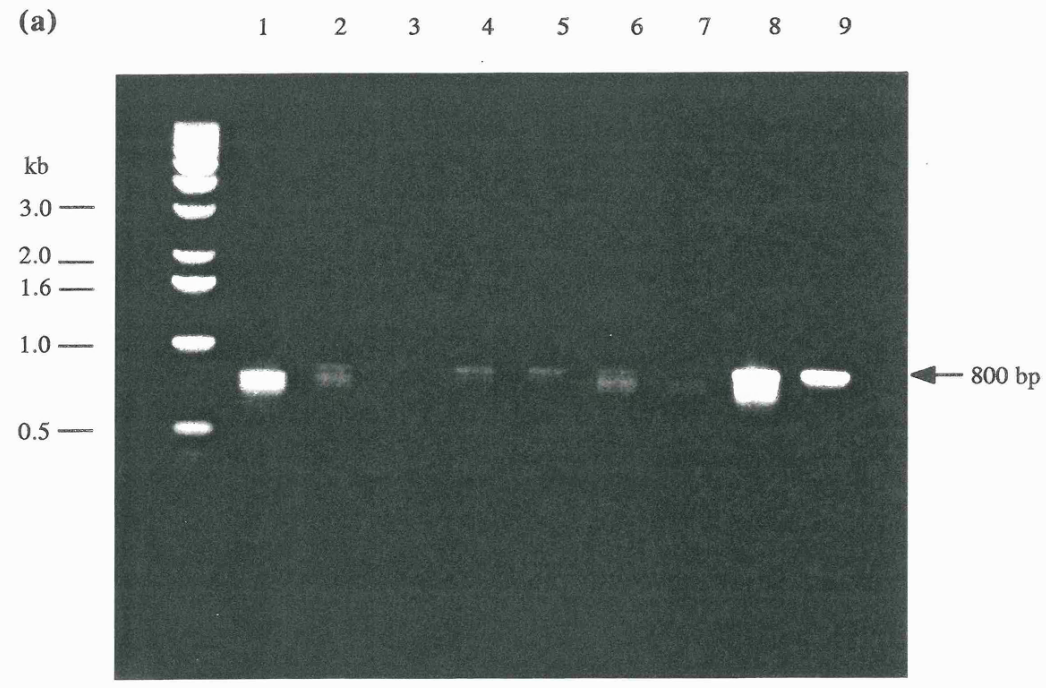
PCR amplification of Δ713 AoPR1 promoter sequence from kanamycin-resistant tobacco plants thought to contain the Δ713-*gus* transgene.

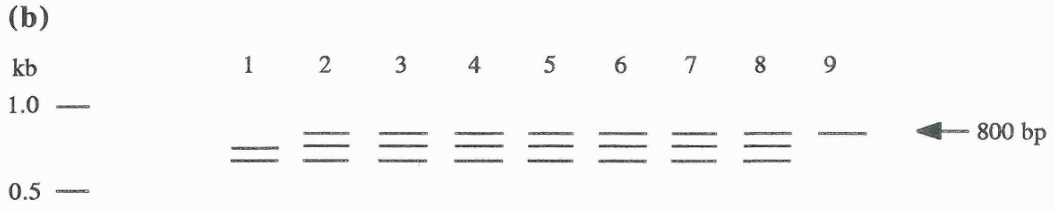
(a)
Lane 1: Untransformed SR1
Lane 2: Line 713.1
Lane 3: Line 713.2
Lane 4: Line 713.5
Lane 5: Line 713.7
Lane 6: Line 713.8
Lane 7: Line 713.9
Lane 8: Positive control plant
Lane 9: Plasmid (10 ng) control (pBI101.1(int.)-'full')

(b) Schematic representation of (a).

(c) Southern analysis of a gel similar to (a) probed with labelled Δ135 fragment.

Lane 1: Untransformed SR1
Lane 2: Line 713.1
Lane 3: Line 713.2
Lane 4: Line 713.5
Lane 5: Line 713.7
Lane 6: Line 713.8
Lane 7: Line 713.9
Lane 8: Positive control plant
Lane 9: Empty
Lane 10: Plasmid (10 ng) control (pBI101.1(int.)-'full')


(a)
1 2 3 4 5 6 7 8 9
kb
3.0
2.0
1.6
1.0
0.5
800 bp


(b)
kb
1 2 3 4 5 6 7 8 9
1.0
0.5
800 bp

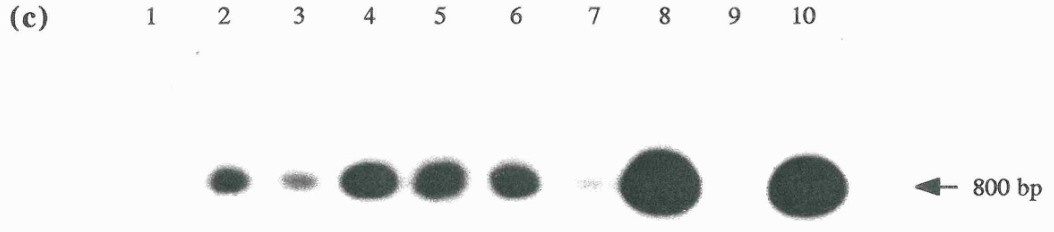
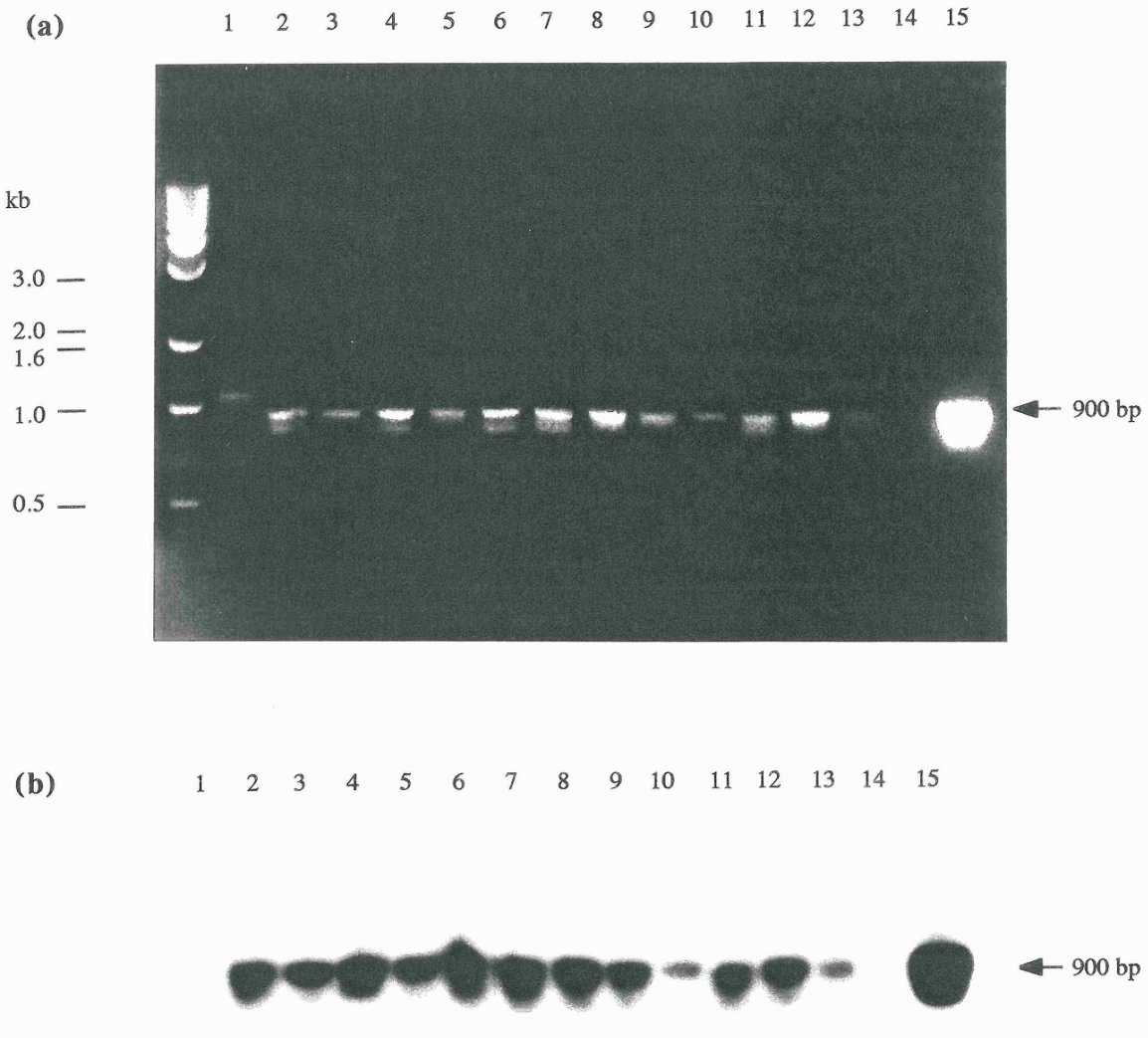

(c)
1 2 3 4 5 6 7 8 9 10
800 bp

Figure 4.31:

PCR amplification of Δ815 AoPR1 promoter sequence from kanamycin-resistant tobaco plants thought to contain the Δ815-*gus* transgene.

(a)
Lane 1: Untransformed SR1
Lane 2: Line 815.2
Lane 3: Line 815.3
Lane 4: Line 815.4
Lane 5 Line 815.5
Lane 6: Line 815.6
Lane 7: Line 815.7
Lane 8: Line 815.8
Lane 9: Line 815.9
Lane 10: Line 815.10
Lane 11: Line 815.11
Lane 12: Line 815.12
Lane 13: Positive control plant
Lane 14: Empty
Lane 15: Plasmid (10 ng) control (pBI101.1(int.)-'full')

(b) Southern analysis of (a) probed with labelled Δ135 fragment.


(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kb
3.0
2.0
1.6
1.0
0.5
900 bp
(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
900 bp

and in all cases yielded a 'fingerprint' characteristic of amplification of SR1 genomic DNA using GUS 1 and Δ primers. The presence of the appropriate transgene sequences was determined by the appearance of a novel fragment only in those samples known to contain template derived from probable transformants positive for the ΔAoPR1-*gus* transgene. In order to confirm the AoPR1-derived nature of amplified sequences gels of relevant PCR products were blotted (section 2.12.1) and probed overnight with labelled AoPR1 Δ135 fragment. Southern analysis confirmed assumptions made on the basis of preliminary PCR analysis. The apparant lack of amplification of AoPR1 promoter sequences observed upon PCR analysis of some extracts (fig.4.25a, lane 3; fig. 4.24a, lane 3) was further investigated by carrying out similar PCR reactions this time using 10 μl of template extract (5 μl were used in the original analysis). This increase in template concentration was sufficient to result in the subsequent amplification of the desired sequences (fig. 4.26c, lane 3; fig. 4.27c, lane 2) which in turn were found to hybridise specifically to labelled AoPR1 promoter sequence (fig. 4.26d, lane 3; fig. 4.27d, lane 2) thereby indicating the transgenic nature of those plants from which this template was derived.

No apparent gross rearrangements were observed to have occurred as determined by the fact that amplified fragments were comparable in size to those amplified from both a plasmid known to contain the 'full' promoter sequence (pBI101.1(int.)-'full') and a plant known to contain a similar construct in most cases. However, in the case of plants containing the Δ815-*gus* construct some rearrangement appears to have occurred in a number of transgenic lines (fig. 4.31a, lanes 2,4,6,7 and 10) as revealed by the presence of two bands, both hybridising to labelled AoPR1 promoter sequence (fig. 4.31b, lanes 2,4,6,7 and 10). The exact nature of this possible rearrangement was not determined precisely, but may be attributable to events occurring either during the introduction of foreign DNA into the tobacco genome or as a result of the PCR analysis of transformed material. Events preceeding *Agrobacterium*-mediated plant transformation may be excluded as all such stages had been monitored for this possibility. Those plants within which this rearrangement may have occurred were, however, taken on for further analysis as it was considered possible that no rearrangement event had

actually occurred ie. that the generation of a doublet on PCR was a consequence of the PCR reaction itself and therefore independent of events occurring within the plant itself. This assumption is possible when one observes that amplification of sequences from the pBI101.1(int)-based plasmid control may also have given rise to two bands (fig. 4.31a, lane 15) although it is difficult to determine this unambiguously from this gel. Hence, the generation of two fragments both of which hybridise to the AoPR1 promoter sequence may be a consequence of sub-optimal PCR reaction conditions and not be a true representation of the situation *in planta*.

4.4.2 The loss of wound-inducibility corresponds to a loss of 167 5' terminal base pairs from the 'full' AoPR1 promoter sequence.

Transgenic tobacco plants harbouring ΔAoPR1-*gus* constructs were analysed for the ability of truncated promoter fragments to drive transcription of the *gus* gene in response to a wound stimulus imposed by stabbing leaf explants several times with a Gilson pipette tip followed by incubation at 25°C for three days. This type of stimulus has previously been shown to induce AoPR1-driven *gus* expression to high levels in transgenic plants containing the 'full' AoPR1 promoter-*gus* construct (Warner *et al.*, 1993). Both histochemical and fluorometric assay methods failed to reveal any activity of the truncated promoter under such conditions. Wound-inducible activity was lost upon removal of the first 167 bp from the 5' terminus of the 'full' promoter and this loss of activity was maintained with subsequent removal of further 5' terminal sequences (fig. 4.32).

4.4.3 SA-responsiveness and wound-inducibility of the AoPR1 promoter appear to reside within the same region of the 'full' promoter.

Having previously demonstrated the ability of concentrations of SA greater than 1 mM to induce AoPR1 promoter activity in a dose-dependent manner (fig. 4.5) the effect of 4 mM SA was investigated for its ability to induce activity of the truncated promoter fragments. Leaf explants from transgenic tobacco plants into which T-DNA carrying the ΔAoPR1-*gus* construct had been introduced were assayed for SA-dependent induction as previously

Figure 4.32:

Effect of promoter deletions on the level of GUS activity in response to wounding.

Each point represents the average of at least three replica assays carried out on each individual transformed line. Variance between replicates of the same line was negligible, with between 7-10 independent lines assayed per construct.

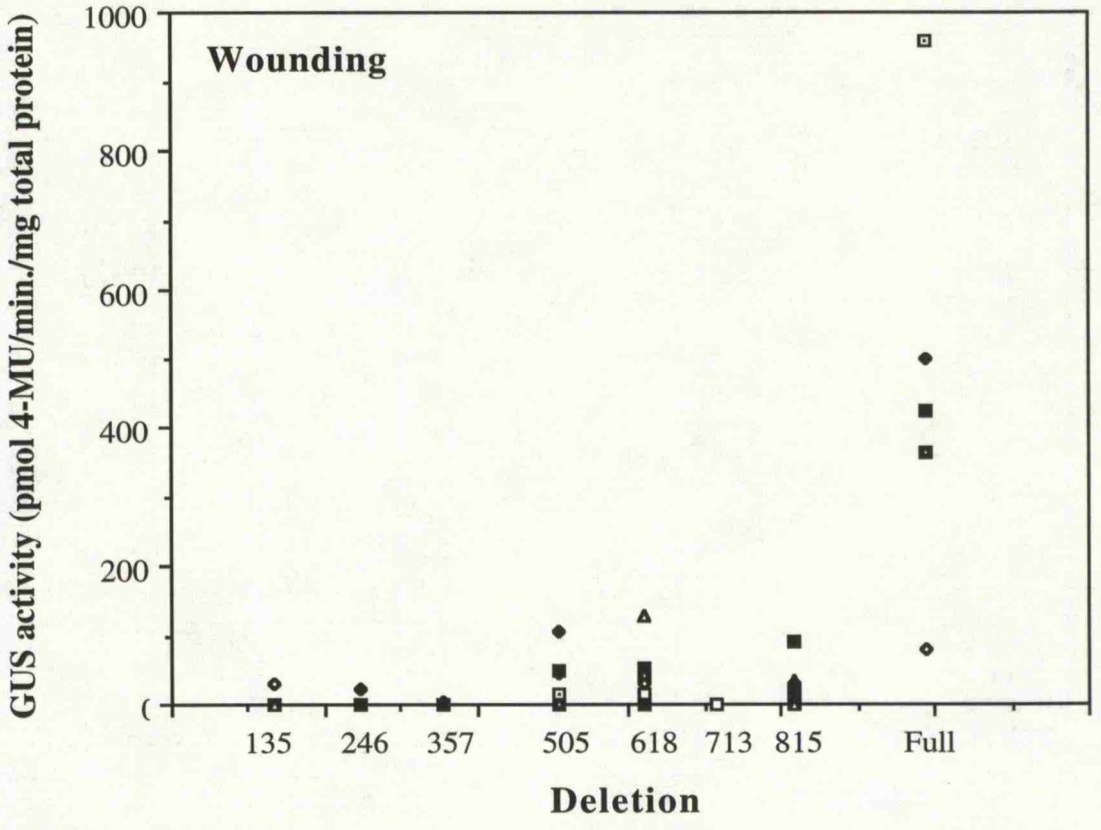
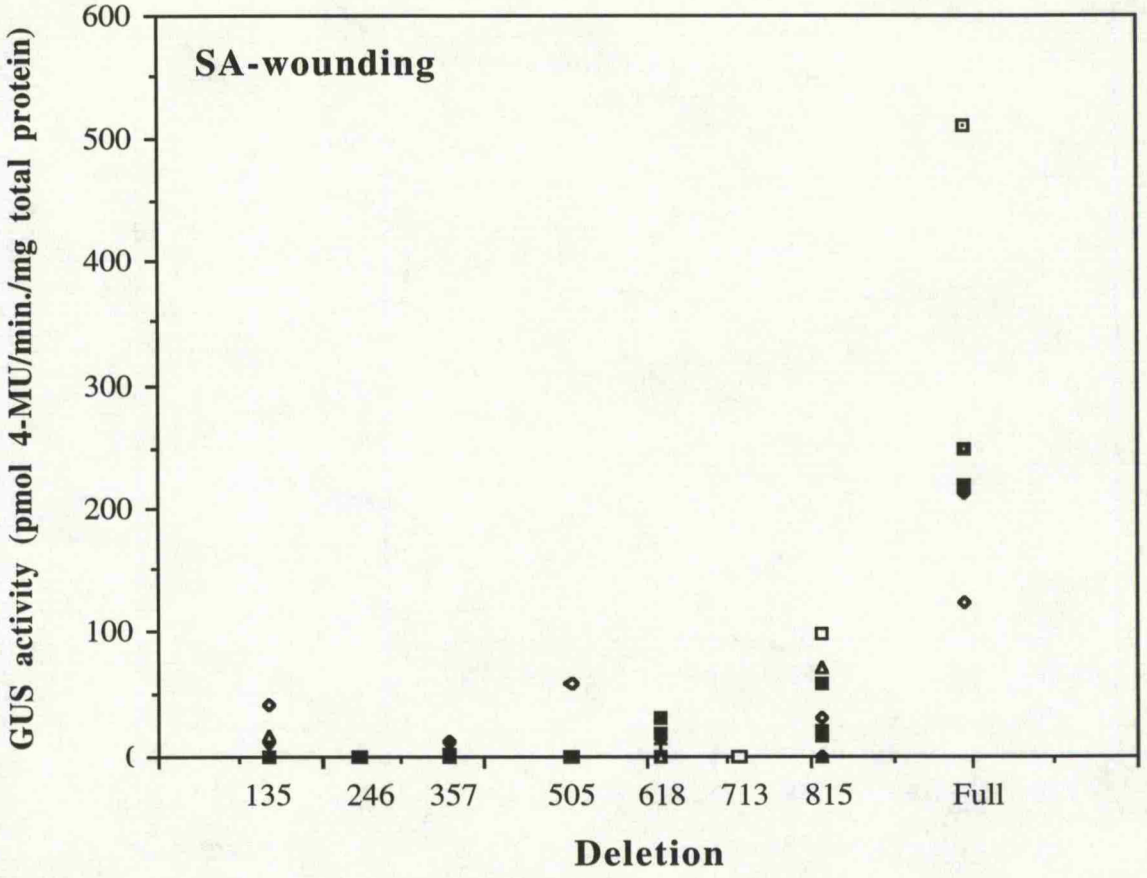

Wounding
GUS activity (pmol 4-MU/min./mg total protein)
1000
800
600
400
200
0
135
246
357
505
618
713
815
Full
Deletion

Figure 4.33:

(a) Effect of promoter deletions on the level of GUS activity in unwounded explants in response to exogenously supplied 4 mM salicylic acid.

