Salicylic Acid in Plant Defense Responses

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Doctor of Philosophy
at the University of Leicester

by

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Publications

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L.A.J. Mur, Y.M.Bi, R.M.Darby, S.Firek & J.Draper Early, local accumulation of salicylic acid is essential for development of necrotic lesions which contain and destroy invading plant pathogens. Manuscript in preparation

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R.M.Darby, S.Firek, L.A.J.Mur, Y.M.Bi & J.Draper (1994) Characterisation of a PR-5 gene from Asparagus officinalis. Journal of Experimental Botany 45, 12

D.Martin, S.Firek, M.Roberts, F.Guerineau, Y.M.Bi, R.Scott & J.Draper (1994) Gametophytic transposition of the maize Ds element in heterologous plant species. Journal of Experimental Botany 45, 8

POSTERS

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J.Draper, Y.M.Bi, R.Darby, S.Doig, S.Firek, L.Mur & S.Warner (1994) Local signalling and gene expression at the wound surface and in cells adjacent to sites of attempted pathogen invasion. Abstracts of 4th International Congress of Plant Molecular Biology, Amsterdam

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Abbreviations

(w/v)	weight ; volume ratio
bp	base pair
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylenediaminetetraacetic acid (disodium salt)
g	gram
GUS	ß-glucuronidase, encoded by the *uidA* gene from *E. coli*
gus	*uidA* coding sequence
h	hour
kb	kilobase pair
l	litre
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
SDS	sodium dodecyl sulphate

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Abstract

The importance of salicylic acid (SA) in both local resistance to pathogens and the subsequent establishment of systemic acquired resistance has been investigated in tobacco. In order to assess the role of SA in the plant defense response, it was decided to try to stop the accumulation of SA by expression of an enzyme able to degrade it. A gene encoding salicylate hydroxylase (*SH-L*) was cloned from *Pseudomonas putida* and shown to be functional in *E. coli*. This gene was fused to various plant promoters which should allow temporal and spatial alteration of salicylate accumulation in pathogen-challenged tobacco. The promoters chosen included the CaMV35S promoter which allowed constitutive expression of SH-L and thus total inhibition of SA accumulation. An AoPR1 promoter was used to inhibit the early accumulation of SA around developing lesions. The tobacco acidic PR1a gene promoter was used to drive SH-L expression in response to endogenous SA accumulation both locally and systemically following localised pathogen attack.

Two pathogen systems, one viral, tobacco mosaic virus (TMV) and one bacterial, *Pseudomonas syringae*, were used to analyse how their interactions with tobacco were altered in various SH-L backgrounds by examining lesion phenotypes, defence gene expression and SA levels. It was found that local PR protein (at least PR1a) induction is dependent on salicylate-mediated signalling and that SA is absolutely required in the development of HR to limit virus spread and kill bacteria. It was also confirmed that SA is required for the establishment of systemic acquired resistance (SAR).

A recent hypothesis on the mechanism of action of SA is that SA may function in plants by inhibiting catalase thus allowing the accumulation of hydrogen peroxide (H_2O_2) which can then act as a second messenger to switch on defence gene expression and activate SAR. The theory was tested using transgenic plants unable to accumulate salicylate. It was concluded that SA does not function downstream of hydrogen peroxide in the induction of PR proteins.

Chapter 1
General Introduction

1.1 Plant defence responses

Plants defend themselves from pathogen infection through a wide variety of mechanisms that can be either constitutive or inducible. The inducible defence responses may occur locally or systemically (Dixon, 1986; Keen, 1990; Ryals *et al.*, 1994).

Before attempting to explain plant defense responses, it is important to make some terms clear.

1.1.1 Terminology

Most of the terms explained here are taken from reviews by Collinge and Slusarenko (1987), Sigee (1993) and Dixon *et al.* (1994).

A **pathogen** is an organism or virus able to cause disease in a specific host or range of hosts. **Resistance** is the ability of the host to suppress or retard the activity of a pathogen and can take many forms. **Virulence** describes two concepts. One is the degree of **pathogenicity**, a definition of the term which refers to the severity of the disease caused by the pathogen. The other has more specific meaning in relation to host range. When two physiological variants of a pathogen (eg. *Pseudomonas syringae* pv. *tabaci* and *Pseudomonas syringae* pv. *syringae*) cause different reactions in the same host cultivar (eg. tobacco), one variant (*Pseudomonas syringae* pv. *tabaci* causing disease on tabacco) is said to be **virulent** on the cultivar whereas the other (*Pseudomonas syringae* pv. *syringae* not causing disease on tobacco) is described as **avirulent.** Where a strain or race of a pathogen that is normally virulent on a particular host plant gives an incompatible response with a specific cultivar (eg. *Pseudomonas syringae* pv. *phaseolicola* race 1 on *Phaseolus*

vulgaris cv. *Red Mexican*), it is referred to as **race-specific resistance** and the plant as a **resistant host**. When a pathogen of a particular host plant species comes into contact with a different species on which it does not normally cause disease, the type of resistance expressed is called **non-host resistance** (eg. *Pseudomonas syringae* pv. *tabaci* on bean). Although there is a distinction between race-specific and non-race-specific resistance in experimental terms, these two types of reaction elicited from the plant are regarded as being fundamentally similar.

The interaction between a virulent race and a susceptible cultivar can be described as **compatible** (eg. *Pseudomonas syringae* pv. *tabaci* and tobacco)whereas the interaction between an avirulent race and the resistant cultivar is described as **incompatible** (eg. *Pseudomonas syringae* pv. *syringae* with tobacco). In a compatible interaction, the pathogen replicates and frequently spreads throughout the plant, often causing considerable damage and even death of the host. In contrast, in an incompatible interaction, the pathogen is restricted to a small zone around the infection sites and unable to spread to the rest of the plants.

Genetic analysis of incompatible interactions between pathogens and resistant host plant cultivars has shown that some of them have a "**gene-for-gene**" interaction. That is, resistance in host cultivars to distant physiological races of a pathogen is determined by pairs of corresponding genes in the host and pathogen (Ellingboe, 1981; Briggs and Johal, 1994). In such "gene-for-gene" interactions, a resistance gene (*R* gene) in a particular host cultivar confers resistance against physiological races that express the matching avirulence gene (*avr* gene).

Some *avr* genes have already been identified such as *avrBs3* found in many *Xanthomonas* pathovars (Bonas *et al.*, 1989; Bonas *et al.*, 1993), *avr9, avr4* that elicit resistance on *Cf-9* tomato and *Cf-4* tomato respectively (van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994;) and *avrD* required for resistance mediated by the soybean *Rpg4* gene (Keen *et al.*, 1991;

Yucel *et al.*, 1994). Among many *avr* genes isolated, some of the products could interact with a plant recognition factor directly such as *avr9*, or act indirectly, such as the *avrD* product which does not interact directly with the plant but enzymically converts a normal cellular constituent to produce a secreted elicitor which is recognised by the non-host (Lindsay *et al.*, 1993).

Some *R* genes have also been isolated (reviewed recently by Dangl, 1995), such as the maize *Hm1* (resistance to fungus *Cochliobolus carbonum*, Johal *et al.*, 1992), tomato *Pto* (resistance to *avrPto*-bearing strains of *Pseudomonas syringae* pv *tomato*, Martin *et al.*, 1993), tobacco *N* (resistance to tobacco mosaic virus (TMV), Whitham *et al.*, 1994), *Arabidopsis RPS2* (resistance to *avrRpt2*-bearing strains of *P. syringae* pv *tomato*, Bent *et al.*, 1994; Mindrinos *et al.*, 1994), tomato *Cf-9* (resistance to the fungus *Cladosporium fulvum* carrying *avr9*, Jones *et al.*, 1994) and flax L^6 (resistance to the leaf rust fungus races, Ellis *et al.*, 1995). Sequence analysis has shown that the products of the *R* genes share some similarities, such as the proteins encoded by tomato *Pto* , tobacco *N* , *Arabidopsis RPS2* , and tomato *Cf-9* all contain leucine-rich repeats (LRRs), which are thought to play a role in mediating protein-protein interactions (Kobe *et al.*, 1993). These similarities suggest that many resistance genes will share similar structural elements and, perhaps, related mechanisms of pathogen recognition and signal transduction. With strategies like transposon tagging or map-based cloning and also novel cloning approaches being devised (Chasan, 1994), a number of other resistance genes should soon be cloned and the mechanisms of these genes should be more clearly elucidated in the near future.

The initial recognition event mediated by *R* and *avr* gene products is generally believed to elicit various defense responses in the host locally at the point of infection which ultimately lead to resistance. These responses often include the programmed cell death believed to restrict pathogen spread (the hypersensitive response), cell wall strengthening as a result of lignification and cross-linking of cell wall proteins and the production of antimicrobial compounds, all of which will be explained in detail in the next section.

1.1.2 Induced local defence responses

Once the avirulent pathogen penetrates its host and is recognized by the resistance mechanism, plants can undergo a wide variety of defense responses locally.

1.1.2.1 Hypersensitive response

The hypersensitive reaction (HR) is one of the most important defense mechanisms in plants and can be described as a rapid localised necrosis associated with limitation of pathogen spread. It occurs only in incompatible combinations of host plants and potential pathogens. After infection, infected cells in resistant varieties rapidly become necrotic, while no rapid cell death occurs in infected cells of susceptible varieties (Keen, 1990). This rapid cell death has been shown to be closely associated with an oxidative burst, which is one of the most striking early defense responses (Mehdy, 1994). Proteins, such as lipoxygenase (LOX) involved in lipid peroxidation, were induced quickly after inoculation and further peroxidation continued due to subsequent liberation of active oxygen species (Sutherland, 1991), which caused general damage to cell constituents (Slusarenko *et al.*, 1989). Levels of superoxide dismutase (SOD) and peroxidase (POX) are believed to rise afterwards as a specific response to the increase in active oxygen radicals and may be important in limiting HR necrosis to a localised region of the tissue (Slusarenko *et al.*, 1989).

Hypersensitive cell death appears to require a period of host protein synthesis before it can occur (Keen *et al.*, 1981). In other words, the dead cells only indicate that HR has occurred. What is important is that the cells are dying in a co-ordinated way, which is associated with a characteristic set of changes in gene expression in the infected and surrounding cells (Daniels *et al.*, 1987). Recent studies have found that H_2O_2 from the oxidative burst plays a central role in the orchestration of this defense response (Levine *et*

al., 1994; Tenhaken *et al.*, 1995). H_2O_2 is believed to be the substrate driving the cross-linking of cell wall structural proteins to slow microbial ingress prior to the development of transcription-dependent defenses and to trap pathogens in cells destined to undergo hypersensitive cell death (Brisson *et al.*, 1994). It also serves as a diffusible signal for the induction in adjacent cells of genes encoding cellular protectants such as glutathione S-transferase and glutathione peroxidase (Levine *et al.*, 1994) which presumably help to stop spread of oxidative damage.

The mechanism of this HR response is still unclear at present, and research in this field continues intensively. Other changes associated with HR are discussed in section 1.1.2.2 and section 1.1.2.3.

1.1.2.2 Phenylpropanoid pathway induction

The phenylpropanoid pathway has long been known to have a close relationship with plant defense (Legrand,. 1983). Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropanoid pathway, which leads to the production of the antimicrobial substances (phytoalexins) and the cell-wall-strengthening materials such as lignin and suberin. It was found that PAL mRNA in intact leaves of French bean (*Phaseolus vulgaris*) undergoing a HR to *Pseudomonas syringae* pv. *phaseolicola* increased 6 h after inoculation and peaked around 12 h while necrosis occurred in this system from 21 h to 25 h after inoculation (Slusarenko and Longland, 1986).

Also, it was reported that mRNA level of PAL, 4-coumarate:CoA ligase (4CL) and chalcone synthesis (CHS) (4CL and CHS are other two important enzymes in phenylpropanoid pathway) transiently increased before the accumulation of glyceollins 1,2 and 3, the most important phytoalexins in soya bean for fungal plant pathogens (Schinelzer *et al.*, 1984).

1.1.2.3 The role of induced synthesis of defense-related proteins

Pathogen attack appears to induce alterations in protein synthesis in the plant that can lead to the development of various degrees of resistance at and around infection sites (for review see Bowles, 1990).

Some proteins, such as hydroxyproline-rich glycoproteins (HRGPs), glycine-rich proteins (GRPs) and enzymes involved in the lignin, callose synthesis, directly affect the defense status of the plant by strengthening, repairing, or altering the wall environment (Wilson and Fry, 1986).

Another big family of proteins correlated with HR are pathogenesis-related (PR) proteins. PR proteins were first described by Van Loon and Van Kammen and independently by Gianinazzi and co-workers in leaves of Samsun NN and Xanthi-nc tobacco plants, respectively, in the 1970s (Van Loon *et al.*, 1970; Gianinazzi *et al.*, 1970) and they were characterized by their low molecular weight, solubility at low pH, resistance to proteolytic degradation and TMV inducibility. Subsequently, basic counterparts of these acidic proteins were isolated. These proteins have been divided into five or more unrelated families based on their electrophoretic and immunological properties. Two of these encode the hydrolytic ß-1,3-glucanases (PR-2) and chitinases (PR-3), which can attack microbial cell walls (Dixon 1986), while the functions of the other families are poorly understood. PR protein classification, biochemistry and function have been extensively reviewed by Bowles (1990); Bol *et al.* (1990); Dixon and Harrison (1990); Linthorst (1991); White and Antoniw (1991); Cutt and Klessig (1992) and Stintzi *et al.* (1993).

PR gene(s) induction in tobacco is not confined to TMV. Their accumulation has been correlated with resistance to a large variety of viral, bacterial and fungal pathogens (Ward *et al.*, 1991; Carr *et al.*, 1989). Further, transgenic tobacco plants overexpressing PR1

(unknown function)(Alexander *et al.*, 1993) or PR3 (chitinase)(Broglie *et al.*, 1991) were demonstrated to have increased resistance to pathogens. As a result, the expression of PR genes is often used as a marker for induction of disease resistance.

1.1.3 Systemic acquired resistance

Apart from local defense response, there are also defense-related changes in systemic non-infected leaves.

1.1.3.1 Concept of SAR

While plants are undergoing local defense responses, a long distance signal is generated and a long-lasting, broad-spectrum, systemic resistance to subsequent infections can be induced. This enhanced level of resistance can be manifested throughout the plant and is generally termed systemic acquired resistance (SAR) (Chester, 1933; Ross, 1961a; Ross, 1961b; Ryals *et al.*, 1994).

1.1.3.2 SAR induced by pathogen infection

The phenomenon of SAR has been known for more than 90 years. The first systematic study of SAR was published in 1961, when Ross used TMV on a local lesion host (Xanthi-nc tobacco) and found that subsequent infections, even with pathogens to which the host was susceptible, were reduced in severity (Ross, 1961a; Ross, 1961b). In the past 30 years, SAR has been demonstrated in many plant species and the spectrum of resistance has been broadened to include not only viruses, but also bacteria and fungi (Staub and Kuc, 1980; Kuc, 1982; Cameron *et al.*, 1994; Uknes *et al.*, 1993b).

1.1.3.3 SAR induced by chemical application

Several chemicals have been reported to induce resistance to pathogens when applied to plants , such as aspirin, salicylic acid (White, 1979) and 2,6-dichloroisonicotinic acid (INA) (Kessman *et al.*, 1994). They were found to be able to induce resistance to the same spectrum of pathogens as biological inducers of SAR. A further breakthrough came with the demonstration that the same chemicals could induce the expression of PR proteins.

1.1.3.4 Signals for SAR

The diversity of the defense responses induced by pathogen attack suggests that they may be controlled by multiple signals acting through several pathways. A lot of work has focused on a search for the signals during the past 15 years.

Some molecules can induce the systemic expression of some defense-related proteins and this makes them the candidates of signal molecule for SAR. These molecules include systemin, an 18 aa polypeptide isolated from the leaves of tomato plants (McGurl *et al,* 1992), Jasmonic acid (JA) and its methyl ester, methyl-jasmonate (Me-JA), ethylene and salicylic acid (SA) (reviewed by Enyedi *et al.*, 1992a).

The expression of the systemin gene can be induced quickly by wounding and consequently results in systemic accumulation of systemin. Application of exogenous systemin can induce proteinase inhibitors (Pearce *et al.*, 1991), which by inhibiting gut proteases of grazing insects protect the plant against excessive leaf-loss. Systemin is capable of systemic movement through the phloem (Pearce *et al.*, 1991). However, it is still not known if systemin expression is induced following the HR or if the response is limited solely to wounding. Further, it is not known if the increase in systemin throughout

the plant is solely the result of systemin export from the wounded leaf either. Also, in any case systemin does not induce PR proteins when applied externally.

JA and Me-JA are found in many plant species (Vick and Zimmerman, 1984) and to have various physiological effects (Staswick, 1992). When one tomato leaf was treated with JA or Me-JA, defence-related proteinase inhibitor accumulated both in treated and untreated leaf. JA might also move in the phloem (Anderson, 1985). This makes JA another candidate signal molecule for SAR. However, JA does not induce either PR proteins nor SAR when applied exogenously.

Ethylene is a gaseous plant hormone that regulates many physiological processes in plants (Mattoo and Suttle, 1991). Ethylene increases when plants are inoculated with pathogens (Boller, 1991) and ethylene can induce several PR proteins as well (Bol *et al.*, 1990). Ethylene readily diffuses from infection sites. However, ethylene treatment does not trigger SAR and it has been demonstrated recently that SAR signal transduction in *Arabidopsis* is ethylene independent (Lawton *et al.*, 1994).

If a molecule can induce the systemic expression of some defense-related proteins, it does not mean that it can also induce SAR. In fact, SA is the only compound shown to stimulate resistance to viral, bacterial, and fungal pathogens at a physiologically relevant concentration. Details concerning SA signalling are discussed in the next section.

1.2 SA biochemistry and plant defense responses

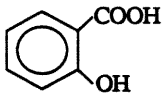
1.2.1 History of salicylates

For more than 2,000 years, people have been using the leaves and bark of the willow tree to cure fevers and to relieve pain. In 1828, Johann Buchner, working in Munich, successfully isolated a tiny amount of salicin - a salicyl alcohol glucoside (Fig.1.1.c), which is the major salicylate in willow bark. The name salicylic acid (SA, Fig.1.1.a), derived from the Latin *Salix*, a willow tree, was given to the active ingredient by Raffaele Piria in 1838. The first commercial production of synthetic SA began in Germany in 1874. Aspirin, a trade name for acetylsalicylic acid (Fig.1.1.b), was introduced by the Bayer Company in 1898 and rapidly became one of the world's best-selling drugs. Aspirin was as effective as SA and caused much less irritation of the human digestive system. It is known that aspirin undergoes spontaneous hydrolysis to SA in aqueous solutions (Mitchell *et al.*, 1967). Americans consume over 16,000 tons of aspirin tablets annually at a cost of about $ 2 billion a year (Weissmann, 1991).

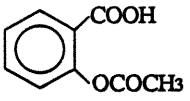
1.2.2 General properties of SA

SA is a natural plant phenolic. It had already been found that many phenolic compounds play an essential role in the regulation of plant growth, development, and interaction with other organisms (Harborne, 1980). Free SA is a crystalline powder that melts at 157-159^{0}C. It is moderately soluble in water and very soluble in polar organic solvents. The pH of a saturated aqueous solution of SA is 2.4. SA fluoresces at 412 nm when excited at 310 nm, and this property is used to detect this compound by HPLC analysis (Raskin *et al.*, 1989; Yalpani *et al.*, 1991).

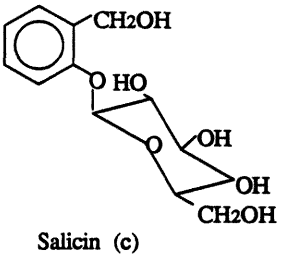
Figure 1.1 Structures of SA and related compounds



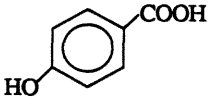
Salicylic acid (a)
(2-hydroxybenzoic acid)



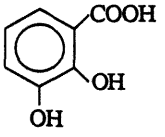
Aspirin (b)



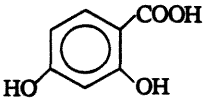
Salicin (c)



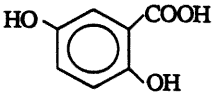
4-hydroxybenzoic acid (d)



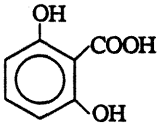
2,3-dihydroxybenzoic acid (e)



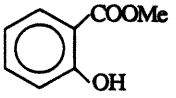
2,4-dihydroxybenzoic acid (f)



2,5-dihydroxybenzoic acid (g)



2,6-dihydroxybenzoic acid (h)



methyl salicylate (i)

1.2.3 Flowering, heat production and SA

SA has long been connected with plant flowering. Cleland and coworkers (Cleland *et al.*, 1974a, Cleland *et al.*, 1974b) found that honeydew from aphids feeding on *Xanthium strumarium* contained an activity that induced flowering in duckweed (*Lemna gibba*) grown under a non-photoinductive light cycle. The flower-inducing factor could be extracted directly from the *Xanthium* phloem and was identified as SA. However, the possibility that SA is an endogenous signal for flowering remains in question since SA did not induce flowering when applied exogenously to *Xanthium*. Moreover, the endogenous levels of SA were the same in the phloem of vegetative and flowering *Xanthium*, and other substances were as effective as SA in flower induction (Raskin *et al.*, 1992).

SA has also been associated with thermogenesis (heat production) in certain plants (Raskin *et al.*, 1987; Raskin *et al.*, 1989). The spadix of the voodoo lily is thermogenic and exhibits dramatic increases in temperature during flowering which releases volatile chemicals that attract pollinating insects. There are two periods of temperature increases in the spadix; a large, transient rise in endogenous SA levels was found to precede both periods. Furthermore, thermogenesis and the production of aromatic compounds associated with thermogenesis could be induced by treatment of spadix explants with SA (Fig.1.1.a), aspirin (Fig.1.1.b), or 2,6-dihydroxybenzoic acid (Fig.1.1.h) but not with 31 structurally similar compounds such as 4-hydroxybenzoic acid (Fig.1.1.d), 2,3-dihydroxybenzoic acid (Fig.1.1.e), 2,4-dihydroxybenzoic acid (Fig.1.1.f) or 2,5-dihydroxybenzoic acid (Fig.1.1.g) (Raskin *et al.*, 1989).

1.2.4 Biosynthesis and metabolism of SA

1.2.4.1 Biosynthesis and metabolism of SA in microbes

Various micro-organisms produce SA (Haslam, 1974; Weiss *et al.*, 1980) via chorismic acid - an important intermediate of the shikimic acid pathway (Fig.1.2), which was demonstrated in *Mycobacterium smegmatis* (Marshall *et al.*, 1972). SA is also an intermediate in the degradation of naphthalene which is carried out by certain *Pseudomonas* strains (Fig.1.3). Genes encoding enzymes in this pathway are grouped into linked plasmid-borne (*nah*) operons. Following its production, SA is then further metabolised by *nah* encoded enzymes (Cane and Williams, 1986). The reaction is shown in Figure 1.3.

Siderophores, produced by bacterial and fungal phytopathogens, are thought to be involved in phytopathogenesis (for review see Loper and Buyer, 1991). SA is known to be able to form very strong complexes with Fe^{3+} (Dawson *et al.*, 1986) and could possibly play a role in siderophore-mediated iron competition.

1.2.4.2 Biosynthesis and metabolism of SA in plants

The biosynthesis and metabolism of SA is recently reviewed by Lee *et al.* (1995).

In plants, it has been proposed that SA is synthesized from phenylalanine (Chadha *et al.*, 1974), which is converted to *trans*-cinnamic acid (CA) by phenylalanine ammonia lyase (PAL). The conversion of cinnamic acid to SA is likely to proceed via the pathway outlined in Figure 1.4.

Figure 1.2 Final steps in the microbial biosynthesis of salicylic acid

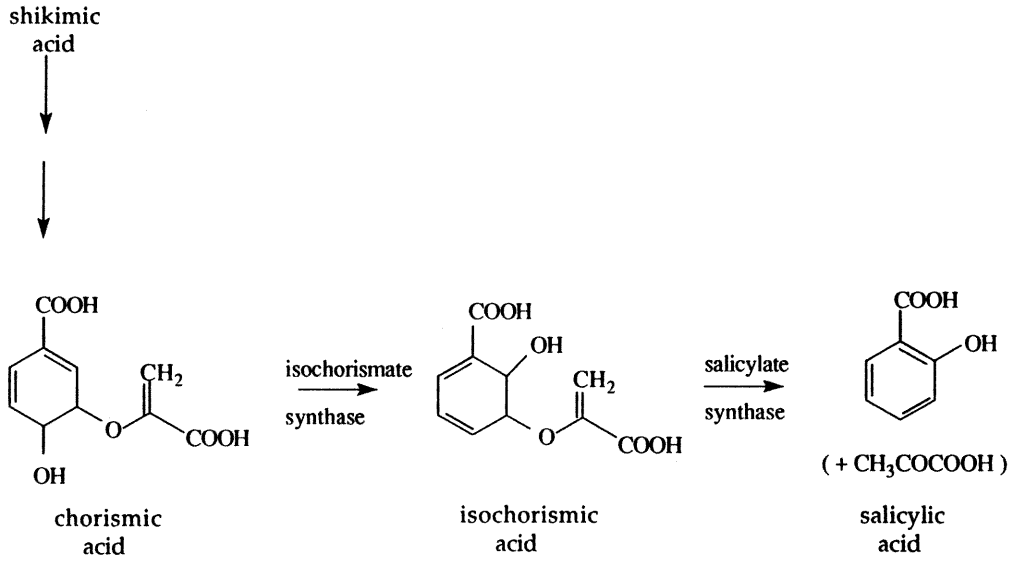

shikimic
acid
COOH
CH2
O
COOH
OH
chorismic
acid
isochorismate
synthase
COOH
OH
CH2
O
COOH
isochorismic
acid
salicylate
synthase
COOH
OH
(+ CH3COCOOH)
salicylic
acid

Figure 1.3 Function of *nahG*

Biochemical degradation of naphthalene by *P. putida* strain NCIB9816 occurs in 13 steps encoded by the bacterial *nah* genes (Cane and Williams, 1986).
The letters A to M represent the enzymes encoded by the genes *nahA* to *nahM*. The *nahG* gene encodes salicylate hydroxylase which degrades SA to catechol.

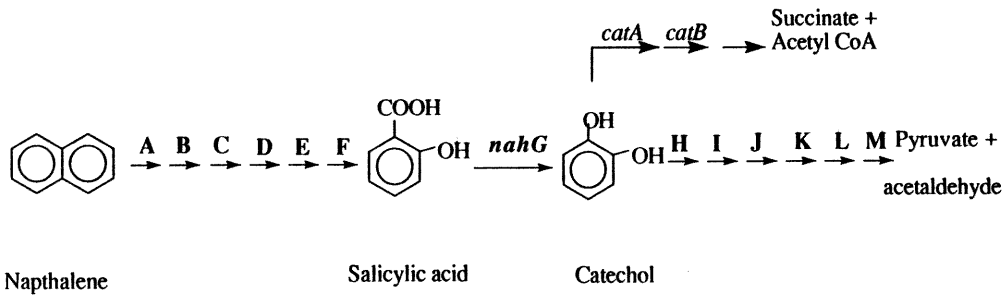
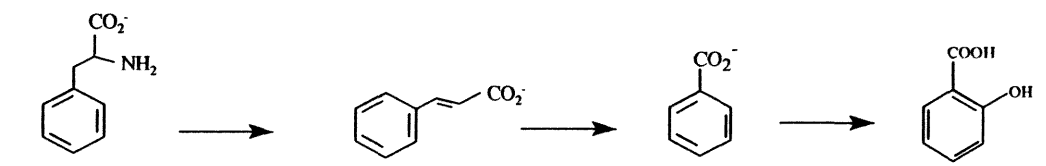

catA
catB
Succinate +
Acetyl CoA
COOH
OH
OH
A B C D E F
nahG
OH
OH
H I J K L M Pyruvate +
acetaldehyde
Napthalene
Salicylic acid
Catechol

Figure 1.4 Possible pathway of SA biosynthesis in plants


CO2-
NH2
CO2-
CO2-
COOH
OH
phenylalanine
cinnamate
benzoate
salicylic acid

Labelling studies by Raskin and coworkers (Yalpani *et al.*, 1993) indicated that in TMV-infected tobacco, SA is predominantly synthesized from benzoic acid (BA). The enzymatic activity responsible for converting BA to SA, BA 2-hydroxylase, was induced four- to five-fold by TMV infection (Leon *et al.*, 1993). BA treatment of tobacco plants also increased BA 2-hydroxylase activity, suggesting this gene was substrate-induced. This latter result, together with the magnitude and timing of BA increases in TMV-infected plants, suggested that an increase in the BA pool is the primary cause of increased BA 2-hydroxylase activity. Thus, the rate-limiting step in SA production may be the formation of BA from *trans*-cinnamic acid or from a conjugated form of BA. However, questions still remain such as why feeding tobacco plants with CA does not lead to the synthesis of SA?

Tobacco leaves metabolize SA rapidly. Several reports have demonstrated that the SA produced after TMV infection of tobacco is rapidly conjugated to glucose to form SA ß-glucoside (SAG) (Enyedi *et al.*, 1992b; Malamy *et al.*, 1992a). SAG does not seem to be an active form in the resistance response (Enyedi *et al.*, 1992b; Chen *et al.*, 1993a). Metabolic inactivation of SA may result from additional hydroxylation of the aromatic ring and the subsequent glucoside (Chadha *et al.*, 1974).

1.2.5 SA appears to be a signal for plant defence responses

The first hint that SA might be involved in plant defense was provided by White (White, 1979) who found that injection of aspirin or SA into tobacco leaves enhanced resistance to subsequent infection by tobacco mosaic virus (TMV). This treatment also induced PR protein accumulation (Antoniw *et al.*, 1980). The specificity of induction of PR proteins is restricted to SA, 2,6-dihydroxybenzoic acid and aspirin, while other related chemicals (such as 4-hydroxybenzoic acid) have no biological activity.

Since SA treatment of tobacco induces several of the same responses as TMV infection such as acquired resistance and PR gene expression, it was postulated that SA acts by

mimicking an endogenous phenolic signal that triggers PR gene expression (such as PR1a) and resistance (van Loon *et al.*, 1983). However, by monitoring endogenous levels of SA in TMV-infected tobacco, Malamy and co-workers (Malamy *et al.*, 1990) provided evidence that strongly suggested a role for SA itself as a signal molecule. Infection of a TMV-resistant cultivar resulted in a dramatic increase (20-50-fold) in levels of endogenous SA in the TMV-inoculated leaves and a substantial rise (5-10-fold) in uninoculated leaves of the same plant. These increases were not seen in a susceptible cultivar. Since the rise in endogenous SA paralleled or preceded the induction of PR-1 gene expression in both inoculated and uninoculated leaves of a TMV-infected resistant plant (Malamy *et al.*, 1990), SA appears to play a role in the pathway leading to resistance responses.

Parallel studies in cucumber indicated that SA signalling is not unique to tobacco. A dramatic rise in SA levels (10-100-fold) was detected in the phloem exudates from cucumber leaves inoculated with tobacco necrosis virus, *C. lagenarium* (Metraux *et al.*, 1990) or *Pseudomonas syringae* pv. *syringae* (Rasmussen *et al.*, 1991; Smith *et al.*, 1991). These SA increases in the phloem preceded both the appearance of SAR and the induction of a specific peroxidase enzyme (the SAR PR protein marker in this experiment).

Ward *et al.* (1991) provided more evidence for the involvement of SA in disease resistance in tobacco. The expression of thirteen families of genes encoding peroxidase, acidic PR-1 through to PR-5 protein and their basic counterparts as well as several previously uncharacterized proteins were investigated. Expression of all thirteen genes was induced in TMV-inoculated leaves of resistant tobacco, while nine showed enhanced expression in uninoculated leaves of TMV-infected plants. These same nine genes, which included the acidic PR-1 through PR-5, basic PR-1, basic and acid class III chitinase, and PR-Q' (a ß-1,3-glucanase) gene, were induced by SA treatment. Thus, SA induced the same spectrum of genes activated during development of SAR upon TMV infection.

Further support for a signalling role of SA was provided by temperature shift experiments in the tobacco-TMV system. When TMV-resistant cultivars (Xanthi nc and Samsun NN) are inoculated and maintained at elevated temperatures (>28^{0}C), they fail to synthesize PR proteins and the infection becomes systemic (Gianinazzi *et al.*, 1970). However, when these infected plants were then transferred to lower temperature (22-25^{0}C), PR gene expression was induced and resistance (HR) was restored. It was shown that temperature increase which blocked the ability of tobacco to resist viral infection also inhibited increases in SA levels (Malamy *et al.*, 1992a; Yalpani *et al.*, 1991). When the resistance response was restored by shifting plants to lower temperatures, endogenous SA levels increased dramatically and preceded both PR-1 gene expression and necrotic lesion formation associated with resistance (Malamy *et al.*, 1992a).