(b) Effect of promoter deletions on GUS activity in wounded explants in response to 4 mM salicylic acid.

In both cases each point represents the average of at least three replica assays carried out on each of between 7-10 independent transformed lines. Variance between replicates of the same line was negligible.


SA-wounding
GUS activity (pmol 4-MU/min./mg total protein)
600
500
400
300
200
100
0
135
246
357
505
618
713
815
Full
Deletion

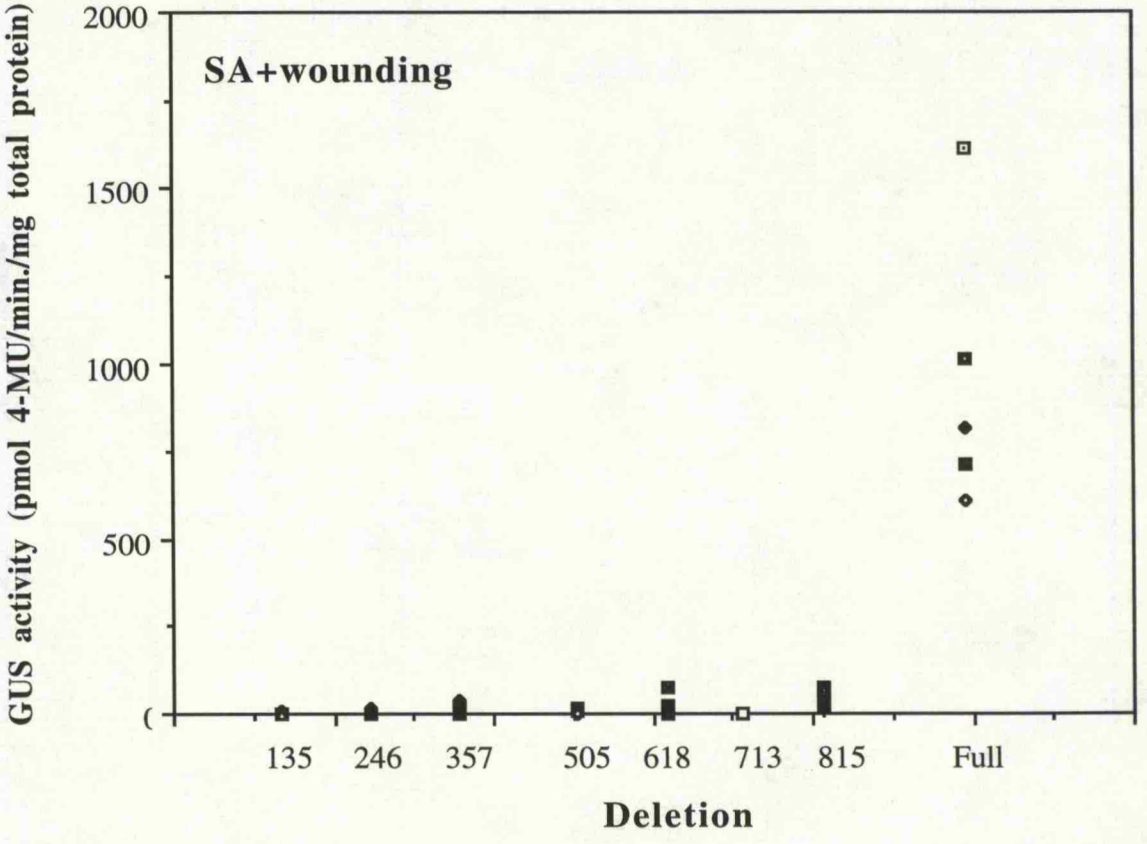
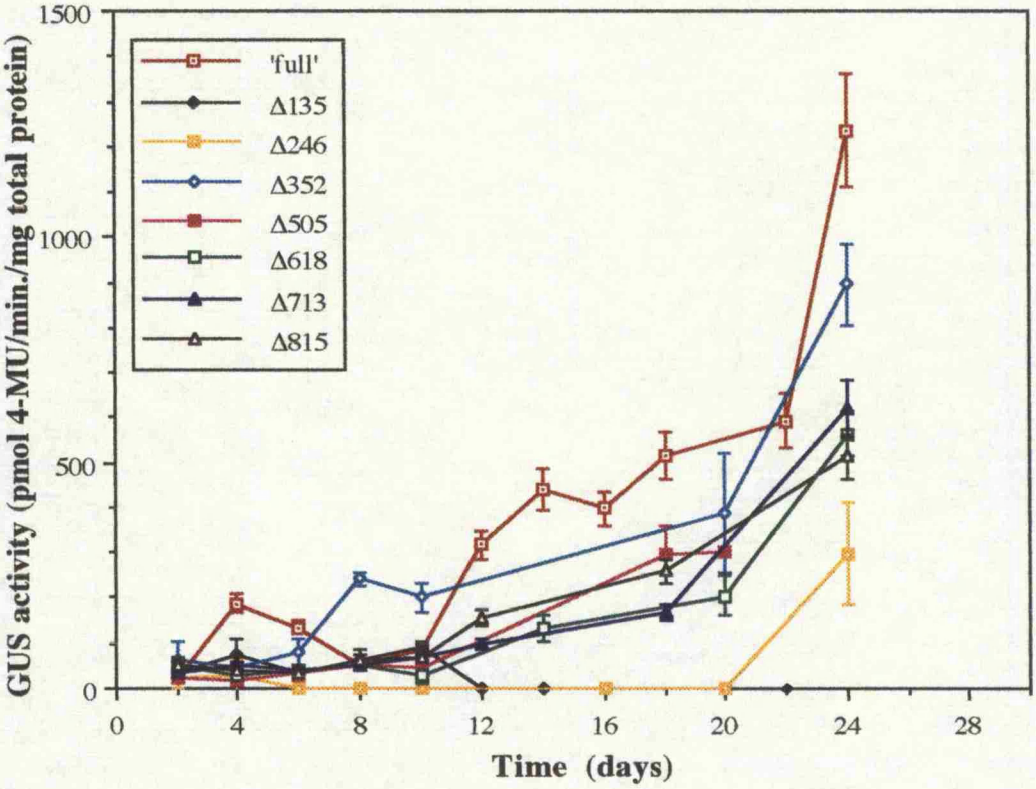

SA+wounding
GUS activity (pmol 4-MU/min./mg total protein)
2000
1500
1000
500
0
135
246
357
505
618
713
815
Full
Deletion

Figure 4.34:

Effect of promoter deletions on GUS activity in callus material at different times post-transformation.

Each point represents the mean of at least three assays for each transformation. Error bars represent the standard deviation ot these values.


GUS activity (pmol 4-MU/min./mg total protein)
1500
1000
500
0
0
4
8
12
16
20
24
28
Time (days)
'full'
Δ135
Δ246
Δ352
Δ505
Δ618
Δ713
Δ815

(section 4.2.3), investigating the potential for induction by SA both in the presence and absence of a wound stimulus. It was observed, as in the case of the wound stimulus alone, that induction of expression of the *gus* gene under the control of deleted versions of the AoPR1 promoter was not detected in any case (fig. 4.33a). SA-responsiveness was lost with the removal of 167 bp from the 5' terminus of the 'full' promoter. No subsequent deletions were observed to restore activity indicating that those sequences responsible for conferring SA-responsiveness on the AoPR1 promoter, either independently or in conjunction with some other sequences further downstream within the promoter sequence, reside within these terminal 167 bp. Similar results were obtained when wounded explants were exposed to 4 mM salicylic acid (fig. 4.33b).

4.4.4 The ability of the AoPR1 promoter to respond to dedifferentiation-related stimuli is determined by sequences 135 to 246 bp upstream of the transcription start site.

It has been hypothesised in a previous chapter of this thesis that the dedifferentiation-specific aspect of AoPR1 expression is intrinsically linked with the ability of this promoter to respond to the wound stimulus (section 3.3). This idea appears appropriate as it has previously been shown that the AoPR protein (DD1) does not accumulate in response to exposure to either auxin or cytokinin (Fioroni, 1989). Thus, it was predicted that the loss of the wound-responsive element(s) would correlate with a loss of dedifferentiation-specific expression. This was not, however, found to be the case. It was seen that while removal of the first 167 bp from the 5' terminus of the 'full' promoter was sufficient to result in a loss of wound-inducibility callus-specific expression was still retained. Indeed this expression profile was evident in the case of all truncated promoter constructs except that of Δ135 (fig. 4.34). Results corresponding to expression as a consequence of the Δ246 promoter construct indicate that, while expression levels are rather low over the period 0-20 days post-inoculation, this region of the AoPR1 promoter is sufficient to drive *gus* expression under these conditions as expression is detected after 24 days. It is therefore proposed that the minimum AoPR1 promoter length required for callus-specific expression is 246 bp, and that further deletion of

this fragment may result in the loss of this induction. Deletion to -135 completely removes all dedifferentiation-specific expression.

4.4.5 Pollen-specific expression is conferred by sequences towards the 3' terminus of the 'full' promoter sequence.

The ability of the AoPR1 promoter to drive transcription in mature pollen grains has been reported (section 4.2.8.4; Warner, 1992; Warner *et al.*, 1994). Unlike Uknes and co-workers (Uknes *et al.*, 1993) the expression of the *gus* gene is a direct consequence of the activity of the AoPR1 promoter and not a result of artifactual expression of this reporter gene. Analysis of pollen from transgenics harbouring the AoPR1 promoter fused to the luciferase coding sequence showed that the expression profile observed correlated with that observed for AoPR1 promoter-*gus* plants (Warner *et al.*, 1994). Thus the nature of those sequences responsible for conferring such expression were further investigated. Both histochemical and fluorometric GUS analysis of plants containing ΔAoPR1-*gus* constructs revealed that pollen-specific expression was conferred by sequences contained within the region -135 to +1. Levels of expression, although low, appeared comparable to those of the 'full' promoter in most cases (fig. 4.35). It is possible, however, that some slight repression may have occurred in the case of constructs Δ815, Δ713 and Δ618, although the rather low activity values observed in all cases and the scatter of values within each population makes any further speculation difficult. To draw any conclusion other than that sequences within the 3' terminal 135 bp of the AoPR1 promoter are responsible, in the main, for pollen-specific expression of genes under the control of this promoter in transgenic tobacco is not possible.

4.4.6 Petal-specific expression involves at least three different regions of the AoPR1 promoter sequence.

Deletion analysis of the AoPR1 promoter with respect to its ability to drive transcription in the pigmented regions of tobacco petal collars revealed the interaction between three distinct sequence regions to be involved in the regulation of expression of genes under the control of this promoter. It was found that the removal of the first 167 bp from the 5' terminus of the

'full' promoter resulted in the loss of petal-specific expression as determined by the absence of any detectable GUS activity. However, deletions resulting in the removal of a further 197 bp from the 5' region of the promoter restored AoPR1-driven *gus* expression thereby indicating the presence of a potential enhancer enhancer element within the region -880 to -815 and a repressor element between positions -713 and -618. Activity was lost again, however, on subsequent deletion to position -416, revealing the presence of a second petal-specific enhancer element within that sequence between positions -505 to -416. These results were obtained both by histochemical and fluorometric methods and data in both cases was in agreement. A comparison of the effect of sequential deletion of the 'full' AoPR1 promoter with the behaviour of the 'full' promoter in petal tissue of transgenic tobacco is presented in fig. 4.36.

4.4.7 A truncated AoPR1 promoter drives *gus* expression in stem tissue, albeit to significantly reduced levels.

The activity of the AoPR1 promoter in stem tissue was previously found, visually, to correlate with sites of lignification (section 4.2.8.1). Those sequences involved in maintaining this expression profile were determined as residing within that sequence 135 bp immediately upstream of the transcriptional start site by deletion analysis (fig. 4.37). It was evident, however, that the levels of activity observed upon deletion of the 'full' promoter to position -815 were significantly reduced relative to that observed for the 'full' promoter. This reduction in expression levels may be observed upon removal of a 167 bp fragment from the 5' terminus of the full' promoter and is maintained through all subsequent deletions. This implies that, although the Δ135 fragment is sufficient to drive stem-specific expression, 'wild-type' expression levels are only obtained in the presence of an enhancer element located between positions -880 to -815.

4.4.8 Stigma-specific activity of the AoPR1 promoter is determined by sequences within 135 bp of the start of transcription.

Reduced stigma-specific *gus* expression was observed in plants containing truncated AoPR1 promoter-*gus* constructs upon comparison to plants within which the 'full' promoter-gus construct had integrated (fig. 4.38). All ΔAoPR1-*gus* constructs were observed to impart reduced levels of AoPR1 promoter activity in stigma tissue of open, pre-dehiscence flowers. This is presumably a direct consequence of the removal of those sequence element(s) responsible for directing 'wild-type' stigma-specific expression from the promoter sequence. It therefore appears that the smallest fragment analysed, Δ135, contains sufficient sequence information to direct this type of expression but that the interaction of elements contained within this region with some element(s) present between positions -880 to -815 is necessary for full activity within this tissue type.

4.4.9 Seed-specific AoPR1 activity is retained upon removal of 745 bp from the 5' terminus of the 'full' promoter.

In the case of the seed-specific aspect of AoPR1 promoter activity, it appears again that it is the interaction between elements located at positions -135 to +1 and -880 to -815 that is responsible for directing full promoter activity in immature seed (5 days post-pollination). Data from fluorometric and histochemical analysis of transgenic tobacco plants containing Δ AoPR1-*gus* constructs revealed that, as with pollen, stem and stigma, a fragment of 135 bp in length derived from the 3' terminus of the 'full' promoter is sufficient to confer the tissue-specificity of expression, while a further element at the 5' terminus of the 'full' promoter is required to determine the level of expression obtained. All deletion constructs exhibited reduced activity in comparison to the 'full' promoter when assayed in transgenic tobacco (fig. 4.39).

Figure 4.35:

Effect of promoter deletions on GUS activity in mature, dehisced pollen grains.

Each point represents the average of at least three replica assays carried out on each independent transformed line. Between 7-10 independent transformed lines were assayed per construct.

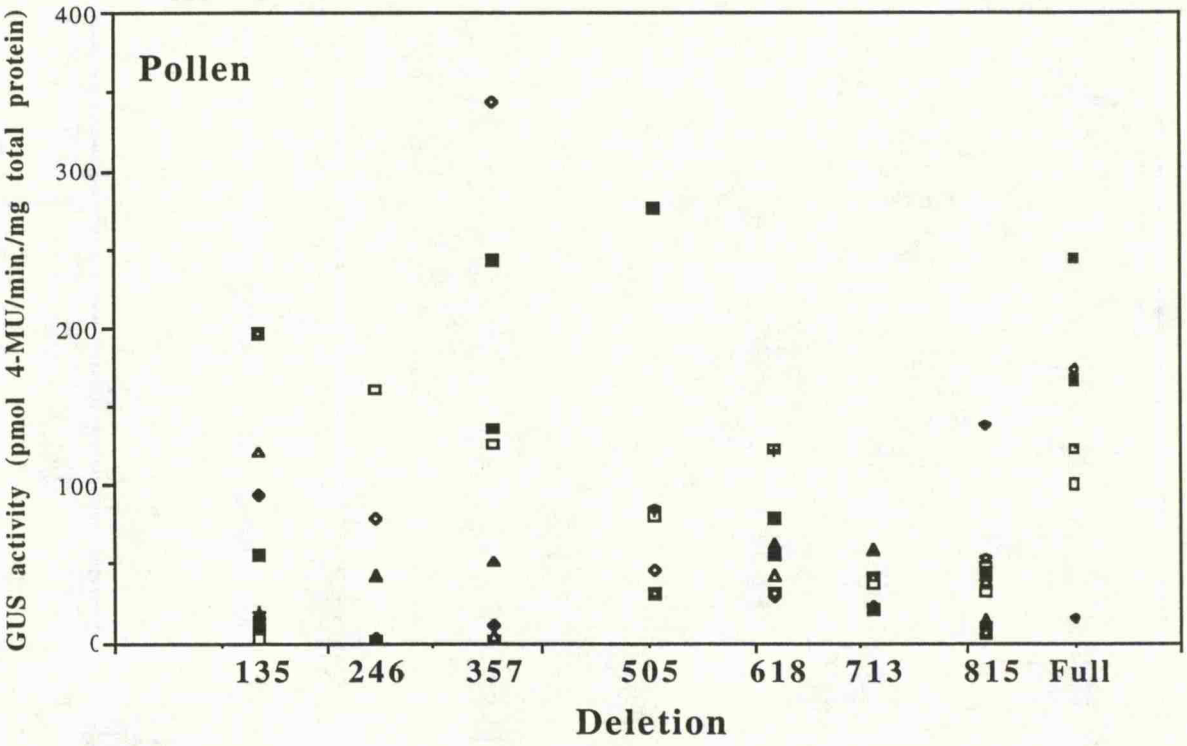

Pollen
GUS activity (pmol 4-MU/min./mg total protein)
400
300
200
100
0
135
246
357
505
618
713
815
Full
Deletion

Figure 4.36:

Effect of promoter deletions on GUS activity in petal collars of pre-dehiscence flowers.