Increases in SA levels recently have also been documented in *Arabidopsis thaliana* after infection with turnip crinkle virus (Uknes *et al.*, 1993b) or *P. syringae* (Summermatter *et al.*, 1995) and in tobacco infected by tobacco necrosis virus, *P. syringae* and *Peronospora tabacina* (Silverman *et al.*, 1993) and *Erwinia carotovora* (Palva *et al.*, 1994). PR proteins can be induced by SA in a wide range of both dicotyledonous and monocotyledonous plants including tomato (White *et al.*, 1987), potato (White, 1983), cucumber (Metraux *et al.*, 1989), rice (Matsuta *et al.*, 1991; Simmons *et al.*, 1992) and *Arabidopsis thaliana* (Uknes *et al.*, 1992). SA also induced acquired resistance against many other necrotizing or systemic viral, bacterial and fungal pathogens in a variety of plants (Malamy *et al.*, 1992b).

Taken together, these studies provide very strong support for the involvement of SA in disease resistance.

1.2.6 SA - the translocated signal for SAR?

The experiments described above establish that SA plays a critical role in resistance and the development of SAR. However, it is unclear whether SA is the primary signal that travels from the inoculation site to systemic non-infected tissues. Initial experiments suggested that SA might fulfill this function. First, the rise in SA levels preceded PR gene induction in uninoculated leaves of TMV-infected resistant tobacco (Malamy *et al.*, 1990). Secondly, the large increase in SA in phloem exudates from infected cucumber leaves preceded the development of SAR and induction of peroxidase activity in uninoculated leaves (Metraux *et al.*, 1990; Rasmussen *et al.*, 1991; Smith *et al.*, 1991). Finally, the appearance of chitinase (PR-3) in *P. lachrymans*-infected cucumber was preceded by an increase in SA levels in the upper, uninoculated leaves as well as in inoculated leaves (Meuwly *et al.*, 1995). These observations, together with the report that SA was found in the phloem sap of TMV-infected tobacco (Yalpani *et al.*, 1991), suggested that SA might be a primary mobile signal.

In contrast, another experiment by Hammerschmidt and co-workers (Rasmussen *et al.*, 1991; Smith *et al.*, 1991) in the cucumber-*P. syringae* system argued that SA is not the translocated signal for SAR. When only one leaf on a cucumber plant was inoculated with *P. syringae*, increases in SA levels, peroxidase gene expression (resistance marker gene in this system) and resistance were detected in the uninoculated leaves even if the inoculated leaf was removed as early as 4-6 h after infection. In contrast, SA was not detected in the phloem sap from the inoculated leaf until 8 h after infection. The result suggested a model in which local infection leads to the production of an unidentified mobile factor or signal, which in turn requires SA synthesis in distal tissue for the establishment of SAR.

1.2.7 Mechanism of action of SA

A model for the mechanism of action of SA was suggested recently by Klessig and co-workers. They identified and characterized a soluble SA-binding protein (SABP) from tobacco (Chen *et al.*, 1991; Chen *et al.*, 1993a). Sequence analysis of the purified protein and a cDNA clone encoding the 57 KDa subunit indicated that SABP is highly homologous to catalase (Chen *et al.*, 1993b). In *in vitro* experiments high levels of SA was found to reduce SABP's catalase activity while related benzoic acid such as 4-hydroxybenzoic acid will not induce PR protein induction and were not found to inhibit catalase. They then suggested that SA acts by blocking catalase activity, which results in elevated H_2O_2 levels, H_2O_2 then, acting as a second messenger, activates defense-related genes on the pathway to disease resistance.

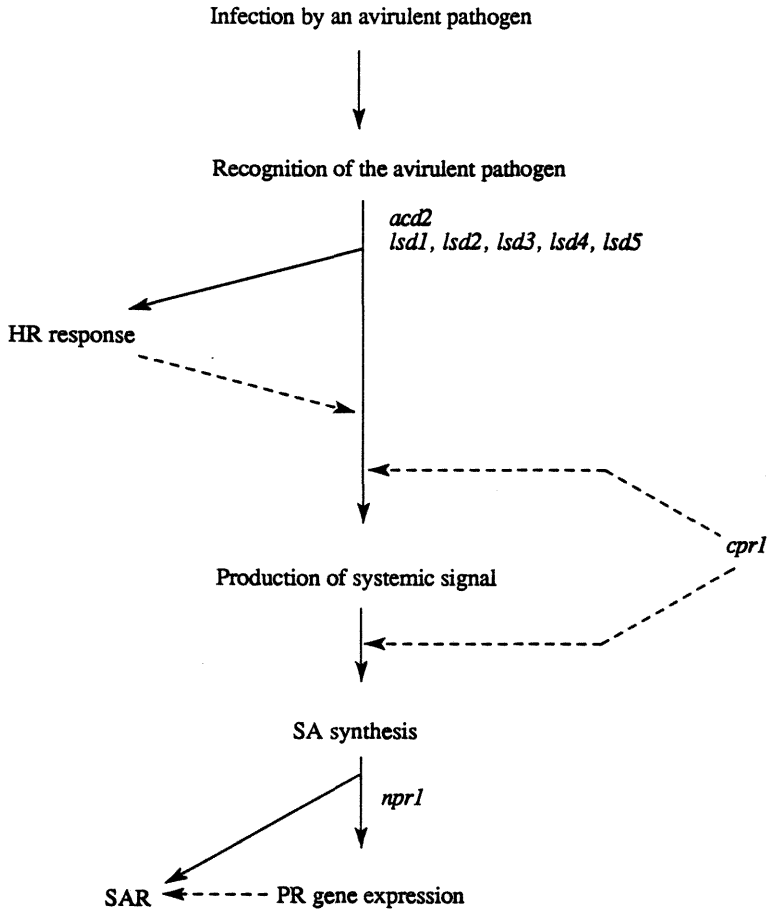
1.2.8 SA signal transduction pathway(s)

The genetic approach to defining components in a signalling pathway is very powerful and has been used in plants, particularly *Arabidopsis thaliana* which has a relatively rapid generation time, a small well-mapped genome, and the ability to be transformed (Meyerowitz, 1989). Previous studies have verified that *Arabidopsis* exhibits the characteristics of SAR, inducing development of SAR after a hypersensitive response (HR) to an avirulent pathogen (Dempsey *et al.*, 1993; Uknes *et al.*, 1993b; Cameron *et al.*, 1994; Mauch-Mani and Slusarenko, 1994), responsiveness to SA and INA induction (Uknes *et al.*, 1992) and expression of PR genes (Uknes *et al.*, 1992; Dempsey *et al.*, 1993). A number of mutants have already been isolated (Dietrich *et al.*, 1994; Greenberg *et al.*, 1993; Greenberg *et al.*, 1994; Cao *et al.*, 1994; Bowling *et al.*, 1994). In one class of mutants, lesions resembling an HR form spontaneously, followed by induction of SAR, these include *acd2* (accelerated cell death, Greenberg *et al.*, 1994), *lsd1, lsd2, lsd3, lsd4 and lsd5* (lesion simulating disease, Dietrich *et al.*, 1994). Another class of mutants (*npr1, cpr1*) have been identified affecting signal transduction downstream of the HR. Dong *et al*

transformed *Arabidopsis* with a reporter gene containing the promoter of a ß-1,3-glucanase-encoding PR gene (BGL2) and the coding region of ß-glucuronidase (GUS). The resulting transgenic line (BGL2-GUS) was mutagenized. Mutants *npr1* (nonexpresser of PR genes) and *cpr1* (constitutive expresser of PR genes) were identified. The former exhibited reduced resistance and the latter showed increased resistance to pathogen attack. The endogenous level of SA is elevated in the *cpr1* mutants and it is proposed that the CPR1 gene functions as a negative regulator acting upstream of SA in the SAR signalling pathway. (See Figure 1.5 from Bowling *et al.*, 1994).

Cloning and further characterization of the regulatory genes associated with these mutants will help to elucidate the molecular basis of SAR.

Figure 1.5 **Proposed placement of mutants in the SAR signaling pathway**
(From Bowling *et al.*, 1994)


Infection by an avirulent pathogen
Recognition of the avirulent pathogen
acd2
lsd1, lsd2, lsd3, lsd4, lsd5
HR response
cpr1
Production of systemic signal
SA synthesis
npr1
SAR
PR gene expression

1.3 Aims of the project

At the start of the project, it was clear that synthesis of SA around HR lesions was associated with systemic induction of acquired immunity. Many questions remained and the following formed the basis of this thesis:

i) Is SA a local signal for defence response?
ii) Is SA involved in local HR lesion development?
iii) Is SA really the mobile signal?
iv) Does SA function by affecting H_2O_2 level, via inhibition of catalase?

The approach I decided to take in mid 1992 was to try and use the activity of a bacterial enzyme to block SA accumulation in specific cell types so as to examine the importance of SA in defense responses.

Salicylate hydroxylase (E.C.1.14.13.1), encoded by the *nahG* gene of *Pseudomonas putida* pWW60-1, can catalyze the decarboxylative hydroxylation of salicylic acid, converting it to catechol. By fusing specific promoters to *nahG*, I hoped to express *nahG* constitutively or only at local infection sites or in systemic tissue, so as to prevent SA accumulation constitutively, locally or systemically. By doing so, it was hoped that I would be able to investigate the role of SA in the local and systemic defense responses.

In late 1993 when my experiments were well underway, a group based in Ciba Geigy reported the use of a different *nahG* for similar experiments (Gaffney *et al.*, 1993). Our *nahG* gene (SH-L, L stands for Leicester) was obtained from a different source 6 months before Gaffney's work was published and has proved to have somewhat different properties which will be elucidated in chapter 3. Since 1994, I have been collaborating with Ciba in the development of a hypothesis concerning the function of SA in disease resistance.

Chapter 2 Materials and Methods

2.1 Plant DNA extraction for PCR analysis

This method was modified from that of Konieczny and Ausubel (1993). A small piece of leaf material was removed and placed in an Eppendorf tube. 250 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 50 mM EDTA, 10 mM mercaptoethanol, 1.4% SDS) was added and the material was ground using a micro-homogeniser (Biomedix, Pinner, U.K.). The samples were incubated at 65^{0}C for 10 minutes, after which time 65 µl of 5 M potassium acetate was added. After 5 min incubation on ice, the precipitate was pelleted by centrifugation at 13,000 g for 10 minutes, and then the supernatant was transferred to a fresh tube. Nucleic acids were precipitated from the supernatant by the addition of 320 µl isopropanol and 30 µl 3 M sodium acetate. Following incubation at -20^{0}C for 10 minutes, the nucleic acids were recovered by centrifugation for 10 minutes at 13,000 g, dried under vacuum and resuspended in 50 µl of sterile, distilled water. 1 µl of this solution was routinely used as template for PCR analysis.

2.2 Plant RNA extraction

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

Plant material was ground to a fine powder with liquid nitrogen in a pre-cooled pestle and mortar. Extraction buffer (6% 4-aminosalicylate, 1% triisopropyl naphthalene sulphonate, 6% phenol, 50 mM Tris-HCL pH 8.4) was then added at a ratio of 2 ml, per gram fresh weight of tissue, and ground further, again with liquid nitrogen. The resultant powder was transferred to a 50 ml polypropylene tubes and thawed on ice. Once thawed an equal volume of phenol-chloroform (tris-buffered liquefied phenol / chloroform / isoamyl alcohol; 50:44:2) was added, mixed thoroughly by vortex, before centrifugation at 3,600 g for 10

minutes. The upper, aqueous phase was removed to a fresh tube and re-extracted twice or until all precipitated protein at the interface was removed. At this stage the aqueous phase was transferred to a 30 ml siliconised Corex tube (Corex, U.S.A.) and the total nucleic acids precipitated by the addition of 1/20th volume 4 M sodium acetate (pH 6.0), 2.5 volumes of absolute ethanol, followed by storage at -20^{O}C overnight. Total nucleic acids were recovered by centrifugation at 10,000 g for 20 minutes at 4^{0}C in a Sorvall RC-5B (Du Pont, U.S.A.) centrifuge fitted with a SS-34 fixed angle rotor (Du Pont, U.S.A.). The resultant pellet was dried under vacuum and resuspended in sterile distilled water at a ratio of 0.5 ml per gram fresh weight of tissue. RNA was differentially precipitated by the addition of 3 volumes of 4 M sodium acetate (pH 6.0), followed by incubation on ice for 1 hour. RNA was pelleted by centrifugation at 10,000 g for 20 minutes at 4^{0}C, the supernatant was removed and the pellet dissolved in 400 µl sterile, distilled water. The RNA was then precipitated by the addition of 1/20th volume 4 M sodium acetate (pH 6.0), 2.5 volumes of absolute ethanol, stored at -20^{O}C for 1 hour, centrifuged at 13,000 g, supernatant removed, dried under vacuum and resuspended in 50 µl sterile distilled water.

2.3 Nucleic acid quantitation by spectrophotometry

Concentrations of DNA and RNA were determined using the ultra-violet light absorbing properties of nucleic acids. Both molecules absorb ultra-violet light maximally at a wavelength of approximately 260 nm. Samples were scanned over a wavelength range from 200-300 nm, using a Hewlet Packard diode array spectrophotometer (Hewlet Packard, U.S.A.), and nucleic acid concentration determined by measurement of the absorbance value at 260 nm. It was assumed that an absorbance unit of 1.0 was produced by RNA at a concentration of 40 µg per ml and DNA at a concentration of 40 µg per ml. A ratio of Abs.260nm:Abs.280nm gave an estimate of the purity of the sample, a figure greater than 2.0 being acceptable.

2.4 DNA manipulation and modification

2.4.1 DNA ligation

T4 DNA ligase (Life Technologies, U.S.A.) was used in all ligation reactions. Ligations reactions were carried out essentially as manufacturers instructions, either at 37^{0}C for 1 hour or overnight at 16^{0}C. Generally for efficient ligation of blunt ends or sticky ends a molar ratio of one to three respectively, for ends of insert to ends of vector, was used.

2.4.2 Restriction digests

Restriction digestions were carried out in the appropriate buffers and at the appropriate temperatures as suggested by manufactures and generally up to 1μg of plasmid DNA in a volume of 20 μl.

2.4.3 Purification of DNA fragments

Digested DNA was separated by agarose gel electrophoresis (2.5.1). The desired fragments were excised from the gel and the DNA electroeluted into high salt solution (3 M sodium acetate pH 7.9, 0.01% bromophenol blue) at 100 V for 1 hour. DNA was then precipitated by addition of three volumes of absolute ethanol, incubated at -20^{0}C for 10 minutes and then centrifuged at 13,000 g for 10 minutes. The pellet was washed in 70% ethanol, dried under vacuum, and resuspended in 10 μl water.

2.4.4 DNA amplification by the polymerase chain reaction (PCR)

PCR reactions were routinely carried out in a 25 μl volume, with a mineral oil (Sigma, U.K.) overlay, as follows:

Template (1ng/µl)	1 µl
11 x reaction buffer	2.2 µl
primer 1 (100ng/µl)	0.5 µl
primer 2 (100ng/µl)	0.5 µl
Taq polymerase (1U/µl, Promega)	0.5 µl
H_2O	to 25 µl

To make 676 µl 11 x reaction buffer, it needs 167 µl 2M Tris-HCl pH 8.8, 83 µl 1M ammonia sulphate, 33.5 µl 1M $MgCl_2$, 3.6 µl 2-mercaptoethanol, 3.4 µl 10 mM EDTA pH 8.0, 75 µl each dNTP 100 mM stock and 85 µl 10 mg/ml BSA.

Denaturation of template was achieved by heating to 94^0C for 30 seconds.
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 approximate annealling temperature was calculated by subtracting 5^0C from the T_m of the primer. The Tm of each primer was determined as follows:

$$T_m\ (^0C) = [(A+T) \times 2] + [(G+C) \times 4]$$

where A, T, G, C are the numbers of each individual type of nucleotide within each primer.

Generally, annealling was carried out for 30 seconds.
Extension were carried out at 72^0C for varying lengths of time depending on the size of the final product. In general an extension time of 1 minute at this temperature was considered sufficient for products of up to 2 kb in length. If the PCR product was to be used for cloning then an additional four minutes at 72^0C was included at the end to ensure complete formation of product

2.5 Nucleic acid electrophoresis

2.5.1 DNA agarose gel electrophoresis

DNA samples were routinely analysed by eletrophoresis through agarose (Life Technologies, U.S.A.). Gels were prepared by dissolving agarose in 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA), containing 10 ng/ml ethidium bromide. The concentration of agarose used was dependant upon the size of fragment under investigation, as discussed by Sambrook *et al.* (1989).

Gels were run at 100-150 V in 1x TAE buffer containing 0.2 mg/l ethidium bromide. 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. 1 kb ladder DNA size standards (Life Technologies, U.S.A.) were co-electrophoresed with the samples in order to determine fragment length. DNA was visualised on a UV transilluminator (UVP Inc, U.S.A.). A photographic record of the gels was obtained using a video camera (UVP Inc.) and processor (Mitsubishi).

2.5.2 RNA gel

RNA samples (6 ul final volume) were incubated at 65^{0}C for 5 minutes in the following solution: 12.5 µl deionised formamide, 2.5 µl 10x MOPS buffer (0.2M MOPS, 0.05M NaOAc, 0.01M Na_2EDTA, pH 7.0 adjusted by the addition of NaOH) and 4.0 µl 37% Formaldehyde.

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% agarose gel. Visualisation of RNA samples was facilitated either by adding ethidium bromide (final

concentration 0.1 mg/ml) to the running buffer prior to electrophoresis or, preferably, staining the gel with ethidium bromide following electrophoresis.

2.5.3 DNA sequencing gels

The following stock solutions were prepared and stored in the dark at 4^{0}C until required:

40% acrylamide solution:

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

10x TBE buffer:

Tris base	108 g
Boric acid	55 g
EDTA	5.8 g
Distilled water	to 1000 ml

6% acrylamide/urea gel mix

40% acrylamide solution	150 ml
10x TBE	100 ml
Urea	420 g
Distilled water	to 1000 ml

For separation of samples a BioRad 20 cm x 60 cm gel kit fitted with 0.5 mm spacers was used. The kit was assembled as per manufacturer's instruction and the gel poured using the 60 ml 6% acrylamide/urea gel mix solution, polymerized with 750 µl 10% ammonium persulphate and 125 µl TEMED (N,N,N',N'- tetramethylethylenediamine).

Prior to loading samples the gel was pre-run at 2,500V until a gel temperature of 50^{0}C was attained. The running buffer used was 1x TBE and gels were routinely run at a voltage sufficient to maintain an average temperature of 50^{0}C.
Following electrophoresis, the gel kit was dismantled, the gel was then fixed in 10% methanol, 10% acetic acid for 20 minutes, and then transfered to a piece of Whatman 3MM filter paper. This was then covered with cling film and dried at 80^{0}C for 1 hour using a Bio-Rad gel drier.

2.6 Cloning and manipulation of plasmids

2.6.1 Preparation of competent cells

A single colony of the *E. coli* strain XL-1Blue was 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^{0}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^{0}C having first precooled 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), dispensed into 400 µl aliquots and flash-frozen in liquid nitrogen. Cells were stored at -80^{0}C until required.

2.6.2 Transformation of *E.coli*

Approximately 10-100 ng plasmid was added to 100 µl of competent *E.coli* XL 1-Blue cells and incubated on ice for 30 minutes. Cells were then heat-shocked at 42^{0}C for 2 minutes. 1 ml of LB, prewarmed to 37^{0}C, was added and the cells incubated at 37^{0}C for 15 minutes. Cells were collected at the bottom of tube by brief centrifugation at 6,500 g, supernatant

removed, gently resuspended in 100 µl LB and then spread onto LA plates containing the appropriate antibiotic for selection of transformed cells. For plasmids containing the ß-galactosidase fusion colour selection system, 20 µl 2% X-gal (5-bromo, 4-chloro-galactoside, Melford Laborateries, U.K.)(prepared in dimethyl formamide) and 20 µl 0.5 M IPTG (isopropyl-ß-D-thiogalactopyranoside, Melford Laborateries, U.K.) was added to each plate by spreading on the surface prior to the application of the cell suspension. Plates were incubated overnight at 37^0C.

2.6.3 Small scale (mini-prep) plasmid preparations

Recovery of plasmid DNA from 1.5ml 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 13,000 g for 5 minutes in a microcentrifuge to pellet the bacterial cells. After removing the supernatant 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, 2M glacial acetic acid) the tube was gently shaken and then centrifuged for 5 minutes at 13,000 g, to pellet precipitated protein and genomic DNA. The supernatant was transfered to a fresh tube and 1 µl (50 µg/ul) RNase (DNase free) was added and incubated at room temperature for 15 min. After phenol-chloroform extraction (2.2) the upper aqueous phase was transfered to fresh tube, and the nucleic acids were precipitated by the addition of an equal volume of propan-2-ol. Following centrifugation at 13,000 g for 5 minutes, the supernatant was removed and the remaining pellet resuspended in 200 µl of sterile distilled water. The nucleic acids were then reprecipitated by the addition of 2.5 volumes of ice-cold absolute ethanol and 1/20th volume of sodium acetate pH 6.0. The plasmid DNA was then recovered by centrifugation at 13,000 g for 5 minutes, the supernatant discarded, and then the pellet dried under vacuum,

prior to resuspension in 20 µl sterile distilled water. 2 µl of this final suspension were normally used in restriction digests.

2.6.4 Large scale plasmid preparations

Large-scale plasmid preparations were routinely carried on cultures of bacteria greater than 100ml and up to 1l, according to the method of Sambrook *et al.* (1989).

The culture, grown for approximately 18 hours, was centrifuged at 6,000 g for 5 minutes in a Sorval RC-5B centrifuge, fitted with GSA rotor. After removal of the supernatant from the pellet, the cells were thoroughly resuspended in 30 ml of solution I (2.6.3) and incubated on ice for 5 minutes. The cells were then lysed by the addition of 60 ml of freshly prepered solution II (2.6.3), and this solution was gently agitated until liquid appeared clear. Protein and genomic DNA were precipitated by the addition of 30 ml of solution III (2.6.3). The tube was gently shaken to ensure adequate mixing and left on ice for 15 minutes. The precipitate was then pelleted by centrifugation for 10 minutes at 12,000 g. To ensure efficient removal of any remaining bouyant precipitate the supernatant was filtered through 4 layers of muslin. Nucleic acids were precipitated by the addition of an equal volume of isopropanol to the filtrate prior to centrifugation at 12,000 g for 10 minutes. Following removal of the supernatant the nucleic acid pellet was resuspended in 3 ml sterile distilled water. Large amounts of contaminating ribosomal RNA usually remained at this stage, thus this was precipitated by the addition of 3 ml of 5 M ice-cold LiCl, and then removed by centrifugation at 10,000 g for 10 minutes. The supernatant was transfered to a fresh tube and plasmid precipitated using 1 volume of isoprapanol. The precipitate was recovered by centrifuging at 10,000 g for 10 minutes. The supernatant was removed and the pellet resuspended in 400 µl sterile distilled water. The plasmid solution was transfered to a 1.5 ml Eppendorf tube and any remaining RNA was digested by incubation for 15 minutes at room temperature with 1 µl DNase free RNase (50 µg/µl).The RNase was denatured and removed by vortexing briefly with an equal volume of phenol/chloroform (2.2). Following

centrifugation at 13,000 g for 3 minutes, the upper aqueous phase was removed to a fresh tube and the plasmid DNA precipitated by the addition of 2.5 volumes of ice-cold absolute ethanol, and 1/20th volume sodium acetate pH 6.0. The precipitate was recovered by centrifugation at 13,000 g for 5 minutes, the supernatant discarded, and the pellet dried under vacuum prior to resuspension in 50 µl sterile distilled water. 2 µl of this final suspension were normally used in restriction digests.

2.7 DNA Sequencing

2.7.1 Preparation of single-stranded template

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 5 µl helper phage (VCS M13, Stratagene, U.S.A.). The culture was incubated for 2 hours at 37^{0}C. After this time kanamycin was added to a final concentration of 50 µg/ml and the culture incubated at 37^{0}C overnight. The following day the whole culture was centrifuged at 3,600 g for 5 minutes, and then 1 ml of supernatant was removed to a 1.5 ml Eppendorf tube and centrifuged for a further 10 minutes to ensure total removal of cells. Following transfer of the supernatant to a fresh Eppendorf tube, the phagemid was precipitated by the addition of 200 µl of phage precipitation buffer (20% PEG 6,000, 2.5 M NaCl). After 10 minutes incubation at room temperature the phagemid was recovered by centrifugation at 13,00 g for 10 minutes. The supernatant was discarded and the pellet briefly re-centrifuged to ensure removal of any remaining PEG solution. The pellet was then resuspended in 100 µl distilled water and the phagemid deproteinized by extraction with phenol/chloroform. The upper aqueous phase was removed to a fresh tube and the phagemid DNA precipitated by the addition of 1/20th volume 4 M sodium acetate, and 2.5 volumes absolute ethanol, followed by incubation at -80^{0}C for 20 minutes. After centrifugation at 13,000 g for 10 minutes the supernatant was removed, the pellet dried

under vacuum and then finally resuspended in 20 µl sterile distilled water. 5 µl aliquot of this solution was analysed on a TAE/agarose gel to estimate concentration.

2.7.2 Preparation of double-stranded template

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 minutes at room temperature by the addition of 2 µl of 2 M sodium hydroxide. The alkali was neutralised with 4 µl of 5 M ammonium acetate pH 4.8, and then the DNA precipitated by the addition of 3 volumes of ethanol prior to incubation at -80^{0}C for 10 minutes. After centrifugation, the pellet was washed in 70% ethanol, dried under vacuum, and resuspended in 14 µl of distilled water.

2.7.3 Manual sequencing of single- and double-stranded templates

Sequencing was carried out using Sequenase 2.0 kits (USB Inc.) according to the manufacturer's instructions. 7 µl of template (single- or double-stranded) prepared as in sections 2.6.1 and 2.6.2 were used in each reaction.

2.7.4 Analysis of DNA sequences

Sequence analysis and database searches were carried out using the University of Wisconsin GCG programmes. (Genetics Computer Group, 1991).

2.8 *Agrobacterium* manipulations

2.8.1 Preparation of electroporation-competent cells

A 36 hour old 5 ml starter culture of *Agrobacterim*, strain GV2260, was diluted into 500 ml fresh NB and grown to an OD_{600} of 0.6. The cells were recovered by centrifugation at 5,000 g for 5 minutes, supernatant removed, and the cells then resuspended in an equal volume of ice-cold 10% glycerol. This was repeated three times resuspending cells in 0.5, 0.02 and finally 0.012 volume ice-cold 10% glycerol. Cells were dispensed into 40 µl aliquots and stored at -80^{0}C until required.

2.8.2 Transformation of recombinant plasmids into *Agrobacterium* by electroporation

Plasmid DNA from either mini or large scale preparations (2.6.3-4) was diluted to a concentration of 2 µg/µl. This solution was then diluted 100 fold. 1 µl of which was used for electroporation.

1µl of DNA was added to 40µl competent cells (2.8.1) and placed in 0.2 cm cuvette.

The parameters of the electroporator (BioRad) were set as follows:

Capacitance (C) = 25 µF

Resistance (R) = 600 Ω

Voltage (V) = 2.5 kV

Time constant (RC) = 13.8ms

Immediately following electroporation 1ml of NB was added to the cuvette. The solution was transferred to an Eppendorf tube and incubated at 30^{0}C with shaking for 3 hours to increase recovery. Cells were briefly centrifuged, resuspended in 100µl NB, and then plated onto selection plates.

2.8.3 Transformation of recombinant plasmids into *Agrobacterium* by triparental mating

Matings were carried out as described in Draper *et al.* (1988).

Single colony of donor (XL1-Blue, containing the binary plasmid to be transferred) and helper (pRK2013, carrying the mobilisation and transfer functions) strains of *E. coli* were grown at 37^{0}C overnight in 5 ml NB with selection. Similarly, a single colony of the recipient *Agrobacterium* strain LBA4404 was grown for 48 h at 28^{0}C. Triparental mating was achieved by mixing 100 µl of each (donor, helper and recipient) strain and then spreading onto a NA plate. This was then incubated at 28^{0}C until a lawn of bacterial growth appeared. An inoculum was removed from this plate using a sterile inoculating loop and streaked onto a NA plate containing selective antibiotics for both the *Agrobacterium* and for the binary vector. After incubating for a further 48 h individual colonies of recombinant *Agrobacterium* were observed.

2.9 Tobacco transformation and regeneration

Transformation were carried out essentially as described in Draper *et al.* (1988).

Tobacco (cv Samsun) leaves were surfaced-sterilised in 10% bleach (Domestos, Lever, U.K.), for 20 minutes and then washed several times with 2 l 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 (see section 2.25) for 15-30 min. Inoculated explants were then transfered to MSD 4x2 (see section 2.25) plates, the plates sealed with sealing film (Whatman, U.K.), and incubated at 25^{0}C for two days. After this period of time had elapsed the explants were transfered to MSD 4x2 plates containing 400 µg/ml augmentin and 100 µg/ml kanamycin. After approximately two weeks in culture,

transformed cells began to proliferate forming callus tissue around the edge of the explants. After a further period of approximately one week the callus began to generate shoots. Such shoots were aseptically excised from the explant, taking great care to remove all callus material first, and transfered to solid MSO containing kanamycin and augmentin as above. In the absence of phyto hormones the shoots to produce roots. Once rooted, regenerated tobacco plants were planted in soil and grown under standard glasshouse conditions.

2.10 *Arabadopsis* transformation and regeneration

Arabidopsis thaliana plants were transformed as described by Clarke *et al.* (1992).

2.11 Screening transgenic plants by PCR

DNA extracted from transgenic plants (section 2.1) was analysed for transgene insertion by PCR. 2 µl of DNA extract were subjected to PCR analysis as in section 2.4.4. Primer selection was dependent on the context of the sequence of interest within the T-DNA. Annealing and extension times were selected appropriately.

2.12 Screening transgenic plants by kanamycin-resistance

Tobacco seeds were germinated on GM medium containing 100 µg/ml kanamycin to select the kanamycin resistant ones. *Arabidopsis* seeds were germinated on GM medium containing 35 µg/ml kanamycin to select the resistant ones.

2.13 Screening transgenic plants by ß-glucuronidase activity

2.13.1 Histochemical localization of GUS (ß-glucuronidase) activity in plant sections

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, 10 mM EDTA, 1 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (solublized in dimethyl formamide)(X-gluc, Melford Laboratories, U.K.) at 37^0C 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 plant line. Generally staining was monitored regularly for up to four hours, after which time staining was left to progress overnight. Material thus stained was cleared by transferring to ethanol and incubating at room temperature overnight. Samples were then stored in ethanol indefinitely.

2.13.2 Fluorometric assay for GUS activity

Fluorometric determination of GUS activities followed the Jefferson *et al* . (1987) protocol with the modifications of Topping *et al.* (1991). Assays were carried out on soluble-protein extracts from discs removed from leaves using a 14 mm cork-borer. The samples were then homogenised in 300 µl GUS extraction buffer (GEB) (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.7 µl/ml ß-mercaptoethanol , 0.1% sarkosyl) and clarified by centrifugation at 13,000 g for 10 minutes, the supernatant transferred to a fresh tube and maintained on ice until required for subsequent reactions. Reactions were initiated by the addition of 20 µl of the extract to 180 µl of reaction mix (1 mM methyl umbelliferyl glucuronide (MUG) in GEB). Once all reactions were set up a 20 µl aliquot was removed from each and added to a well of an opaque microtitre plate (Dynatech, U.S.A) containing 180 µl of STOP solution (0.2 M Na_2CO_3). This sample represented the fluorescence at time zero for the reaction. Similar aliquots were then taken and treated in asimilar manner for subsequent time points, generally at 20, 40 and 60

minutes. 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 known concentrations of 4-methyl umbelliferone (4-MU, Sigma, U.K.).4-MU is the fluorogenic compound released when MUG is cleaved by ß-glucuronidase.

2.14 Salicylate hydroxylase assay

In planta salicylic acid hydroxylase activity was measured essentially as described by Yamamoto *et al* (1965).

Plant material was ground mechanically in a precooled mortar with 33 mM potassium phosphate buffer, pH 7.0. The homogenate was centrifuged at 13,000 g for 10 min. The supernatant was used for enzyme assay. Since the rate of salicylate-dependent NADH oxidation was strictly proportional to that of catechol formation from salicylic acid (Katagiri *et al.*, 1962), the amount of salicylic acid hydroxylated was estimated by the decrease of the absorbance of NADH at 340 nm. For the routine assay, the decrease of the absorbance at 340 nm was followed with a dual beam recording spectrophotometer (Hewlett-Packard diode array model) in a cuvette with a 1cm light path. Each cuvette contained 20 nmoles of FAD, 200 nmoles of sodium salicylate, 100 nmoles of NADH, 60 nmoles of Tris-HCl buffer, pH 8.0, and 100 µg crude protein extract in a total volume of 3.0 ml. The reaction was allowed to proceed at room temperature and was monitored for 10 minutes. The protein concentration was determined by Bradford assay (2.16.2).

2.15 cDNA synthesis and RT-PCR

The method was based on that described by Guerineau *et al.* (1991).

Single-stranded cDNA was synthesized from 3-5 μg of total RNA prepared from wild type and transgenic tobacco leaves (2.2), using a standard cDNA synthesis kit (Amersham) and nahG 3' primer. One-twentieth of the cDNA synthesized was susequently used in a standard PCR reaction using both 3' and 5' nahG primers (see chapter 3). Some RNA was also used in the PCR reaction for control just in case there is contamination by genomic DNA. A quarter of the DNA synthesised following PCR was then analysed by agarose gel eletrophoresis.