Each point represents the mean of at least three replica assays carried out on each independent transformed line. Between 7-10 independent transformed lines were assayed per construct.

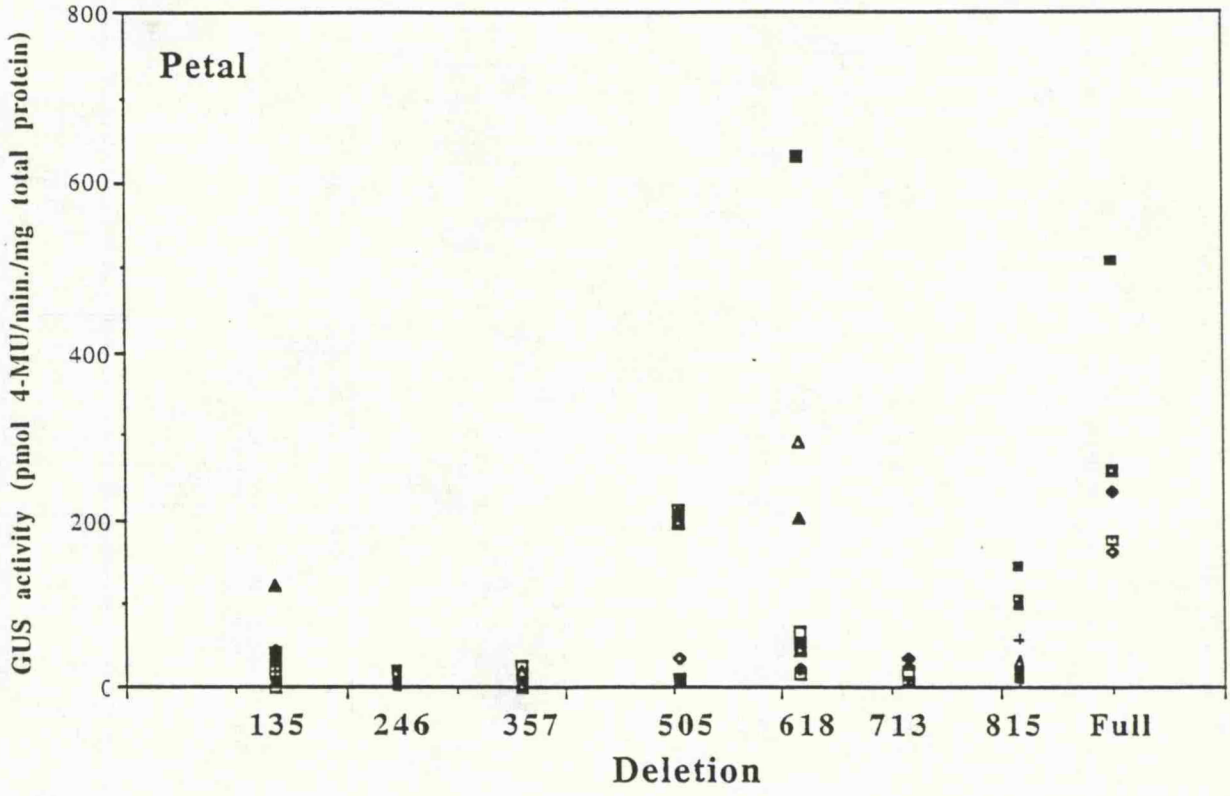

Petal
GUS activity (pmol 4-MU/min./mg total protein)
800
600
400
200
0
135
246
357
505
618
713
815
Full
Deletion

Figure 4.37:

Effect of promoter deletions on GUS activity in stem tissue of transformed tobacco plants.

Each point represents the average of at least three replica assays carried out on each independent transformed line. Between 7-10 independent transformed lines were assayed per construct.

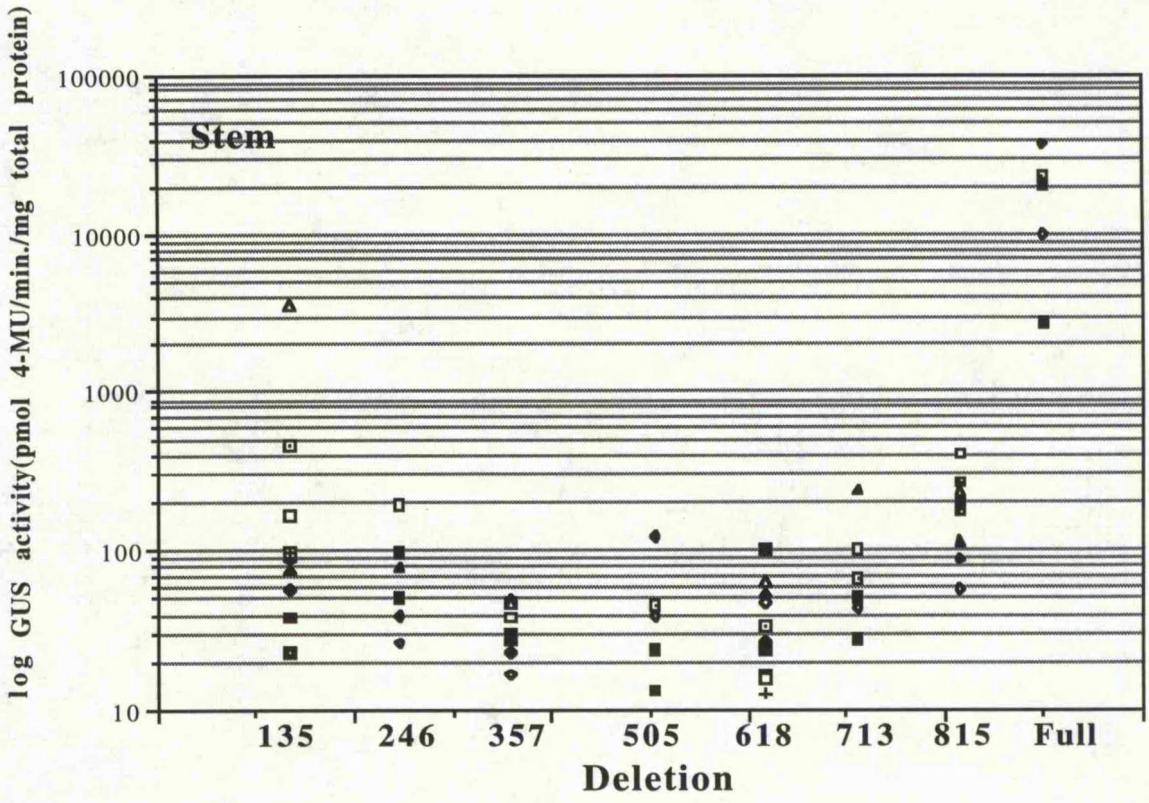

log GUS activity(pmol 4-MU/min./mg total protein)
100000
10000
1000
100
10
Stem
135
246
357
505
618
713
815
Full
Deletion

Figure 4.38:

Effect of promoter deletions on GUS activity in stigmas of pre-dehiscence flowers.

Each point represents the average of at least three replica assays carried out on each independent transformed line. Between 7-10 independent transformed lines were assayed per construct.

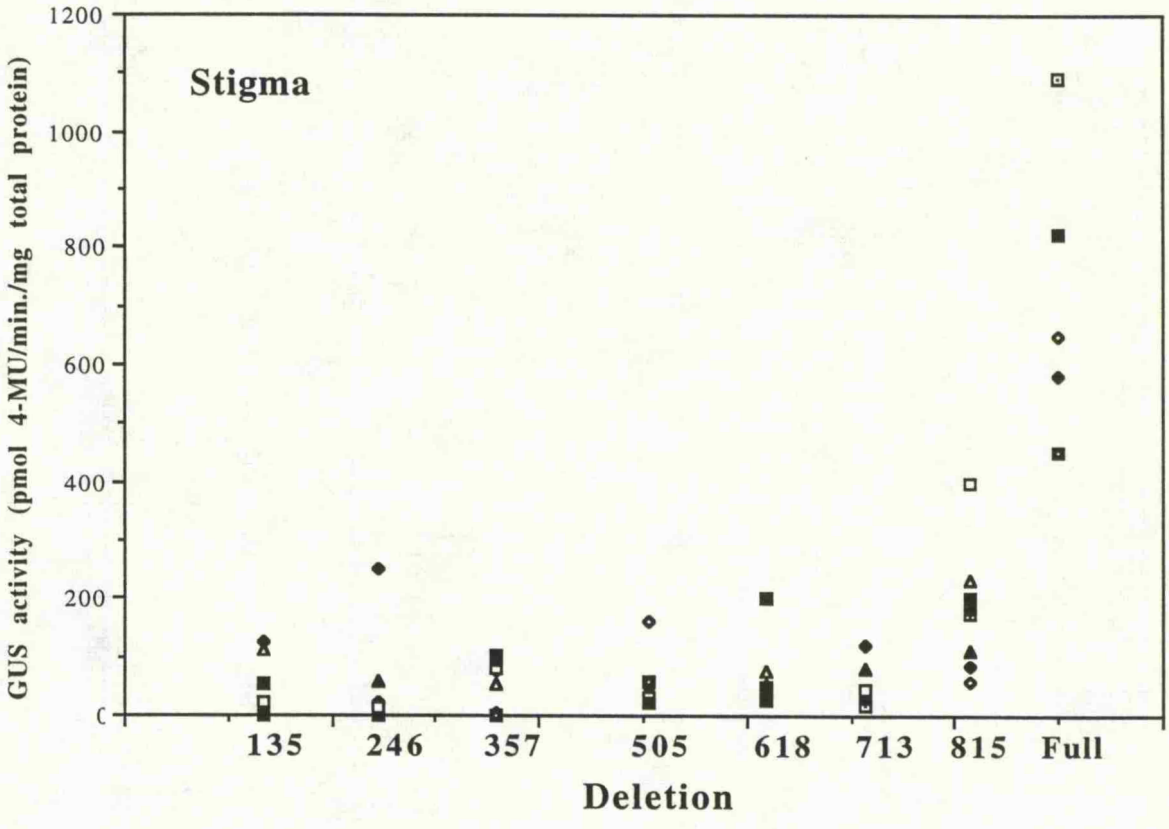
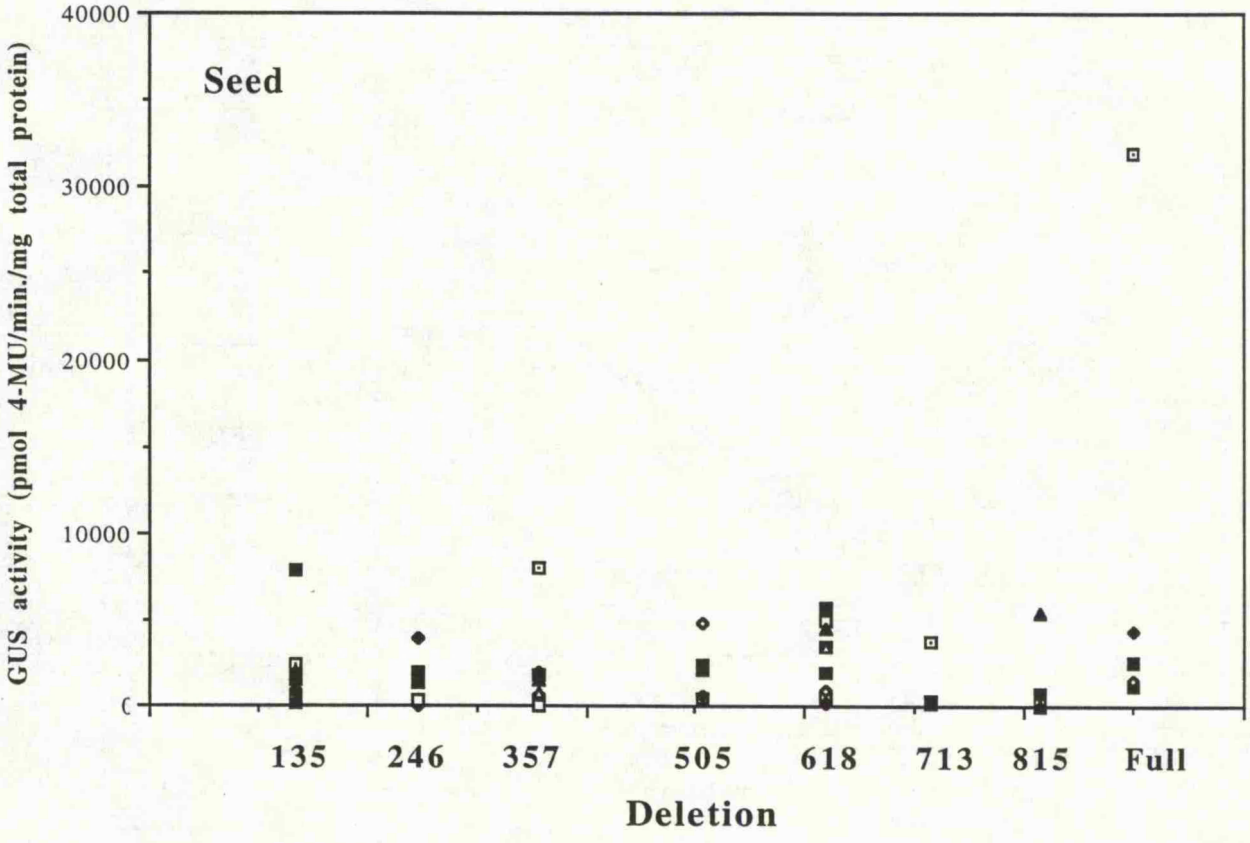

Stigma
GUS activity (pmol 4-MU/min./mg total protein)
1200
1000
800
600
400
200
0
135
246
357
505
618
713
815
Full
Deletion

Figure 4.39:

Effect of promoter deletions of GUS activity of seed material five days post-pollination.

Each point represents the mean of at least three replica assays carried out on each independent transformed line. Between 7-10 independent transformed lines were assayed per construct.


Seed
GUS activity (pmol 4-MU/min./mg total protein)
40000
30000
20000
10000
0
135
246
357
505
618
713
815
Full
Deletion

4.5 Discussion:.

4.5.1 Quantitative GUS activity is influenced by endogenous factors.

Analysis of the effects of increasing concentrations of tissue extract on GUS activity revealed the presence of potential promoters or inhibitors within extracts of different tissues. In all cases it was observed that as little as 1 μg of tissue extract protein was sufficient to result in a significant ($P<0.05$) alteration in GUS activity when compared with activity in the absence of any extract. Some extracts were found to promote activity upon the addition of this concentration of total cell protein to the reaction mix (petal, stem, seed and stigma), while repression of activity was observed in the presence of 1 μg leaf extract. Increasing extract concentration to 5 μg resulted in no significant changes in enzyme activity in the case of extracts where enhancement was previously observed, although graphical data appears to indicate some slight enhancement in the presence of 5 μg of stem and seed extract. The addition of 5 μg of pollen extract did, however, result in a significant ($P<0.05$) enhancement of GUS activity which was maintained upon increasing extract concentration to 10 μg. In all cases 20 μg of extract was observed to significantly inhibit measurable GUS activity. From this data optimum extract concentrations were determined for use in future GUS fluorometric assays. Protein concentrations of between 1 and 5 μg were generally considered to be appropriate for analysis of all tissues except leaf, where an extract protein concentation of no more than 1 μg was suitable. GUS activity values (pmol 4-MU/min./mg total protein) thus obtained should not be artificially high or subject to significant repression, and thus may be interpreted as being an accurate representation of GUS activity *in planta*.

4.5.2 The suspension culture environment is not condusive to enhanced AoPR1 expression.

In marked contrast to observations of AoPR1 expression in suspensions of freshly isolated asparagus mesophyll cells where AoPR1 expression is markedly enhanced, established suspension cultures of transgenic tobacco containing the 'full' AoPR1-*gus* transgene were found to exhibit negligible levels of detectable GUS activity. This data is in agreement with

observations by Fioroni (1989) where it was noted that AoPR1 (DD1) expression decreases with time in culture to a relatively low basal level as determined by 2-D gel analysis. Thus such material may provide an ideal assay system for examination of the ability of certain chemical stimuli to enhance AoPR1 promoter activity. However, as a consequence of the decision to further investigate the developmental regulation of AoPR1 gene expression, it was decided not to pursue this avenue of investigation further but rather to assay stimuli for AoPR1 induction/repression in stable transformants.

4.5.3 Salicylic acid as an inducer of AoPR1 promoter activity.

The ability of salicylic acid to influence expression of genes under the control of the AoPR1 promoter in tobacco is manifest only at concentrations greater than 0.1 mM. This appears to indicate a certain threshold level of salicylic acid which is esential for the perception of this stimulus by the AoPR1 promoter. Below this concentration AoPR1 promoter activity in response to this stimulus alone is negligible. Indeed super-imposing a wound stress upon salicylic acid-stressed material enhances rather than overrides this effect. The dose-dependency of the response to salicylic acid is retained while the wound stimulus further enhances activity to levels greater than those observed in the presence of either stimulus alone. This would possibly imply the operation of either two independent but compatible induction events or of a single induction event arising from two independent stimulation events. By 'induction event' I refer to those protein-DNA interactions directly causing gene expression, while the term 'stimulation event' is used to mean those events by which the primary signal or stimulus is transduced. In the latter case the term 'event' may be seen to refer in many cases to one or more pathways involved in perception and transmission of the initial stimulus.