2.16 Protein extraction and quantitation

2.16.1 Extraction of protein from plant material

Plant material was homogenised in approximately 1 volume of extraction buffer in a precooled morter and pestle at 4^0C. The homogenate was then transferred to an appropriate volume centrifuge tube and clarified by centrifugation for 10 minutes at 4^0C. The supernatant was then transferred to a fresh tube. For extraction of protein from small amounts of material homogenisation was carried out in a 1.5 ml Eppendorf tube using a micro-homogeniser.

2.16.2 Protein quantitation

Protein quantitation was carried out essentially as described by Bradford (1976).
A protein calibration curve was prepared using known concentrations of BSA (Sigma, U.K.) over a range of 0 - 1.0 mg/ml as follows:

20 µl of standard protein solution was pipetted into each well of a flat-bottomed microtitre plate and 180 µl of Bradfords reagent (600 mg Coomassie R-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 an accurate a measurement as possible. After all samples had been thus treated the microtitre plate was read at 595 nm in a Dynatech MR5000. Protein concentration was determined by comparison of sample absorbance readings with those of the protein standards and expressed as mg/ml.

2.17 SDS-PAGE analysis of protein samples

2.17.1 Electrophoresis of protein samples

Electrophoresis of protein samples was essentially as described by Laemmli (1976).

Electrophoresis of protein samples was carried out using the Bio-Rad Protein II kit following the manufacturer's guidelines.

Separation of protein was routinely achieved by electrophoresis of samples through a 5% stacking gel (5% acrylamide, 0.1% SDS, 0.25M Tris-HCl pH 6.8), overlaid on a 15% resolving gel (15% acrylamide, 0.1% SDS, 0.25 M Tris-HCl pH 8.8).
Acrylamide was made at a stock concentration of 30% by dissolving 30 g acrylamide, and 0.8 g bis-acrylamide in 100 ml of distilled water.
Electrophoresis was generally carried out at 100 V in 1 x Tris-glycine running buffer (25 mMTris-HCl, 250 mM glycine, 0.1% SDS).
Usually, 10 µg protein was loaded per lane. Prior to loading, protein samples were mixed with an equal volume of 2 x cracking buffer (0.25% bromophenol blue, 0.5 M Tris-HCl pH 6.8, 0.1% SDS, X M ß-mercaptoethanol) and then boiled for 3 minutes.

2.17.2 Coomassie-staining of protein gels

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

2.18 Western Blot

Western blotting was carried out as previously described (Warner *et al.*, 1993).

2.18.1 Blotting of protein gels

Protein gels were generally transferred to Immobilon-P (Millipore, U.S.A.) membranes using a semi-dry blotter (Millipore, U.S.A.). A single gel was blotted at 80 mA for 1 h, while two gels were transferred together at 150 mA for a similar length of time. The apparatus was assembled as follows:

3 pieces of Whatman 3 MM filter paper were cut to the same dimensions as those of the gel to be blotted. One piece was soaked in anode 1 buffer (0.3 M Tris-HCl pH 10.4, 10% methanol), one in anode 2 buffer (25 mM Tris-HCl pH10.4, 10% methanol), and one in cathode buffer (25 mM Tris-HCl pH 9.4, 40 mM 6-amino hexanoic acid, 20% methanol). The filter paper soaked in anode 1 buffer was layered onto the blotter anode, followed by the piece soaked in anode 2 buffer. A piece of Immobilin-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 cathode buffer-soaked 3 MM piece was then layered onto the gel and the blotter lid secured. Transfer was carried out as above.

2.18.2 Ponceau staining of membranes

The efficiency of transfer could be estimated by visualisation of transferred protein. Membranes were incubated with Ponceau stain (0.6% Ponceau-S, 1% acetic acid) for 5-10 minutes before washing away excess stain with distilled water. Protein samples became visible as the stain was rinsed off. Remaining stain was removed by rinsing in TBS buffer (20 mM Tris-HCl pH 7.6, 138 mM sodium chloride).

2.18.3 Detection of protein using polyclonal antibodies

Following blotting and ponceau staining the membrane was then blocked with TBS-Tween (TBS, 0.1% Tween 20 (Sigma, U.K.)) containing 3% Marvel (Cadbury-Schweppes, U.K.) at 4^0C overnight. It was then incubated with the primary antibody diluted 1 in 5000 with a solution of TBS, 10% Marvel, 10% glycerol, in a sealed bag for 2 hours at room temperature. The filter was then washed three times with TBS-Tween, each wash of 10 minutes duration. The filter was then incubated for 1 hour at room temperature with a 1:5000 dilution of alkaline phosphatase-conjugated secondary antibody (Dako, Denmark) diluted in TBS supplemented with 1% Marvel. The filter was the washed three times with TBS-Tween as described before. The blot was then developed in 5 ml BCIP developing solution (0.1M Tris-HCl, pH 9.5, 1 mM $MgCl_2$, 5 µg/ml BCIP (5-bromo-4-chloro-3-indoyl-phosphate, Sigma, U.K.), 3 µg/ml NBT (nitro blue tetrazolium, Sigma, U.K.). Development was arrested by washing the filter with distilled water.

2.19 SA extraction

SA was extracted using the method described by A. Crozier, University of Glasgow, (unpublished). Frozen tissue was ground with 90% methanol, 5 mM sodium dithiocarbamate and the centrifuged at 7,000 g in a Sorval RC5-B. The supernatant was transferred to a clean tube and the remaining pellet was resuspended in 100% methanol and

re-centrifuged. The two supernatant fractions were combined, dried in a Speedvac (Savant, U.S.A.), and then resuspended in 20 mls 100 mM sodium phosphate buffer (pH 8.0). The pH was then adjusted to 2.5 by the addition of hydrochloric acid. This solution was then partitioned 3 times against ECI (ethyl acetate: cyclopentane : isopropanol, 50:50:1, v/v). The organic phases were combined and dried.This fraction contained free salicylic acid. To measure the salicylic acid conjuhgated to sugars it was necessary hydrolyse the remaining aqueous phase with acid. To do this the aqueous phase was adjusted to pH 1.0 by the addition of HCl and then boiled for 30 minutes prior partitioning against ECI as described above. Samples were thoroughly resuspended in 40 µl of methanol, and then made upto 400 µl with a solution of 5% acetonitrile. 50 µl of this solution was then subjected to HPLC analysis.

To determine SA concentration in the material for catalase activity, leaf disks were treated as stated in the section referring to catalase assays. The supernatant remaining, following that removed for catalase assays, was made up to 20 mls with 100 mM sodium phosphate pH 8.0, and thoroughly mixed. The following procedures are the same as described above: the mixture was then acidified to pH 2.5 by the addition of HCl, and partitioned 3 times against an equal volume of ECI (ethyl acetate:cyclopentane:isopropanol / 50:50:1). The combined organic phases were reduced to dryness in a Speedvac (Savant). Samples were resuspended in 500 µl methanol:acetonitrile:water (10%:4.5%:85.5%) and 50µl was subjected to HPLC analysis.

Recovery of SA was estimated by addition of 50,000 dpm of 7-^{14}C SA (American Radio Chemicals, U.K.) to the starting supernatant, and then calculating the amount of radioactivity remaining following the extraction.

2.20 HPLC analysis

SA was analysed by HPLC using the method described by A. Crozier, University of Glasgow, (unpublished). Samples were analysed using an ATI Unicam HPLC system. Samples were separated by reverse phase analysis utilising a gradient of acetonitrile/water increasing from 5-40% acetonitrile in 30 min. An ODS Techsphere 5 μm 250 mm x 4.5 mm column was used in all assays. SA was determined by fluorescence with an excitation of 304 nm and emission of 440 nm using a Thermo Separations FL2000 fluorescence detector. Detection limit was 10 ng/gfw.

2.21 Trypan blue staining

Cell death was identified by trypan blue staining essentially as described by Koch (1990).

Plant material to be stained was placed into wells of a microtiter plate. Trypan blue stain solution (10 ml lactic acid, 10 ml glycerol, 10 g phenol and 10 mg trypan blue dissolved in 10 ml distilled water) was pre-warmed to 65^{0}C and added to the wells. To ensure good penetration of the stain throughout the sample the material was subjected to 5 minutes vacuum infiltration. The material was then boiled for 2 minutes or cooked in a microwave for 8 seconds and left to stain overnight. The next day, the stain was removed and chloral hydrate (25 g in 10 ml water) was used to clear the stain for at least a few hours or overnight.

2.22 Catalase assay

The assay method used was based on that of Storrie and Madden (1990). 1.5 cm leaf discs were ground in 200μl of ice-cold 20mM imidazole buffer (pH 7.0) containing 0.2% Triton X-100 (catalase extraction buffer). Whole leaves were ground using a pestle and mortar containing sand in ice-cold catalase extraction buffer. Samples were then centrifuged for 1

min at 11 600 g. Protein content was determined by the method of Bradford (2.16.2). Assays were performed in 96 well microtitre plates. Unless stated otherwise in figure legends, standard assay conditions were 20µl of sample (diluted to 0.2 $mg.ml^{-1}$ in catalase extraction buffer) to which was added 50µl of catalase extraction buffer containing 1 $mg.ml^{-1}$ bovine serum albumin and 0.06% H_2O_2 (catalase assay buffer). In all experiments less than 12 mins elapsed from extract dilution to end of assay period. Three reactions per sample were set up containing H_2O_2, H_2O_2 plus 10mM 3AT and a peroxide-free background incubation. The reaction was allowed to proceed for 2 min after which 200µl of catalase stop solution (2.25$g.L^{-1}$ $TiOSO_4$ in 0.1N H_2SO_4) was added. Absorbance was then read at 405nm. Following subtraction of background, absorbance units were converted to H_2O_2 content by reference to a H_2O_2 standard curve and the difference between 3AT-containing and 3AT-free incubations taken as 3AT-sensitive catalase activity. The unit definition used is: 1 unit = 1µmol H_2O_2 degraded. min^{-1}. The standard curve was linear up to 0.82 mmol H_2O_2 , had a limit of detection of at least 13 nmol H_2O_2 and a sensitivity of 1.44 absorbance units per mmol H_2O_2. Under standard conditions, the assay was linear for at least three min. In some cases involving samples taken from infected tissue, the protein concentration of the sample assayed was below 0.2 $mg.ml^{-1}$.

2.23 Vectors and bacterial strains

2.23.1 Vectors

pBluescript I SK^+ (Stratagene, U.S.A.)
pBin19 (Bevan, 1984)

2.23.2 Bacterial strains

E. coli strain: XL1-blue (Stratagene, La Jolla, California, USA)

Agrobacterium tumefaciens strain: PGV2260, binary vector host strain, rif^r (Deblaere *et al.*, 1985)

2.24 Bacterial media

Luria-Bertani (LB) medium: Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g pre litre. Adjust pH to 7.0 with 5 M NaOH prior to autoclaving.

LB agar: As for LB, but with the addition of 15 g/l agar prior to autoclaving.

NA (nutrient agar): NA was purchased from Difco and reconstituted according to the manufacturer's instructions.

NB (nutrient broth): NB was purchased from Difco and reconstituted according to the manufacturer's instructions.

2.25 Plant media

MSO: MS salts 4.71 g/l, sucrose 30 g/l. Adjust 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 (GM): Half-strength MS, 10 g sucrose/l. Adjust pH to 5.8 with 1 M KOH. 0.8% agar was added prior to autoclaving.

2.26 Chemicals

Unless otherwise stated all chemicals were supplied by Fisons, U.K.. 2,6-dichloro-isonicotinic acid (INA) was a gift of Dr Helmut Kessmann (Ciba-Geigy Ltd., Basel, Switzerland). Rabbit antiserum to PR1a was a gift of Dr R.F. White (Rothamsted Experimental Research Station, UK).

Restriction endonucleases and DNA modifying enzymes were purchased from Life Technologies, U.S.A. unless otherwise stated. *Taq* DNA polymerase and dNTP stocks were purchased from Promega, U.K..

2.27 Plant growth conditions and treatments

Tobacco plants were maintained under standard glasshouse conditions. Chemical treatment of tobacco leaf material involved either, injection of intercellular leaf spaces *in planta* with a 2ml syringe fitted with a 27G needle, or utilised 14 mm diameter leaf discs cored with a cork-borer and incubated in multi-well dishes (Nunc), with appropriate solutions. Injected samples were isolated from leaves as 14 mm diameter discs prior to assay.

2.28 Pathogens and infection techniques

Tobacco Mosaic Virus strain U1 was the gift of R. F. White (Rothamsted Experimental Research Station, UK). TMV infections involved rubbing viral suspensions (1μg/ml) mixed with 20 mg/ml carborundum carmine in water on to the leaf lamina After abrasion, carborundum carmine was removed by spraying with water. *Pseudomonas syringae* pathovar *syringae* strain 2774 was the gift of John Taylor, HRI, Wellesbourne, UK. *Pseudomonas syringae* was grown overnight in nutrient broth (Oxoid), centrifuged and then resuspended to an approximate concentration of 1 x 10^8 colony forming units/ml in 10mM phosphate buffer (pH 7.0). Tobacco leaves were infected with *P. syringae* by

injection of leaf intercellular spaces using a 2 ml syringe fitted with a 27G needle. Each infiltrated region covered an area of approximately $2cm^2$ and typically resulted in the application of approximately 100µl bacterial suspension.

2.29 Tissue Blotting

The method was described in Warner *et al.* (1994).

Chapter 3
Cloning and Expression of a Bacterial Salicylate Hydroxylase Gene in Transgenic Tobacco

3.1 Introduction

The approach I used to investigate the relationship between SA and defense responses was to remove SA from the plant tissue by expressing in transgenic plants a bacterial gene encoding salicylate hydroxylase. Salicylate hydroxylase (E.C.1.14.13.1) is a flavoenzyme. Currently, there are at least two distinct salicylate hydroxylase enzymes reported in the literature. One is the *Pseudomonas putida* salicylate hydroxylase which contains one mole of flavin adenine dinucleotide (FAD) and is a monomer with an approximate molecular weight of 54 kD (Yamamoto *et al.*, 1965; Takemori *et al.*, 1974) and the other is the *Pseudomonas cepacia* salicylate hydroxylase which contains two moles of FAD and two identical subunits in a total molecular weight about 91 kD (Tu *et al.*, 1981). Genes coding for salicylate hydroxylase (*nahG*) were found to be carried on several different plasmids of naphthalene degrading *Pseudomonas* strains, such as *P. putida* PpG7 (Dunn and Gunsalus, 1973) and NCIB9816 (Davies & Evans, 1964).

Salicylate hydroxylase is one of the enzymes in the naphthalene degradation pathway (see Fig.1.3). It catalyzes the conversion of SA into catechol, which has not been reported to be an inducer of defence genes. Since SA is postulated as an endogenous signal molecule required for SAR induction (See 1.3.1), it was expected that SAR should not be developed if SA accumulation is blocked in plants. The first objective therefore was to clone the salicylate hydroxylase gene from bacteria and generate transgenic tobacco plants constitutively expressing salicylate hydroxylase.

3.2 Results

3.2.1 Cloning of salicylate hydroxylase from *Pseudomonas putida*

The plasmid pWW60-3022 carrying a 4.7 kb *Xho*I fragment containing *nah*R, G and H genes, components of the naphthalene (*nah*) catabolism operon of *Pseudomonas putida* strain NCIB9816 (Assinder *et al.*, 1988), was a gift of Prof. Peter Williams (University of Wales, Bangor, UK). Two PCR primers, 5'-CG GGATCC AGCATGAAAAACAATAAA CTTGG (containing a *Bam*HI site) and 3'-CG GAATTC CGTTGTCACCCTTGACG (containing an *Eco*RI site) were designed from the published sequence of a *nah*G gene coding for a related salicylate hydroxylase (SH) gene (You *et al.*, 1991) and used to amplify the entire *nah*G (designated SH-L; Salicylate Hydroxylase-Leicester) open reading frame (Fig.3.1). Following a standard polymerase chain reaction (PCR) using these primers and pWW60-3022 as the template, the resulting c1300 bp PCR product was digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI-*Eco*RI-digested pBluescript to produce pSK-*SH-L* (Fig.3.2).

3.2.2 SH-L expression in *E. coli*

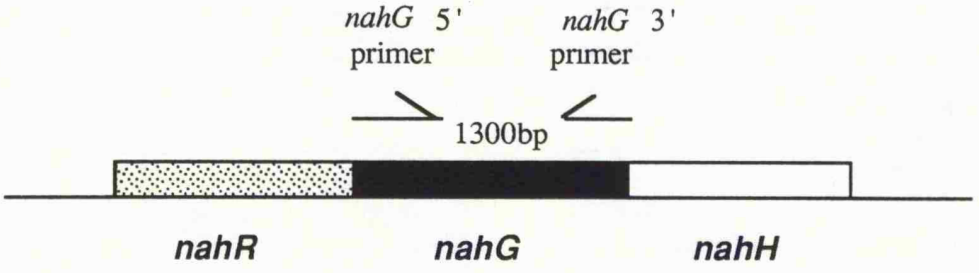
E. coli strains harbouring pSK-*SH-L* produced a brown discoloration around colonies when grown on media containing 1mM IPTG and 1mM SA, due to conversion of SA to catechol by SH-L and its subsequent oxidation (Fig.3.3), hence demonstrating that the SH-L enzyme was functional. This was particularly important as the gene was cloned by PCR and may have contained sequence errors.

3.2.3 Sequencing of SH-L

As a preliminary to fusing the *SH-L* gene to a plant promoter, a suitable subcloning strategy needed to be decided. According to the *nahG* sequence published by You *et al.*

Figure 3.1 Primers used to amplify the *nahG* (*SH-L*) gene

Part of plasmid pWW60-3022 and the primers for *nahG* (SH-L) PCR amplication is shown. The plasmid carries a 4.7 kb *Xho*I fragment containing *nahR, G* and *H* genes, which are components of the naphthalene (*nah*) catabolism operon (see Fig.1.3) of *Pseudomonas putida* strain NCIB 9816 (Assinder and William, 1988).
The underlined residues are *Bam*HI (5') and *Eco*RI (3') sites respectively used for cloning the amplified gene.



nahG 5' primer = CGGGATCCAGCATGAAAAACAATAAACTTGG

nahG 3' primer = CGGAATTCCGTTGTCACCCTTGACG

Figure 3.2 Restriction map of pSK-*SH-L*

A *Bam*HI / *Eco*RI fragment containing the *SH-L* gene (see Fig.3.1) was cloned into *Bam*HI / *Eco*RI digested pSK to generate pSH-*SH-L*.

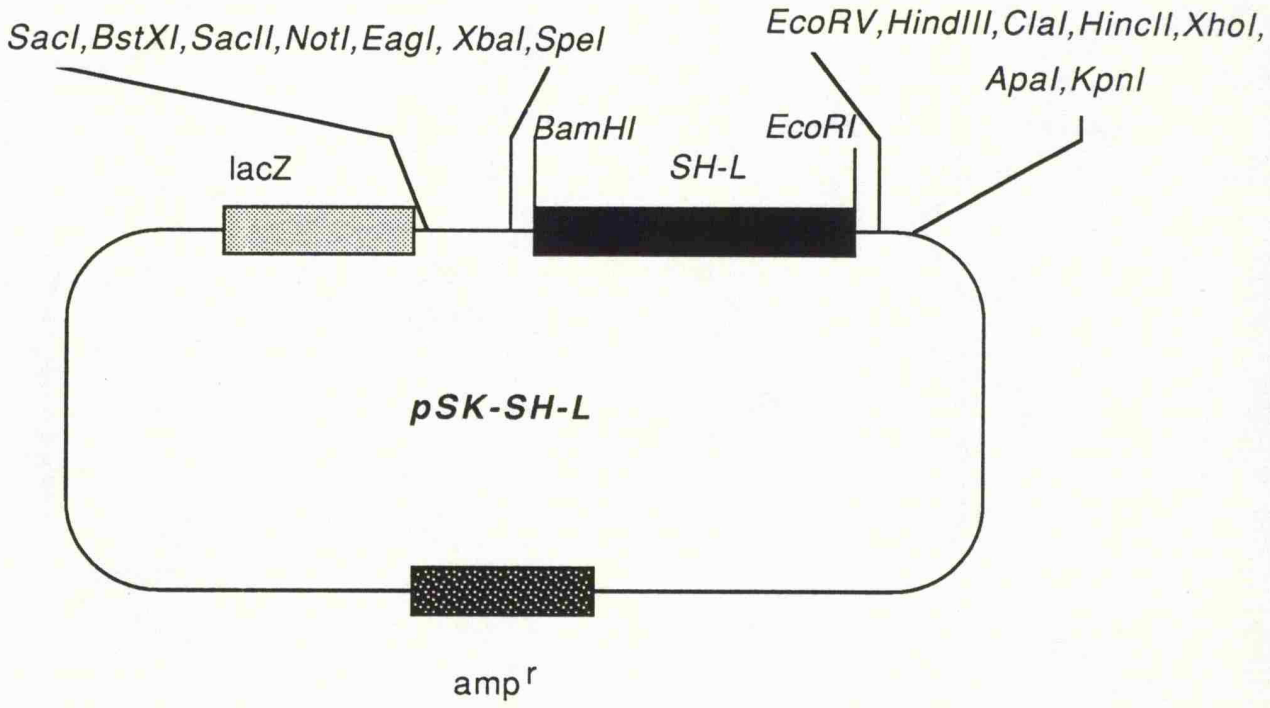
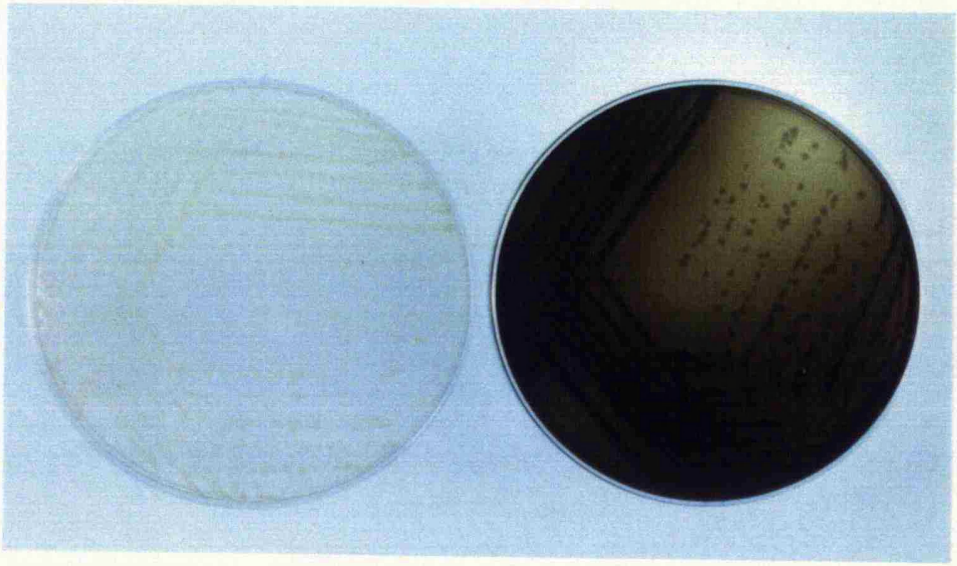

SacI,BstXI,SacII,NotI,EagI, XbaI,SpeI
EcoRV,HindIII,ClaI,HincII,XhoI,
ApaI,KpnI
BamHI
EcoRI
lacZ
SH-L
pSK-SH-L
amp r

Figure 3.3 SH-L expression in *E. coli*.

E. coli harbouring pSK or pSK-*SH-L* was streaked on LA plates containing 1mM SA and 1mM IPTG and incubated at 37^0C overnight. *E. coli* harbouring pSK showed normal colour (left) while *E. coli* harbouring pSK-*SH-L* went brown (right).



(1991), there were no recogntion sites for restriction enzymes *Kpn*I and *Sac*I. However, *Kpn*I digestion of pSK-*SH-L*, resulted in the production of two DNA fragments. Assuming that this suggested that DNA sequence of *SH-L* differed from that of *nahG*, a different cloning strategy was tried using *Sac*I but it was found that *SH-L* also contained a *Sac*I site as well. As an alternative to cloning *SH-L* by partial digestion, it was decided to sequence *SH-L* to determine its relationship with the published *nahG* gene and thereby allowing a redesigning of the cloning strategies.

The c1,300bp fragment in pSK-*SH-L* (Fig.3.2) was subcloned and sequenced. The subcloning strategy is shown in Figure 3.4.

The sequence of *SH-L* is shown in Figure 3.5. It has been registered in the EMBL database and the accession number is: X83926 *P. putida* nahG gene.

The predicted peptide sequence of SH-L is shown in Figure 3.6.

3.2.4 Comparison of nahG and SH-L

There is a 92% similarity between *nahG* from *P. putida* PpG7 (You *et al.*, 1991) and *SH-L* DNA sequences (Fig.3.7) and so it appears that the two genes are very closely related.

There is a 95% similarity comparing nahG and SH-L peptide sequences (Fig.3.8) reflecting the closeness of the two gene products.

The other predicted differences between SH-L and nahG are shown in Table 3.1.

Differences do exist between *nahG* and *SH-L*, but it is still difficult to know how these may influence the efficiency and stability of the enzyme, although it is known that the salicylate hydroxylase from *P. putida* PpG7 appears to be unstable compared to the

Figure 3.4 ***SH-L*** **subcloning strategy**

The c1300 bp SH-L gene was cut using restriction enzymes into 7 subclones. The restriction sites were chosen according to Cane and William (1986). Arrows indicate the direction of sequencing.

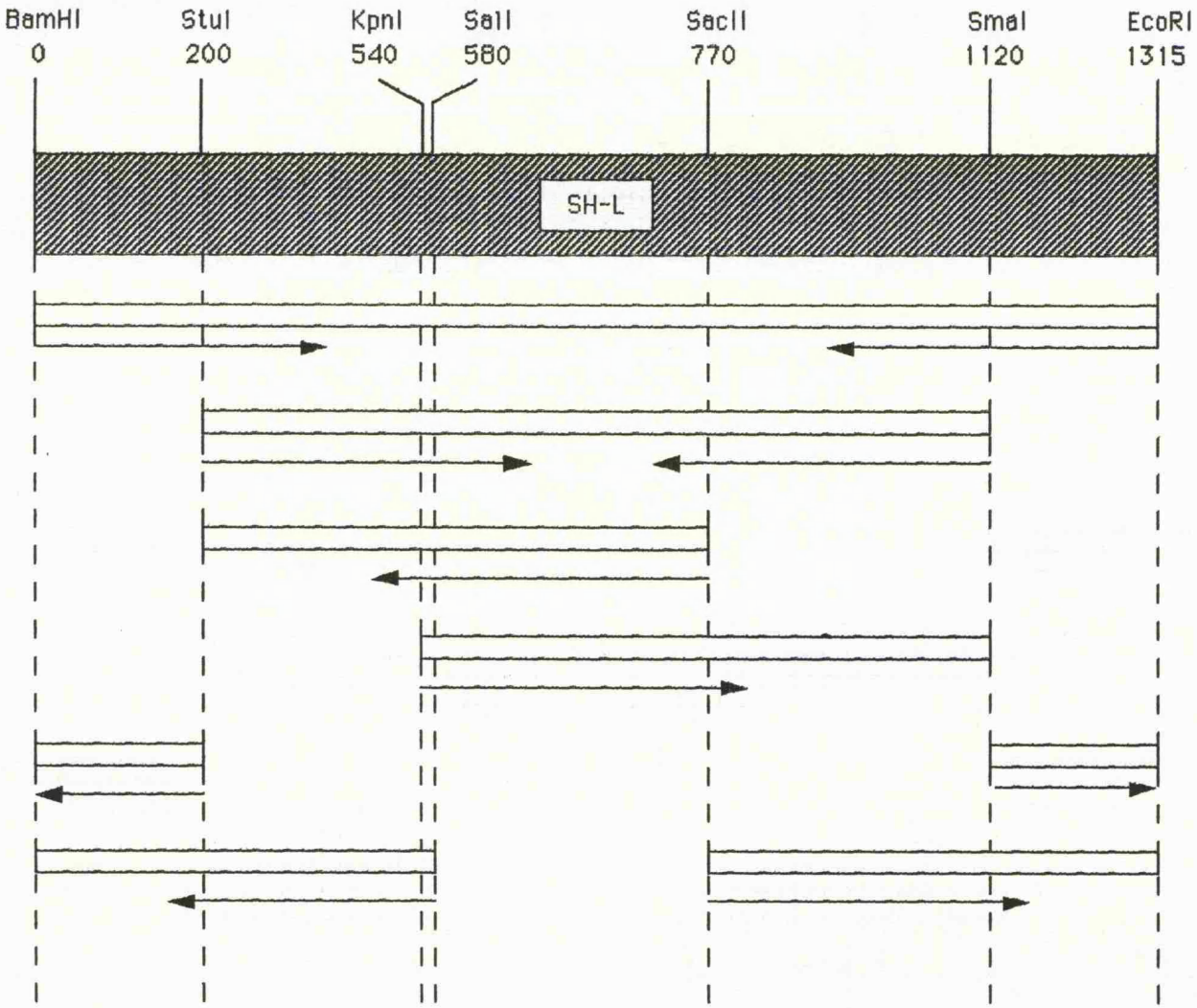

BamHI
0
StuI
200
KpnI
540
SalI
580
SacII
770
SmaI
1120
EcoRI
1315
SH-L

Figure 3.5 ***SH-L*** **DNA sequence and some restriction sites in SH-L**

a. *SH-L* sequence is shown. Translation stop and start codons have been underlined.

b. Some common restriction sites found in *SH-L* and the comparison to *nahG*

Fig.3.5a

1 ATGAAAAACA ATAAACTTGG CTTGCGCATC GGTATCATCG GCGGCGGGAT
51 TTCCGGCGTT GCCTTGGCTC TGGAGCTCTG TCGCTACTCC CATCTCCAAG
101 TACAGCTGTT CGAGTGCGCG CCGGCTTTTG GTGAGGTCGG TGCAGGAGTT
151 TCCTTTGGCC CCAACGCGGT GGCCGCCATT GTCGGCCTAG GCTTGGGCGA
201 GGCCTACTTG CAGGTCGCCG ACCGTACCTC GGAGCCCTGG GAGGACGTGT
251 GGTTCGAGTG GCGGCGCGGC AGGGATGCCA GCTATCTGGG AGCGACCATC
301 GCTCCGGGCG TGGGCCAGTC CTCGGTACAC CGGGCGGATT TCCTCGATGC
351 CCTAGTAAAT CACCTCCCAA AAGGTATCGC CCAATTCAGG AAGCGCGCCA
401 CACAGGTCGA GCAAAAGGGG GGCGAAGTGC AAGTGCTATT CGCCGACGGC
451 ACAGAGCACC GCTGTGACCT TCTAATCGGT GCCGACGGAA TCAAGTCAGC
501 GCTGCGTAGT CATGTGCTGG AAGGTCAGGG GCTGGCCCCA CAGGTACCGC
551 GCTTCAGCGG CACCTGTGCC TATCGGGGGA TGGTCGACAG CCTACACCTG
601 CGCGAAGCCT ATCGAGCCCA GGGCATCGAC GAGCACTTGG TGGACGTACC
651 GCAGATGTAC CTAGGGCTTG ACGGCCATAT CCTCACTTTT CCGGTGAGGA
701 ATGGCCGCCT CATCAACGTT GTGGCCTTCA TTTCCGACCG TAGTGAGCCG
751 AAGCCGAACT GGCCCGCGGA TGCCCCTTGG GTGCGCGATG TGAGCCAGCG
801 AGAGATGCTC GATGCCTTCG CGGGTTGGGG TGATGCCGCG CGCACCCTGC
851 TGGAGTGCAT CCCGACACCA ACTCTCTGGG CACTGCACGA CCTGGCGGAG
901 CTGCCGGGCT ACGTGCACGG GCGGGTCGTC CTGATCGGCG ACGCAGCTCA
951 CGCCATGCTG CCGCACCAAG GCGCCGGTGC CGCCCAAGGA CTTGAGGACG
1001 CCTACTTCCT CGCCCGCCTG TTGGGCGATA CCCAAGTCGA TGCAGACAAC
1051 CTCGCCGAGC TGCTTGAAGC CTACGACGAC CTGCGCCGCC CTCGTGCCTG
1101 TCGCGTGCAG CGAACCTCCC GGGAGACCGG CGAGTTATAC GAGTTTCGCG
1151 ACCCCGTCGT AGGTGCGAAC GAGCATCTGC TGGGGGAAAA CCTGGCGACC
1201 CGCTTCGACT GGCTGTGGAG CCACGACCTC GACGCCGACC TGGCCGAGGC
1251 ACGTGCGCGC CTGGGTTGGG AAAATGGTAG CCGGGGTGTG CTACGTCAAG
1301 GGTGA

Fig.3.5b

	SH-L	nahG
*Bam*HI	-	-
*Eco*RI	-	-
*Eco*RV	-	-
*Hind*III	-	-
*Kpn*I	+	-
*Sac*I	+	-
*Sac*II	+	-
*Sal*I	+	+
*Sma*I	+	-
*Xba*I	-	-
*Xho*I	-	-

Figure 3.6 Predicted SH-L peptide sequence

1 MKNNKLGLRI GIIGGGISGV ALALELCRYS HLQVQLFECA PAFGEVGAGV

51 SFGPNAVAAI VGLGLGEAYL QVADRTSEPW EDVWFEWRRG RDASYLGATI

101 APGVGQSSVH RADFLDALVN HLPKGIAQFR KRATQVEQKG GEVQVLFADG

151 TEHRCDLLIG ADGIKSALRS HVLEGQGLAP QVPRFSGTCA YRGMVDSLHL

201 REAYRAQGID EHLVDVPQMY LGLDGHILTF PVRNGRLINV VAFISDRSEP

251 KPNWPADAPW VRDVSQREML DAFAGWGDAA RTLLECIPTP TLWALHDLAE

301 LPGYVHGRVV LIGDAAHAML PHQGAGAAQG LEDAYFLARL LGDTQVDADN

351 LAELLEAYDD LRRPRACRVQ RTSRETGELY EFRDPVVGAN EHLLGENLAT

401 RFDWLWSHDL DADLAEARAR LGWENGSRGV LRQG*

Figure 3.7 DNA sequence comparison between *nahG* and *SH-L*

NahG stands for *SH-L* and M6055 stands for *nahG*.

```
NAHG            ATGAAAAACAATAAACTTGGCTTGCGCATCGGTATCATCGGCGGCGGGATTTCCGGCGTT
M60055          ATGAAAAACAATAAACTTGGCTTGCGCATCGGTATCGTCGGCGGCGGAATTTCCGGCGTT
                ************************************ ********** ************

NAHG            GCCTTGGCTCTGGAGCTCTGTCGCTACTCCCATCTCCAAGTACAGCTGTTCGAGTGCGCG
M60055          GCCTTAGCACTGGAACTCTGCCGCTACTCCCATATCCAGGTACAGCTGTTCGAGGCTGCG
                ***** ** ***** ***** ************ **** ***************   ***

NAHG            CCGGCTTTTGGTGAGGTCGGTGCAGGAGTTTCCTTTGGCCCCAACGCGGTGGCCGCCATT
M60055          CCGGCTTTCGGTGAGGTCGGTGCCGGCGTGTCCTTTGGCCCCAACGCGGTGCGCGCCATT
                ******** ************** ** ** **********************  *******

NAHG            GTCGGCCTAGGCTTGGGCGAGGCCTACTTGCAGGTCGCCGACCGTACCTCGGAGCCCTGG
M60055          GTCGGCCTGGGCTTGGGCGAGGCCTACCTGCAGGTTGCCGACCGTACTTCGGAGCCCTGG
                ******** ****************** ******* *********** ************

NAHG            GAGGACGTGTGGTTCGAGTGGCGGCGCGGCAGGGATGCCAGCTATCTGGGAGCGACCATC
M60055          GAGGACGTGTGGTTCGAATGGCGGCGCGGCAGCGATGCCAGCTATCTGGGAGCCACCATC
                ***************** ************** ******************** ******

NAHG            GCTCCGGGCGTGGGCCAGTCCTCGGTACACCGGGCGGATTTCCTCGATGCCCTAGTAAAT
M60055          GCTCCGGGCGTGGGCCAGTCCTCGGTACACCGGGCGGATTTCATCGACGCCCTAGTAACT
                ****************************************** **** ********** *

NAHG            CACCTCCCAAAAGGTATCGCCCAATTCAGGAAGCGCGCCACACAGGTCGAGCAAAAGGGG
M60055          CACCTCCCAGAAGGTATCGCCCAATTCGGGAAGCGCGCCACCCAGGTCGAGCAGCAGGGT
                ********* ***************** ************ ***********  ****

NAHG            GGCGAAGTGCAAGTGCTATTCGCCGACGGCACAGAGCACCGCTGTGACCTTCTAATCGGT
M60055          GGCGAAGTGCAAGTGCTGTTCACCGACGGCACAGAGTACCGCTGCGACCTTCTGATCGGT
                ***************** *** ************** ******* ******** ******

NAHG            GCCGACGGAATCAAGTCAGCGCTGCGTAGTCATGTGCTGGAAGGTCAGGGGCTGGCCCCA
M60055          GCCGACGGAATCAAGTCAGCGCTCCGTAGCCATGTGCTGGAAGGTCAGGGGCTGGCCCCA
                ********************** ***** ******************************

NAHG            CAGGTACCGCGCTTCAGCGGCACCTGTGCCTATCGGGGGATGGTCGACAGCCTACACCTG
M60055          CAAGTGCCGCGATTCAGCGGCACCTGTGCCTATCGGGGGATGGTCGACAGCCTGCATCTG
                ** ** ***** ****************************************** ** ***

NAHG            CGCGAAGCCTATCGAGCCCAGGGCATCGACGAGCACTTGGTGGACGTACCGCAGATGTAC
M60055          CGAGAAGCCTATCGGGCCCATGGCATCGACGAGCACTTGGTGGACGTGCCGCAGATGTAC
                ** *********** ***** ************************** ************

NAHG            CTAGGGCTTGACGGCCATATCCTCACTTTTCCGGTGAGGAATGGCCGCCTCATCAACGTT
M60055          CTAGGGCTCGACGGCCATATCCTCACCTTTCCAGTGAGGAATGGCGGCATCATCAACGTG
                ******** ***************** ***** ************ ** **********

NAHG            GTGGCCTTCATTTCCGACCGTAGTGAGCCGAAGCCGAACTGGCCCGCGGATGCCCCTTGG
M60055          GTGGCTTTCATCTCCGACCGTAGCGAGCCGAAGCCGACCTGGCCTGCGGATGCCCCTTGG
                ***** ***** *********** ************* ****** ***************

NAHG            GTGCGCGATGTGAGCCAGCGAGAGATGCTCGATGCCTTCGCGGGTTGGGGTGATGCCGCG
M60055          GTGCGTGAGGCGAGCCAGCGCGAGATGCTCGATGCCTTCGCGGGTTGGGGGGATGCCGCG
                ***** ** * ********* ****************************** *********

NAHG            CGCACCCTGCTGGAGTGCATCCCGACACCAACTCTCTGGGCACTGCACGACCTGGCGGAG
M60055          CGCGCCCTGCTGGAGTGCATCCCGGCACCAACTCTCTGGGCACTGCATGACCTGGCGGAG
                *** ******************** ********************** ************

NAHG            CTGCCGGGCTACGTGCACGGGCGGGTCGTCCTGATCGGCGACGCAGCTCACGCCATGCTG
M60055          CTGCCGGGCTACGTGCACGGTCGGGTCGTCCTGATCGGCGACGCAGCTCACGCCATGCTG
                ******************** ****************************************
```

```
NAHG            CCGCACCAAGGCGCCGGTGCCGCCCAAGGACTTGAGGACGCCTACTTCCTCGCCCGCCTG
M60055          CCGCACCAAGGTGCCGGTGCTGGCCAAGGGCTTGAGGACGCCTACTTCCTCGCCCGCCTG
                *********** ******** * ****** ******************************

NAHG            TTGGGCGATACCCAAGTCGATGCAGACAACCTCGCCGAGCTGCTTGAAGCCTACGACGAC
M60055          TTGGGCGATACGCAGGCCGATGCCGGCAACCTCGCCGAGCTGCTTGAAGCCTACGACGAC
                *********** ** * ****** * ***********************************

NAHG            CTGCGCCGCCCTCGTGCCTGTCGCGTGCAGCGAACCTCCCGGGAGACCGGCGAGTTATAC
M60055          CTGCGCCGCCCTCGTGCCTGTCGCGTGCAGCAAACCTCCTGGGAGACCGGCGAGTTATAC
                ******************************** ******* ********************

NAHG            GAGTTTCGCGACCCCGTCGTAGGTGCGAACGAGCATCTGCTGGGGGAAAACCTGGCGACC
M60055          GAGTTGCGCGACCCCGTCGTCGGTGCGAACGAGCAGCTGCTGGGGGAAAACCTGGCGACC
                ***** ************** ************** ************************

NAHG            CGCTTCGACTGGCTGTGGAGCCACGACCTCGACGCCGACCTGGCCGAGGCACGTGCGCGC
M60055          CGCTTCGACTGGCTGTGGAACCACGACCTCGACACTGACCTGGCCGAGGCCCGTGCGCGG
                ******************* ************* * ************** ********

NAHG            CTGGGTTGGGAAAATGGTAGCCGGGGTGTGCTACGTCAAGGGTGA
M60055          CTGGGTTGGGAGCATGGTGGCGGGGGTGCGCTACGTCAAGGGTGA
                ***********  ***** ** ****** ****************
```

Figure 3.8 Peptide sequence comparison between nahG and SH-L

Peptide sequence comparisons were performed using Clustal V programme.

Amino acids with a dot beneath means a conserved substitution.

nahG stands for *SH-L* and M6055 stands for *nahG*.

```
NAHG            MKNNKLGLRIGIIGGGISGVALALELCRYSHLQVQLFECAPAFGEVGAGVSFGPNAVAAI
M60055          MKNNKLGLRIGIVGGGISGVALALELCRYSHIQVQLFEAAPAFGEVGAGVSFGPNAVRAI
                ************.******************.****** ****************** **

NAHG            VGLGLGEAYLQVADRTSEPWEDVWFEWRRGRDASYLGATIAPGVGQSSVHRADFLDALVN
M60055          VGLGLGEAYLQVADRTSEPWEDVWFEWRRGSDASYLGATIAPGVGQSSVHRADFIDALVT
                ****************************** **********************.****

NAHG            HLPKGIAQFRKRATQVEQKGGEVQVLFADGTEHRCDLLIGADGIKSALRSHVLEGQGLAP
M60055          HLPEGIAQFGKRATQVEQQGGEVQVLFTDGTEYRCDLLIGADGIKSALRSHVLEGQGLAP
                *** ***** ********.********.**** ****************************

NAHG            QVPRFSGTCAYRGMVDSLHLREAYRAQGIDEHLVDVPQMYLGLDGHILTFPVRNGRLINV
M60055          QVPRFSGTCAYRGMVDSLHLREAYRAHGIDEHLVDVPQMYLGLDGHILTFPVRNGGIINV
                *************************.**************************** .***

NAHG            VAFISDRSEPKPNWPADAPWVRDVSQREMLDAFAGWGDAARTLLECIPTPTLWALHDLAE
M60055          VAFISDRSEPKPTWPADAPWVREASQREMLDAFAGWGDAARALLECIPAPTLWALHDLAE
                ************ *********. *****************.******.************

NAHG            LPGYVHGRVVLIGDAAHAMLPHQGAGAAQGLEDAYFLARLLGDTQVDADNLAELLEAYDD
M60055          LPGYVHGRVVLIGDAAHAMLPHQGAGAGQGLEDAYFLARLLGDTQADAGNLAELLEAYDD
                ***************************.***************** **.************

NAHG            LRRPRACRVQRTSRETGELYEFRDPVVGANEHLLGENLATRFDWLWSHDLDADLAEARAR
M60055          LRRPRACRVQQTSWETGELYELRDPVVGANEQLLGENLATRFDWLWNHDLDTDLAEARAR
                **********.**.*******.*********.**************.****.*********

NAHG            LGWENGSRGVLRQG
M60055          LGWEHGGGGALRQG
                ****.*. * ****
```

Table 3.1 Other comparisons between nahG and SH-L

	SH-L (*P. putida* NCIB9816) (nahG)	nahG (*P. putida* PpG7) (M60055)
molecular weight	47444.21	46963.48
residues	434	434
average residue weight	109.318	108.211
charged	-15	-22
isoelectric point	5.49	4.88
extinction coefficient	70060	76970

enzyme from *P. putida* NCIB9816 (You *et al.*, 1990). Perhaps the major differences are related to the charged residues and the effects on isoelectric point (see Table 3.1).

3.2.5 CaMV 35S-SH-L fusion and generation of transgenic tobacco plants expressing high levels of SH-L

The CaMV35S promoter in the binary vector pROK2 (Bevan, 1984) was fused to SH-L to allow constitutive expression of salicylate hydroxylase. As the analysis of *SH-L* sequence indicated that sites for *Xba*I and *Eco*RV were suitable enzymes to generate a CaMV 35S-SH-L transcriptional fusion, the *SH-L* gene in pSK-*SH-L* was then digested with *Xba*I and *Eco*RV (see Fig.3.2) and cloned into *Xba*I-*Sma*I-digested pROK2 to create a transcriptional fusion (Fig.3.9). After growth in *E. coli*, the p35S-*SH-L* plasmid was purified and then electroporated (Shen and Forde, 1989) into *Agrobacterium tumefaciens* strain pGV2260. Transgenic tobacco (*Nicotiana tabacum*) Samsun NN lines harbouring 35S-SH-L transgenes were generated using standard leaf disc transformation procedures (Draper *et al.*, 1988). A total of 20 kanamycin resistant plantlets were used for further study.

3.2.6 Analysis of transgenic tobacco plants

DNA was extracted from different lines (2.1) and PCR reactions were performed using SH-L primers. The plants from which a c1300bp DNA fragment band (see 3.2.1) was amplified were chosen for further study.

3.2.6.1 Identification of high expressing SH-L lines

The activity of salicylate hydroxylase was measured in crude leaf extracts of several transgenic lines (those showing the appropriately sized band after PCR) using the assay

Figure 3.9 *SH-L* fusion to CaMV35S promoter

The *SH-L* gene in pSK-*SH-L* was digested with *Xba*I and *Eco*RV (see Fig.3.2) and cloned into *Xba*I-*Sma*I-digested pROK2 to create pROK2-*SH-L*.

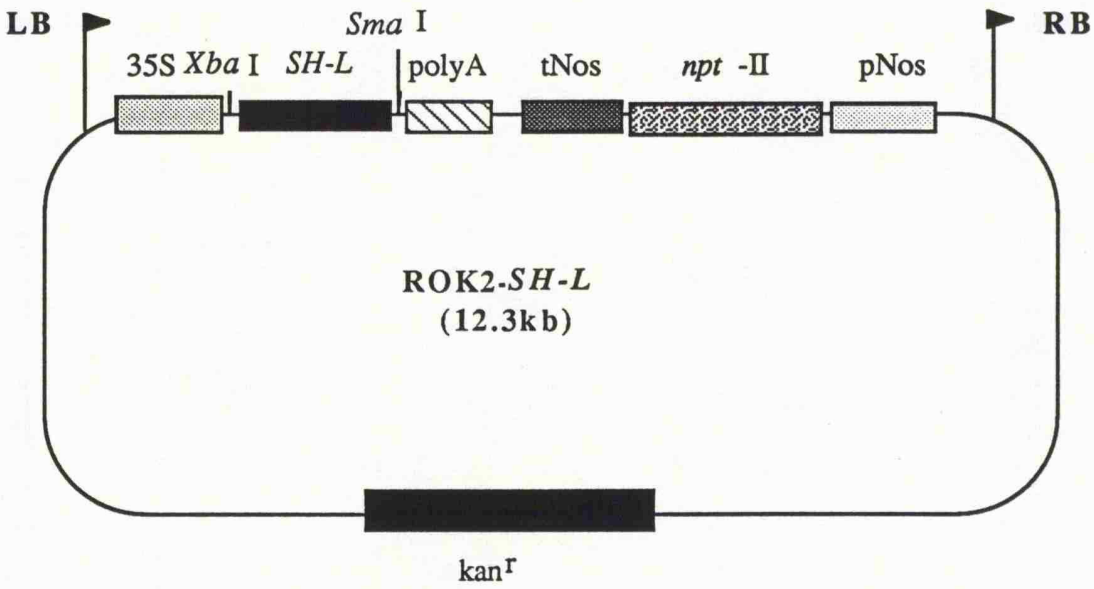

LB
Sma I
RB
35S Xba I
SH-L
polyA
tNos
npt -II
pNos
ROK2-SH-L
(12.3kb)
kanr

Figure 3.10 SH-L expression in tobacco

Wild type tobacco seedling germinated on MS medium with 1 mM SA remained white (left), while 35S-SH-L tobacco seedling went brown (right).

developed by Yamamoto *et al.* (1965). Three lines (1, 4 and 7) showed high SH-L specific activity (> 0.3 U/mg of total protein) and were selected for further analysis.

Explants from line 1, 4 and 7 were treated with 1 mM SA. After 2 days, PR1a protein level was determined by western blotting using PR1a antisera. There was reduced PR1a protein accumulation in line 1 and line 7 but no PR1a induction was detected in line 4. Therefore, Line 4 was selected for further study.

3.2.6.2 Progeny of 35S-SH-L line 4

35S-SH-L line 4 seeds (T1) were germinated on MS medium containing 100 μg/ml kanamycin and approximatlely 75% of progeny exhibited resistance to kanamycin, implying a single transgene was segregating in the population. 10 kanamycin resistant seedlings were transplanted and seeds (T2) from these 10 plants were collected individually. We predicted that transgenic lines producing T2 progeny which were all kanamycin resistant were homozygous for the 35S-SH-L construct; these were identified and used for further experiments.

3.2.7 Metabolism of ring ^{14}C labelled SA in wild type and 35S-SH-L tobacco

In preliminary experiments, the seeds of SH-L were germinated on MS medium containing 1mM SA, the roots and surrounding medium exhibited a brown discolouration (probably due to the oxidation of catechol) while the wild type tobacco roots remained white (Fig.3.10). Therefore, as catechol accumulation could be a significant effect in SH-L transgenic tobacco, SA metabolism was studied in the detached leaves to determine whether the plants can degrade high levels of SA and to see if catechol accumulated.

Wild type and 35S-SH-L-4 plants were fed through the petiole with uniformly ring-labelled ^{14}C SA and the leaves were harvested 4 hours later. The leaf material was extracted in 90% methanol and centrifuged. The pellet was resuspended with 100% methanol and centrifuged. The two supernatant fractions were combined, dried and resuspended in 1 ml methanol. Samples containing equal amounts of radioactivity were loaded into the HPLC (2.20) and separated by reverse phase analysis utilising a gradient of acetonitrile/water increasing from 5-40% acetonitrile in 30 min. ^{14}C SA and its metabolites were monitored using a radioactive monitor. It was found that ^{14}C SA had been converted completely to some unknown, highly polar compound (retention time about 3.5 min) within 4 hours in 35S-SH-L tobacco, while SA still remained intact in free (retention time 24 min) and conjugated (retention time 11 min and 13 min; identified by Dr. Alan Crozier in Botany Dept. of Glasgow University) forms in wild type tobacco (Fig.3.11).

It seems quite clear that catechol is not accumulated in SH-L plants because the retention time of catechol is about 10.5 minutes but the retention time of the unknown compound is at 3.5 minutes (Fig.3.11).

3.2.8 Comparison of nahG 10 and SH-L 4 plants

At about the time I was screening my 35S-SH-L plants, the experiments utilising *nahG* from Ciba were published (Gaffney *et al.*, 1993). In their material, they found that only very few of their transgenic plants have low levels of SA after TMV infection. Some seeds of their best line 35S-nahG 10 were kindly sent to us by the Ciba group. This made it possible for us to do a few experiments to compare nahG 10 (best line of 35S-nahG) and SH-L 4 (best line of 35S-SH-L).

3.2.8.1 Transcription level in nahG 10 and SH-L 4 plants

RNA was extracted from nahG 10 and SH-L 4 plants using the method described in 2.2 and RT-PCR was performed (method 2.15). The result is shown in Figure 3.12. The experiments were repeated twice and the same results were obtained. Although RT-PCR is not a very quantitative measurement, it suggests that the *nah*G transcript accumulation is relatively poor as compared to *SH-L*.

3.2.8.2 Efficiency of clearing SA in nahG 10 and SH-L 4 plants

NahG 10 plants were also fed with ring-labelled ^{14}C SA (see 3.2.7) and it was found that SA was still detected sometimes in nahG 10 plants, while SA was completely degraded in SH-L 4 plants (Fig.3.13). This data could imply that SA is degraded more efficiently in SH-L 4 plants than in nahG 10 plants.

Figure 3.11 Metabolism of ^{14}C labelled SA in wild type and 35S-SH-L tobacco

Ring ^{14}C labelled SA was fed to wild type and 35S-SH-L tobacco through the petiole for 4 h. Some free SA remained (retention time 24 min) and some was converted to conjugated SA derivatives (retention time 11 and 13 minute) in wild type tobacco (lower), but all free SA was converted to some unknown high-polar compound (retention time 3.5 minute) in 35S-SH-L tobacco (upper).

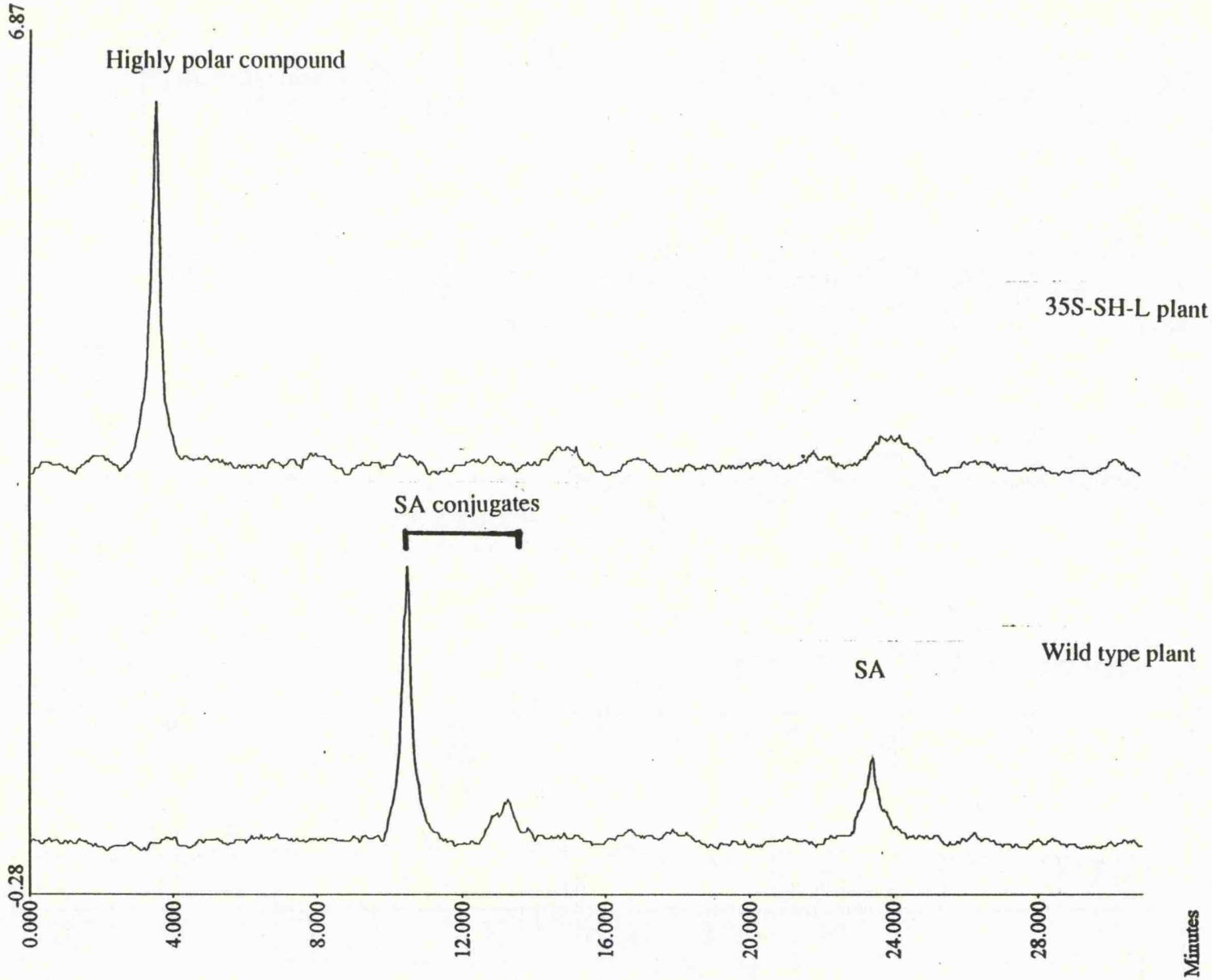

6.87
Highly polar compound
35S-SH-L plant
SA conjugates
Wild type plant
SA
-0.28
0.000
4.000
8.000
12.000
16.000
20.000
24.000
28.000
Minutes

Figure 3.12 Transcription level of salicylate hydroxylase in 35S-nahG 10 and 35S-SH-L 4

Total RNA was extracted and subjected to RT-PCR (see 2.15). Amplified DNA was run on a 1% agarose gel with ethidium bromide and photographed under UV light.

Lane 1 RT-PCR result in 35S-SH-L 4 tobacco

Lane 2 RT-PCR result in 35S-nahG 10 tobacco

Lane 3 RT-PCR result in wild type tobacco

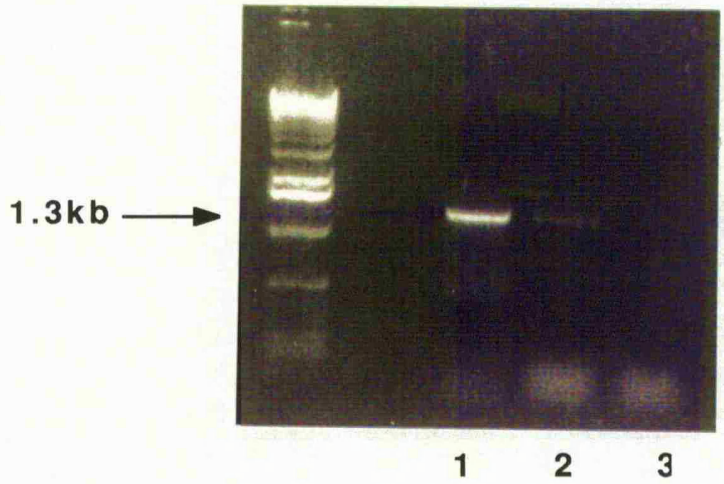
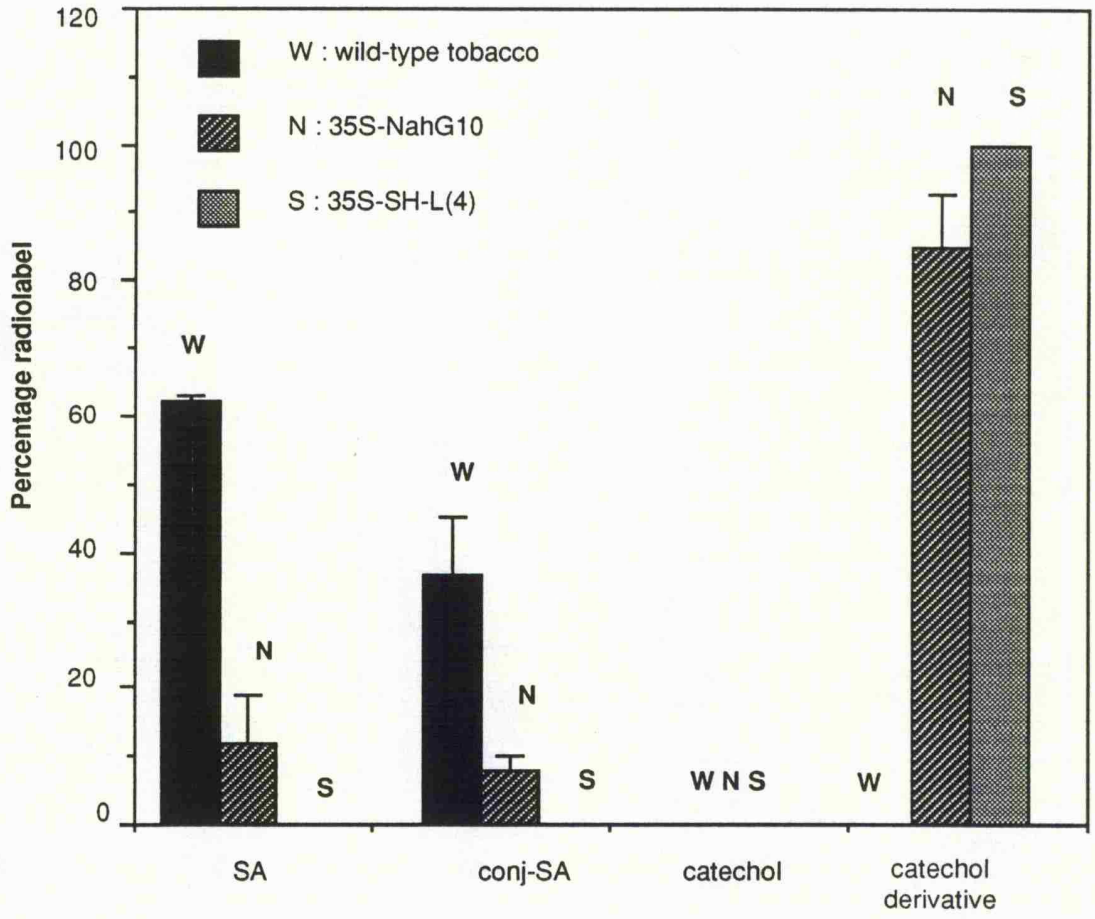

1.3kb
1
2
3

Figure 3.13 Metabolism of ^{14}C labelled SA in wild type, 35S-nahG 10 and 35S-SH-L 4 tobacco

Ring ^{14}C labelled SA was fed to wild type, 35S-nahG 10 and 35S-SH-L 4 tobacco through the petiole for 4 h. The percentage of the conversion of ^{14}C labelled SA to conjugates, catechol and other products is presented. Means ± SE shown, n=3. Catechol derivative referred to the highly polar compound shown in Fig.3.11.


120
100
80
60
40
20
0
Percentage radiolabel
W : wild-type tobacco
N : 35S-NahG10
S : 35S-SH-L(4)
W
N
S
W
N
S
W N S
W
N
S
SA
conj-SA
catechol
catechol derivative

3.3 Conclusion and discussion

3.3.1 Differences exist between *SH-L* and *nahG* gene.

The *SH-L* gene coding for salicylate hydroxylase has been cloned from *Pseudomonas putida* and proved to be functional in *E. coli*. The gene has been sequenced and it has been found that differences do exist between *SH-L* and the published *nahG* sequence, though the two genes are very closely related. Whether these differences could lead to different specific activities in plant tissue and/or influence the stability of the two enzymes still remains unknown.

3.3.2 Differences exist between SH-L and nahG plants.

Transgenic tobacco plants constitutively expressing a high level of salicylate hydroxylase (SH-L) activity have been identified. Analysis clearly showed that SH-L is able to degrade a large amount of SA in transgenic tobacco. However, a continuous high level of SA supplied to the SH-L transgenic tobacco could lead to the abnormal growth of the plants (see Fig.3.9, stunted brown roots). It remains unknown whether SH-L is more stable or more efficient than nahG. However, it seems that we have at least obtained a transgenic line expressing stabily a high level of salicylate hydroxylase. The differences between nahG 10 and SH-L 4 plants are: first, *nah*G transcript accumulation was relatively poor as compared to SH-L (Fig.3.12); second, SA seems to be metabolised more efficiently in SH-L plants than in nahG plants (Fig.3.13). In the next chapter, I have investigated the effect of SH-L expression on plant defence responses.

Chapter 4
Effects of constitutive expression of salicylate hydroxylase on plant defence responses

4.1 Introduction

Since transgenic tobacco plants constitutively expressing SH-L have been generated and the high expressing line has been identified (Chapter 3), it is feasible to test the role of SA in the defence responses. In particular, a major aim was to test the effect of SA depletion on PR protein expression, the development of HR lesions and the growth and containment of pathogens in wild type and SH-L plants.

4.1.1 Pathogens used in the experiments

Two types of pathogens were used in experiments; TMV was chosen as an example of an intracellular pathogen, whilst *Pseudomonas syringae* pathovars were used as typical extracellular pathogens. Both types of pathogen have a long history of use for basic pathology and SAR studies in tobacco (Sigee, 1993) and each is introduced below.

4.1.1.1 TMV

The TMV-tobacco *N* gene interaction has long served as a classical model system for the study of plant resistance responses to pathogens. TMV is a mechanically transmitted positive-sense RNA virus that encodes four proteins: two are required for viral replication, one is for viral cell-to-cell movement and one coat protein is required for viral RNA encapsidation (Dawson, 1992). An antibody of the TMV coat protein can be used as a sensitive detector of viral presence. TMV infection of tobacco plants containing the *N* gene induces HR within 48 hr postinfection, and TMV is restricted to the region immediately surrounding the necrotic lesions (Culver *et al.*, 1991; Padgett *et al.*, 1993). The *N* gene is a

single locus, dominant gene, and is believed to be a resistance gene. In tobacco plants which do not contain the *N* gene (tobacco cultivars *nn*), TMV is allowed to spread systemically and mosaic symptoms can develop. The *N* gene has recently been isolated by transposon tagging (Whitham *et al.*, 1994).

TMV-mediated HR is accompanied by induction of defense responses which are rapidly activated in *NN* plants but not in *nn* plants (Lamb *et al.*, 1989). A second challenge of TMV on non-infected systemic leaves a week after the primary infection causes much fewer and smaller lesions due to the onset of SAR (see 1.2.3). Figure 4.1 shows the lesions of first and second challenge of TMV on *N*-tobacco leaves. Hence, lesion diameter measurements can provide an accurate indication that a SAR state has been achieved.