Investigations into the rate of induction of AoPR1-driven gene expression in response to both the wound stimulus and salicylic acid revealed marked differences in initial induction rates of wounded material in the presence and absence of 4 mM salicylic acid. Unfortunately the effect of salicylic acid alone was not investigated; however, it may be concluded from the

data available that the observed differences in induction rates are attributable to the effect(s) of salicylic acid on gene expression. Given previous data relating to AoPR1 promoter activity in the presence and absence of salicylic acid (section 4.1.4) one may speculate that induction rates in the presence of salicylic acid alone would be comparable with, if not slightly lower than, rates observed in response to wounding. Enhancement of induction rates in the presence of both stimuli may be seen, in all probablilty, to be synergistic, the result of the simultaneous response of the promoter to both stimuli.

4.5.4 AoPR1 promoter activity correlates with sites of phenylpropanoid metabolism but is not influenced by phenylpropanoid pathway intermediates.

The observation that AoPR1-driven *gus* expression was apparently correlated with sites of active phenylpropanoid metabolism led to the speculation that the AoPR1 gene product may, in some way, be involved in the phenylpropanoid pathway, either directly or indirectly. Expression was found to occur at sites of accumulation of products of branch pathways derived from the 'core' steps of the phenylpropanoid pathway. Thus any direct relationship, if it indeed exists, must in all probability lie with those few 'core' steps of the pathway. The ability of intermediates of this pathway to influence AoPR1 promoter activity either by induction or repression was examined in order to investigate the nature of the relationship between AoPR1 promoter activity and phenylpropanoid metabolism. Any alterations in AoPR1 promoter activity resulting from exposure to intermediates of this pathway would therefore indicate whether observed correlations between expression profiles were the result of a causative relationship between the two or simply the product of the simultaneous induction of both AoPR1 expression and phenylpropanoid metabolism. No significant efffect upon AoPR1 promoter activity was detected following exposure to the two phenylpropanoid pathway intermediates, *trans*-cinnamic acid and *p*-coumaric acid. Thus it seems likely that AoPR1 expression is not modulated by the phenylpropanoid pathway itself, but is induced in response to similar, if not the same, stimuli, both developmental and otherwise.

While agreeing with previous data concerning the developmental regulation of AoPR1-driven *gus* expression data presented here provides further insight into visually observed correlations between GUS activity and accumulation of phenylpropanoid pathway products. As expected pollen and seed activities exhibited regulation profiles similar to those already documented by Warner (Warner *et al.*, 1992). Stigma-specific expression, however, failed to show any temporal regulation, being expressed to relatively high levels throughout plant development. A significant correlation ($P<0.05$) was found to occur between levels of detectable GUS activity and extractable anthocyanin (as measured by Abs_{535nm}). No time lag was observed in the relative accumulation of GUS or anthocyanin i.e. the onset of accumulation of both products was simultaneous, thus indicating the absence of any causal relationship between the two. It it therefore probable that both anthocyanin and GUS accumulation occur in response to different stimuli present in a simultaneous manner in petal tissue. While the relationship between lignin accumulation and GUS activity was further investigated by histochemical methods thereby revealing similar developmental expression profiles correlations based on actual lignin content were not investigated. It is feasible to hypothesise, therefore, that stimuli causing induction of the phenylpropanoid pathway may also result in increased activity of the AoPR1 promoter. These results agree with the speculation that AoPR1 may be a component of the generalised plant defence response, where induction of the phenylpropanoid pathway has previously been shown to play a major role (section 1.2). The developmental aspects of AoPR1 expression also agree with data pertaining to expression profiles of a number of other defence-related genes (sction 1.3).

4.5.6 Generation and sequence analysis of ΔAoPR1 promoter constructs.

Having thus compiled a comprehensive analysis of the behaviour of the 'full' AoPR1 promoter in transgenic tobacco it was decided to explore the nature of this behaviour further. A series of 5' promoter deletions was constructed and assayed for altered expression in comparison to the 'full' promoter in order to investigate the sequence-specificity of the characterised AoPR1 expression profile.

Initial attempts to generate a 5' deletion series of the AoPR1 promoter were conducted using the exonuclease III method of Henikoff (1987). However, the failure to achieve successful re-circularisation of exonuclease III digested pSKII$^+$-WIP-*gus* led to the consideration of alternative means of generating a deletion series of the AoPR1 promoter. Among the many advantages of the PCR-based approach subsequently taken is the ability to 'direct' the deletions in relation to specific sequences while simultaneously deleting progressively from the 5' terminus of the promoter i.e. it was possible to generate a deletion series designed such that each deletion removed/retained specific sequence elements. This 'targeting' of deletions, it must be remembered, is constrained somewhat by the relative positions of specific sequence elements within the promoter, and rearrangements of elements relative to each other was not undertaken in this study. Working within restrictions determined in the main by the AoPR1 promoter sequence itself a set of primers was designed such that it was possible to amplify specifically the desired deletion series from the 'full' AoPR1 promoter. Using this technology it was possible to engineer specific restriction enzyme recognition sites onto the termini of amplified fragments to facilitate subsequent cloning and manipulation of deleted promoter fragments.

Although a powerful technique, PCR amplification of DNA sequences has been shown in some cases to introduce base mismatch and other amplification-related errors into amplified sequences. Thus it was considered wise to sequence the products of the AoPR1-based reactions prior to their introduction into plants. Since the objective of undertaking the deletion analysis of the AoPR1 promoter was to investigate those sequence elements responsible for conferring a specific expression profile any introduced changes within that sequence could also, potentially, affect the observed expression profile. Sequence analysis of all constructs except one (pSKII$^+$Δ357) showed no sequence alterations introduced as a consequence of the PCR process. In the case of Δ357 it was decided to continue with the introduction of the amplified sequence into tobacco bearing in mind that all subsequent interpretation of data obtained from such transgenics must be made on the basis of the introduced sequence and not on the predicted amplification product sequence. This deletion,

as previously described (section 4.3.5), essentially represents a deletion to position -416 with the introduction of CC at positions -394 and -395 in place of TT. In other words, amplification of this fragment has resulted in the retention of the overall repeat sequence structures found between positions -416 and -288 bp. These repeats however have been transformed from the original "Rpt.A-Rpt.B"-type (illustrated in fig. 4.19) into two identical sequence repeats by the base pair substitutions introduced. The term Δ357 will thusforth be used in reference, not to sequences downstream of position -357, but to those sequences cloned following amplification of promoter sequence using the Δ357 and CONST. primers. Hence all future data obtained from analysis carried out on plants harbouring this construct must be seen as arising from the presence of a promoter deletion to position -416 and not to position -357.

PCR-amplified deletions were introduced into tobacco as T-DNA inserts derived from the modified binary transformation vector pBI101.1(intron). All regenerants were phenotypically normal. As a direct consequence of the nature of the introduced transgene it was not possible to select transformants on the basis of GUS activity. Rather kanamycin-based selection methods were employed with the assumption being that expression of the introduced *npt*II gene would correlate with the presence of the ΔAoPR1-*gus* transgene. Subsequent experiments were undertaken to ascertain whether or not this, in fact, was the case, and to analyse in detail the effect of each deletion on AoPR1-driven *gus* expression under certain conditions and in response to various developmental stimuli.

4.5.7 The ΔAoPR1-*gus* transgene was present in the progeny of all regenerated lines.

Having ascertained the stable integration of the *npt*II transgene into a transcriptionally active region of tobacco genomic DNA through selection of germinated seedlings on kanamycin-containing medium it was also necessary to demonstrate the presence of the ΔAoPR1-*gus* transgene in a stably integrated form within the tobacco genome. As a direct consequence of the nature of the AoPR1 transgene i.e. a promoter deletion-*gus* construct it was necessary to

demonstrate the presence of this sequence directly rather than *via* measurements of promoter activity as determined by the accumulation of gene product. This was achieved using the technique of PCR, where it was possible to amplify those sequences corresponding to the AoPR1 promoter sequence while also demonstrating the presence of the *gus* coding sequence by the use of the GUS 1 primer. Thus fragments amplified in this manner represent sequences homologous to both the AoPR1 promoter and the *gus* coding sequence, and are thus indicative of the presence of the desired transgene within the tobacco genome. To further characterise thus amplified sequence Southern analysis was carried out, probing blots with labelled promoter sequence. In all cases only those fragments specifically amplifying in putative transgenics were found to hybridise to this probe. Thus, it appears that all plants analysed for the presence of the ΔAoPR1-*gus* transgene were indeed positive. Only in the case of plants containing the Δ815 construct were any rearrangements observed, although, as previously discussed (section 4.5.6) the origin of these rearrangements may possibly be the PCR amplification of transgene sequences itself, and hence these plants were included in further analysis of ΔAoPR1 promoter activity. In the case of Δ357-containing plants two hybridising fragments were observed. These correspond to fragments amplified from two Δ 357 primer sites generated during the initial generation of this deletion (section 4.3.4). It was found, therefore, that selection of regenerants on kanamycin-containing media and germination of resultant progeny on similar selection was correlated with the selection of plants found to contain the desired transgene in a stably integrated form. All such plants were subsequently analysed in detail for AoPR1 promoter activity as measured by GUS activity and a comprehensive picture of the regulation of expression of genes under the control of this promoter in tobacco built up.

4.5.8 A proposed model for the regulation of transcriptional activity of the AoPR1 promoter in transgenic tobacco.

All the data accumulated from the GUS analysis (histochemical and fluorometric) of Δ AoPR1-*gus* transgenics is compiled in tabulated form in fig. 4.40a. This table was then used to compile a detailed picture of the relative positions of regulatory elements within the AoPR1

promoter and this is represented graphically in fig. 4.40b. The 'full' promoter sequence referred to in this thesis and which formed the basis of all experimental design and work, it must be noted, actually comprises a deleted version of the AoPR1 published sequence (Warner *et al.*, 1993). The generation of the construct used initially in this thesis as the source of the 'full' promoter (pJIT60-WIP-GUS) is described fully in Ozcän (1993). Thus the term 'full' promoter referrs to an 880 bp fragment derived from the original promoter sequence by PCR. This 880 bp promoter was found to exhibit the same expression profile as the original sequence and therefore was considered as the 'full' complement of sequence necessary to direct AoPR1 promoter activity in the observed manner.

The regulation of the AoPR1 promoter may be seen to be mediated by a number of sequence elements residing in the delineated regions of the promoter (fig. 4.40c). The presence of a general enhancer element at the extreme 5' end of the promoter was suggested by the consistent observation that removal of the terminal 167 bp resulted in a dramatic reduction in detectable GUS expression levels in most tissues. It appears that this region is also responsible for mediating the wound- and salicylic acid- response of the promoter. Callus-specific expression, on the other hand, appears to reside in that region located between positions -135 and -246, indicating the ability of the promoter to respond to dedifferentiation signals other than the wound stimulus. It has previously been demonstrated, however, that the AoPR1 protein (DD1) does not accumulate to significant levels in response to the presence of either of the hormones auxin or cytokinin in the medium (Fioroni, 1989). Neither has the AoPR1 promoter been shown to respond to elevated levels of sucrose in the medium. Thus the exact nature of the dedifferentiation signal perceived by the promoter remains elusive. It may, however, be the case that it is in fact the wound stimulus that triggers the dedifferentiation response of the promoter. It is possible that those elements responsible for directing wound-induced gene expression do reside within the same region of the promoter as those responsible for perceiving the dedifferentiation signal. The absence of the general enhancer element may result in levels of GUS activity as a consequence of the wound stimulus being undetectable, although those levels observed would indicate no activity

Figure 4.40:

Summary of GUS data obtained following analysis of transgenic tobacco plants containing ΔAoPR1-*gus* constructs.

(a) Tabulated summary of GUS data obtained using both histochemical and fluorometric GUS assay methods.

Key:		
	+:	Positive
	(+):	Expression, but a markedly reduced levels
	-:	Negative.

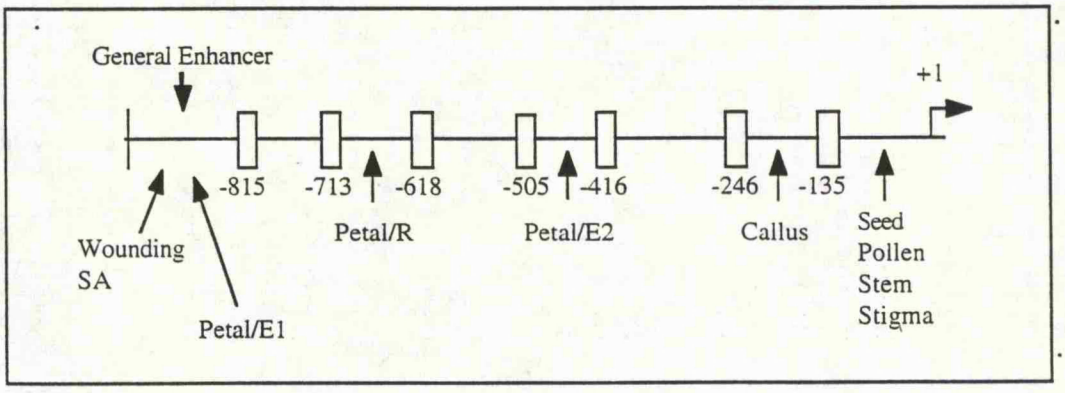
(b) Schematic representation of a model for the possible location of regulatory elements within the 'full' AoPR1 promoter sequence.

(c) Summary of regions of homology between the AoPR1 promoter sequence and that of other defence-related genes.

(A)

Construct	Callus	Wounding	SA	Petal	Pollen	Seed	Stem	Stigma
'full'	+	+	+	+	+	+	+	+
Δ815	+	-	-	-	+	+	(+)	+
Δ713	+	-	-	-	+	+	(+)	(+)
Δ618	+	-	-	+	+	+	(+)	(+)
Δ505	+	-	-	+	+	+	(+)	(+)
Δ357	+	-	-	-	+	+	(+)	(+)
Δ246	(+)	-	-	-	+	+	(+)	(+)
Δ135	-	-	-	-	+	+	(+)	(+)

(B)



(C)

Sequence	Homologies
+1 : -135	H-box (CHS); wun1 box; lat52 box
-135 : -246	no known homologies
-246 : -357	G-box; PcPR1-1 (cF); H-box
-357 : -505	H-box (PAL); PcPR1-1 (iF); G-box
-505 : -618	no known homologies
-618 : -713	PR1-3 box
-713 : -815	PR-1 hd site; H-box (CHS)
'full' : -815	no known homologies

whatsoever in response to wounding. Thus it appears that the sequence elements determining wound- and dedifferentiation- responsiveness are not located within the same region of the AoPR1 promoter and may perceive different stimuli.

Those elements of the promoter sequence responsible for mediating the developmental regulation of the AoPR1 promoter appear to reside within sequences located towards the 3' terminus of the 'full' promoter. Although levels of expression of the *gus* gene were somewhat reduced activity of the GUS enzyme was detected in the presence of as little as 135 bp of the promoter sequence. The Δ135 fragment was found to be sufficient to drive expression in seed, pollen, stem and stigma tissue. Petal expression, however, was found to be regulated by at least three independent sequence elements found between positions -880 to -815, -713 to -618, and -505 to -416. One of these elements, between -713 and -618, appeared to act as a repressor of petal-specific expression, while the other two elements appear to function as enhancers of expression. Histochemical observations of this nature were reflected in fluorometric analysis data.

4.5.9 The presence of two AGC box-like elements may confer enhancer-like properties on the 5' terminal 167 bp of the AoPR1 promoter.

While those regions shown to play a role in determining AoPR1 promoter responsiveness do, in some cases, show homology with elements found to be involved in the regulation of other defense-related promoters (fig. 4.40c) no definitive conclusions may be drawn regarding the effect of these elements within the AoPR1 promoter based on data presented here alone. While it is interesting to speculate on the possible conservation of regulatory function between genes of related function further work is necessary to delineate those sequences within defined regions responsible for determination of promoter specificity and sensitivity to different stimulation events.

Two sequence elements homologous to the AGC-box found in virtually all chitinase and glucanase promoters studied (section 1.3.6) are found immediately upstream of the Δ815 primer site. Removal of these sequence elements by deletion of the AoPR1 promoter to

position -815 has been shown to significantly reduce levels of detectable GUS activity in all tissues, while also apparently abolishing wound- and salicylic acid-inducibility.

5' AGCCGCC 3'	AGC-box (Hart *et al.*, 1993)
5' AGCAGCC 3'	AoPR1 (-821/-815)
5' AGCCTCC 3'	AoPR1 (-818/-812)
5' AGC(C/A)(G/T)CC 3'	Consensus

Interestingly, a 61 bp promoter fragment derived from the basic tobacco glucanase (GLB) promoter containing three AGC-like motifs exhibits enhancer-like activity in tobacco protoplast transient assay studies (Hart *et al.*, 1993). In the case of the AoPR1 promoter, the presence of these elements correlates with the proposed presence of some element(s) capable of functioning as a 'general' enhancer, being responsible for the quantitative determination of levels of gene expresion. While not present in tandem but overlapping substantially, both AGC-like motifs found in the AoPR1 promoter may, it might be envisaged, function in a manner similar to the observed behaviour of such sequences within other defense-related promoters, where these elements have been demonstrated to bind specific leaf nuclear proteins from ethylene-treated material (Hart *et al.*, 1993).

4.5.10 A *trans*-acting factor activating phenylpropanoid biosynthetic genes in flowers recognises sequence motifs with a proposed involvement in the modulation of petal-specific AoPR1 promoter activity.