4.1.1.2 *Pseudomonas syringae* pathovars

The other pathogens we used for investigating SA and local defense responses are *Pseudomonas syringae* pathovars (*P. s.* pv.). The ability of bacteria to elicit the HR was discovered 30 years ago when Klement and coworkers injected the intercellular spaces of tobacco leaves with high levels of a range of bacterial species and observed three main types of result (Klement, 1963; Klement *et al.*, 1964): One was that the areas infiltrated with *P. s.* pv *syringae* collapsed and desiccated within 24 hr, with no spread of bacteria to surrounding tissues (a typical incompatible reaction); another was that those infiltrated with *P. s.* pv *tabaci* produced slowly developing and progressively spreading water-soaked lesions and resulted in disease (a typical compatible reaction); the last was that no observable reaction occured after infiltration of the saprophytic bacterium *Pseudomonas fluorescens*. At lower levels of inoculum, *P. s.* pv *syringae* caused no visible reaction, whereas *P. s.* pv *tabaci* again caused disease. Further, it was found that new gene products were observed as early as 4 h after inoculation in the incompatible relationship whereas no detectable effect on gene expression was found up to 9 h in the compatible relationship (Somlyal *et al.*, 1988). Later on, it was found that unlike the *N* gene-TMV interaction, the

ability of *P. s.* pv *syringae* to elicit the HR in non-host tobacco is controlled by multiple recognition events and not by a single dominant gene (Niepold *et al.*, 1985; Lindgren *et al.*, 1986; Willis *et al.*, 1991; He *et al.*, 1993).

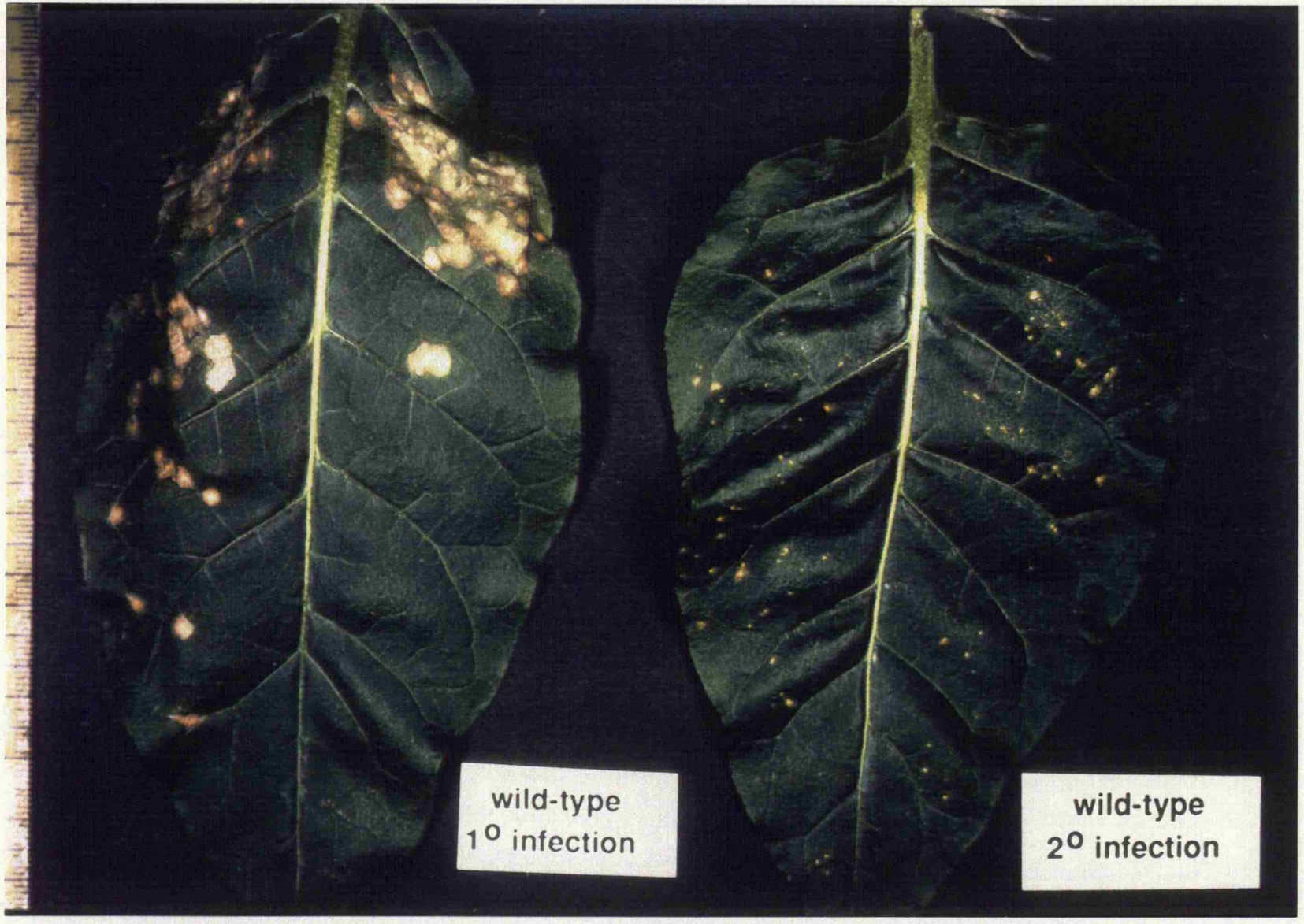
4.1.2 Aims of the chapter

As an initial aim, the role of SA in SAR was investigated by preventing SA accumulation, through the constitutive expression of SH-L. The induced expression of PR1a protein was used in my experiments as a marker for activation of the systemic signalling pathway for SAR establishment.

At the same time, the role of SA in the local response to pathogen attack was investigated. The levels of free and conjugated salicylic acid rise more than fifty fold in the immediate vicinity of developing HR lesions and reach concentrations nearly 70μM (Malamy *et al.*, 1990). This is rather high for a chemical thought to be a signal molecule. Thus, I expected that inhibiting SA accumulation may effect PR protein synthesis around developing lesion and could also have some role in HR.

Figure 4.1 TMV lesions in unchallenged leaves and in plants exhibiting SAR

TMV was inoculated into Samsun NN leaves and lesions photographed after 7 days (left). Secondary TMV infection was performed on systemic non-infected tobacco (Samsun NN) leaves 14 days after first infection (right). Lesions are fewer and smaller in secondary infected leaf. The picture was taken 7 days after secondary infection.


wild-type
1° infection
wild-type
2° infection

4.2 Results

4.2.1 Catechol-mediated effects on PR1a induction

Before carrying out detailed experiments on the SH-L plants, it was important to be certain that catechol, the product of SA degradation, would not interfere with experiments.

4.2.1.1 The effect of catechol on PR1a induction by SA

There are no reports of catechol being a SAR inducer. However, it is possible that catechol can inhibit PR1a induction particularly since it seemed very harmful to plants when leaf discs were treated with 1 mM catechol (unpublished observation, pers. comm. with Dr. L. Mur). The effect of catechol was investigated using transgenic PR1a-GUS plants supplied by Dr. Simon Firek (Leicester). PR1a induction by SA can be estimated by the rise in GUS activity at different time points. When SA and catechol were applied to plants together at different concentrations, it was found PR1a induction by SA was inhibited by catechol at 1000 μM but at concetrations lower than 500 μM catechol had no effect on PR1a induction (Fig.4.2). Maximum SA concentrations in wild type tobacco after TMV infection are about 70 μM. At an equivalent level, catechol seems unable to either induce PR proteins or exhibit any general toxic effects.

4.2.1.2 Catechol in the TMV-infected 35S-SH-L tobacco leaf tissue

Although catechol has no effect on PR1a induction by SA at the concentration equivalent to SA in TMV-infected tobacco, I attempted to determine the exact amount of catechol in SH-L plants in TMV infected tobacco as there may be a high concentration of catechol in some cellular compartment.

Figure 4.2 The effect of catechol on PR1a induction by SA

Leaf intercellular spaces of PR1a-GUS Samsun NN tobacco plants were infiltrated with increasing levels of catechol and SA. GUS activity was measured after 2 days. Means ± SE shown, n = 3.

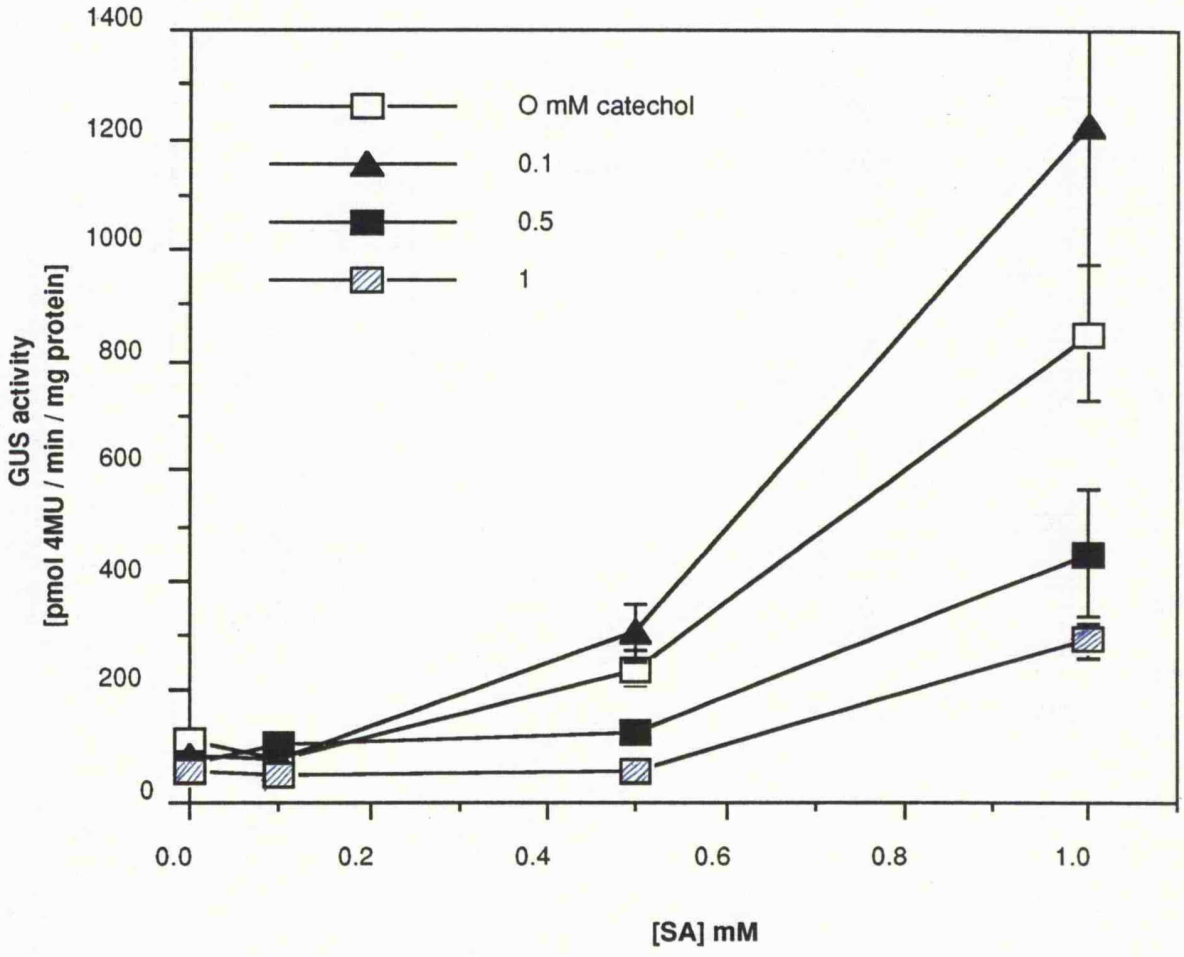
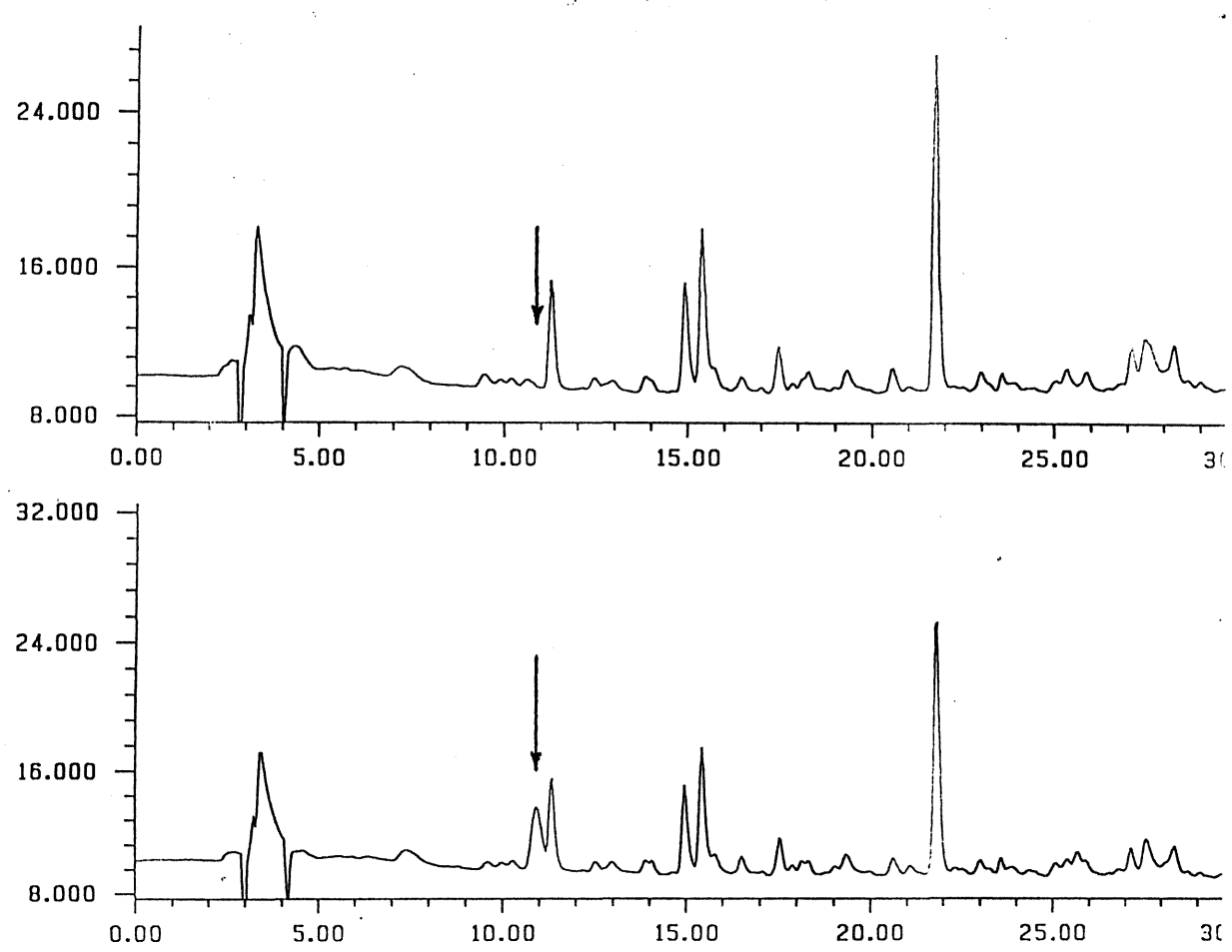

1400
1200
1000
800
600
400
200
0
GUS activity
[pmol 4MU / min / mg protein]
O mM catechol
0.1
0.5
1
0.0
0.2
0.4
0.6
0.8
1.0
[SA] mM

Figure 4.3 Catechol in the TMV-infected 35S-SH-L tobacco leaf tissue

UV traces of methanol extracts from TMV-infected 35S-SH-L tobacco leaf tissue material (upper) and an identical sample spiked with 100 ng standard catechol (lower) are shown. Arrows indicate the position of catechol.


24.000
16.000
8.000
0.00
5.00
10.00
15.00
20.00
25.00
32.000
24.000
16.000
8.000
0.00
5.00
10.00
15.00
20.00
25.00

Catechol has a retention time about 10.5 min in the UV trace when analysed by HPLC (pers. comm. with Dr. Alan Crozier, Glasgow University), and as expected a new peak appeared at about 10.5 min after spiking the TMV-infected 35S-SH-L sample with catechol (Fig.4.3). The traces show clearly that catechol is not accumulated in TMV-infected 35S-SH-L tobacco.

4.2.2 Primary TMV lesion development in 35S-SH-L plants

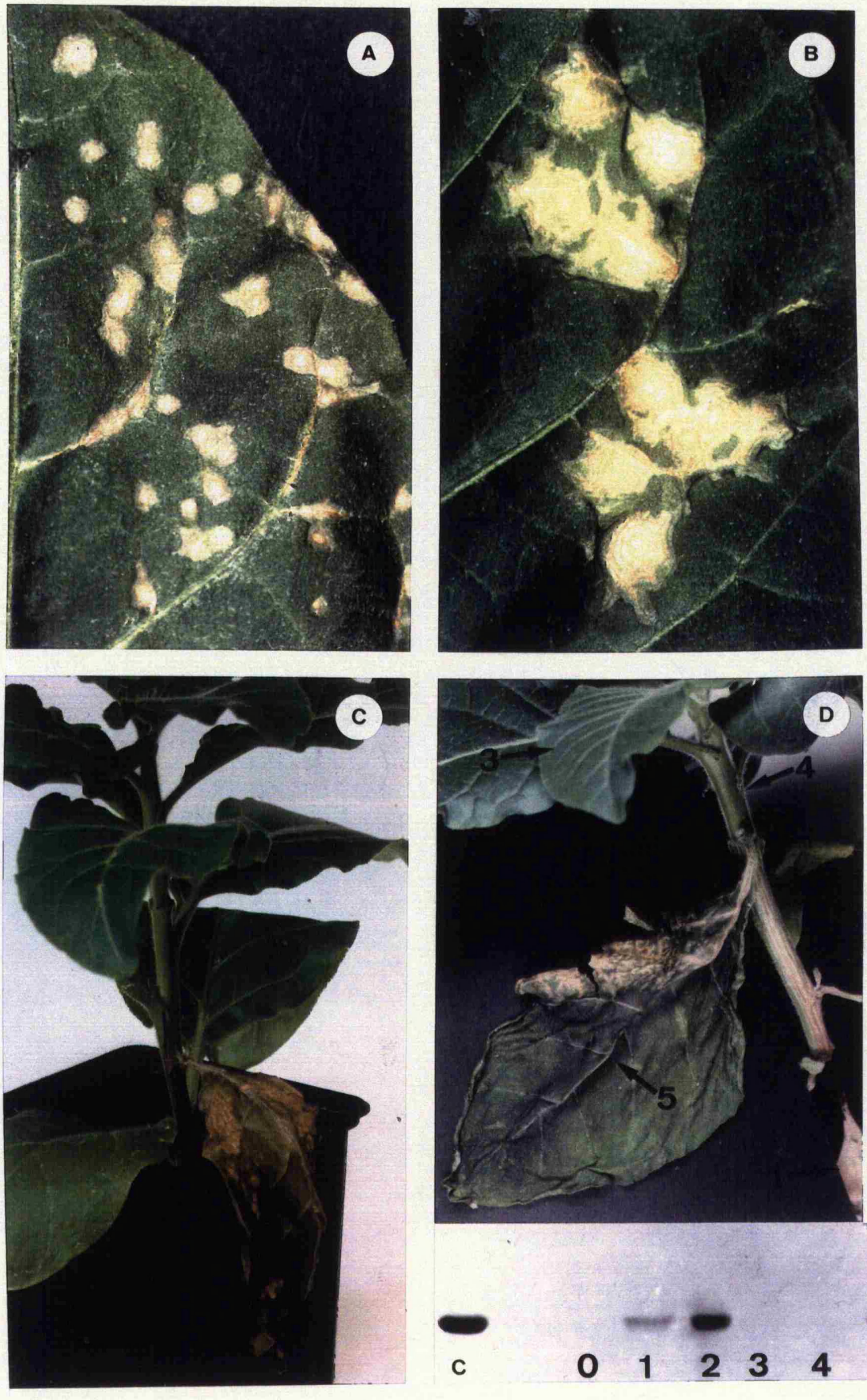
4.2.2.1 TMV lesion phenotype in wild type and 35S-SH-L tobacco plants

TMV strain U1, the gift of R. F. White (Rothamsted Experimental Research Station, UK), was applied to wild type *NN* and 35S-SH-L *NN* tobacco by the method described in 2.28. HR lesions appeared about 3 days after inoculation. Phenotypically, lesions forming following TMV infection of CaMV 35S-SH-L plants were much larger than normal (Fig.4.4a & 4.4b). In addition, within the lesions on CaMV35S-SH-L plants there appeared many concentric necrotic rings of increasing diameter. (Fig. 4.4b). This may indicate that lesions grow in diameter in a discontinuous fashion.

Following TMV infection of wild type tobacco plants expressing the *N*-gene, the virus remains confined to the lesion within the primary infected leaf. In contrast, TMV-infected CaMV35S-SH-L tobacco lesions appeared not to be limited within the infected leaf, and spreading lesions coalesce. By 14 days, such "spreading-necrosis" had entered the stem and occasionally appeared on other non-inoculated leaves (Fig.4.4c). These symptoms are reminiscent of cell-to-cell viral-dispersal that occurs following temperature shift experiments (Ray White, Luis Mur, pers. comm). Protein samples from various tissue exhibiting spreading necrosis and also appearently healthy tissues were tested for the presence of TMV using TMV-coat protein anti-sera. This revealed, (Fig. 4.4d) that the appearance of the spreading necrosis phenotype could be correlated with the presecence of TMV virus, implicating viral "escape" to be one cause of the loss in lesion limitation.

Figure 4.4 TMV lesion phenotype in wild type and 35S-SH-L tobacco

a. Day 7 TMV lesion in wild type tobacco
b. Day 7 TMV lesion in 35S-SH-L tobacco
c. Cell-to-cell spreading lesion in 35S-SH-L tobacco
d. TMV presence detected by western blotting using TMV coat protein antibody
   Lane c = positive control
   Lane 0 = negative control
   Lane 1 - 5 = material from corresponding areas in photograph


A
B
C
D
3
4
5
C
0 1 2 3 4 5

4.2.2.2 TMV lesion size in wild type and 35S-SH-L tobacco

Actual lesion size was measured in wild type and 35S-SH-L tobacco at different time points after inoculation. Ten lesions per plant (3 plants each line) were taken into account. Lesion size in 35S-SH-L day 7 after TMV infection was more than twice that of lesions in wild type tobacco (Fig.4.5).

4.2.2.3 SA levels in TMV-infected wild type and 35S-SH-L tobacco plants

SA levels in wild type and 35S-SH-L line 4 plants were measured using the method described in 2.19 and 2.20. After 7 days post TMV infection, the SA level in local, infected leaves increased to 2.1 ± 0.3 ug/g fresh weight ($n = 3$) in wild type Samsun NN tobacco. In contrast SA was not detected in 35S-SH-L line 4 plants (Fig.4.6) used in all experiments.

4.2.2.4 PR protein induction in wild type and 35S-SH-L tobacco plants after TMV infection

The levels of PR1a protein in wild type and 35S-SH-L line 4 plants were estimated by western blotting. Figure 4.7 showed that infection of wild-type tobacco with TMV resulted in the expression of endogenous PR1a in infected tissue within 2 days. In contrast, PR1a was almost undetectable up to 7 days after TMV infection in 35S-SH-L transformants (Fig.4.7). The data suggests that SA is essential for the activation of PR protein gene transcription at sites of necrotic lesion formation.

Just as I had finished these experiments, the Ciba group (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994) reported that PR1a expression was absent in systemic leaves but only slightly reduced in TMV-infected lesions after 3 days. For comparison, I tested, in plants grown under the same conditions, PR1a induction in infected leaves in nahG 10 and SH-L 4. As

described previously by the Ciba laboratory, we saw only a reduction in PR1a expression in TMV-infected leaves of 35S-nahG 10 plants (data not shown), whilst PR1a was absent around lesions induced on 35S-SH-L leaves (Fig.4.7).

4.2.2.5 PR induction by INA in SH-L plants

Externally supplied INA has been shown to induce systemic acquired resistance and elevate expression of SAR-associated genes (1.1.3.3). Application of INA to 35S-SH-L plants induced the same levels of PR1a accumulation as compared to INA treatment of wild-type tobacco (Fig. 4.8). This indicated that INA is not a substrate for SH-L and could induce PR1a production in the plants absence of SA. As a control, Figure 4.8 shows that infiltration with 4hBA was ineffective as an inducer of PR proteins and the PR1a protein was not present in SA-treated leaves from transgenic tobacco plants expressing the SH-L protein. This result indicated clearly that some steps downstream of SA in mechanisms activating PR gene expression are still functional in the absence of salicylate accumulation.

4.2.3 Systemic effect of local TMV infection in 35S-SH-L plants

4.2.3.1 PR1a induction in wild type and 35S-SH-L systemic uninfected leaves

14 days after first infection, PR1a levels in wild type and 35S-SH-L systemic uninfected leaves were measured by western blotting. Figure 4.9 clearly shows that no PR1a upregulation occurred in 35S-SH-L tobacco while PR1a protein did accumulate in wild type tobacco.

4.2.3.2 Lesion size of second TMV infection in wild type and 35S-SH-L tobacco

A second TMV infection was applied to systemic leaves 14 days after the first local infection and lesion size was measured. Figure 4.10 shows that the average secondary

Figure 4.5 Primary TMV lesion size in wild type and 35S-SH-L tobacco

Local TMV lesion size (diameter) is shown. 10 lesions per plant, 3 plants each line were taken into account.

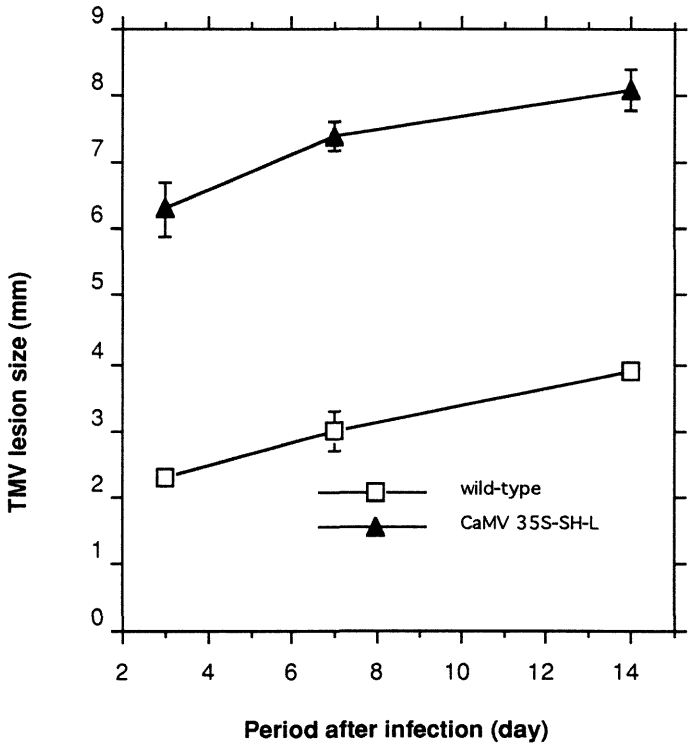

TMV lesion size (mm)
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
14
Period after infection (day)
wild-type
CaMV 35S-SH-L

Figure 4.6 SA levels in TMV-infected leaves from wild type and 35S-SH-L plants 7 days after inoculation

SA was measured as described in 2.19 and 2.20. Means ± SE shown, n = 3.

	wild type	35S-SH-L
SA levels (μg / gfw)	2.1± 0.3	n.d.*

* n.d. = not detected

Figure 4.7 PR1a induction around local TMV lesions in wild type and 35S-SH-L tobacco plants

Wild-type Samsun NN (WT) and Samsun NN 35S-SH-L (SH-L) tobacco plants were infected with TMV. Samples before infection and 3 or 7 days post-infection were taken. PR1a protein accumulation was determined by PR1a anti-serum after western blotting.

Lane 1 PR1a level in wild type tobacco at day 0

Lane 2 PR1a level in wild type tobacco at day 3

Lane 3 PR1a level in wild type tobacco at day 7

Lane 4 PR1a level in 35S-SH-L tobacco at day 0

Lane 5 PR1a level in 35S-SH-L tobacco at day 3

Lane 6 PR1a level in 35S-SH-L tobacco at day 7

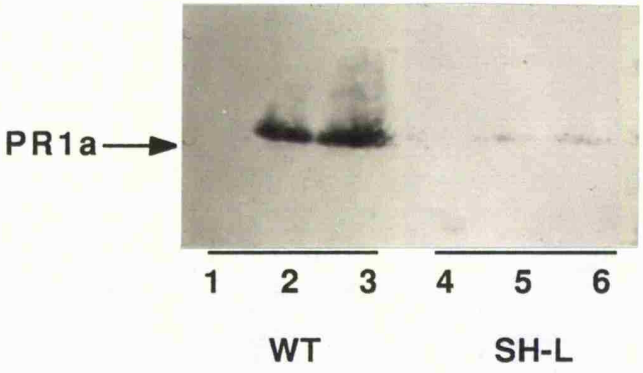

PR1a
1
2
3
4
5
6
WT
SH-L

Figure 4.8 Accumulation of PR1a protein following application of 2,6-dichloroisonicotinic acid (INA) in wild type and 35S-SH-L tobacco plants

Wild-type Samsun NN (WT) and Samsun NN 35S-SH-L (SH-L) tobacco plants were infiltrated with 1mM 4-hydroxybenzoic acid (4-hBA), 1mM salicylic acid (SA) or 0.5 mM INA by injection. After 2 days, PR1a protein accumulation was determined by PR1a anti-serum.

Lane 1 PR1a level in wild type tobacco infiltrated with 4-hBA

Lane 2 PR1a level in wild type tobacco infiltrated with SA

Lane 3 PR1a level in wild type tobacco infiltrated with INA

Lane 4 PR1a level in 35S-SH-L tobacco infiltrated with 4-hBA

Lane 5 PR1a level in 35S-SH-L tobacco infiltrated with SA

Lane 6 PR1a level in 35S-SH-L tobacco infiltrated with INA

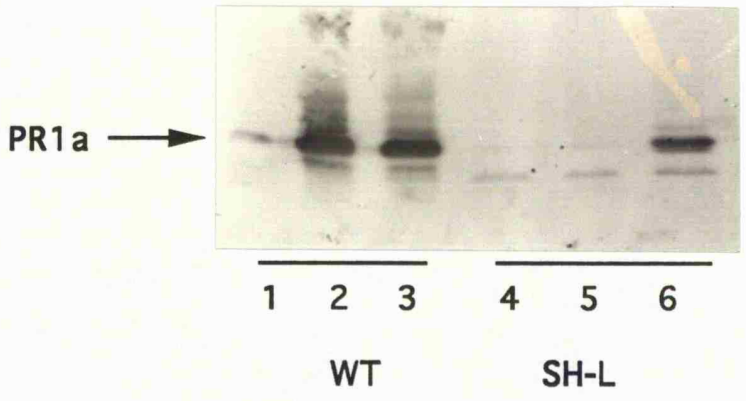

PR1a
1 2 3 4 5 6
WT
SH-L

Figure 4.9 PR1a induction in wild type and 35S-SH-L systemic uninfected leaves

Accumulation of PR1a protein in systemic uninfected leaves of Samsun NN and 35S-SH-L Samsun NN tobacco plants 14 days after primary TMV infection was determined by PR1a antiserum. 3 replicates. One mock-inoculation was used as control.

Lane 1 Systemic PR1a level in mock-inoculated wild type tobacco plant

Lane 2 Systemic PR1a level in TMV-infected wild type tobacco plant 1

Lane 3 Systemic PR1a level in TMV-infected wild type tobacco plant 2

Lane 4 Systemic PR1a level in TMV-infected wild type tobacco plant 3

Lane 5 Systemic PR1a level in mock-inoculated 35S-SH-L tobacco plant

Lane 6 Systemic PR1a level in TMV-infected 35S-SH-L tobacco plant 1

Lane 7 Systemic PR1a level in TMV-infected 35S-SH-L tobacco plant 2

Lane 8 Systemic PR1a level in TMV-infected 35S-SH-L tobacco plant 3

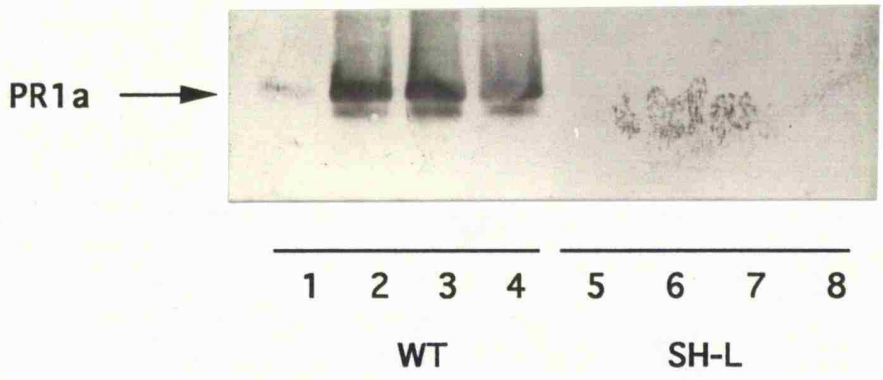
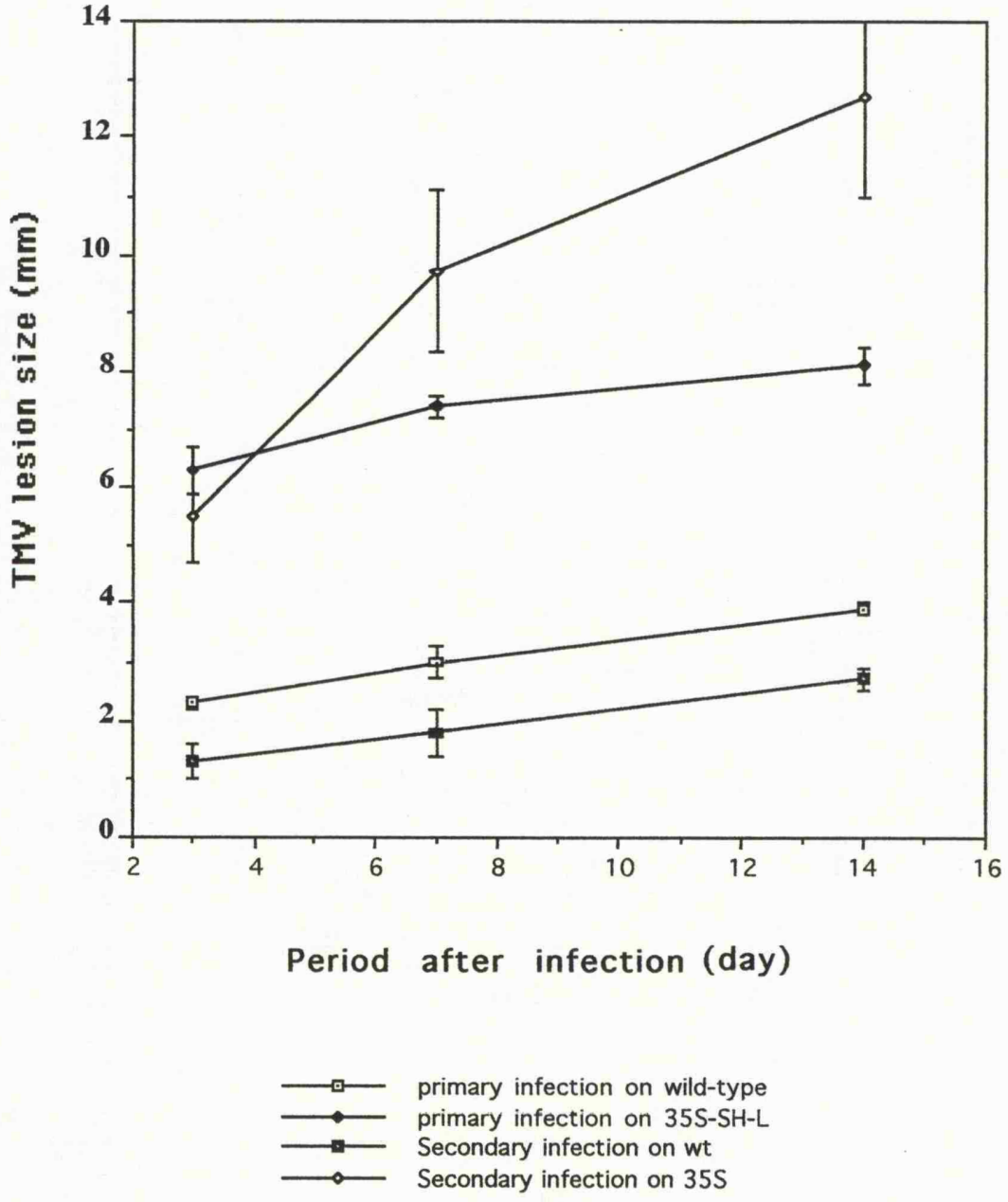

PR1a
1 2 3 4 5 6 7 8
WT
SH-L

Figure 4.10 Primary and secondary TMV lesion sizes in wild type and 35S-SH-L tobacco

Primary and secondary TMV lesion sizes (diameter) are shown. 10 lesions per plant, 3 plants each line were taken into account.

Secondary TMV infection was applied 14 days after first infection.

Means ± SE shown, n = 3.


TMV lesion size (mm)
0
2
4
6
8
10
12
14
2
4
6
8
10
12
14
16
Period after infection (day)
primary infection on wild-type
primary infection on 35S-SH-L
Secondary infection on wt
Secondary infection on 35S

lesion size in wild type plants was reduced severely, while secondary lesion size in 35S-SH-L tobacco was about the same as that of first infection. The data showed clearly that no SAR was exhibited in plants unable to accumulate SA.

4.2.4 Effects of bacterial infection in 35S-SH-L plants

4.2.4.1 Expression pattern of 35S-GUS transgenic tobacco following challenge with *P. s.* pv. *syringae* and *P. s.* pv. *tabaci*

There has been a report that the 35S promoter is inducible by SA (Qin *et al.*, 1994) and so I investigated the expression pattern of a 35S-GUS fusion to see if this effect could be visualised easily. In these experiments I collaborated with Dr. Luis Mur to study the role of SA in the local defense response following *Pseudomonas syringae* infection.

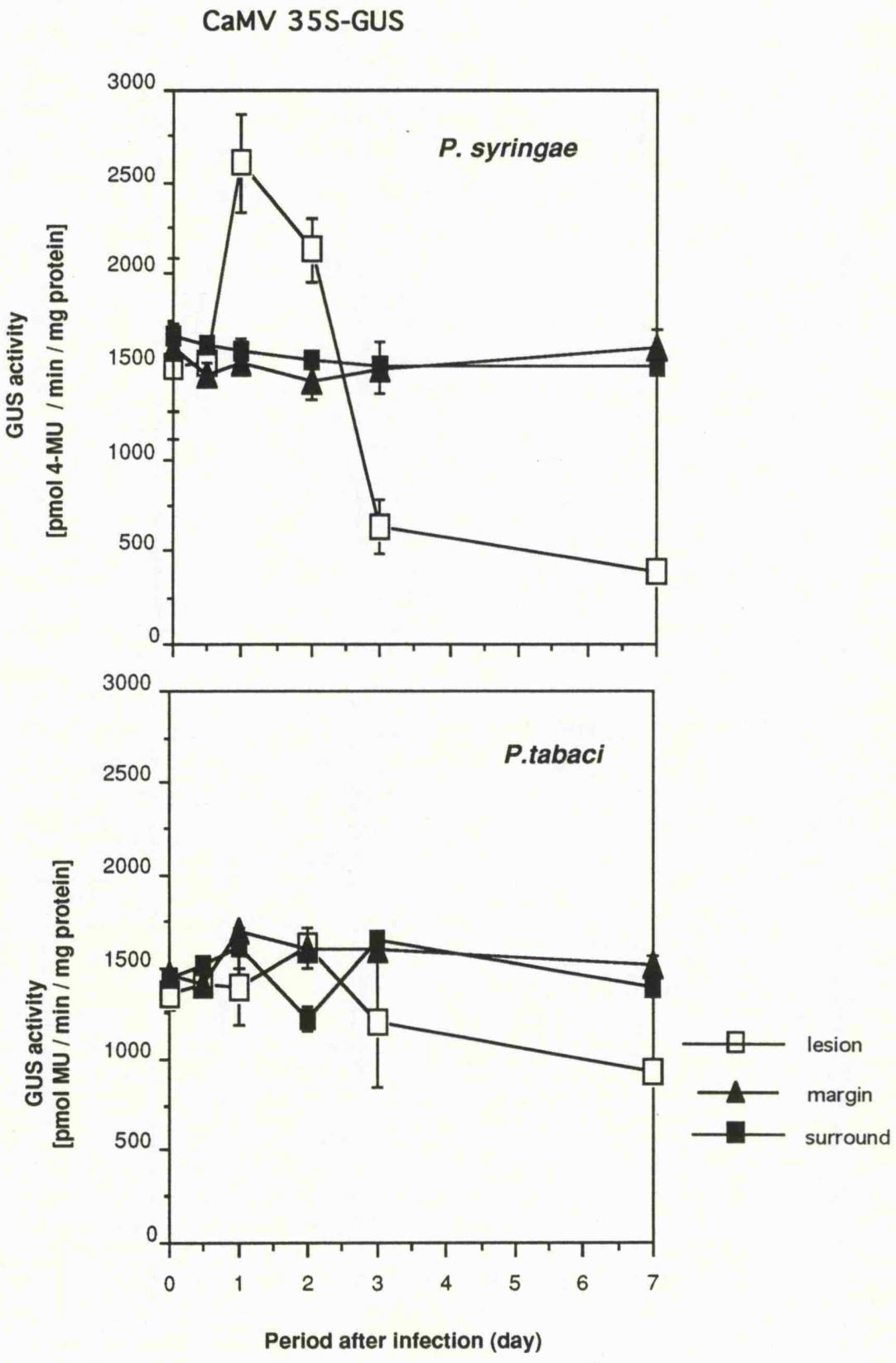
GUS activities were measured after challenging 35S-GUS transgenic tobacco with *P. s.* pv. *syringae* and *P. s* . pv. *tabaci*. Figure 4.11 showed that GUS activity basically remained the same in the margin and surrounding area after infection with *P.s.* pv *syringae* and *P. s.* pv *tabaci*. However, in the lesion there was some increase in GUS activity after infection with *P. s.* pv *syringae* which then dropped off probably due to rapid cell death. Plants infected with *P.s.* pv *tabaci* showed slightly decreased GUS activity in the lesion area. These experiments established that it is possible to monitor 35S promoter activity even in the centre of developing HR lesions and that this promoter can drive SH-L expression during the infection process.

4.2.4.2 Lesion phenotype in wild type and 35S-SH-L tobacco after bacterial infection

Wild type tobacco plants inoculated with *P. s.* pv *syringae* or *P. s.* pv. *tabaci* (Fig.4.12a and b) produced lesions typical of incompatible and compatible responses respectively.

Figure 4.11 Expression pattern of 35S-GUS transgenic tobacco following challenge with *P. s.* pv. *syringae* and *P. s.* pv. *tabaci*

Leaves of 35S-GUS Samsun NN tobacco were infiltrated with *P. s.* pv. *syringae* or pv *tabaci*. Samples were periodically taken from necrotic lesions (—□—) and from margin (area just adjacent to lesions) (—▲—) and surrounding tissue (—■—). Means ± standard errors shown. n = 3.


CaMV 35S-GUS
P. syringae
P.tabaci
GUS activity
[pmol 4-MU / min / mg protein]
GUS activity
[pmol MU / min / mg protein]
3000
2500
2000
1500
1000
500
0
0
1
2
3
4
5
6
7
Period after infection (day)
lesion
margin
surround

Lesions started to form 6 h after *P. s.* pv *syringae* infection on wild type tobacco and became very dry and were still restricted to a defined region after 7 days. Lesions appeared 24 h after *P. s.* pv. *tabaci* infection on wild type tobacco and became water-soked, chlorotic and thereafter spread throughout the leaf. The method to infect plants was described in 2.28. 35S-SH-L transgenic tobacco inoculated with the normally incompatible *P. s.* pv. *syringae* strain exhibited disease symptoms more typical of a compatible interaction (Fig.4.12c). The lesions formed 1 to 2 days after inoculation and the infected leaf became watersoaked and chlorotic, with evidence of spreading-necrosis after 7 days.

4.2.4.3 PR1a induction in wild type and 35S-SH-L tobacco after *P. s.* pv. *syringae* infection

PR1a was detected in wild type plants as little as 12 h after *P. s.* pv. *syringae* infection but PR1a accumulation was delayed until 24 h after *P. s.* pv. *syringae* infection in 35S-SH-L plants (Fig.4.13). A possible explanation for PR1a production in SH-L tobacco could be that a large amount of SA is produced in the vicinity of developing lesions which saturates the SH-L enzyme and this allows the expression of the PR1a gene.

4.2.4.4 Bacterial population in lesions arising in 35S-SH-L tobacco following infection with *P. s.* pv. *syringae*

The bacterial population decreased rapidly in the incompatible interaction of *P. s.* pv *syringae* with wild type plants (Fig.4.14). In contrast, a decrease in bacterial numbers was not observed in the inoculated zone (Fig.4.14) after inoculation with *P. s.* pv. *tabaci.* The bacterial population decreased to some extent when 35S-SH-L transgenic plants were inoculated with the normally incompatible *P. s.* pv. *syringae* strain (Fig.4.14) but there were still a large number of bacteria surviving (several logs more) compared to the number in wild type.

4.2.4.5 Bacterial escape around lesions arising following infection with *P. syringae* in wild type and 35S-SH-L tobacco

Figure 4.15 shows that there was no invasion of bacteria into adjacent tissue in wild type tobacco after infection with *P. s.* pv. *syringae* while bacterial numbers steadily increased in the surrounding tissue after inoculation with *P. s.* pv. *tabaci*. However, when 35S-SH-L transgenic plants were inoculated with the normally incompatible *P. s.* pv. *syringae* strain, the pathogen was found not to be confined to the original inoculated zone and the bacterial numbers increased with time in the surrounding area (Fig.4.15).

4.2.4.6 *P. s.* pv. *tabaci* lesions in 35S-SH-L tobacco

Superficially, inoculation of 35S-SH-L plants with *P. s.* pv. *tabaci* caused symptoms similar to those formed in interactions with wild type tobacco. In lesions, bacterial numbers rose at the same speed (Fig.4.16a), however, the invasion of these surrouding tissue occured much more quickly in 35S-SH-L tobacco than in wild type tobacco (Fig.4.16b). Due to the massive necrosis caused by 10^8 cfu/ml inoculum, 10^4 cfu/ml was used in this experiment.

Figure 4.12 Lesion phenotype in wild type and 35S-SH-L tobacco after bacterial infection

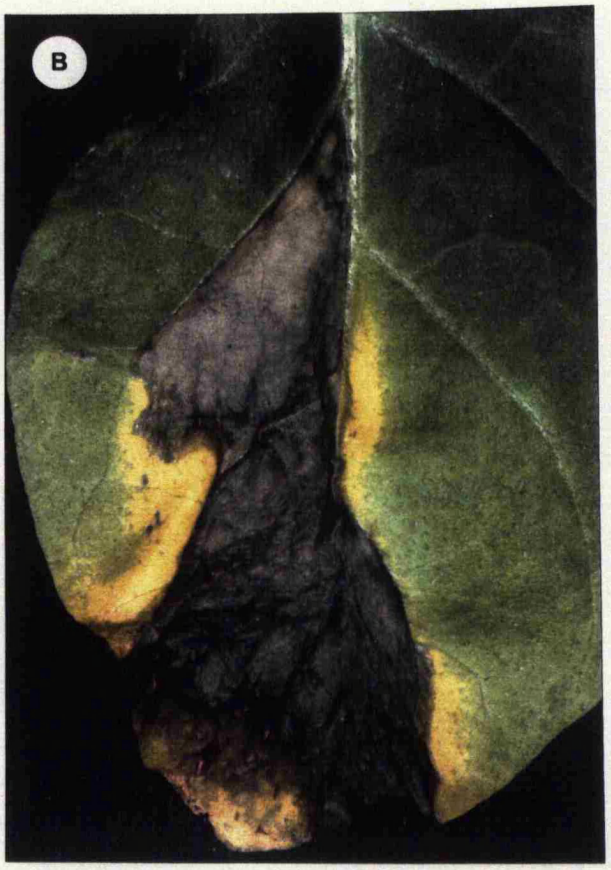
a. Wild type tobacco day 7 after inoculation with 10^8 cfu/ml *P.s.* pv. *syringae*

b. Wild type tobacco day 7 after inoculation with 10^8 cfu/ml *P.s.* pv. *tabaci*

c. 35S-SH-L tobacco day 7 after inoculation with 10^8 cfu/ml *P.s.* pv. *syringae*

d. Wild type tobacco (left) and 35S-SH-L tobacco (right) day 7 after inoculation with 10^4 cfu/ml *P.s.* pv. *tabaci*


A


B


C


D

Figure 4.13 PR1a induction after bacterial infection

Wild-type and 35S-SH-L tobacco plants were infected with *P.s.* pv. *syringae* . Samples at the inoculation zone before infection and after 6, 12, 24, 48 h post-infection were taken. PR1a protein accumulation was determined by PR1a anti-serum.

Lane 1	PR1a level in wild type tobacco at 0 h
Lane 3	PR1a level in wild type tobacco at 6 h
Lane 5	PR1a level in wild type tobacco at 12 h
Lane 7	PR1a level in wild type tobacco at 24 h
Lane 9	PR1a level in wild type tobacco at 48 h
Lane 2	PR1a level in 35S-SH-L tobacco at 0 h
Lane 4	PR1a level in 35S-SH-L tobacco at 6 h
Lane 6	PR1a level in 35S-SH-L tobacco at 12 h
Lane 8	PR1a level in 35S-SH-L tobacco at 24 h
Lane 10	PR1a level in 35S-SH-L tobacco at 48 h

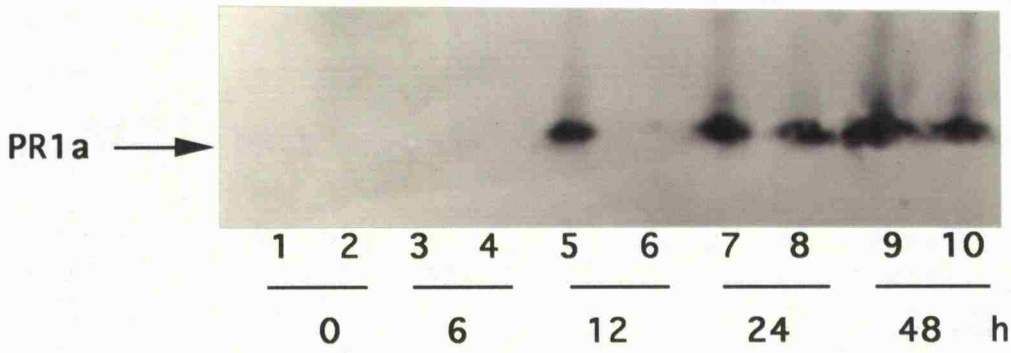
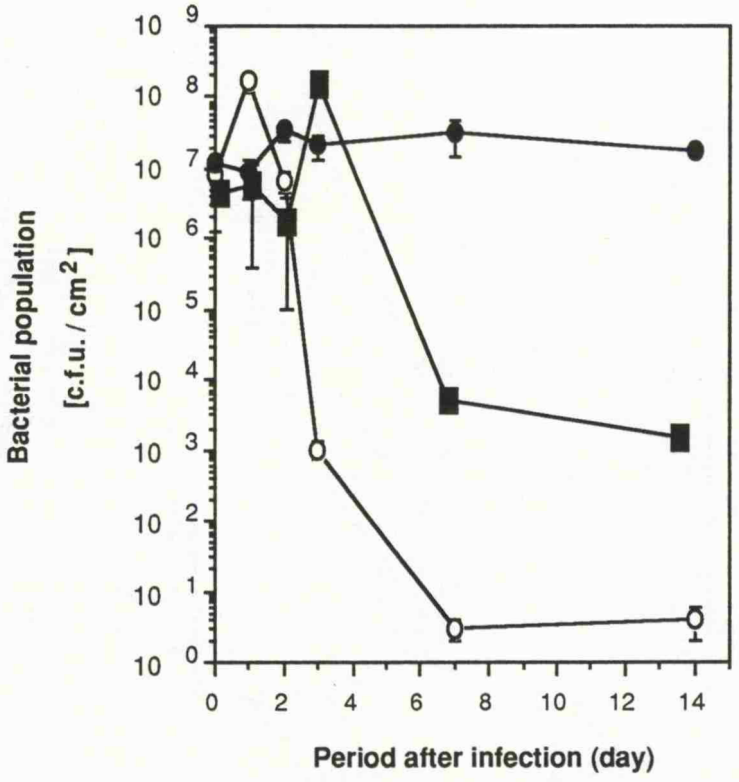

PR1a
1 2 3 4 5 6 7 8 9 10
0 6 12 24 48 h

Figure 4.14 Bacteria population in wild type and 35S-SH-L tobacco following infection with *P. s.* pathovars

P. s. pathovars were grown overnight in nutrient broth, centrifuged and then resuspended to an approximate concentration of 1 x 10^8 colony forming units (cfu) per ml in 10 mM phosphate buffer (pH 7.0). Tobacco leaves were infected with bacteria by injection of leaf intercellular spaces (see 2.28). Lesion materials were taken at different time points. Because the *P. s.* bacteria have a rif^R gene, it could selected following growth on a NA plate containing 50 μg/ml rifampicin. The number of cfu was counted at different time points.

Means ± SE shown, n = 3.



wild type infected with P. s. pv. syringae

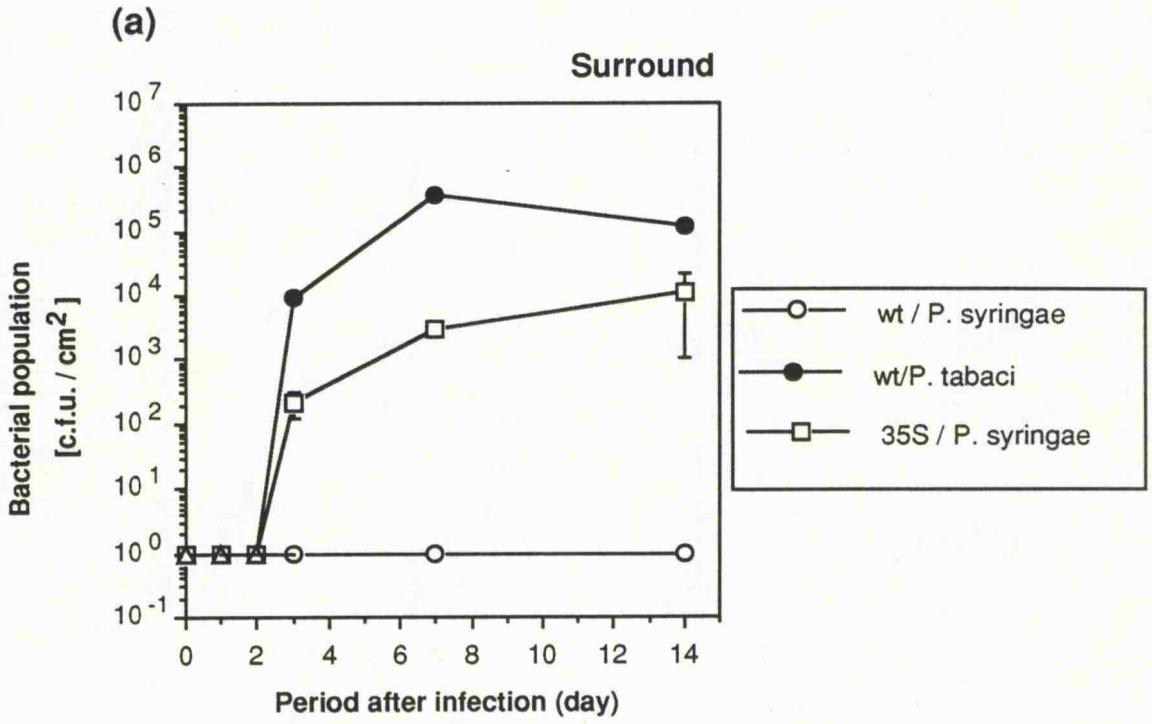
wild type infected with P. s. pv. tabaci

35S-SH-L infected with P.s. pv. syringae

Figure 4.15 Bacterial escape around lesions arising following infection with *P. s.* pv. *syringae* in 35S-SH-L tobacco

a. *P. s.* pathovars were grown overnight in nutrient broth, centrifuged and then resuspended to an approximate concentration of 1 x 10^8 colony forming units (cfu) per ml in 10 mM phosphate buffer (pH 7.0). Tobacco leaves were infected with bacteria by injection of leaf intercellular spaces (see 2.28). Tissue surounding inoculation zone were taken at different time points. Bacteria were extracted after leaf grinding and the number of cfu was counted after bacteria growth on NA plates containing rifampicin. Means ± SE shown, n = 3.

b. Bacterial number was counted in different areas in 35S-SH-L plants after *P. s.* pv. *syringae* infection. Means ± SE shown, n = 3.


(a)
Surround
Bacterial population
[c.f.u. / cm2]
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
10 -1
0 2 4 6 8 10 12 14
Period after infection (day)
wt / P. syringae
wt/P. tabaci
35S / P. syringae

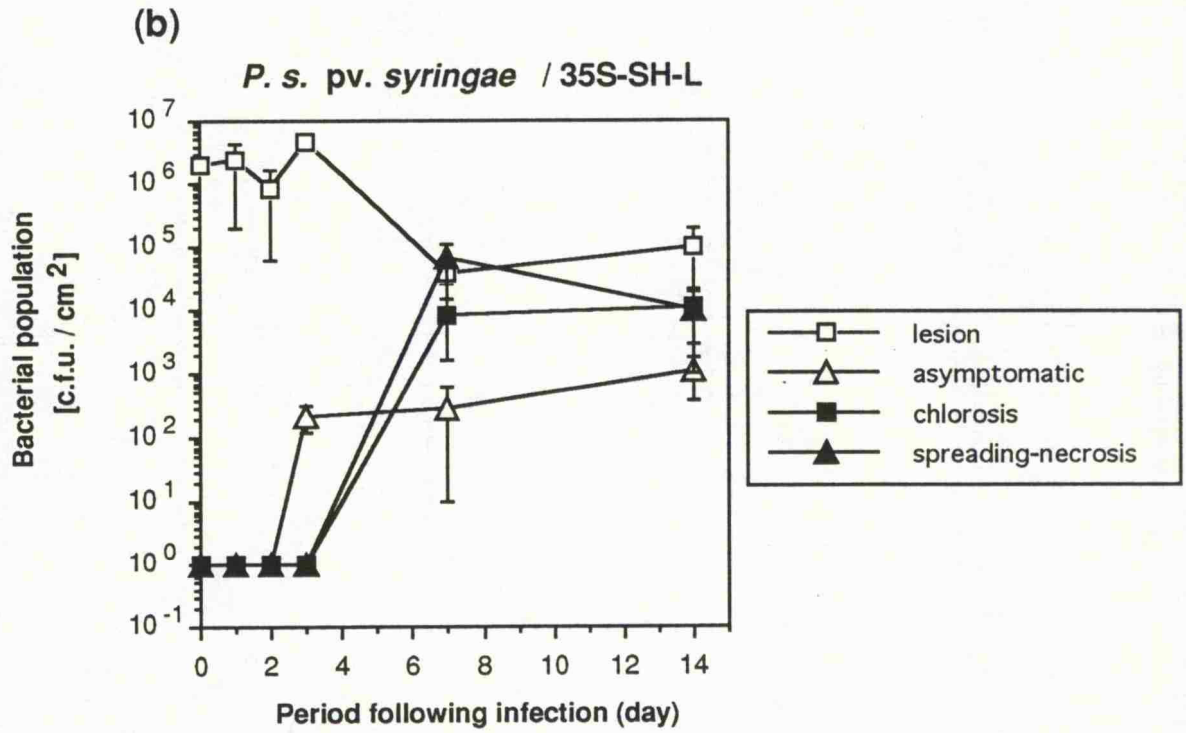
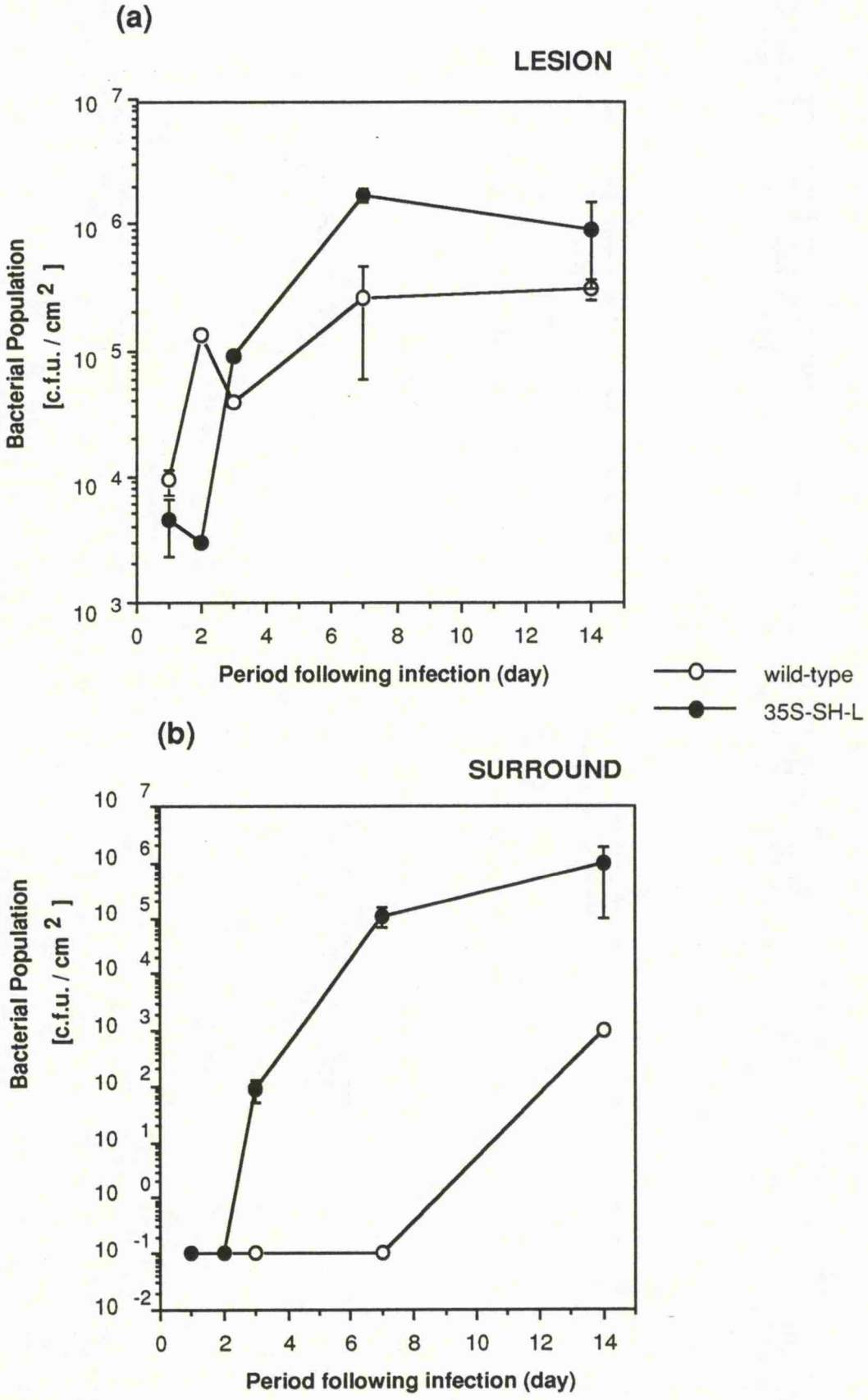

(b)
P. s. pv. syringae / 35S-SH-L
Bacterial population
[c.f.u. / cm 2]
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
10 -1
0 2 4 6 8 10 12 14
Period following infection (day)
lesion
asymptomatic
chlorosis
spreading-necrosis

Figure 4.16 Characterising *P. s.* pv. *tabaci* lesions in 35S-SH-L tobacco

The pathogen infection procedure is the same as described in the legend of Fig.4.15 apart from using a concentration 1×10^4 colony forming units per ml.

a. Bacterial number in lesion area. Means ± SE shown, n = 3.

b. Bacterial number in surrounding area. Means ± SE shown, n = 3.


(a)
LESION
Bacterial Population
[c.f.u. / cm 2]
Period following infection (day)
wild-type
35S-SH-L
(b)
SURROUND
Bacterial Population
[c.f.u. / cm 2]
Period following infection (day)

4.3 Conclusions and Discussion

4.3.1 Catechol is not accumulated in SH-L plants and has no effect on PR1a induction

SA is converted to catechol by SH-L in 35S-SH-L plants. Catechol is unable to induce PR proteins (at least PR1a) (Bi *et al.*, 1995) and from our results, we know that catechol has no effect on PR1a induction by SA at a concentration that would be achieved with equal molar conversion of SA to catechol in TMV-infected wild type *N*-tobacco (Fig.4.2). Neuenschwander *et al* actually have coinfiltrated tobacco leaves with 1 mM catechol and 0.1 mM SA and they have found that PR1a transcription level is not affected (pers. comm. with Neuenschwander). Catechol would not therefore interfere with interpretation of the results. Delaney also found that catechol has no effect on PR induction and SAR (unpublished data, Delaney,1994). What is more, catechol does not accumulate in SH-L plants (Fig.3.11 & Fig.4.3). Thus, the effect of catechol in these experiments is minimal and recent data from Dr. Simon Warner has demonstrated that concurrent expression of a catechol-degrading enzyme does not alter the phenotype of the SH-L plants (pers.comm.). It is still unknown what the compound is at retention time 3.5 min (Fig.3.11) and what is the effect of this unknown compound on plants. Since some other compounds like 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate are also the substrates of salicylate hydroxylase (Yamamoto *et al.*, 1965), it is still not clear what the levels of these compounds were in 35S-SH-L plants and what the effect might be on plants if these compounds are metabolised to some extent.