Recent work by Sablowski and colleagues (Sablowski *et al.*, 1994) has demonstrated the isolation and characterisation of a nuclear protein from petal extracts specifically interacting with H-box-like sequence elements present within the promoters of several genes the products of which are involved in phenylpropanoid metabolism. The sequence recognised by this factor is also found within the AoPR1 promoter sequence, between positions -491 and -484. Deleting the AoPR1 promoter to position -416 removes this sequence, with a concommittent loss of petal-specific promoter activity previously restored following removal of a potential

petal-specific repressor located between positions -713 and -618, thereby implying a possible role for this sequence motif in the mediation of AoPR1 petal-specific expression.

5' AGAACCTAACTT 3'	(Sablowski *et al.*, 1994)
5' TGAACCTAGCCT 3'	AoPR1 (-491/-484)

In transient assays investigating the activity of the above element activity was found to require the presence of a G-box-like element, suggesting a co-operative interaction with other elements and factors. Such an interaction may also occur in the case of the AoPR1 promoter, where a single G-box-like motif (CACTTG) is found located between positions -225 and -220.

4.5.11 Sequences responsible for tissue-specific and developmental expression of AoPR1 show homology to known *cis*-acting elements.

The AoPR1 promoter appears to exhibit a modular organisation. Results from deletion analysis indicate the involvement of sequence element(s) residing within the terminal 167 bp of the promoter in the determination of promoter responsiveness to external environmental stimuli, while those elements responsible for directing tissue-specific and developemtnal expression appear to be located, except in the case of petal-specific expression, within the 3' terminal 135 bp. Petal-specific expression, as previously discussed, appears to be mediated by interactions between at least three elements, two showing apparent enhancer-like activity and one exhibiting a repression effect. The first enhancer (E1) is possibly located within that region of the promoter thought to contain a general enhancer, although the loss of petal-specific expression upon removal of this region may reflect removal of this general enhancer rather than removal of a petal-specific enhancer. Promoter activity in petal tissue is restored following removal of sequences upstream of position -618, with a possible repressor being located between -713 and -618. Between these two positions no significant homology is observed to *cis*-elements previously identified within other promoter sequences and demonstrated to have activity either in functional or gel-shift assays. The second petal-specific enhancer (E2) has been described above (section 4.5.10).

A number of sequence motifs found to exhibit homology to previously reported *cis*-acting regulatory elements are found towards the 3' terminus of the AoPR1 promoter, that region found to mediate developmental, dedifferentiation- and tissue-specific expression. Within the 3' terminal 246 bp of the AoPR1 promoter sequence homology has been found with H- and G-box motifs, elements of importance in the regulation of gene expression in response to light, elicitors and developmental stimuli (section 1.3.6). An H-box-like element (CCTCACC), differing from the H-box consensus sequence (Loake *et al.*, 1992) only in the presence of a central C interrupting the CCTACC core, is found between 225 and 231 bp upstream of the transcription start site, a region implicated in callus-specific expression. Within this region also one observes the presence of a G-box-like motif (CACTTG) (-225/-220), overlapping the H-box element by the 5' C.

Sequences implicated in the determination of tissue-specific and developmental expression i.e. the 135 TATA-proximal base pairs, include an H-box motif (CCTACC; -51/-46) conforming exactly to the H-box core consensus and sequences homologous to regions of the *wun*1 (CACCTACC; -53/-46) and pollen-specific *lat*52 (AAATAA; -74/-69) promoters. In the latter two instances no functional significance has been assigned to the sequence motifs described in the context of their native promoters. The presence of a conserved H-box core sequence, however, is of some interest. Similar sequence motifs have been implicated, not only in environmental induction of gene expression, but also in the regulation of tissue-specific and developmental promoter activity (section 1.3.7), and may, thus, be of potential importance in the regulation of AoPR1 gene expression in a similar menner.

The possibility exists, therefore, that sequence elements conserved in promoter sequences of other defence-related genes and implicated in the regulation of gene expression may also be involved the regulation of AoPR1 gene expression, at least in transgenic tobacco. The mere presence of such sequences and motifs derived therefrom, however, does not necessarily infer any functional significance. Detailed experimental analysis concerned with further delineation of regulatory elements within the AoPR1 promoter and investigation into the ability of such promoter sequence to interact with nuclear proteins under specific conditions is

necessary to allow conclusive assignation of a regulatory role to specific DNA sequence motifs found within the AoPR1 promoter sequence.

Chapter 5:

Conclusions and suggested further research.

The plant defence response (chapter 1), a complex, highly sensitive and coordinated sequence of events invoked by the plant in response to an array of stimulation events, both physical and biological, is of crucial significance in plant development. The ability to withstand and counter environmental and biological stresses is fundamental to the plants potential to survive; any compromise of this response whatsoever will drastically reduce the survival opportunity of the plant. This research programme is concerned with the characterisation of AoPR1 gene expression, a component of the defence response in asparagus.

A cDNA, cWIP, corresponding to the AoPR1 coding sequence encodes a protein of predicted molecular weight 16.9 kDa and neutral pI. No significant homology was found upon comparison of this DNA sequence with sequences in a number of databases. Computer-aided translation of the cDNA sequence and analysis of the predicted amino acid sequence, however, revealed strong homology with members of a protein family described by Walter *et al.* (1990). This protein family was found to represent a previously uncharacterised family of pathogenesis-related proteins, and comprised PR proteins of parsley (PcPR1-1, PcPR1-3: Somssich e*t al.*, 1988; PcPR2: van de Löcht *et al.*, 1990), bean (PvPR1, PvPR1: Walter *et al.*, 1990) birch (*Betv*1: Breiteneder *et al.*, 1989) and potato (Marineau *et al.*, 1987). Subsequent surveys of databases continue to reflect the ever-growing number of proteins showing homology with members of this protein family, a family recently designated PR-10 (Somssich, 1994). At the time of writing this thesis the PR-10 protein family comprises at least eighteen members, having been expanded to include IPR proteins from soybean (SAM22, H4: Crowell *et al.*, 1992), pea (ABR17, ABR18: Barratt and Clark, 1991), rice (CO375A: unpublished sequence) and ginseng (Moiseyev *et al.*, 1994). While, as yet, no common function has been assigned to the PR-10 family that IPR protein isolated from ginseng callus cultures has demonstrated ribonuclease activity (Moiseyev *et al.*, 1994). A

second such protein (ginseng RNase 2) has also been isolated from this material showing, as with the ginseng RNase 1, homology with members of the PR-10 protein family, in particular with the parsley IPR proteins (J. Beintema, pers. comm.). Attempts to assign a similar function to other members of the IPR protein family have met with little success (S. Warner, pers. comm.; I. Somssich, pers. comm.; J. Beintema, pers. comm.), although not conclusively discounting the possibility that some members of this protein family may possess RNase activity.

Members of the PR-10 family have been isolated from a number of different systems, both tissue culture and explant (section 3.3.1), with the AoPR1-encoding cDNA being isolated following differential screening of a λzap cDNA library generated using transcript isolated from mechanically isolated asparagus mesophyll cells over a period of three days post-cell isolation. A second asparagus protein, DD1, had previously been characterised on 2-D gels of *in vitro*-translated protein from message isolated over a period of time subsequent to mechanical cell isolation, and a large amount of data accumulated concerning the behaviour of this protein in a culture system (Fioroni, 1989). Given similarities observed between both the predicted AoPR1 protein and DD1 i.e. their apparent similarity in size and identity of source, further investigation into the characteristics of expression of both was considered appropriate, while exploring the extent of the relationship, if any, existing between the two proteins.

Immunological investigations revealed, in fact, that the cWIP-encoded AoPR1 protein is one member of the DD1 protein family, a family shown to comprise at least five serologically-related proteins (Fioroni, 1989; this thesis section 3.2.5). Members of this protein family were found to be immunologically related to the potato STH-2 protein, a member of the PR-10 family, the first evidence of a serological relationship between members of this PR protein family, and to accumulate in an apparently identical manner in response to both mechanical cell isolation and wounding. One-dimensional electrophoretic and immunological analysis revealed accumulation of gene product following exposure to both salicylic acid and the phytohormone ABA, agents previously documented as exerting modulatory effects over PR

gene expression (section 1.3.3). In contrast to the STH-2 gene product, however, AoPR1 protein does not appear to accumulate following exposure to arachidonic acid; exposure of asparagus seedlings to the related compound methyl jasmonate similarly failed to result in increased levels of AoPR1 protein accumulation.

AoPR1 gene expression was observed to be regulated in response to developmental signals, showing a tissue-specific expression profile, a feature of gene expression proviously reported for a number of PR- and defence-related genes including genes encoding members of the PR-10 protein family (section 1.3.3). Surveying the observed expression profile revealed a striking correlation between the location of sites of AoPR1 protein accumulation and of active phenylpropanoid metabolism, with AoPR1 protein being detected in root and stem tissue, as well as a possible immunoreacting protein being detected in tobacco petal material.

Analysis of AoPR1 promoter activity, conducted using transgenic tobacco plants harbouring the AoPR1 promoter fused to the *gus* coding sequence, confirmed this correlation with sites of phenylpropanoid metabolism. Detectable GUS activity was determined, using both fluorometric and histochemical assay methods, as being present in extracts from pollen, petal collars, stem and stigma tissue (section 4.2.8), and found to be developmentally regulated. These data have recently been published (Warner *et al.*, 1994). Thus speculation arose as to the possible involvement of the AoPR1 gene product in the phenylpropanoid pathway. The potential of intermediates of this pathway, *p*-coumaric acid and *trans*-cinnamic acid, to influence AoPR1 gene expression, as manifest in perturbations of promoter activity, was therefore explored and revealed no apparent causal relationship between AoPR1 gene expression and activity of the phenylpropanoid pathway. It is therefore most likely that any observed correlations are the result of simultaneous induction in response to related, or perhaps the same, stimulation events, and not a consequence of some form of interdependence.

Speculation as to the possible function of the AoPR1 protein, based on observations detailed in this thesis, leads to the conclusion that expression of the AoPR1 gene is intrinsically linked

with expression of the plant defence response, possibly implicating this protein in the manifestation of this response. A number of striking correlations are observed between expression of the AoPR1 gene and of genes encoding proteins known to comprise aspects of the defence response (section 4.1.6), observations which support the involvement of the AoPR1 protein in this response. The question remains, however, 'What is the exact function or role of the AoPR1 protein in the defence response?'. While no precise function may be assigned to the AoPR1 protein on the basis of expression profile alone it is possible to speculate, on this basis, as to the possible role of this protein in plant defence and development. Moiseyev and colleagues (1994) have recently isolated a protein demonstrated to possess ribonuclease activity from ginseng callus cultures. Preliminary amino acid sequence analysis of this protein revealed significant homology with the parsley PR-10 proteins, and led to speculation that members of the PR-10 protein family were, in fact, ribonucleases. Subsequently, parsley and asparagus IPR proteins have been assayed for such activity. Results from these experiments have, however, failed to show any RNase activity associated with the proteins encoded by the respective cDNAs (I. Somssich, pers. comm.; J. Beintema, pers. comm.; S. Warner, pers. comm.). Such data does not, it must be stated, conclusively discount the possibility that members of the PR-10 protein family do possess RNase activity. Further work is necessary to isolate and assay such proteins from the source plant material, since the possibility exists that proteins isolated as recombinant forms from bacterial systems may not display *in vivo* function, some form of *in planta* modification being necessary for activity. Moiseyev *et al.* (1994) suggest, however, that a possible ribonuclease activity of IPR proteins may actually be an intrinsic aspect of a more specialised, as yet undetermined, function.

It has also previously been suggested (section 4.1.6) that the AoPR1 protein may play a role in sub-cellular stabilisation during the dedifferentiation process. This hypothesis may be further extrapolated to encompass an involvement in cellular stabilisation necessitated by the intracellular accumulation of phenolics and other phenylpropanoid pathway derivatives induced by environmental and developmental stimuli. Accumulation of such compounds may

exert a detrimental effect over the maintenance of cellular integrity, an effect possibly counteracted by a simultaneous induction of AoPR1 protein accumulation. Should such a role be assigned to the AoPR1 protein it appears likely that this function is manifest as a component of a cellular stabilisation complex, a set of proteins acting in concert to restore and maintain cellular integrity.

Further work concerned with exploring possible functions of the AoPR1 protein must address the characterisation of the protein itself, either by its isolation and in vitro analysis or through the use of genetic engineering approaches. Selectively knocking out AoPR1 gene expression through the use of antisense technology, and assessment of alterations in all processes known to involve induction of this gene could provide some clues as to AoPR1 function *in planta*. The main obstacle to undertaking this approach, however, is the need to knock out AoPR1 gene expression in its native host plant asparagus, a plant not easily manipulated in this way. Techniques for the transformation and regeneration of asparagus are not sufficiently established to merit undertaking this approach. An alternative to this could be to explore the function of AoPR1 homologues using this approach. The possible function of, for example, the potato IPR protein STH-2 could be investigated in this way, as could that of any tobacco IPR proteins, the existence of which is suggested by the presence of an immunoreacting band on western blots of total cell protein extracted from tobacco petal material (section 3.2.12).

Over-expression of the encoded IPR protein may also assist in the determination of protein function. This approach has previously been addressed by Brisson and colleagues in the analysis of STH-2 function in planta (Constabel *et al.*, 1993). Introduction of the STH-2 coding sequence into potato plants under the control of the CaMV 35S promoter resulted in constitutive expression of the STH-2 protein as determined by immunoassay. Constitutive expression of the STH-2 gene did not, however, reduce the susceptibility of the transgenic potato plants to either *P. infestans* (previously shown to induce STH-2 protein accumulation; Constabel and Brisson, 1992) or to potato virus X when compared with similarily infected non-transgenic plants. All regenerated plants in this study appeared phenotypically normal, and did not differ appreciably from control plants in biochemical events known to be

associated with the defence response. Similar experimental approaches have also been undertaken in the study of the involvement of a number of other defence-related proteins in the determination of disease- (Broglie *et al.*, 1991; Linthorst *et al.*, 1989) and insect-resistance (Neuhaus *et al.*, 1991).

It may be seen, thus, that in order to address the question 'What is the function of the AoPR1 protein?' one must explore avenues of investigation afforded by both molecular and biochemical techniques. While valuable information may be gained from either analysis in isolation a function may be definitively assigned to the protein only after consideration of evidence presented by both approaches.

The question now posed was 'What features of the AoPR1 promoter are concerned with the determination of the observed profile of gene expression?'. Comparison of the AoPR1 promoter sequence with that of other defence- and pathogenesis-related gene promoters revealed the presence of a number of homologous sequence elements, some of which have been implicated or shown to be involved in the regulation of gene expression in response to specific stimuli (section 4.3.1). While inference of function in the basis of homology alone is not feasible the presence of such conserved sequence motifs could indicate a possible conservation of function and a potential role for these elements in the regulation of AoPR1 gene expression. A series of 5' AoPR1 promoter-*gus* constructs were generated and introduced into tobacco, the aim being to investigate any possible correlations that may exist between AoPR1-driven *gus* expression and the presence of potential conserved regulatory elements, while also delineating regulatory motifs the presence of which had not previously been detected by homology.

Results generated from this analysis revealed the AoPR1 promoter to be organised in a modular fashion with regard to the location of regulatory elements. Sequences found to determine developmental regulation of gene expression were found to reside within the 3' terminal 135 bp of the promoter sequence, with the exception of elements involved in the regulation of petal-specific expression. In this case at least three separate domains of the

promoter were found to exert a modulatory effect over petal-specific AoPR1-driven *gus* expression, indicating the presence of at least three regulatory elements. The deletion series employed in this analysis effectively delineated the promoter sequence into eight domains, each one being approximately 100-150 bp in length and being delineated by 5' and 3' Δ primer sites. Thus, each successive deletion results in the removal of successive domains. Any loss or gain of promoter activity observed upon removal of certain domains therefore indicates the presence of at least one regulatory element within that particular domain. Removal of the 5' terminal domain resulted in the abolition of both wound- and SA-induced gene expression, and correlated with a marked decrease in detectable levels of GUS activity in tissues showing developmentally regulated *gus* expression. Thus, the presence of a 'general enhancer' element within that sequence delineated by the 5' terminus of the promoter sequence and the Δ815 primer was proposed. Removal of this domain also abolished petal-specific expression, indicating the possible presence of a petal-specific enhancer although it appears likely that the proposed 'general enhancer' also functions in this capacity. A second element, exerting a repressive effect on AoPR1 promoter activity in pigmented petal collars was detected within the Δ713/Δ618 domain, the removal of which restores activity, in some cases to 'full' promoter levels. The loss of petal-specific expression was again observed upon deletion to position -416 (a deletion generated using the Δ357 primer: section 4.3.5), presumably reflecting the location of a second enhancer-like element within the Δ505/Δ416 domain.

Interestingly, that element responsible for induction of AoPR1 promoter activity in response to dedifferentiation-related stimuli appears to be located at some distance from that apparently involved in the perception of the wound stimulus. Previous observations of AoPR1 expression in response to both stimuli (sections 3.2.7 and 3.3.1) led to the hypothesis that induction of AoPR1 gene expression in response to dedifferentiation-related stimuli arises as a direct consequence of the wound stimulus imposed during mechanical cell isolation. This, in turn, would suggest that an identical series of molecular events occurs as a consequence of exposure to each stimulus, and indicates the potential involvement of a single, or the same complement of, regulatory element(s) within the promoter sequence. Data obtained, however,

does not appear to support this hypothesis. A number of possibilities exist to potentially explain this apparent discrepancy. First, it is possible that a common regulatory mechanism is operating under both conditions. Removal of the general enhancer may not have completely abolished wound-induced gene expression, but resulted in a reduction in promoter activity such that GUS activity, although present, was below the threshold necessary for detection. A second hypothesis suggests that both elements are, indeed, separate. In this case, however, one may speculate that the initial stimulation event necessary for induction of AoPR1 promoter activity is dependent on a wounding event. The persistence of this response, however, as manifest in callus material may be dependent on the presence of other regulatory elements, in this case located within the Δ246/Δ135 domain. Analysis of protein-DNA interactions has revealed the apparent involvement of sequences between -618 and -663 in wound-induced AoPR1 promoter activity (L. Mur, pers. comm.), supporting the first hypothesis i.e. that despite apparent abolition of wound-inducibility upon removal of sequences located between -982 and -815, the actual wound-responsive element(s) reside further downstream, removal of a general enhancer resulting in the reduction of promoter activity in response to the wound stimulus to levels below the detection threshold.