4.3.2 Local PR1a induction is dependent on salicylate-mediated signalling

A major aim of the present study was to examine the role of SA in defensive signalling in tobacco (specifically PR1a expression) by preventing its accumulation via the constitutive expression of a salicylate hydroxylase enzyme (SH-L).

In similar experiments, using *nah*G to stop SA accumulation in transgenic tobacco, PR1a expression was inhibited in systemic, non-infected tissue, but only slightly reduced in pathogen challenged leaves (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994). In contrast, by expressing the *nah*G isoenzyme SH-L in tobacco we demonstrated for the first time that PR1a expression in infected tissues is dependent on SA accumulation (Fig.4.6 & Fig.4.7). The result suggests that at least local induction of this PR protein operates via salicylate-dependent signalling.

It was interesting to note that INA (which is not a substrate for SH-L) could still induce PR protein expression in plants expressing the SH-L gene (Fig.4.8). A further paper from Neuenschwander *et al.* (1995) showed that SA treatment of *nah*G plants does not induce PR1a mRNA accumulation, whilst INA is effective in this respect, thus confirming that the effect of SA and INA are at the level of transcription. These data suggest that PR protein genes in a *nah*G background are still capable of responding to signals that lie downstream of SA, or are responding to signals that activate a separate signalling pathway. A similar observation has been published recently where it was found that INA can actually restore SAR in nahG tobacco and *Arabidopsis* plants (Vernooij *et al.*, 1995).

4.3.3 Local SA accumulation is essential for containment of invading pathogen

We have been trying to elucidate the importance of SA in the local defense responses in this chapter. Apart from the signalling role in SAR, SA seems to induce plant defences which actively play a part in pathogen containment.

In tobacco plants expressing SH-L, SA and PR1a protein are not detectable in the local TMV lesions (Fig.4.6 & Fig.4.7). The lesions are much larger than wild type *N*-tobacco plants (Fig.4.5) and eventually TMV lesions expanded from the leaf to the stem (Fig.4.4).

The virus moved in a cell-to-cell manner but did not move systemically through the phloem as it would do in genetically susceptible tobacco plants and so SA seems to have functions which limit virus spread. Similar data has been reported recently by Delaney *et al.*(1994) but they found that some SA and PR1a was still present around local TMV lesions in nahG 10 plants (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994).

It was observed that an incompatible bacterial pathogen invaded the adjacent tissue (Fig.4.12 & Fig.4.15) in plants lacking SA so that SA appeared to be involved in processes which could kill bacteria. Delaney *et al.* (1994) also found that nahG plants exhibited increased susceptibility when infected by an incompatible bacterial pathogen but they didn't find the incompatible bacterial pathogen escaping. The reasons for these differences in data could be the result of the differences between the levels of salicylate hydroxylase activities in 35S-nahG and 35S-SH-L (see 3.2.8). It seems that the less SA accumulation in the plants, the more susceptible the plants are to pathogen attack.

In a recent paper, Vernooij *et al.* (1995) showed that although INA can reverse the effect of nahG of inhibiting SAR, it does not totally compensate. The size of the lesions in both control and induced plants were larger in nahG plants while the percent reduction of lesion size was similar in Xanthi and nahG tobacco. The explanation they gave was that SA may have a role in the hypersensitive response apart from its involvement in SAR. This matches the results we obtained in this chapter.

4.3.4 SA is required in SAR

Systemic PR1a was not induced and SAR was not developed (4.2.3) in the plants unable to accumulate SA so that it is very clear that SA is definitely required in the development of SAR.

Similar results have been published (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994). In grafting experiments, Vernooij *et al.* (1994) found that when the scion (upper, grafted portion of the plant) was derived from the transgenic nahG plants, neither SAR nor PR-1 expression were induced in the scion after inoculation of the rootstock leaves with TMV, regardless of the origin of the rootstock. In contrast, an untransformed Xanthi nc scion grafted onto a nahG rootstock expressed PR-1 genes and showed SAR to a secondary infection by TMV. These results confirmed that SA was required in the uninoculated leaves in the scion to mediate the translocated systemic signal and also indicated that a signal other than SA might move from the rootstock to the scion after infection.

Chapter 5
Effects of differential, temporal and spatial expression of salicylate hydroxylase on plant defence responses

5.1 Introduction

In the first chapter, it was mentioned that infection of TMV into a resistant cultivar resulted in a dramatic increase (20-50-fold) in levels of endogenous SA in the TMV-inoculated leaves and a substantial rise (5-10-fold) in uninoculated leaves of the same plant (1.3.1). We have been using the CaMV35S promoter to drive the expression of SH-L so as to block SA accumulation constitutively (chapter 4). These experiments, however, give little information on the importance of the spatial and temporal accumulation of SA in the defense response. In this section, I describe experiments in which specific promoters are used to drive different, temporal and spatial expression of SH-L in order to block SA accumulation, either only at the local infection sites, or to block SA accumulation in tissues undergoing both HR and SAR.

The promoters used for these experiments are described in the next section.

5.1.1 Expression profile of promoters used to drive SH-L expression

5.1.1.1 AoPR1 promoter

The *Asparagus officinalis* intracellular PR1 (AoPR1) gene is expressed locally in response to wounding and pathogen attack and shows no systemic induction. The promoter, isolated by the inverse polymerase chain reaction, is locally inducible by wounding and pathogen attack in heterologous species (Warner *et al.*, 1992; Warner *et al.*, 1993). The promoter is induced within 2-3 hours following a fungal infection, before SA synthesis, and is

therefore ideal to drive SH-L expression at local pathogen attack sites to eliminate local SA accumulation so that we can assess the importance of SA in the local reaction.

Additionally, AoPR1 is active at the site of phenylpropanoid synthesis (Warner *et al.*, 1994) so that SA accumulation at these sites should not occur. As SA is thought to be a phenylpropanoid derivative, this might be an important consideration in studies aimed at investigating the site of SA synthesis in SAR tissue.

5.1.1.2 PR1a promoter

The PR protein-1a (PR1a) belongs to the subclass of acidic, extracellular PR1 proteins from tobacco that is strongly induced by TMV infection during the onset of HR and later systemically as SAR is established. The protein is also synthesized following external treatment with SA (Van Loon *et al.*, 1970; Malamy *et al.*, 1990; Ward *et al.*, 1991). Likewise, plants transformed with a construct consisting of the PR1a promoter linked to ß-glucuronidase (PR1a-GUS) exhibit elevated GUS levels following challenge with TMV or SA (Ohshima *et al.*, 1990; Uknes *et al.*, 1993a). The promoter was therefore used to drive the expression of SH-L in the local tissues undergoing HR and later in systemic tissues undergoing SAR.

5.1.2 Expression patterns of AoPR1-*gus* and PR1a-*gus* after pathogen inoculation

These data were obtained in collaboration with Dr. Luis Mur in the laboratory.

5.1.2.1 Expression patterns of AoPR1-*gus* and PR1a-*gus* after TMV infection

Infection of Samsun NN AoPR1-GUS transformed tobacco with TMV induced a rise in GUS activity around local lesions. GUS activity was not increased in the surrounding and systemic tissue (Fig.5.1a). In contrast, inoculation of Samsun NN PR1a-GUS transformed tobacco with TMV induced a rise in GUS activity around local lesions and the systemic expression of the PR1a-GUS was detectable 7 days after infection (Fig.5.1b).

5.1.2.2 Expression patterns of AoPR1-*gus* and PR1a-*gus* after *P. s.* pv. *syringae* and *P. s.* pv. *tabaci* infection

In this study we have used *P. s.* pathovars, as well as TMV, as a model pathogenic system (see 4.1.2).

Infection of Samsun NN AoPR1-GUS transformed tobacco with *P. s.* pv. *syringae* induced a rapid rise (within 3 hours) in GUS activity reaching its peak at about 12 hours after infection (Fig.5.2c). After infection of Samsun NN AoPR1-GUS transformed tobacco with *P. s.* pv. *tabaci,* GUS activity was slightly induced and very much delayed compared to the incompatible reaction (Fig.5.2d). As with TMV infection, there was no expression in systemic tissues.

Infection of Samsun NN PR1a-GUS transformed tobacco with *P. s.* pv. *syringae* induced a rise in GUS activity (Fig.5.2a). GUS activity was detectable at the infection site after 10-12 hours, rising approximately 50-fold above initial levels by day 3. After infection of Samsun NN PR1a-GUS transformed tobacco with *P. s.* pv. *tabaci,* GUS activity was induced to some extent at a very late stage (Fig.5.2b). Unlike AoPR1-GUS, the PR1a-GUS gene are also upregulated in systemic tissue at a later stage.

Figure 5.1 Responsiveness of AoPR1- and PR1a-GUS promoter fusions in transgenic tobacco to challenge with TMV

a. AoPR1-GUS local, surround & systemic expression after TMV infection

b. PR1a-GUS local, surround & systemic expression after TMV infection

AoPR1-GUS Samsun NN and PR1a Samsun NN tobacco plants were infected by TMV. Samples were taken from lesions, surrounding area and systemic leaves at different time points. Means ± standard errors shown, n = 3.

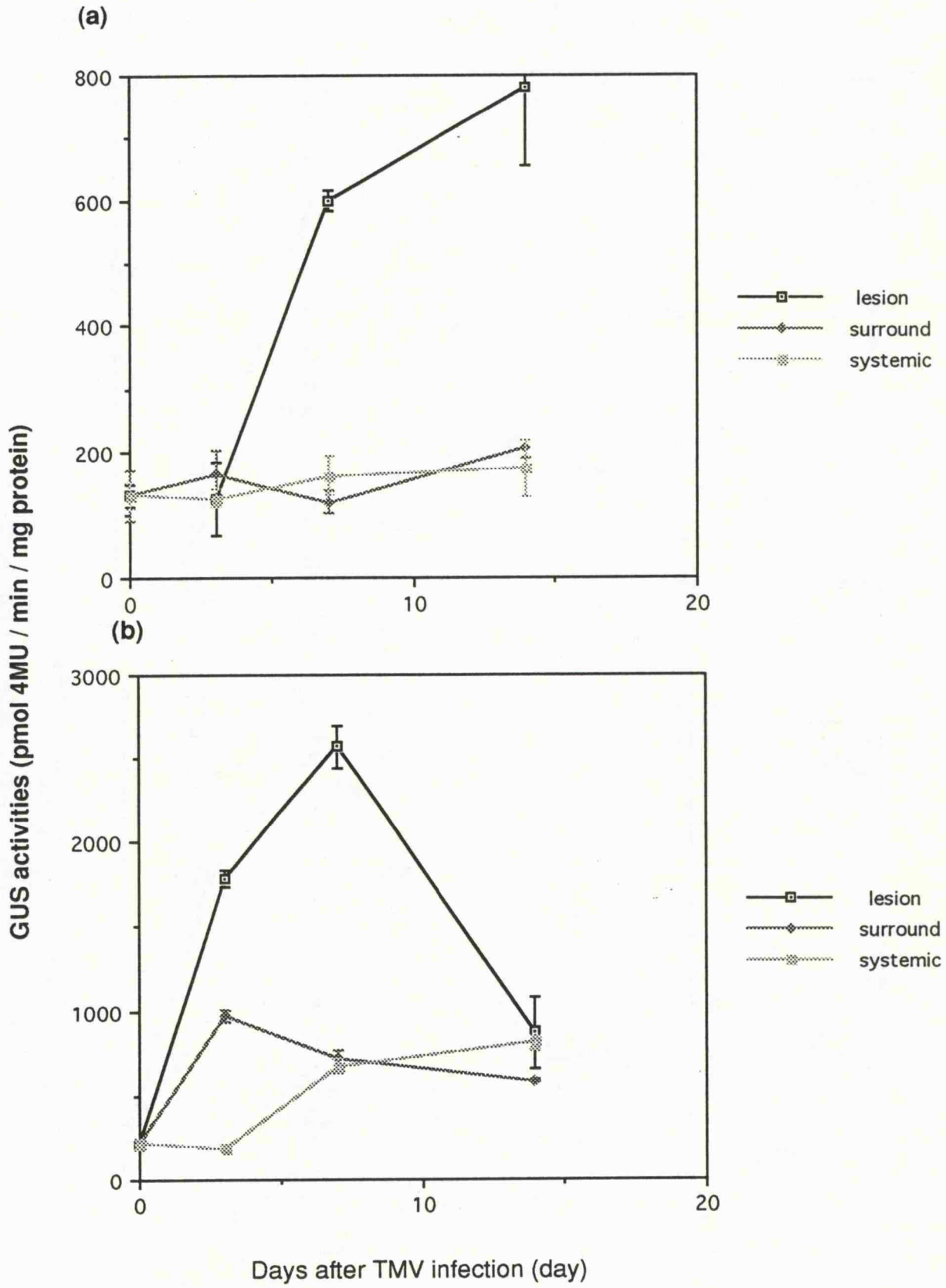

(a)
800
600
400
200
0
0
10
20
lesion
surround
systemic
(b)
3000
2000
1000
0
0
10
20
lesion
surround
systemic
GUS activities (pmol 4MU / min / mg protein)
Days after TMV infection (day)

Figure 5.2 GUS activity in AoPR1-GUS and PR1a-GUS tobacco following challenge with *P.s.* pv. *syringae* or *P.s.* pv *tabaci*

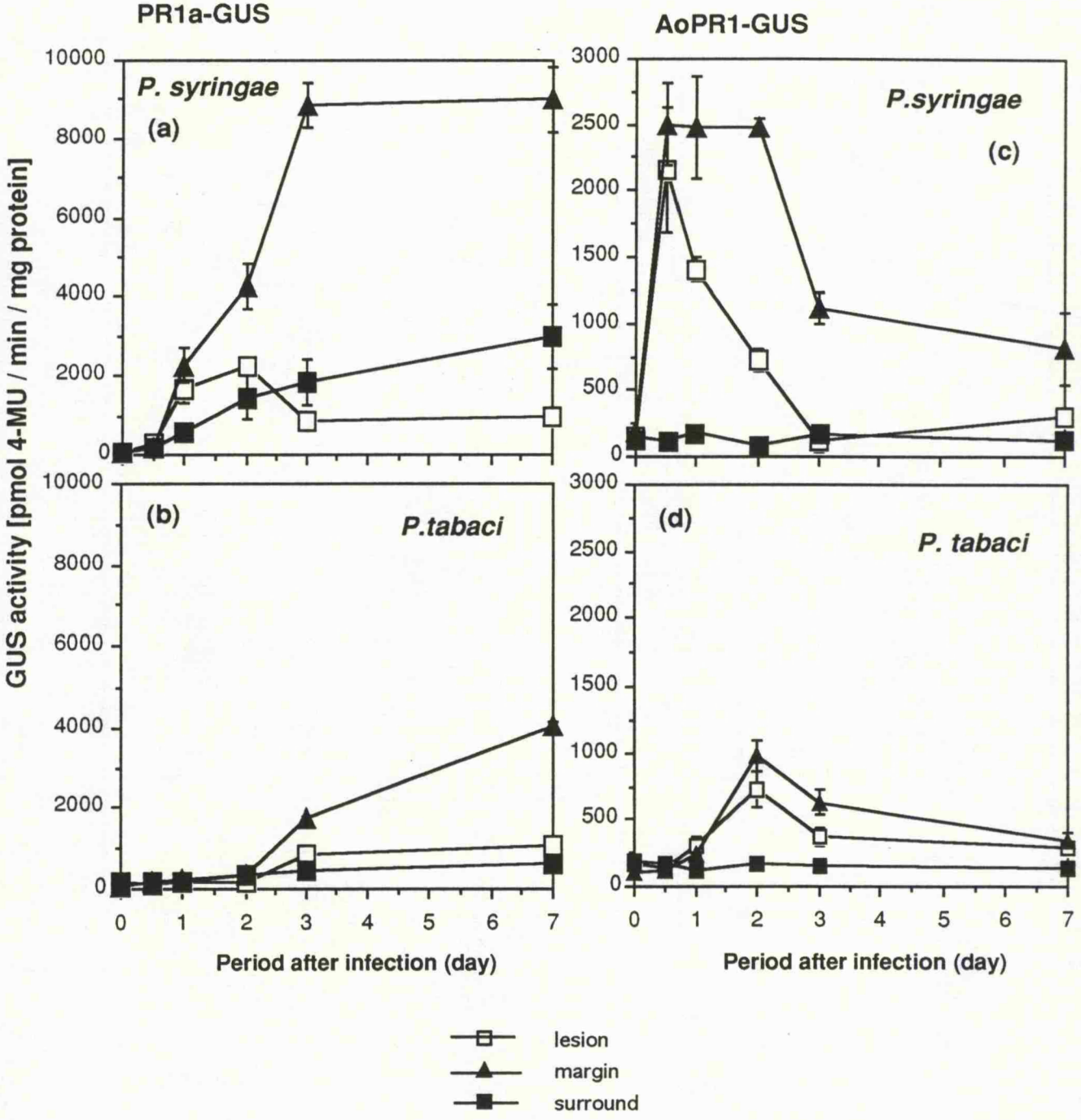
a. PR1a-GUS expression after *P. s.* pv *syringae* infection

b. PR1a-GUS expression after *P. s.* pv *tabaci* infection

c. AoPR1-GUS expression after *P. s.* pv *syringae* infection

d. AoPR1-GUS expression after *P. s.* pv *tabaci* infection

Leaves of AoPR1-GUS Samsun NN and PR1a-GUS Samsun NN tobacco were infiltrated with *P. s.* pv. *syringae* or pv *tabaci*. Samples were periodically taken from necrotic lesions (—□—) and from margin (area just adjacent to lesions) (—▲—) and surrounding tissue (—■—). Means ± standard errors shown. n = 3.


PR1a-GUS
AoPR1-GUS
P. syringae
(a)
P.syringae
(c)
(b)
P.tabaci
(d)
P. tabaci
GUS activity [pmol 4-MU / min / mg protein]
Period after infection (day)
Period after infection (day)
lesion
margin
surround

Mock-inoculated plants failed to show any reporter gene activity in all these experiments (data not shown). The expression pattern of GUS activity following challenge with *P. s.* pv. *syringae* and *P. s* . pv. *tabaci* was used to monitor the effect of SH-L activity in AoPR1-SH-L and PR1a-SH-L tobacco.

5.1.3. Aims of the chapter

We intended to investigate the role of SA in local defense responses by using the AoPR1 promoter to drive the expression of salicylate hydroxylase so as to prevent only the local accumulation of SA.

By using the AoPR1 promotor, we hoped we could then answer the question whether SA is a mobile signal transported from a local infection site to systemic leaf tissue. If some other molecule rather than SA (or a SA derivative) is the primary systemic signal, SAR should still be developed even if the local SA accumulation was blocked (1.3.2).

Conversely, by using the PR1a promoter to drive the expression of SH-L, we tried to address the question whether a continuous, high level supply of SA is needed for the manifestation and maintenance of the HR and the SAR. The establishment of HR should be initiated by TMV infection and a SH-L gene driven by the PR1a promoter should be switched on during this process after a delay of several hours. In systemic leaves, SA should first increase to induce PR protein and then PR1a-SH-L should start to work leading to a decrease of SA.

In summary, the expression of AoPR1-SH-L should stop early accumulation of SA around lesions, but not affect systemic build up; whilst PR1a-SH-L should not affect the early accumulation of SA around developing lesions but may be sufficient to stop any systemic SA accumulation.

5.2 Results

5.2.1 AoPR1-SH-L and PR1a-SH-L gene fusions and generation of transgenic plants

5.2.1.1 AoPR1-SH-L fusion

SH-L was cloned into *Bam*HI-*Eco*RI-digested pJIT60-AoPR1, provided by Dr. S. Firek (Firek *et al.*, 1993), to produce pJIT60-AoPR1-SH-L (Fig.5.3a). The AoPR1 promoter, SH-L gene and CaMV polyA signal was released by *Kpn*I-*Sal*I partial digestion from pJIT60-AoPR1-SH-L and cloned into the *Kpn*I-*Sal*I digested binary vector pBin19.

5.2.1.2 Identification of AoPR1-SH-L transgenic tobacco plants

pBin19-AoPR1a-SH-L was mobilised into *Agrobacterium tumefaciens* GV 2260 using the triparental mating system (2.8.3). Tobacco (*Nicotiana tabacum* Samsun NN) leaf disc transformation and shoot regeneration were performed as standard. Transgenic plants were identified by PCR (2.1 and 2.4.4) and from enzyme activity after wounding (2.14). Lines 2, 3, and 8 were selected for further work.

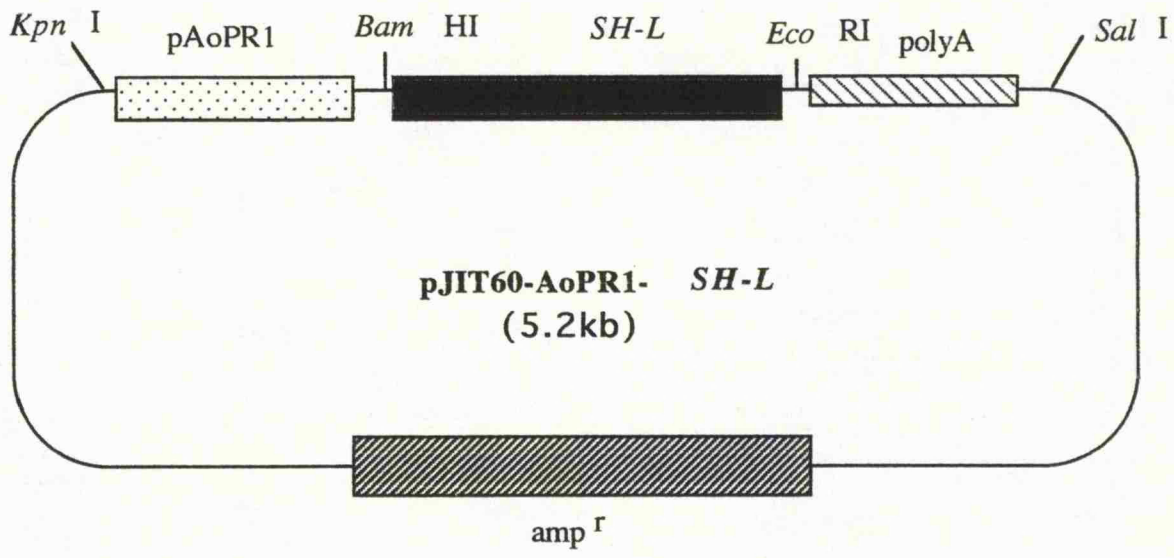
Further experiments using RT-PCR (2.15) showed that SH-L transcript accumulation after wounding in line 2 and line 3 was higher than that in line 8 (data not shown). Thus, line 2 and line 3 plants were used in the experiments described in this chapter.

Figure 5.3 ***SH-L*** **fusion to AoPR1 promoter and PR1a promoter**

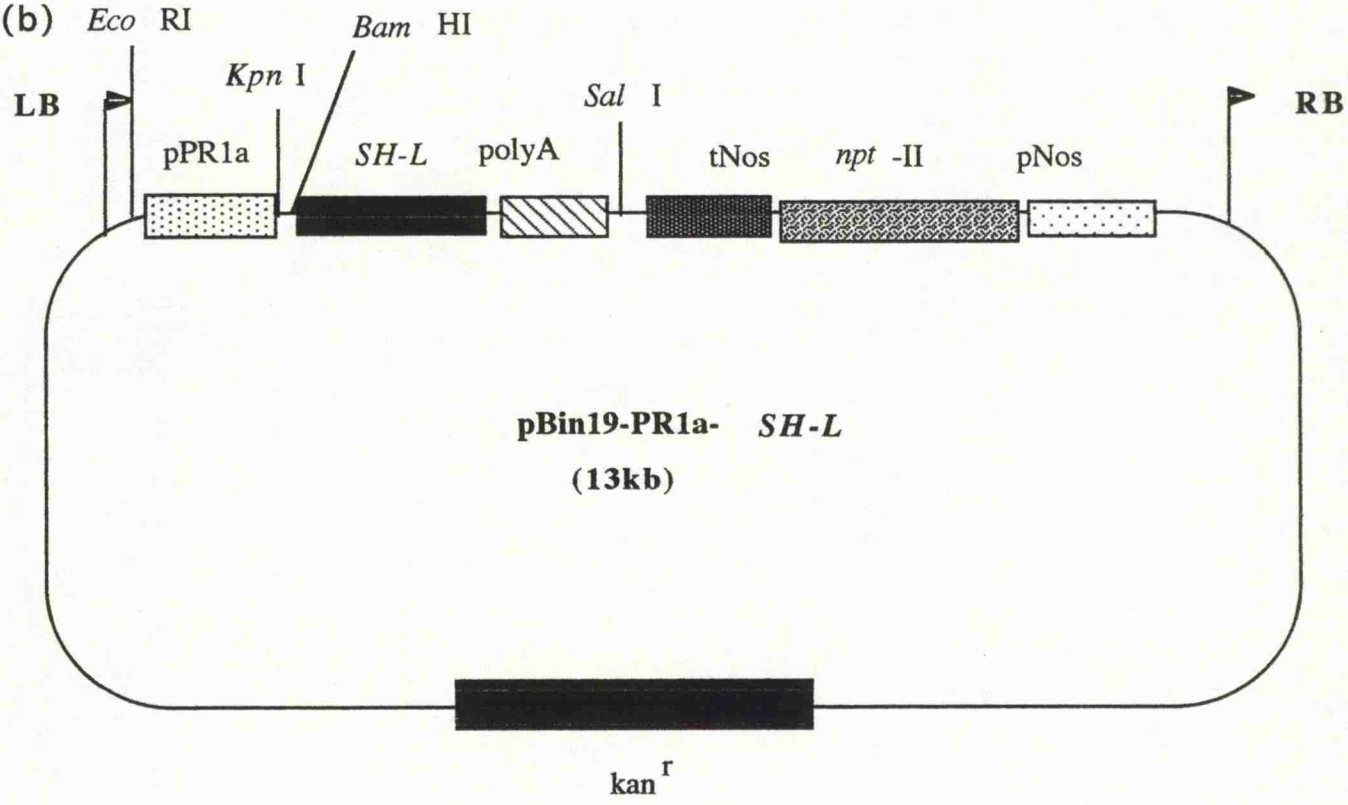
a. SH-L was cloned into *Bam*HI-*Eco*RI-digested pJIT60-AoPR1 to produce pJIT60-AoPR1-SH-L. The AoPR1 promoter, SH-L gene and CaMV polyA signal was released by *Kpn*I-*Sal*I partial digestion from pJIT60-AoPR1-SH-L and cloned into the *Kpn*I-*Sal*I digested binary vector pBin19.

b. The PR1a promoter was released from *Eco*RI-*Kpn*I-digested pSK-PR1a and cloned into *Eco*RI-*Kpn*I-digested pBin19 first to form pBin19-PR1a. A SH-L-polyA fragment released from pJIT60-AoPR1-SH-L (Fig.5.3.a) was then cloned into *Bam*HI-*Sal* I-digested pBin19-PR1a to produce pBin19-PR1a-SH-L.

(a)


Kpn I
pAoPR1
Bam HI
SH-L
Eco RI
polyA
Sal I
pJIT60-AoPR1- SH-L
(5.2kb)
amp r

(b)


Eco RI
Bam HI
Kpn I
Sal I
LB
RB
pPR1a
SH-L
polyA
tNos
npt -II
pNos
pBin19-PR1a- SH-L
(13kb)
kan r

5.2.1.3 PR1a-SH-L fusion

The PR1a promoter, a gift of the Ciba-Geigy corporation (Uknes *et al.*, 1993a), was released from *Eco*RI-*Kpn*I-digested pSK-PR1a and cloned into *Eco*RI-*Kpn*I-digested pBin19 to form pBin19-PR1a. A SH-L-polyA fragment released from pJIT60-AoPR1-SH-L (Fig.5.3a) was then cloned into *Bam*HI-*Sal* I-digested pBin19-PR1a to produce pBin19-PR1a-SH-L (Fig.5.3b)

5.2.1.4 Identification of PR1a-SH-L transgenic tobacco plants

pBin19-PR1a-SH-L was electroporated into *A. tumefaciens* GV 2260. Tobacco (*Nicotiana tabacum* Samsun NN) leaf disc transformation and regeneration were performed as standard. Several transgenic lines were obtained and T1 seeds were germinated and selected by kanamycin resistance. The resistant plants were moved to soil and further identified by PCR (2.1 and 2.4.4). Line 1 and line 5 were selected for further study because TMV systemic escape were observed in these plants. These plants were available only right at the end of my studies.

5.2.2 Local TMV infection in AoPR1-SH-L and PR1a-SH-L tobacco plants

5.2.2.1 TMV lesion phenotype in AoPR1-SH-L and PR1a-SH-L tobacco

TMV strain U1 was applied to wild type, AoPR1-SH-L and PR1a-SH-L tobacco by the method described in 2.28. HR lesions appeared about 3 days after infection. TMV lesions were much bigger in AoPR1-SH-L tobacco (Fig.5.4b) than in wild type tobacco (Fig.5.4a), whilst lesion phenotype in PR1a-SH-L tobacco appeared the same as in wild type tobacco (Fig.5.4c).

Like the CaMV35S-SH-L transgenics, TMV-infected AoPR1-SH-L tobacco exhibited symptoms of cell-to-cell virus movement (Fig.5.4d). In addition, chlorosis was commonly observed around developing lesions. In contrast, lesions on PR1a-SH-L plants stopped expanding after 10 days and cell-to-cell movement of TMV was not seen. Surprisingly, a "systemic necrosis" was observed on the upper-most leaves of PR1a-SH-L tobacco (Fig.5.4e) 2 to 3 weeks after TMV infection in a lower leaf and these plants after failed to flower (Fig.5.4f and Fig.5.4g).

The continuous TMV spreading in AoPR1-SH-L tobacco and the discontinuous TMV spreading in PR1a-SH-L plants was confirmed by trypan blue staining for necrosis and tissue immunoblotting (see 2.29) using an antibody to TMV coat protein (Fig.5.5). Figure 5.5a and 5.5b are negative controls for trypan blue staining and tissue blot. Figure 5.5c, 5.5d and 5.5e showed the staining pattern in AoPR1-SH-L plants and it is clear that necrosis continued from the stem adjacent to the infected leaves and went up through the stem to link with the necrotic tissue in upper leaves. In contrast, there was no staining in the stem sections between infected leaves and top leaves in PR1a-SH-L plants (Fig.5.5f), but the stem sections near top leaves stained heavily (Fig.5.5g) indicating that necrosis happened in a discontinuous way. Figure 5.5h to 5.5l showed the tissue immunoblot results from corresponding materials confirming that TMV was present where necrosis occurred.

5.2.2.2 TMV lesion size in AoPR1-SH-L and PR1a-SH-L tobacco

Actual lesion size was measured in wild type and AoPR1-SH-L and PR1a-SH-L tobacco (Fig.5.6). Ten lesions per plant (3 plants each line) were taken into account. Lesions in AoPR1-SH-L tobacco were much bigger than those in wild type plants whilst lesions in PR1a-SH-L tobacco were only slightly bigger than those in wild type tobacco.