Preliminary delineation of regulatory elements modulating the activity of the AoPR1 promoter in transgenic tobacco indicated the potential involvement of previously described *cis*-acting elements in both quantitative determination and tissue-specificity of expression. Removal of the 5' terminal domain i.e. -982/Δ815, found to function as a potential general enhancer, was found to correlate with the removal of two overlapping sequence motifs homologous to the chitinase and glucanase ACG-box (section 4.5.9). Also, a *myb*-like trans-acting factor involved in the determination of petal-specific PAL expression has been demonstrated to interact specifically with sequences homologous to a motif located in the Δ 505/Δ416 domain, a domain implicated in the regulation of petal-specific AoPR1 promoter activity (section 4.5.10). A number of sequence motifs found to exhibit homology with sequence motifs implicated in the regulation of gene expression are also found located within the 3' terminal 246 bp of the AoPR1 promoter sequence, containing the Δ246/Δ135 domain

and the 3' terminal 135 bp of the promoter (section 4.5.11). It is possible, therefore, that the observed conservation of sequence motifs between the AoPR1 promoter and promoters of other defence-related genes does indeed represent a conservation of similar regulatory function.

In order to address this hypothesis a much more detailed analysis of the AoPR1 promoter is required. The deletion series generated in this analysis delineated potential regulatory elements to within fragments of 100-150 bp in length, and data generated therefrom provides an indication as to the probable location of regulatory elements. Further delineation of such elements may be achieved through the generation and analysis of a series of 3' promoter deletions, allowing, in conjunction with data obtained from the 5' deletion series, the delineation of regulatory elements to within fragments of a much smaller size. The possible presence of regulatory elements within certain promoter domains may be explored by the manipulation of promoter domains relative to each other. For example, the role of the 'full'/Δ 815 domain in determining quantitative AoPR1 promoter activity may be assessed by the fusion of this domain to each other successive 5' deletion, observing any effect on detectable levels of GUS activity. This approach would also facilitate the location of wound- and SA-responsive elements, should they be located downstream of this terminal domain. Each separate domain may also be assayed for regulatory activity through the use of minimal promoter constructs. One further approach currently being employed in the exploration of AoPR1 promoter activity is that of the analysis of protein-DNA interactions. A series of gel shift experiments has been conducted and preliminary results indicate a correlation with results obtained in this thesis. A number of promoter fragments have been found to bind nuclear proteins in extracts of both wounded and unwounded leaf material, presumably reflecting the non-inducible nature of this interaction in response to wounding. Two fragments, -663 to -618 and -606 to -505, exhibit altered electrophoretic mobility following exposure to nuclear extracts from wounded, but not unwounded, material, and to asparagus cell culture-derived extract. Further, each of these fragments may be used to abolish the observed electrophoretic shift of the other, suggesting that the same factor interacts with both

sequences (L. Mur, pers. comm.). A more detailed analysis of this inducible protein-DNA interaction is currently being undertaken at Leicester.

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Appendix

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The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis

Simon A.J. Warner, Aideen Gill and John Draper*
Botany Department, Leicester University, Leicester LE1 7RH, UK

Summary

Previous reports have described the induction, by either wounding or attempted pathogen invasion, of an *Asparagus officinalis* intracellular pathogenesis-related (AoPR1) promoter–GUS gene fusion in transgenic tobacco. Here we describe the unexpected developmental expression pattern of the AoPR1–GUS gene which correlates well, temporally and spatially, with the developmental expression observed for GUS fusions with promoters derived from genes coding for enzymes in the 'core phenylpropanoid pathway'. Analysis of endogenous AoPR1 gene expression in asparagus and both AoPR1–GUS and AoPR1–luciferase gene fusions in transgenic tobacco suggests that the AoPR1 promoter directs similar cell-specific transcription patterns in both asparagus and transgenic tobacco. The AoPR1 promoter contains sequence motifs similar to those implicated as important in the regulation of phenylpropanoid pathway genes and another 'intracellular' PR gene. Treatment with salicylic acid enhances AoPR1 promoter gene activity both in tobacco and in asparagus.

introduction

Plants respond to stimuli applied externally such as wounding or pathogen attack by altering patterns of gene expression (Bowles, 1990; Dixon and Harrison, 1990). Several classes of 'defence-related' genes are known to be regulated developmentally as well as being induced in response to wounding or pathogen attack. Well characterized examples include genes coding for enzymes involved in the phenylpropanoid pathway which catalyse the synthesis of cell wall structural molecules, pigments, UV-light protectants, chemical attractants and anti-microbial phytoalexins (Hahlbrock and Scheel, 1989). Analysis of the promoters of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) genes by fusion with a GUS reporter gene has allowed the histochemical localization of promoter activity in transgenic plants (Bevan *et al.*, 1989; Fritze *et al.*, 1991; Liang *et al.*, 1989; Ohl *et al.*, 1990; Schmid *et al.*, 1990; Shufflebottom *et al.*, 1993). The developmental expression patterns observed for these constructs correlate with the expected locations of phenylpropanoid derivatives synthesized from later points of the pathway, such as in xylem parenchyma cells in lignifing stem tissue (PAL2), or anthocyanin-containing regions (Harborne, 1976; de Vlaming, 1976) of the petal and seed testa (CHS). Other genes that have been implicated in the defence response but which encode gene products of unknown function are also expressed developmentally. For example, the potato *wun 1* gene promoter drives GUS reporter gene expression in mature pollen and in the stomium of anthers in transgenic tobacco (Siebertz *et al.*, 1989). Because the function of these proteins is unknown it has proved difficult to predict any possible developmental role for these defence-related genes.

The AoPR1 gene (Warner *et al.*, 1992) derived from an asparagus wound-induced messenger RNA population (Harikrishna *et al.*, 1991) shares homology with a new class of pathogenesis-related proteins (Walter *et al.*, 1990). Several lines of evidence suggest that this group of PR proteins are intracellular (Somssich *et al.*, 1988; Warner *et al.*, 1992) and thus differ from the 'classical' acidic PR proteins described from tobacco which are generally extracellular (Bol *et al.*, 1990). The AoPR1 promoter was fused to the β-glucuronidase (GUS) reporter gene and used to transform tobacco. Initial studies confirmed that the AoPR1 promoter drives strong GUS expression at wound sites and at sites of pathogen invasion (Warner *et al.*, 1993). Previous studies on other reported 'intracellular' PR (IPR) proteins have also observed induction at sites of wounding as well as pathogen attack (Constabel and Brisson, 1992; Crowell *et al.*, 1992; Matton *et al.*, 1993). The developmental regulation of IPR proteins has yet to be described in detail but reports exist of endogenous intracellular PR protein or transcript expression in developing pea seeds (Barratt and Clark, 1991), soybean roots (Crowell *et al.*, 1992) and birch pollen (Breiteneder *et al.*, 1989). Here we report the first detailed description of the developmentally regulated transcriptional activity of AoPR1 in asparagus and transgenic tobacco.

Received 17 December 1993; revised 10 March 1994; accepted 21 March 1994.
* For correspondence (fax +44 533 522791).

The role of salicylic acid in systemic acquired resistance (SAR) and the induction of PR protein genes such as tobacco PR1a, by exogenously supplied salicylic acid has been the focus of many recent studies (Enyedi *et al.*, 1992; Malamy and Klessig, 1992; Raskin, 1992; Ward *et al.*, 1991; Yalpani and Raskin, 1993). Although there is no similarity at the predicted amino acid level, the mature 'classical' tobacco PR1a protein and the IPR proteins share a similar isoelectric point, have a similar molecular weight and are both induced by pathogen infection. In this study we show that the AoPR1 promoter is inducible by exogenously supplied salicylic acid in asparagus seedlings and in transgenic tobacco leaf disks.

Results

The AoPR1–GUS gene fusion is strongly expressed at sites of active lignification in tobacco stems undergoing secondary thickening and in tobacco roots

AoPR1–GUS tobacco plant stem tissue was examined for reporter gene activity using both fluorometric and histochemical analytical techniques at various times after seed germination. Faint histochemical staining was found localized to the vascular tissue in stem sections taken from seedlings up to 12 weeks old (data not shown). In contrast, intense GUS activity is found in stems taken from mature plants which were undergoing increasing secondary thickening postflowering (Figure 1a). More detailed histochemical analysis (Figure 1c and d) revealed that AoPR1–GUS expression in stems was limited primarily to parenchyma ray cells of the developing secondary xylem. These cells lie radially between the highly lignified tracheary elements and are responsible for the production of the phenylpropanoid precursors required for xylem vessel lignification (Bevan *et al.*, 1989). Autofluorescence of the stem section resulting from the presence of high concentrations of lignin and other cell wall-bound phenolics (Figure 1e) and phloroglucinol staining for lignin (Figure 1a and b) showed clearly that AoPR1–GUS activity sharply mirrors the pattern of stem lignification.

Previous fluorometric analysis of AoPR1–GUS gene expression revealed a low level of expression in roots taken from 6-week-old plants and in seedlings stressed by growth *in vitro* on agar-containing medium, derived from certain transgenic tobacco lines (Firek *et al.*, 1993; Özcan *et al.*, 1993). Histochemical analysis of roots from mature soil grown-tobacco plants revealed that GUS activity was restricted to vascular tissue (Figure 1l), whilst quantitative measurements showed that expression levels were relatively low in comparison with other tissues (see Figure 3a).

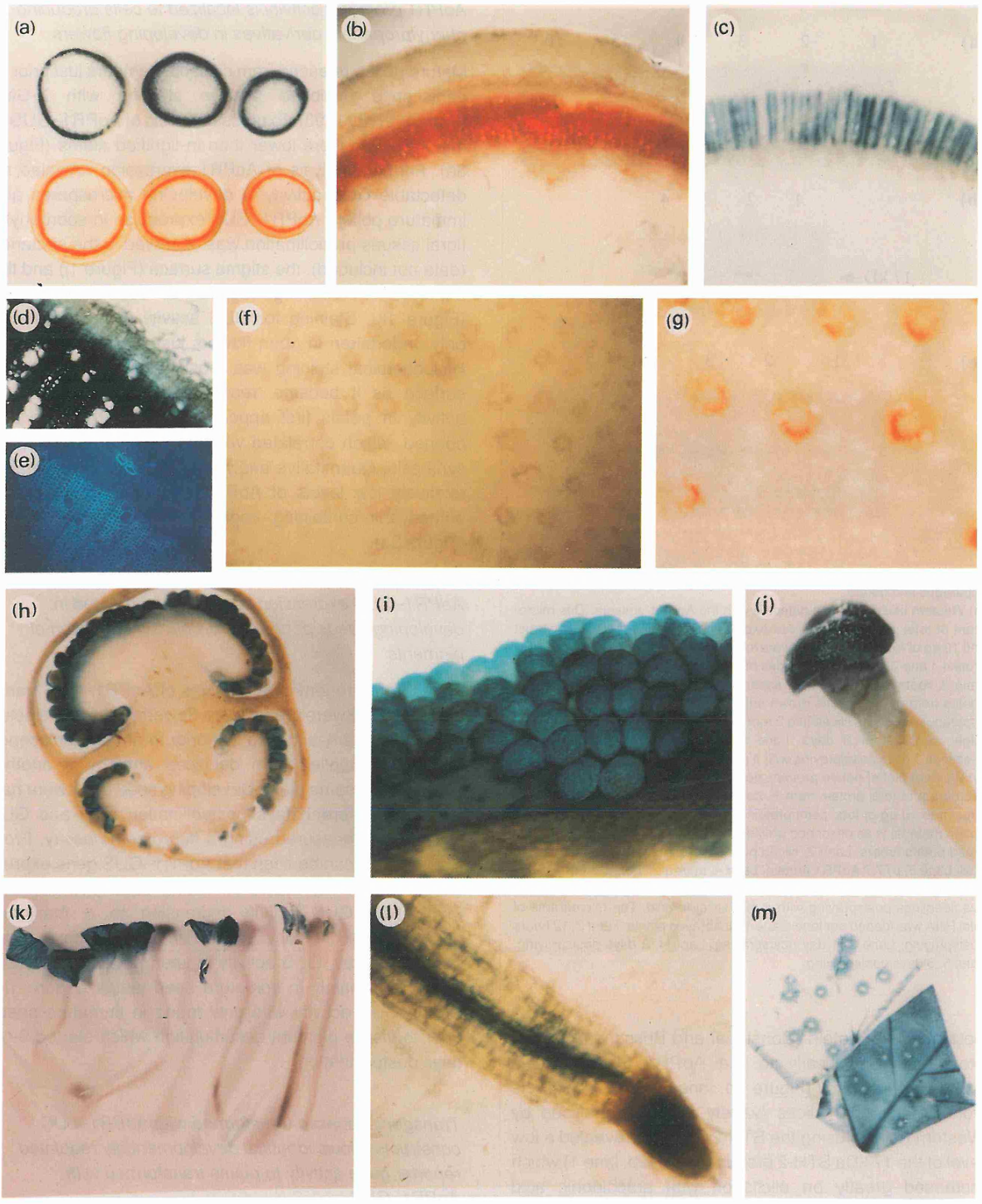
Expression of endogenous AoPR1 protein in asparagus stems is associated with vascular tissue

Endogenous AoPR1 gene expression was studied in asparagus stems using a polyclonal antibody raised to AoPR1 protein that had been expressed in *Escherichia coli*. Protein extracts from 6-week-old, light-grown, unwounded asparagus seedlings, 6-week-old asparagus seedlings cut into 2 mm lengths 3 days postwounding, asparagus roots and mature greenhouse-grown asparagus spears were used for Western blot analysis. As predicted from the experiments in transgenic tobacco, AoPR1 protein was detected by the AoPR1 antisera in wounded asparagus samples and in unwounded stem and root tissue (Figure 2a). Tissue prints performed on mature asparagus spears demonstrated that the AoPR1 antisera cross-reacted with protein associated with the vascular bundles, which again correlated with cells producing lignin as detected by phloroglucinol staining (Figure 1f and g). Pre-immune antisera gave no signal on identically treated tissue prints.

Endogenous intracellular PR proteins are developmentally expressed in potato stems and tubers

We have demonstrated that the antibody raised against the AoPR1 protein will recognise the 17 kD STH-2 intracellular PR protein from potato (Gill *et al.*, manuscript in preparation). Likewise an antibody prepared against the

Figure 1. Expression of AoPR1-GUS in transgenic tobacco and endogenous AoPR1 gene expression in asparagus.
(a) Adjacent stem sections from a flowering mature tobacco plant alternately stained for GUS activity or lignin deposition (X-Gluc staining above and phloroglucinol staining below) (×2).
(b) Stem section from a mature tobacco plant stained with phloroglucinol (×30).
(c) Stem section from a mature tobacco plant stained with X-Gluc for 3 h showing GUS activity in the xylem parenchyma rays (×30).
(d) X-Gluc stained stem section from a tobacco plant (×150).
(e) Identical section to (d) showing UV autofluorence of phenolic compounds.
(f) Tissue print of asparagus spears cross-reacting with the AoPR1 anti-sera around the vascular bundles (×30).
(g) Phloroglucinol staining of asparagus stem showing the location of lignin surrounding the vascular bundles (×60).
(h) Cross-section of a tobacco seed pod 5 days postpollination showing staining of seeds (×7).
(i) Part of a longtitudinal section of a tobacco seed pod 5 days postpollination showing GUS activity in the testa (×50).
(j) GUS activity in a receptive tobacco stigma (×12).
(k) Tobacco petals at various stages from prior to flower opening (right) to fully opened (left).
(l) Root from tobacco seedling showing staining in the vascular tissue (×150).
(m) Wounded tobacco leaf disks placed on filter paper soaked in water (top) and 4 mM salicylic acid (bottom) for 48 h.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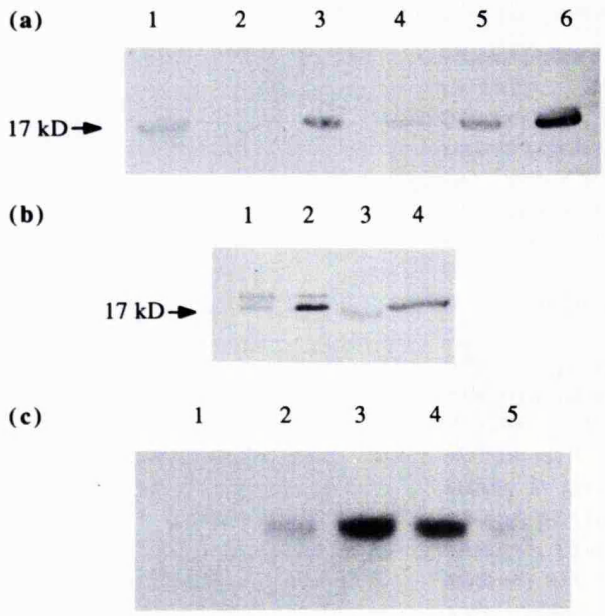

Figure 2. Western and Northern analysis of AoPR1 gene expression in asparagus and potato.
(a) Western blot of proteins detected with the AoPR1 antisera. One microgram of total protein from *E. coli* expressing the pT7.7 AoPR1 construct and 10 μg of total plant protein were loaded per lane. Lane 1, pT7.7 AoPR1 protein. Lane 2, untreated cladodes from 6-week-old asparagus seedlings. Lane 3, roots from 6-week-old asparagus seedlings. Lane 4, asparagus spears from mature plants grown outdoors. Lane 5, etiolated 6-week-old asparagus seedlings sliced into 2 mm lengths and incubated on damp filter paper at 20°C for 3 days. Lane 6, etiolated 6-week-old asparagus seedlings 3 days postspraying with 4 mM salicylic acid.
(b) Western blot of potato proteins detected with the STH-2 antisera. One microgram of total protein from *E. coli* expressing the pT7.7 AoPR1 construct and 10 μg of total plant protein were loaded per lane. Treatment of potato material is as described previously (Marineau *et al.*, 1987). Lane 1, sliced potato tubers. Lane 2, sliced potato tubers treated with arachidonic acid. Lane 3, pT7.7 AoPR1 protein. Lane 4, untreated potato stem.
(c) Northern blot of total RNA extracted from etiolated 6-week-old asparagus seedlings postspraying with 4 mM salicylic acid. Ten micrograms of total RNA was loaded per lane. Lane 1, unsprayed plants. Lane 2, 12 hours postspraying. Lane 3, 1 day postspraying. Lane 4, 3 days postspraying. Lane 5, 5 days postspraying.

potato STH-2 protein (Constabel and Brisson, 1992) will cross-react specifically to the AoPR1 fusion protein expressed in *E. coli* (Figure 2b, lane 3). Protein extracts from potato tuber slices (variety Desiree) analysed by Western blotting using the STH-2 antibody revealed a low level of the 17 kDa STH-2 protein (Figure 2b, lane 1) which increased greatly on elicitation with arachidonic acid (Figure 2b, lane 2). In contrast a second cross-reacting protein of approximately 18 kDa (Constabel and Brisson, 1992) was not inducible by elicitor treatment. Endogenous expression of the STH-2 intracellular PR protein was also detected in mature potato stems (Figure 2b, lane 4).

AoPR1 promoter activity is localized to cells producing phenylpropanoid derivatives in developing flowers

Mature pollen released from dissected anthers just prior to dehiscence exhibited positive staining with X-Gluc (Warner *et al.*, 1993). Expression levels of AoPR1–GUS in mature pollen were lower than in lignified stems (Figure 3a). Further analysis of AoPR1 expression revealed no detectable GUS activity in developing microspores and immature pollen. AoPR1–GUS expression in sporophytic floral tissues prepollination was observed in the nectaries (data not included), the stigma surface (Figure 1j) and the anthocyanin-containing regions of developing petals (Figure 1k). Staining for GUS activity in nectaries was only undertaken in open flowers just prior to pollination. Histochemical staining was most intense at the stigma surface as it became 'receptive' for pollination. GUS activity in petals first appeared in buds that had just opened, which correlated with the onset of anthocyanin synthesis. Quantitative analysis of GUS activity revealed relatively low levels of AoPR1 promoter activity in the anthocyanin-containing regions of developing petals (Figure 3a).

AoPR1–GUS expression is greatly upregulated in developing seeds prior to the visible accumulation of pigments

Plants from progeny of three lines of AoPR1–GUS transgenic tobacco were grown to the flowering stage. Selected flowers were emasculated just prior to anther dehiscence and pollen applied from dehisced anthers of another flower on the same plant. Developing seed pods were harvested at different days postpollination (PP) and GUS activity was measured using a fluorometric assay. From Figure 3(b) it can be seen that AoPR1–GUS gene expression is temporally regulated during seed pod development, with GUS activity increasing to a maximum approximately 3–5 days postpollination. Using a histochemical assay, GUS activity in seed pods was found to be located mainly in immature seed testas (Figure 1h). Strong GUS activity was only found in immature seeds prior to visible pigment accumulation which started 9–10 days postpollination.

Transgenic tobacco transformed with AoPR1–LUC constructs shows identical developmentally regulated reporter gene activity to plants transformed with AoPR1–GUS.

Recently, it has been suggested that artefactual expression in transgenic plants may be associated with the use of GUS as a reporter gene, especially in pollen (Uknes *et al.*, 1993). For this reason we constructed an

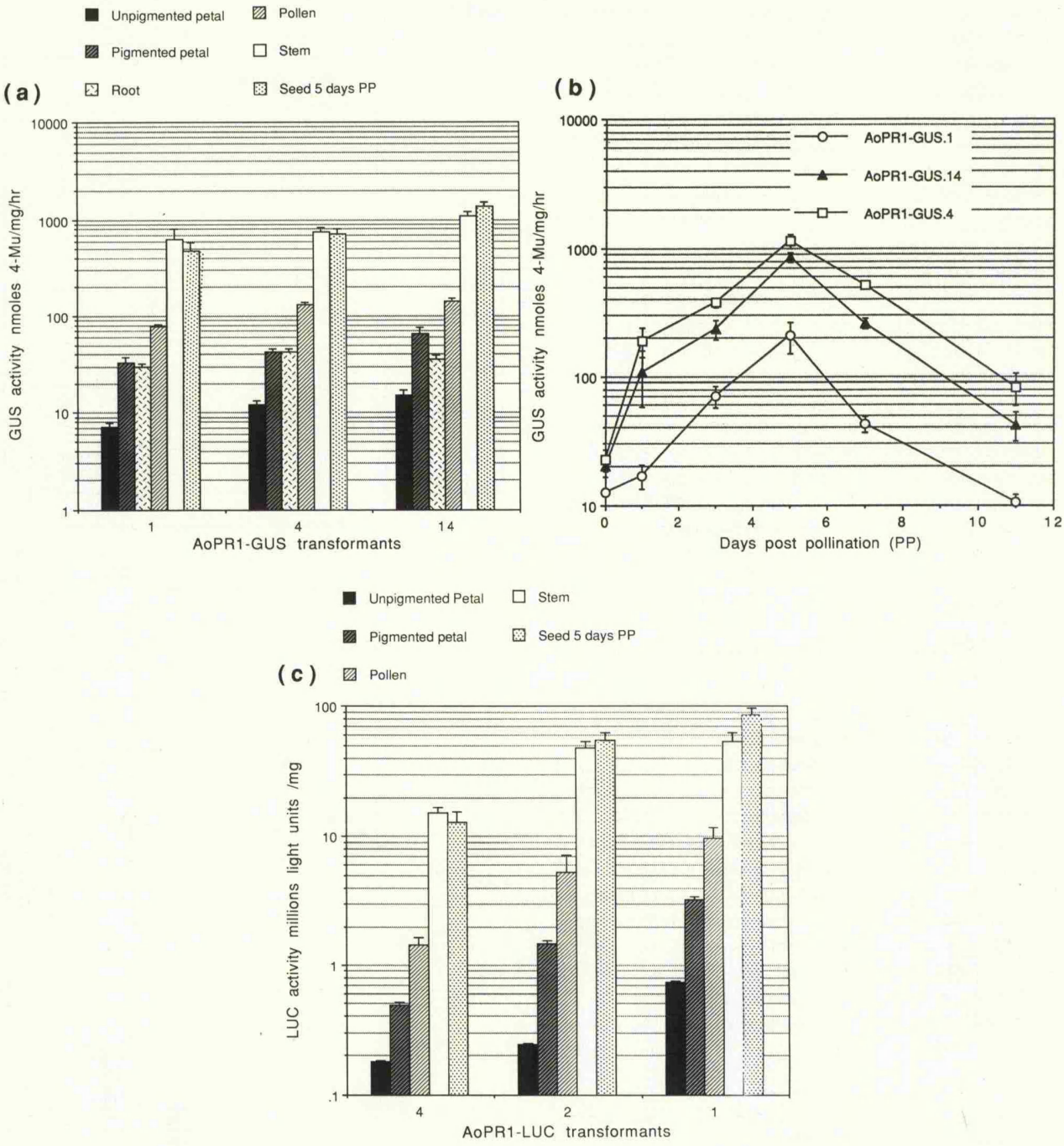

Figure 3. Developmental expression of AoPR1 reporter gene constructs in transgenic tobacco.
(a) AoPR1–GUS activity in three independent plant lines. The tissues analysed are from mature flowering plants. The stem tissue analysed was cut 2 cm from the soil level and seed pods were analysed 5 days post-pollination. Flowers and pollen were analysed when the flower was fully open with dehisced anthers, roots were removed from the soil, and washed prior to analysis.
(b) Temporal regulation of the AoPR1–GUS construct in developing seed pods in three independent lines.
(c) AoPR1–LUC activity in three independent lines. The tissues analysed are as described in (a) except roots were not tested.

AoPR1–luciferase translational fusion differing from the AoPR1–GUS construct only in the nature of the fusion. The AoPR1–LUC fusion was created so that the initiating ATG of the *luc* gene was an integral part of an *Nco*I site used in the fusion. Although firefly luciferase does not offer the same advantages as the GUS reporter gene, because no simple histochemical assay is available for this enzyme, luciferase activity may be simply, sensitively and quickly assayed using a photoluminometer. Standard tobacco transformation techniques were employed to transfer the AoPR1–LUC construct into the tobacco genome and eight independent transformed tobacco lines were obtained. The presence of the transgene was confirmed by PCR (data not shown). Protein extracts were prepared from various organs and tissues and these were assayed for luciferase activity. Figure 3(c) shows that the luciferase activity in the eight independently transformed tobacco lines mirrors the developmentally regulated GUS activity in transgenic tobacco harbouring the AoPR1–GUS constructs (Figure 3a). These data suggest that there is no artefactual expression of the AoPR1–GUS reporter gene in tobacco.

The AoPR1 promoter activity is enhanced by salicylic acid

Salicylic acid inducibility of the classical tobacco PR genes has been well characterized (Antoniw and White, 1986). More recently, the soybean IPR transcript, SAM 22, has been shown to be moderately induced following treatment of detached leaves with 10 mM salicylic acid (Crowell *et al.*, 1992). Initial experiments using tobacco leaf disks harbouring the AoPR1-GUS construct were performed to determine the optimum concentrations of salicylic acid. The leaf disks were placed on filter paper soaked in increasing concentrations of salicylic acid for 48 h at room temperature, harvested and assayed for GUS activity using the procedure described by van de Rhee *et al.* (1990). Figure 4(a) shows that the increase in

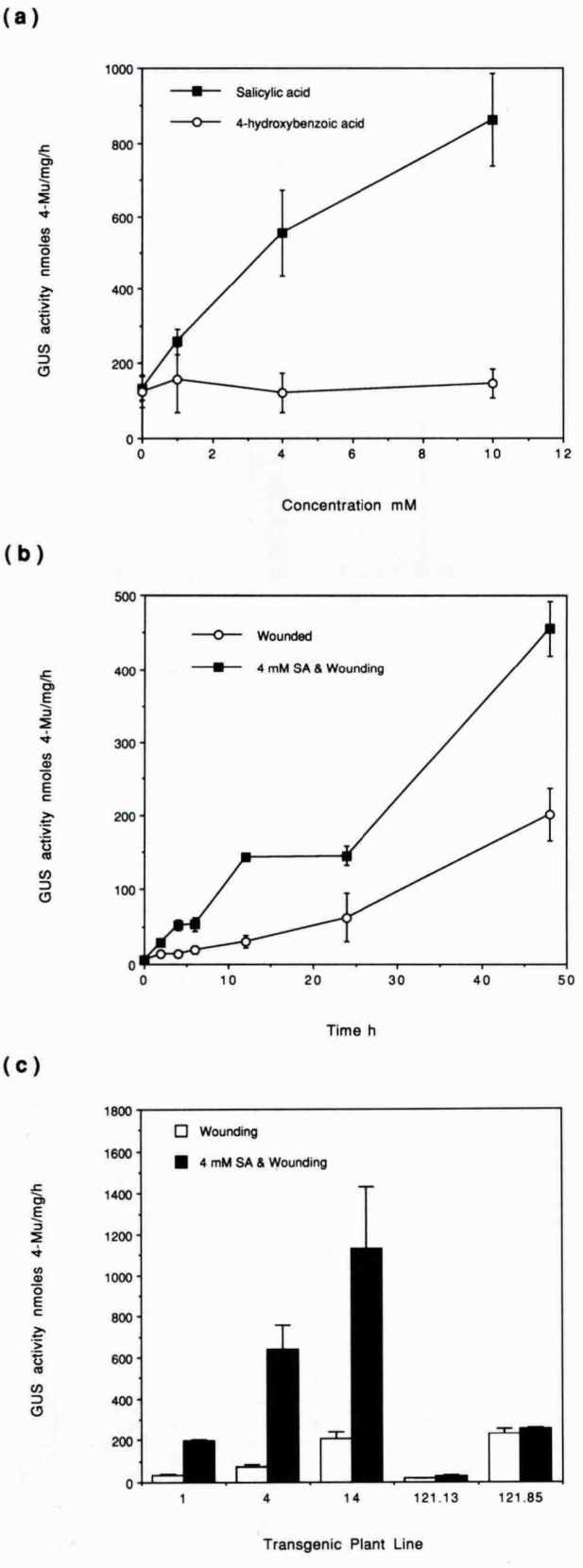

Figure 4. Induction of GUS activity in leaf disks in response to treatment with salicylic and 4-hydroxybenzoic acid.
Error bars represent the standard deviation taken from measurements of a minimum of three independent samples.
(a) Dose dependency of AoPR1–GUS expression following a 48 h time period postspraying with 4 mM salicylic acid or 4-hydroxybenzoic acid.
(b) Time course of GUS activity following wounding alone or following wounding and treatment with 4 mM salicylic acid.
(c) A comparison of GUS activity following wounding alone or following wounding and treatment with 4 mM salicylic acid in three independent transgenic plant lines harbouring the AoPR1–GUS transgene (1, 4 and 14) representing the range of expression levels found for this construct and two plants harbouring the CaMV35–GUS construct (121.13 and 121.85) that exhibit high and low expression levels.

GUS activity is approximately linear up to a salicylic acid concentration of 4 mM. After incubation in 10 mM salicylic acid the leaf disks appeared less healthy and the measured GUS activity ceased to increase linearly with concentration. Treatment of disks with salicylic acid concentrations below 1 mM gave no detectable induction of GUS activity and treatment with 4-hydroxybenzoic acid, a biologically inactive analogue of salicylic acid, had no effect on measurable GUS activity in identically treated leaf disks (Figure 4a). Subsequent experiments were carried out using 4 mM salicylic acid. The induction kinetics of the AoPR1–GUS gene were similar following either wounding or treatment with salicylic acid (Figure 4b). Maximal expression levels varied in independent transgenic lines but in all cases an approximate sixfold induction of the AoPR1–GUS gene was achieved following treatment of leaf explants with salicylic acid (Figure 4c).

Histochemical staining of leaf disks that had been treated with 4mM salicylic acid revealed GUS activity throughout the whole of the explant with stronger staining surrounding the wound sites (Figure 1m). AoPR1–GUS expression in major leaf veins was greater than in surrounding non-wounded tissue in salicylic acid-treated leaf explants (Figure 1m). Control leaf disks, floated on water, revealed characteristic staining only around the wound sites (Figure 1m).

Endogenous AoPR1 protein was undetectable in asparagus cladode tissue using Western blotting (Figure 2a, lane 2). Spraying with salicylic acid induced AoPR1 protein accumulation to levels similar to those achieved following gross wounding of etiolated seedling tissue (Figure 2a, lane 6). The time course of AoPR1 transcript induction (Figure 2c) following spraying with salicylic acid was similar to that achieved after wounding seedlings (Warner *et al.*, 1992, 1993).

The AoPR1 promoter contains elements related to nucleotide sequences known to be important for gene upregulation both in other intracellular PR proteins and in genes coding for enzymes of the phenylpropanoid pathway

PcPR1-1 is a parsley gene coding for a member of the intracellular PR group of proteins which has been shown to be strongly upregulated at sites of fungal attack and following microbial elicitor treatment of established suspension cultured cells (Meier *et al.*, 1991; Somssich *et al.*, 1988). Although there are no significant long stretches of homology between the AoPR1 and PcPR1-1 promoters there is a sequence from –396 to –388 in the AoPR1 promoter that is identical to an inducible footprinted

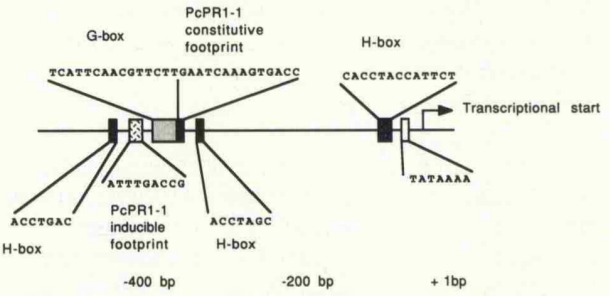

Figure 5. Architecture of sequences that may possibly interact with *trans*-acting factors in the AoPR1 promoter.

sequence of the PcPR1-1 promoter from –244 to –235 which reads ATTTGACCG (Figure 5) . The core sequence TGACCG is within the repeat 2 sequence of the AoPR1 promoter (Warner *et al.*, 1992) but there are no inverted repeat sequences of this motif as in the parsley PcPR1-1 promoter.

Discussion

Previously we have shown that the AoPR1 promoter is strongly and locally induced adjacent to wounded regions or sites of attempted pathogen invasion in leaves of transgenic tobacco plants (Warner *et al.*, 1993). Additionally, as a birch protein related to AoPR1 (*Betv1*) was isolated originally as a pollen allergen, we were not surprised to find AoPR1–GUS expression in mature pollen (Warner *et al.*, 1993). Other members of the intracellular PR protein class exhibit developmental expression in other tissues. For example, the soybean SAM 22 transcript is detectable at high levels in roots, as well as being induced by chemical elicitors such as salicylic acid and methyl viologen (Crowell *et al.*, 1992), whilst the ABR17 and ABR18 proteins from pea are strongly upregulated in developing pea seeds and induced by treatment with exogenous abscisic acid (Barratt and Clark, 1991; unpublished cDNA sequence on GCG Wisconsin DNA database). These findings prompted us to examine the possible developmental expression of the AoPR1–GUS translational fusion in transgenic tobacco plants at various points in the life cycle and in response to exogenously-supplied salicylic acid. In addition, recent evidence of aberrant GUS enzyme expression driven by the tobacco PR1a promoter has been described where ectopic GUS expression in pollen was shown in particular to be artefactual (Beilmann *et al.*, 1992; Uknes *et al.*, 1993). To investigate this possibility we analysed also endogenous AoPR1 expression in asparagus and repeated the promoter analysis in tobacco, this time using an AoPR1 promoter–firefly luciferase reporter construct.

AoPR1–GUS expression is developmentally regulated with expression patterns similar to those documented for other IPR and genes coding for enzymes in the phenylpropanoid pathway

Many genes that are wound-, pathogen-, or elicitor-induced are not only activated following these external stimuli, but are often also subject to developmental control, especially in floral organs. For example, studies on the pathogenesis-related (PR) proteins of tobacco revealed that some are expressed in floral organs (Lotan *et al.*, 1989; Neale *et al.*, 1990), whilst the proteinase inhibitor (*Pin* 2) promoter–GUS gene fusion demonstrated reporter gene activity in developing potato ovules (Peña-Cortés *et al.*, 1991). The reasons for the developmental expression of these defence-related genes are currently unknown. In the case of phenylpropanoid pathway genes, the developmental expression observed for promoter–GUS fusions correlates with cell types that accumulate known phenylpropanoid pathway products. For example, histochemical analysis of the bean PAL-2 and parsley 4CL-1 promoter–GUS fusions in transgenic tobacco (Bevan *et al.*, 1989; Hauffe *et al.*, 1991; Liang *et al.*, 1989; Shufflebottom *et al.*, 1993) revealed developmental expression in the pigmented parts of the petal (anthocyanins and other flavonoids) and developing seed coats (anthocyanins; Harborne, 1976), in mature pollen (4,2',4',6'-tetrahydroxychalcone; de Vlaming and Kho, 1976), in the secondary xylem parenchyma cells of the stem (lignin) and at the stigma surface where volatile phenylpropanoid compounds that attract pollinating insects are produced (Hauffe *et al.*, 1991).

The present data represent the first comprehensive analysis of developmental gene expression driven by an intracellular PR protein gene promoter in transgenic plants. It is clear from the temporal and spatial analysis of the AoPR1 promoter–GUS gene fusion during development in transgenic tobacco and endogenous expression in asparagus that specific expression of the AoPR1 gene occurs in the vast majority of cell types known to accumulate different classes of phenylpropanoid derivatives. These data were somewhat unexpected as it has been shown previously that other promoters of specific genes coding for enzymes involved in the core phenylpropanoid biosynthesis pathway exhibit a much more restricted pattern of developmental expression. For example, the PAL2 gene promoter from bean gives strong expression in tobacco secondary xylem parenchyma but is only very weakly expressed in leaf cells, whilst the PAL1 gene is strongly induced by wounding but weakly expressed in stem tissue (Shufflebottom *et al.*, 1993). Promoters of genes coding for enzymes involved in branches of the phenylpropanoid pathway, such as chalcone synthase (flavonoid synthesis) show very little expression at sites of lignin synthesis and can either be active in all cell types where flavonoids are found (as in the case of snapdragon and *Arabidopsis*, which only have a single CHS gene) or may show differential expression in a range of cell types in different plant species (e.g. bean) which contain several CHS genes (Schmid *et al.*, 1990). Thus, in contrast to these previously analysed promoters the AoPR1–GUS fusion was expressed in the majority of cells producing products of all the main branches of the phenylpropanoid pathway.

Some data on the developmental expression of intracellular PR proteins have been previously presented but these data did not localize IPR protein expression to any particular cell type (Barratt and Clark, 1991; Breiteneder *et al.*, 1989; Crowell *et al.*, 1992). In addition, it has been demonstrated by *in situ* hybridization that the parsley PcPR1-1 transcript is localized to the vascular tissue in the root (Dr Imre Somssich, personal communication) and the bean PvPR1 promoter is active in roots of transgenic tobacco (Dr Michael Walters, personal communication). It was confirmed that the AoPR1 promoter was able to drive GUS and LUC expression in these cell types in transgenic tobacco. However, IPR gene expression in stems and flowers has not been described elsewhere and as such the possibility of unscheduled expression existed. This was examined by testing for endogenous IPR protein expression in asparagus and potato by either Northern or Western analysis. Western blots and tissue prints revealed low levels of endogenous AoPR1 protein in asparagus stem tissues localized to sites of active lignification. The lower amount of AoPR1 expression in asparagus stems as compared with tobacco correlates well with the structural differences between monocot and dicot stems and the corresponding differences in the degree of secondary thickening. Western blot analysis of lignified potato stem protein revealed endogenous STH-2 IPR protein. The IPR protein expression in potato stems or untreated potato tubers has not been reported in previous studies (Constabel and Brisson, 1992).

The observation that the AoPR1 promoter exhibits low expression levels in the pigmented regions of tobacco petals is difficult to correlate with expression patterns in asparagus since asparagus has very small unpigmented flowers. The level of AoPR1–GUS expression in petals correlates well with the activity of the phenylpropanoid pathway in these organs as anthocyanin accumulation only occurs in epidermal cells.

The AoPR1–GUS gene fusion shows by far the strongest developmental expression in the expanding secondary xylem of tobacco stems as they become lignified and in the seed coat, just prior to the appearance of anthocyanins. The PAL2 gene promoter from bean gives strong expression in xylem parenchyma, in an identical manner to that exhibited by AoPR1–GUS fusion

whilst the time course for maximal AoPR1–GUS activity in developing seeds is in approximate agreement with that observed for the snapdragon CHS promoter–GUS fusion in transgenic tobacco (Fritze *et al.*, 1991; Shufflebottom *et al.*, 1993). This temporal and spatial regulation reflects the fact that these latter two genes are being activated in cells required to produce large amounts of lignins and anthocyanins, respectively, and it would perhaps be interesting to speculate why the AoPR1 promoter is strongly activated in both of these cell types, as well as being strongly induced at sites of wounding and attempted pathogen invasion (Warner *et al.*, 1992, 1993).

In conclusion, the AoPR1 promoter is active in transgenic tobacco in all tissues and organs that have been associated with the expression of all the other reported IPR genes and is also expressed in additional cell types such as the pigmented regions of the petal and stems. It is not yet clear whether endogenous IPR genes are developmentally regulated similarly in other species, or whether some gene family members are confined to specific developmental expression in one organ or cell type. Further promoter–reporter analysis in transgenic plants may help answer this question.

The AoPR1 promoter contains sequence elements similar to those identified in promoters of other IPR genes and in genes coding for enzymes of phenylpropanoid biosynthesis

Lignin and flavonoid production represent two separate branches of the phenylpropanoid pathway which diverge after the 'core' part of the pathway which ends at 4-hydroxycinnamoyl CoA. Thus, it is tempting to speculate that AoPR1 either has some function concerned with the core part of the phenylpropanoid pathway, or that perhaps the AoPR1 promoter could be generally activated by phenylpropanoid intermediates, as shown for the CHS15 gene from bean by Loake *et al.* (1991). Several elements in the AoPR1 promoter have been identified previously (Warner *et al.*, 1993) that are similar to the H-box sequences implicated to be important in the regulation of genes coding for enzymes in the early part of the phenylpropanoid pathway (Loake *et al.*, 1991; Lois *et al.*, 1989; Yu *et al.*, 1993). Most of these sequences have been shown to be involved in the induction of transcription by microbial elicitors or UV light stress. However, in the case of the CHS15 gene from bean (Loake *et al.*, 1991; Yu *et al.*, 1993), a further promoter element has been identified which has been shown to increase transcription in response to externally supplied *trans-p*-coumaric acid (4-CA) and the proteins binding these sequences have been biochemically characterized. In preliminary experiments with AoPR1–GUS tobacco seedlings we have not found any evidence for increased GUS activity in UV-stressed plants. Similarly we have no evidence for either up or downregulation of the AoPR1–GUS fusion by externally applied cinnamic or coumaric acid (Gill and Warner, unpublished observations). However, the significance of these latter data is not known as both of these compounds are known to cause non-specific effects on tobacco cell metabolism that are not directly related to gene transcription. It is possible that the elements similar to those described by Lois *et al.* (1989) are involved in AoPR1 induction at wound sites and at sites of pathogen invasion, but further work is required to substantiate any functional link. In this context it should be noted that there are no H-box sequences in the promoter of the pathogen-inducible potato IPR homologue, pSTH2 (Matton *et al.*, 1990), so this sequence motif is not necessarily conserved between all the members of the IPR family.

Although the parsley PR-1 gene promoter sequence ATTTGACCG (shown by *in vivo* DNA footprinting to be protected in elicitor-treated parsley cells) is present in the AoPR1 promoter and a similar sequence is important in the regulation of the potato STH2 promoter (Matton *et al.*, 1993), the functional importance of this element in the AoPR1 promoter is as yet unknown. Other sequence elements that have been shown by other workers to interact with nuclear factors are also present in the AoPR1 promoter. One of these elements is similar to the binding site of the common plant regulatory factors (CPRF) or bZIP proteins which recognize G-box elements (Weisshaar *et al.*, 1991; Williams *et al.*, 1992). These proteins have been shown to be important in the regulation of expression of a wide variety of genes showing tissue specificity or responsiveness to environmental stimuli, such as light, or biologically active compounds such as ABA (Oeda *et al.*, 1991; Schindler *et al.*, 1992; Schmidt *et al.*, 1992). In this context it is interesting to note that preliminary experiments show AoPR1–GUS expression in developing tobacco seed, localized to the embryo (unpublished observations). This developmental expression data correlates well with the expression pattern of the pea IPR protein ABR18 in developing pea embryos (Barratt and Clark, 1991) which is ABA responsive.

The biochemical function of intracellular PR proteins in general is unknown and there have been no other reported detailed promoter–GUS analyses published for other members of this family to corroborate the current findings. The intracellular PR proteins have been described in many species, including both monocots and dicots and are generally transcribed at high levels when activated by elicitors, pathogen attack or (in some cases) wounding. The protein sequences of IPR proteins do not share significant homology with any known phenylpropanoid biosynthesis enzymes or other classes of protein and so they are unlikely to represent a previously unrecognized enzyme of this pathway. The co-induction of

IPR genes and genes of the phenylpropanoid pathway is currently under investigation.

The AoPR1 promoter is responsive to exogenous salicylic acid

Experiments investigating the effects of salicylic acid on transgenic tobacco were carried out using procedures already published (van de Rhee *et al.*, 1990) and demonstrated that the AoPR1 promoter is inducible by salicylic acid in tobacco leaf disks. The levels of induction compare directly with the levels of induction observed for a tobacco 'classical' acidic PR1a promoter reported by van de Rhee *et al.* (1990) and Ohshima *et al.* (1990), but are two to ten-fold lower than those reported by Uknes *et al.* (1993) for essentially the same promoter in experiments where the leaves on an intact plant were painted with 50 mM salicylic acid. In our hands, when greenhouse-grown tobacco plants were sprayed with concentrations greater than 20–30 mM of salicylic acid the leaves wilted within 6–12 h post-treatment rendering further analysis impossible. A possible explanation of why we observed wilting at these levels may relate to the fact that we were using SR1 tobacco, whereas Uknes *et al.* (1993) used Xanthi-nc. In preliminary experiments we found little induction of GUS activity after spraying 4 mM salicylic acid on intact AoPR1–GUS tobacco plants but, in contrast, using PR1a antisera in a Western analysis on protein extracts we confirmed that the endogenous 'classic' secreted PR1a protein accumulated to high levels following this treatment (data not shown). These preliminary observations suggest that classical PR proteins and intracellular PR proteins may be responding differently to the presence of exogenously supplied salicylic acid. Northern analyses confirmed that the AoPR1 transcript was weakly induced following spraying asparagus seedlings with salicylic acid, with message levels at their most abundant 2–3 days post-treatment. It has recently been confirmed that salicylic acid is a phenylpropanoid metabolite, synthesized directly from cinnamic acid via benzoic acid (Yalpani *et al.*, 1993) which again emphasizes the correlation between phenylpropanoid metabolism and AoPR1 expression. In summary, although the AoPR1 promoter is inducible by salicylic acid in tobacco leaf explants and in asparagus seedlings, the levels of induction remain low and are not very high compared with AoPR1 induction by wounding, or in comparison with expression levels observed in lignified stems or developing seeds.

Experimental procedures

Plant growth conditions and treatments

T2 seed was obtained from self-fertilized transgenic SR1 tobacco plants harbouring AoPR1–GUS constructs (Warner *et al.*, 1993). The seeds were germinated on MS0 media (Draper *et al.*, 1989) supplemented with 50 mg ml^{-1} kanamycin and then transferred to compost and grown in greenhouse conditions to maturity. T1 SR1 tobacco plants were obtained for AoPR1–LUC constructs by standard transformation techniques (Draper *et al.*, 1988) and plants were grown to maturity in the greenhouse. Wounding assays were performed on leaf disks as described previously (Warner *et al.*, 1993). Asparagus (*Asparagus officinalis* cv. Conovers Colossal) seeds were germinated in vermiculite and grown in the green house for 6 weeks prior to treatment with 4 mM salicylic acid (Sigma). Mature 3-year-old or greater asparagus plants were grown outdoors in soil beds and spears cut as required. Potato (*Solanum tuberosum* cv. Desirée) plants were grown in the greenhouse to maturity. Tubers were treated according to Marineau *et al.* (1987) and other plant material used for protein extraction with no special prior treatment.

Purification of AoPR1 protein from E. coli *and antibody production*

The AoPR1 cDNA was cloned into the *Eco*RI site of the pT7-7 vector (Tabor and Richardson, 1985). The pT7-7 vector contains the T7 phage RNA polymerase promoter and the translational start site of the gene 10 protein. The pT7-7:AoPR1 construct was then transformed into competent *E. coli* K38 cells that were already harbouring the heat-inducible pGP1-2 plasmid which expresses T7 RNA polymerase. Cultures were grown overnight in LB media at 30°C diluted 1:50 in fresh LB and grown at 30°C to an $OD_{600\ nm}$ 0.8–1.0. The culture was then transferred to a 42°C shaker for 30 min followed by a further incubation of 30 min at 30°C. Inclusion bodies were isolated by the differential centrifugation method as described by Marston (1987) and the inclusion body protein denatured and refolded according to Owen *et al.* (1992). The pT7-7: AoPR1 inclusion body protein products were separated by preparative SDS–polyacrylamide gel electrophoresis and the 17 kDa AoPR1 protein product was excised. The AoPR1 protein was electroeluted from the gel slice, neutralized, dialysed against TBS and used to raise polyclonal antisera in rabbits as described by Harlow and Lane (1988).

Western and Northern analysis of AoPR1 protein and transcript

Protein was extracted from relevantly treated asparagus or potato tissues in protein extraction buffer as described by Constabel and Brisson (1992) and Westerns were carried out using the 1:60 00 dilution of the AoPR1 antisera as described by Harlow and Lane (1988). Tissue prints were developed as for Westerns following pressing of freshly sliced asparagus spears directly on to nitrocellulose. Northern analysis was carried out on asparagus RNA using the AoPR1 cDNA as a probe as described previously (Warner *et al.*, 1992).

Construction of AoPR1–luciferase reporter gene and plant transformation

The pBluescript based plasmid pNBL52 was a gift from Neil Bate and David Twell of Leicester University (manuscript in preparation) and was used as the basis for the construction of the AoPR1 promoter luciferase fusions. The pNBL52 plasmid is engineered to create facile translational fusions between test promoters and the firefly luciferase gene where the initiating ATG codon of the

luciferase gene is encoded in an *Nco*I site. Promoter fragments are ideally cloned into this vector as *Sal*I and *Nco*I fragments. An oligonucleotide primer (5'- GAAGCCATGGTTCCTCTCTGTTGT-TATG-3') was designed to insert an *Nco*I site into the AoPR1 promoter such the *Nco*I site's ATG was in exactly the same position as the AoPR1 gene's natural initiating ATG. This primer was used in conjunction with the T7 sequencing primer in a PCR reaction with 100 ng of the AoPR1 promoter IPCR product cloned into the *Eco*RI and *Sma*I sites of pBluescript for 10 cycles using conditions described previously (Warner *et al.*, 1993). The PCR product was digested with *Nco*I and *Sal*I and cloned into the *Nco*I and *Sal*I sites of pRTL2 creating a translational fusion between AoPR1 and luciferase. The pBI101.1-based plasmid AoPR1–GUS pBI101.1 (Warner *et al.* 1993) was digested with *Sst*I and *Sma*I to remove the GUS reporter gene, the cohesive ends were then end-filled using Klenow in the presence of 2 mM dNTPs, the plasmid was recircularized using T4 DNA ligase, then transformed into *E. coli* and amplified. This plasmid was digested with *Bam*HI and *Sal*I releasing the AoPR1 promoter, gel purified and ligated to a *Bam*HI /*Sal*I AoPR1–LUC fragment so that a pBI101-based plasmid containing the AoPR1–LUC gene fusion was created. This was used to transform LBA4404 *Agrobacterium* and sub-sequently transferred into tobacco by standard techniques (Draper *et al.*, 1988)

Luciferase analysis

Plant tissue was ground up in 0.5 ml of luciferase extraction buffer (100 mM phosphate buffer pH 7.5 and 1 mM dithiothreitol), the debris was spun down for 5 min and the supernatant removed to a fresh tube. Protein extract (50 µl) (adjusted to 0.5 mg ml^{-1} protein) was added to the bioluminescence photometer reaction tube and the tube placed into the photometer (Berthold Clinilumat from Berthold Instruments UK Ltd). ATP buffer (100 µl) (50 mM HEPES pH 7.8, 20 mM $MgCl_2$ and 10 mM ATP) and 100 µl of 0.05 mM D-luciferin (Sigma) made up in luciferase extraction buffer was then added by the machine and the light emission measured as light units. Luciferase activity was expressed as light units per mg of protein. Protein quantitation was carried out essentially as in Bradford (1976).

GUS analysis

Transformed tobacco plant lines 1, 4 and 14, harbouring the AoPR1–GUS construct, were chosen that represented low, medium and high levels of wound-induced GUS expression as determined from initial fluorometric assays. A minimum of three independent samples were assayed for each tissue sample. Fluorometric and histochemical analysis of GUS activity (Jefferson *et al.*, 1987) were carried out as described according to the modifications of Topping *et al.* (1991). Pollen was stained after removal from anthers and washing twice in staining buffer. Petals and stigmas were dissected prior to staining. Developing seeds were stained *in situ* following the bisection of the seed pod to allow entry of the X-Gluc.

Acknowledgements

We would like to thank Ray White and John Antoniw for the gift of the PR1a antisera, Peter Constabel and Normand Brisson for the gift of the STH-2 antisera and Graham Benskin for growing and maintaining transgenic plants. S.A.J.W. is supported by AFRC grant no. PG91/525 and A.G. is supported by a Gatsby Charitable Foundation studentship.

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