Figure 5.4 TMV lesion phenotype in wild type, AoPR1- and PR1a-SH-L tobacco

a. Day 7 TMV lesions in wild type tobacco

b. Day 7 TMV lesions in AoPR1-SH-L tobacco

c. Day 7 TMV lesions in PR1a-SH-L tobacco

d. Continuous TMV spreading in AoPR1-SH-L tobacco after 2 weeks

e. Discontinuous TMV spreading in PR1a-SH-L tobacco after 2-3 weeks

f. Necrosis in top leaf of PR1a-SH-L tobacco after 2-3 weeks

g. Necrosis and chlorosis in top leaf of PR1a-SH-L tobacco after 3 weeks

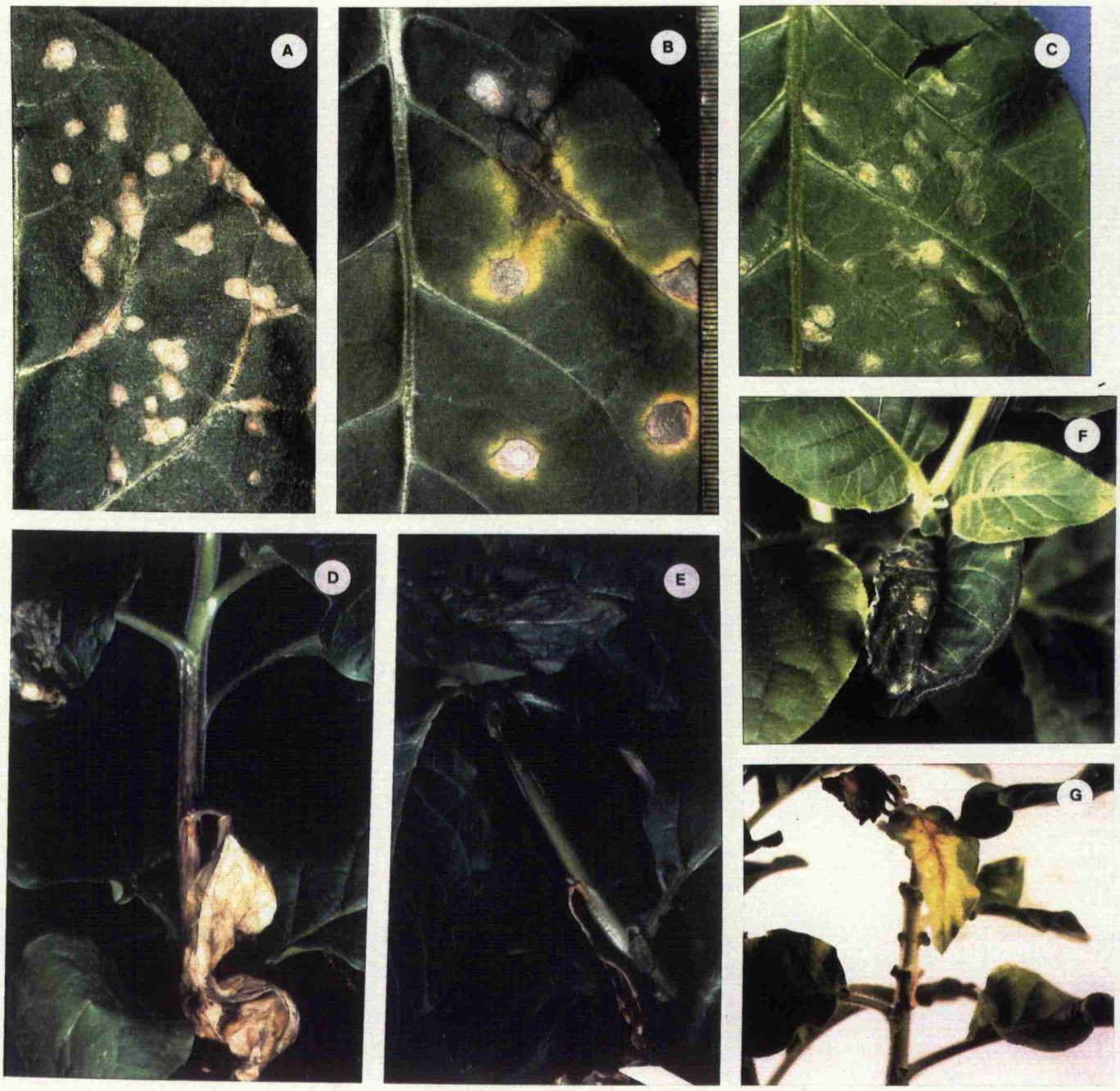

A
B
C
D
E
F
G

Figure 5.5 Continuous and discontinuous TMV spreading in AoPR1- & PR1a-SH-L tobacco

Stems were sectioned from TMV infected plants shown in Fig.5.4. Continuous & discontinuous spreading of TMV in AoPR1 & PR1a-SH-L tobacco was shown by trypan blue staining (dead cells are stained blue, see 2.21) and *in situ* tissue blots of the same material where only virus was detected using a TMV coat protein antibody.

a. negative control for trypan blue staining showing dye in dead cells of vascular tissue
b. negative control for tissue blot
c. stem section next to the infected leaf in AoPR1-SH-L stained by trypan blue
d. stem section in necrotic zone above position (c) in AoPR1-SH-L stained by trypan blue
e. stem section of apparently uninfected tissue above position (d) in AoPR1-SH-L stained by trypan blue
f. stem section next to the infected leaf in PR1a-SH-L stained by trypan blue
g. stem section of necrotic tissue at top of PR1a-SH-L plants stained by trypan blue
h. TMV visualised in stem section next to the infected leaf in AoPR1-SH-L by tissue blotting
i. TMV visualised in necrotic stem section above position (c) in AoPR1-SH-L by tissue blotting
j. TMV is absent in non-necrotic stem section above position (d) in AoPR1-SH-L
k. TMV is not detected in stem section next to the infected leaf in PR1a-SH-L
l. TMV is detected in stem section at top of PR1a-SH-L by tissue blotting

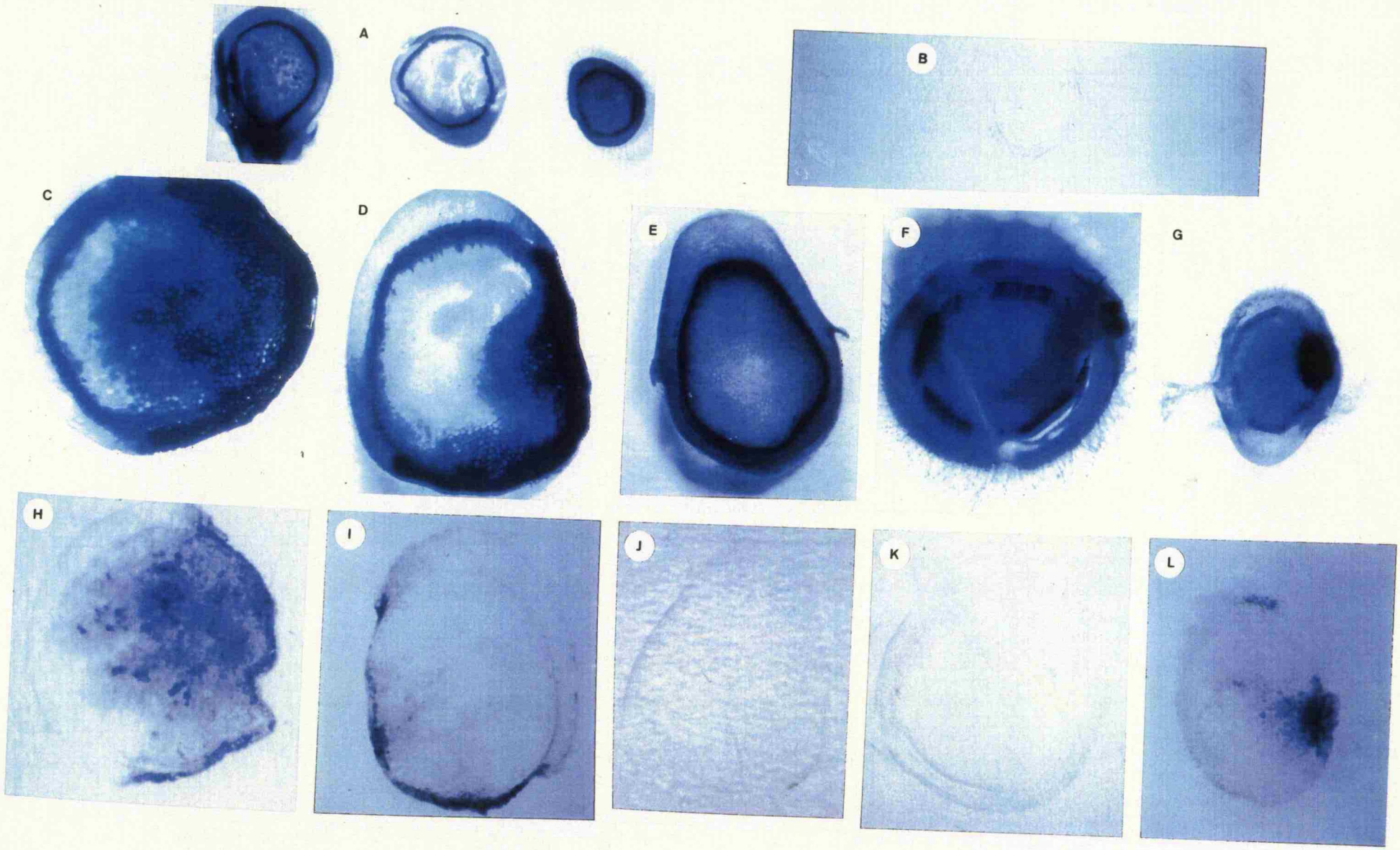
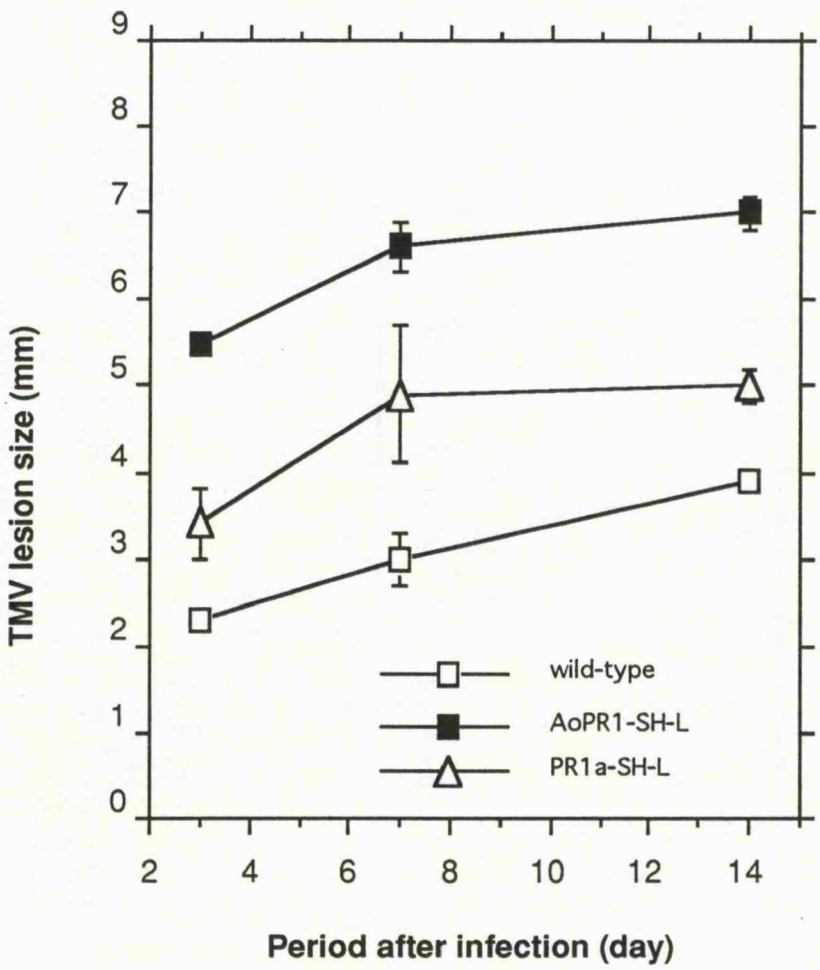

A
B
C
D
E
F
G
H
I
J
K
L

Figure 5.6 Primary TMV lesion size in wild type, AoPR1- & PR1a-SH-L tobacco

Local TMV lesion size (diameter) is shown. 10 lesions per plant, 3 plants each line were taken into account.


TMV lesion size (mm)
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
14
Period after infection (day)
wild-type
AoPR1-SH-L
PR1a-SH-L

5.2.2.3 Local PR1a protein induction in AoPR1-SH-L and PR1a-SH-L tobacco

PR1a protein level was measured by western blotting at different time points after infection with TMV (Fig.5.7). Comparing the result in 35S-SH-L tobacco (Fig.4.7), there is quite a lot PR1a still present in both AoPR1-SH-L and PR1a-SH-L tobacco.

It is easy to understand that PR1a is induced by TMV in PR1a-SH-L tobacco because SA first synthesized after TMV infection will be required to switch on the PR1a promoter. At this time, both PR1a protein and SH-L enzyme synthesis will have started so that there will be already some PR1a protein produced when the SH-L enzyme started to work and PR1a is very stable.

From the expression profile of the AoPR1 promoter, there is delayed expression of AoPR1 promoter after TMV infection as compared to induction by wounding or bacterial pathogen (Fig.5.1a). The reason for PR1a protein expression in AoPR1-SH-L plants could be related to this fact.

5.2.3 Secondary infection of TMV in systemic tissue of AoPR1-SH-L and PR1a-SH-L plants

5.2.3.1 PR1a induction in wild type, AoPR1-SH-L and PR1a-SH-L systemic, uninfected leaves

14 days after first infection, the PR1a level in wild type and AoPR1-SH-L and PR1a-SH-L systemic uninfected leaves was measured by western blotting. Figure 5.8 shows that PR1a protein had accumulated in wild type, AoPR1-SH-L and PR1a-SH-L systemic uninfected leaves, but not in 35S-SH-L plants (see Fig.4.9). It was expected that PR1a protein would be induced systemically in AoPR1-SH-L tobacco as SH-L should not express in the systemic tissue from the expression profile (Fig.5.1.a). A bit surprisingly,

PR1a protein was also induced systemically to quite a large amount in PR1a-SH-L tissue. This could mean that small amount of SA increased in systemic leaves (Malany *et al.*, 1990) can induce quite a lot PR1a protein production in such tissue.

5.2.3.2 Lesion size of secondary TMV infection on wild type and AoPR1-SH-L and PR1a-SH-L tobacco

A second TMV infection was applied to systemic leaves 14 days after first infection and lesion size was measured. Figure 5.9 showed that lesion sizes in secondary infections on AoPR1-SH-L and PR1a-SH-L tobacco were both reduced This data showed that SAR was probably still exhibited in AoPR1-SH-L and PR1a-SH-L plants .

However, it was very interesting to notice that lesion sizes in secondary infections in AoPR1-SH-L tobacco, although smaller than those in the first infection, were still much bigger than that in wild type tobacco.

Also, it was very interesting to notice that systemic TMV escape from primary infected leaves happened in some PR1a-SH-L tobacco whilst the plants seems still to be undergoing SAR.

5.2.3.3 Infection of wild-type Samsun NN with reisolated TMV from spreading lesions in PR1a-SH-L tobacco plants

TMV particles reisolated from PR1a-SH-L tobacco systemic spreading lesions (top necrotic leaf) induced normal, restricted, local lesions on wild-type Samsum NN tobacco plants. This data was confirmed in collaboration with Dr. Ray White at Rothamstand.

Figure 5.7 PR1a induction in AoPR1-SH-L and PR1a-SH-L tobacco

Wild-type Samsun NN (WT), Samsun NN AoPR1-SH-L (AoPR1) and PR1a-SH-L (PR1a) tobacco plants were infected with TMV. Samples before infection and 3 or 7 days after infection were taken. PR1a protein accumulation was determined by PR1a anti-serum.

Lane 1	PR1a level in wild type tobacco at day 0
Lane 2	PR1a level in wild type tobacco at day 3
Lane 3	PR1a level in wild type tobacco at day 7
Lane 4	PR1a level in AoPR1-SH-L tobacco at day 0
Lane 5	PR1a level in AoPR1-SH-L tobacco at day 3
Lane 6	PR1a level in AoPR1-SH-L tobacco at day 7
Lane 7	PR1a level in PR1a-SH-L tobacco at day 0
Lane 8	PR1a level in PR1a-SH-L tobacco at day 3
Lane 9	PR1a level in PR1a-SH-L tobacco at day 7

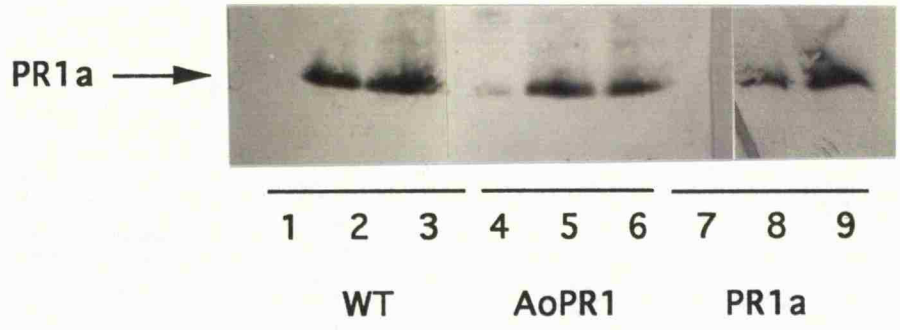

PR1a
1 2 3 4 5 6 7 8 9
WT
AoPR1
PR1a

Figure 5.8 PR1a induction in wild type, AoPR1- and PR1a-SH-L systemic uninfected leaves

Accumulation of PR1a protein in systemic uninfected leaves of Samsun NN, AoPR1-SH-L Samsun NN and PR1a-SH-L Samsun NN tobacco plants 14 days after primary TMV infection was determined by PR1a antiserum. 3 replicates. One mock-inoculation was used as control.

Lane 1	Systemic PR1a level in mock-inoculated wild type tobacco plant
Lane 2	Systemic PR1a level in TMV-infected wild type tobacco plant 1
Lane 3	Systemic PR1a level in TMV-infected wild type tobacco plant 2
Lane 4	Systemic PR1a level in TMV-infected wild type tobacco plant 3
Lane 5	Systemic PR1a level in mock-inoculated AoPR1-SH-L tobacco plant
Lane 6	Systemic PR1a level in TMV-infected AoPR1-SH-L tobacco plant 1
Lane 7	Systemic PR1a level in TMV-infected AoPR1-SH-L tobacco plant 2
Lane 8	Systemic PR1a level in TMV-infected AoPR1-SH-L tobacco plant 3
Lane 9	Systemic PR1a level in mock-inoculated PR1a-SH-L tobacco plant
Lane 10	Systemic PR1a level in TMV-infected PR1a-SH-L tobacco plant 1
Lane 11	Systemic PR1a level in TMV-infected PR1a-SH-L tobacco plant 2
Lane 12	Systemic PR1a level in TMV-infected PR1a-SH-L tobacco plant 3

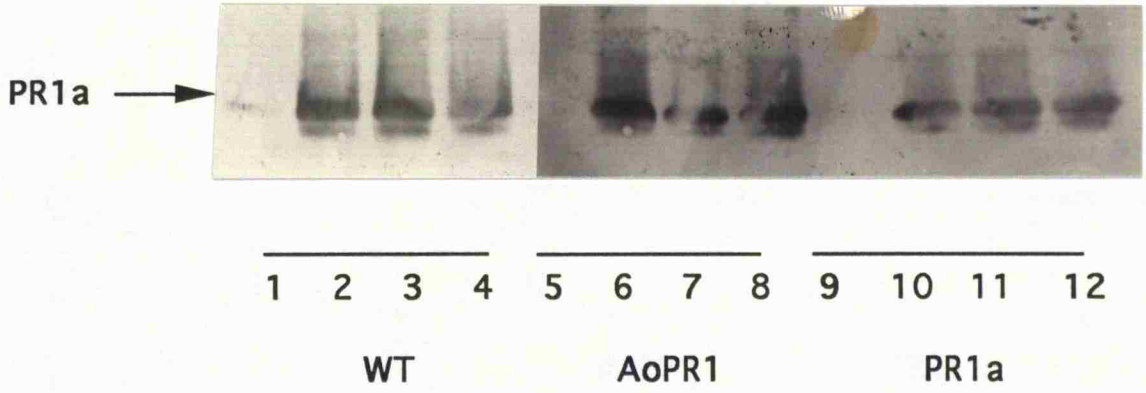
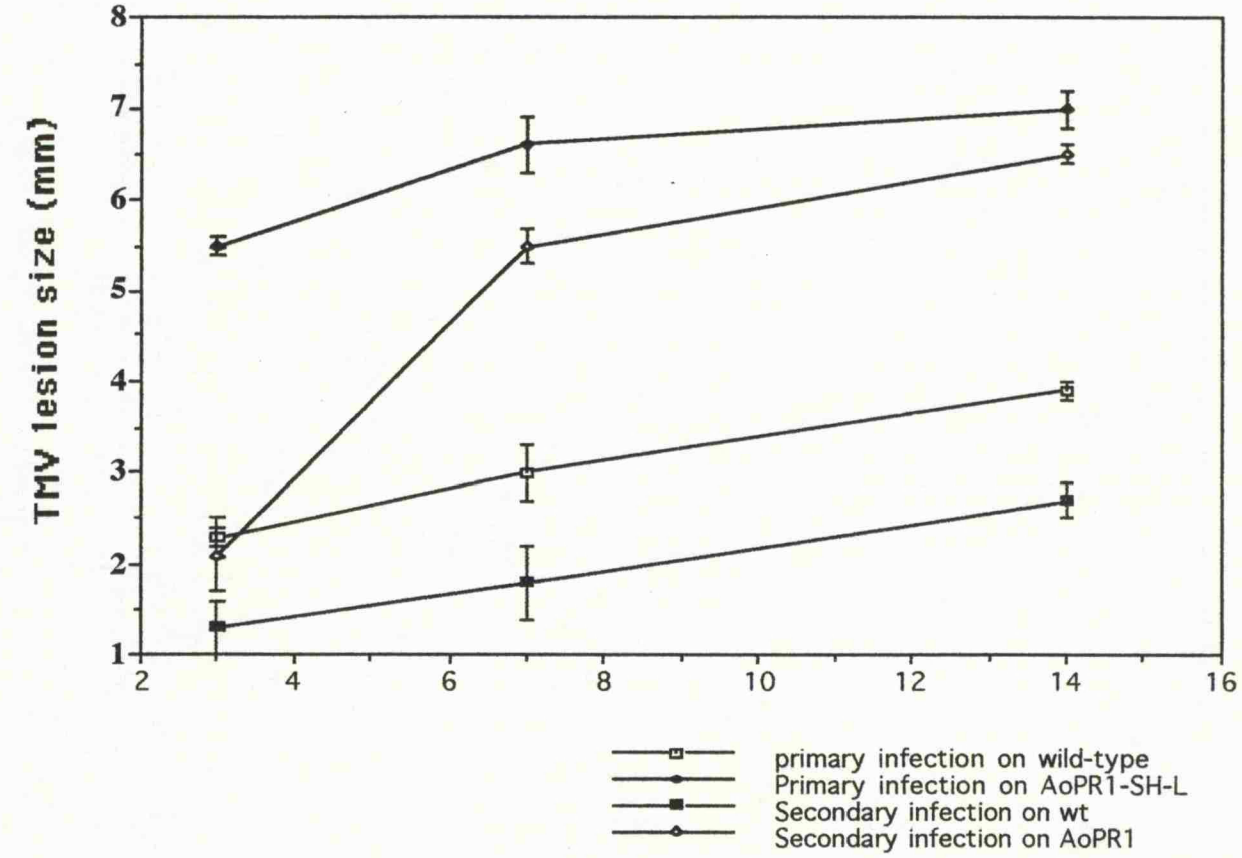
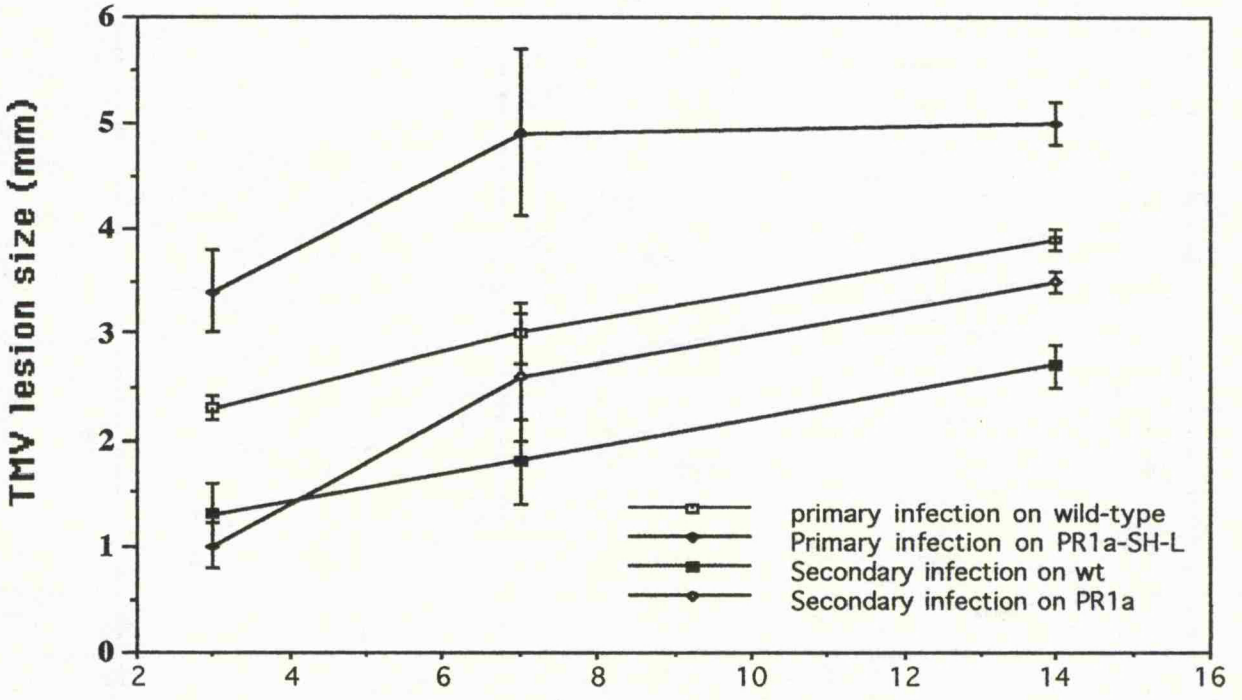

PR1a
1 2 3 4 5 6 7 8 9 10 11 12
WT
AoPR1
PR1a

Figure 5.9 Lesion size of primary and secondary TMV infections in wild type, AoPR1- and PR1a-SH-L tobacco

Primary and secondary TMV lesion sizes (diameter) are shown at different times after inoculation. 10 lesions per plant, 3 plants each line were taken into account. Secondary TMV infection was applied to systemic, non-infected leaves 14 days after first infection of lower leaves.

Means ± SE shown, n = 3.





Period after infection (day)

5.2.4 Bacterial infection on AoPR1-SH-L plants

In collaboration with Dr. Luis Mur, I used *P. s.* pathovars to study the role of SA in the local defense response.

5.2.4.1 Phenotype of bacterial lesion development on infection of AoPR1-SH-L and PR1a-SH-L tobacco plants

Wild type tobacco plants inoculated with *Pseudomonas syringe* pv *syringae* (Fig.5.10a) or *P. s. pv. tabaci* (Fig.5.10b) produced lesions typical of incompatible and compatible responses respectively (See 4.1.1.2). AoPR1-SH-L transgenic plants inoculated with the normally incompatible *P. s.* pv. *syringae* strain exhibited disease symptoms more typical of a compatible interaction (Fig.5.10c) (compare with Fig.4.12). In contrast, PR1a-SH-L transgenic plants inoculated with the incompatible *P. s.* pv. *syringae* strain exhibited normal symptoms (Fig.5.10d).

5.2.4.2 PR1a induction in AoPR1-SH-L and PR1a-SH-L tobacco after bacterial infection

PR1a was normally detectable in wild type plants at least 10-12 hours post infection. PR1a was not detected in AoPR1-SH-L tobacco until 24 hours after *P. s.* pv. *syringae* infection. The PR1a level in PR1a-SH-L tobacco was about the same as in wild type 1 day after infection (Fig.5.11). Previously, it was shown that the AoPR1 promoter was switched on only 3 hours after *P. s.* pv. *syringae* infection (Fig.5.2a) while the PR1a promoter was much delayed compared to AoPR1 (Fig.5.2c). It seems that PR1a protein not produced at early time points in AoPR1-SH-L plants may be due to the blocking of SA accumulation at this time and that in the absence of either SA or PR protein, the mechanisms which kill bacterial pathogens do not operate efficiently.

5.2.4.3 Bacterial number in lesions arising in AoPR1-SH-L and PR1a-SH-L tobacco following infection with *P. s.* pv. *syringae*

Bacterial numbers decreased rapidly in the incompatible combination (Fig.5.12) but remained high in the inoculated zone (Fig.5.12) after inoculation of wild type plants with *P. s.* pv. *tabaci*. As expected from the lesion characteristics, the bacterial population remained at a high level when AoPR1-SH-L transgenic plants were inoculated with the normally incompatible *P. s.* pv. *syringae* strain (Fig.5.12). In contrast, the population decreased rapidly in PR1a-SH-L tobacco, but there were still more bacteria surviving compared to the number in wild type plants (Fig.5.12).

5.2.4.4 Bacterial escape around lesions arising following infection with *P. syringae* in AoPR1-SH-L and PR1a-SH-L tobacco

Figure 5.13 showed that there was no invasion of bacteria into adjacent tissue in wild type and PR1a-SH-L tobacco after infection with *P. s.* pv. *syringae*, while bacterial numbers steadily increased in the surrounding tissue after inoculation with *P. s.* pv. *tabaci*. However, when AoPR1-SH-L transgenic plants were inoculated with the normally incompatible *P. s.* pv. *syringae* strain, bacterial numbers increased and the pathogen was not confined to the original inoculated zone (Fig.5.13).

Figure 5.10 Phenotype of bacterial lesion development after infection of AoPR1- and PR1a-SH-L tobacco plants

a. Wild type tobacco day 7 after inoculation with 10^8 cfu/ml *P. s.* pv. *syringae*

b. Wild type tobacco day 7 after inoculation with 10^8 cfu/ml *P. s.* pv. *tabaci*

c. AoPR1-SH-L tobacco day 7 after inoculation with 10^8 cfu/ml *P. s.* pv. *syringae*

d. PR1a-SH-L tobacco day 7 after inoculation with 10^8 cfu/ml *P. s.* pv. *syringae*

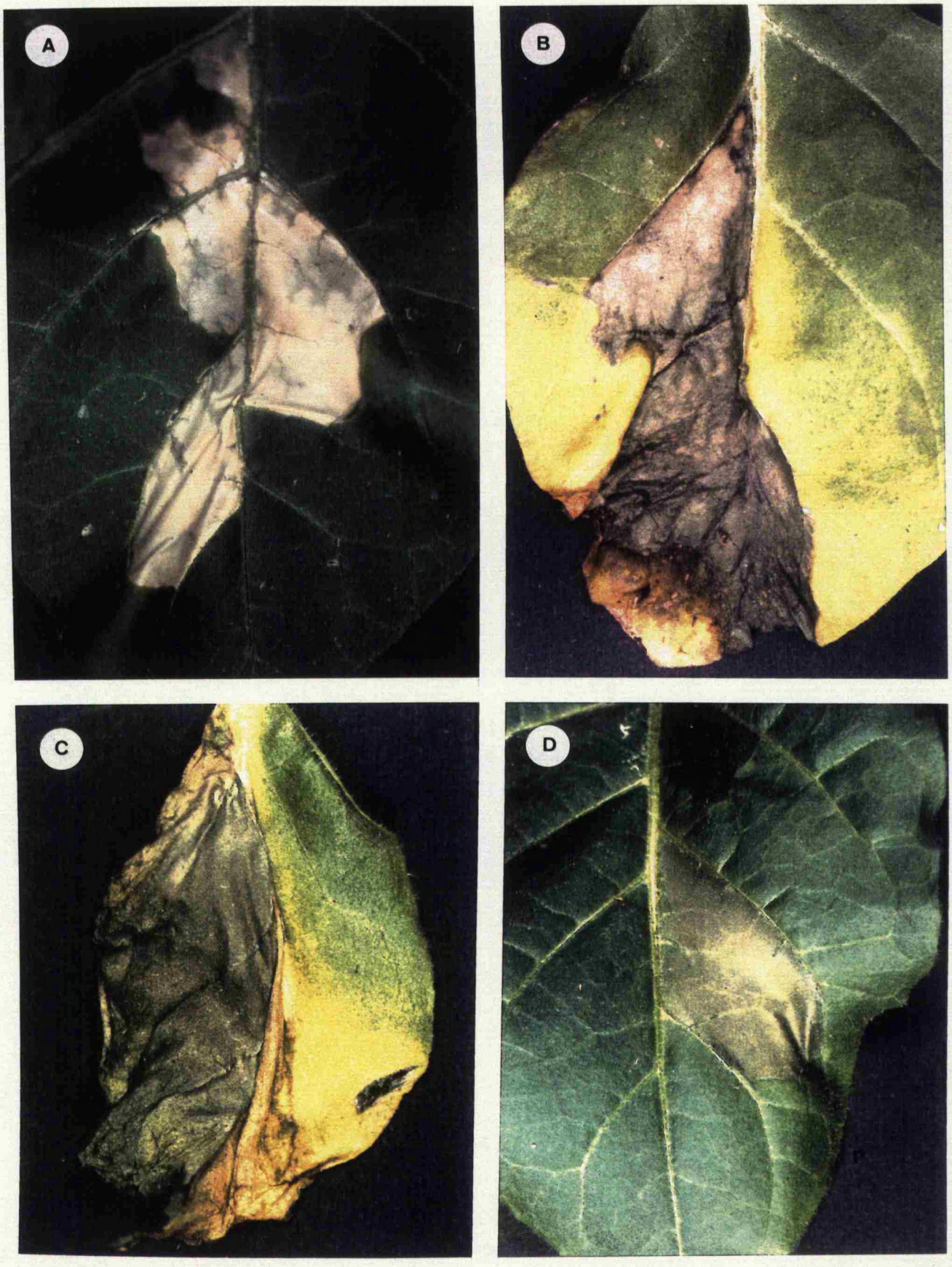

A
B
C
D

Figure 5.11 PR1a induction in AoPR1- and PR1a-SH-L tobacco after bacterial infection

Wild-type, AoPR1- and PR1a-SH-L tobacco plants were infected with *P. s.* pv. *syringae* . Samples before infection and after 6, 12, 24, 48 h infection were taken. PR1a protein accumulation was determined by PR1a anti-serum.

Lane 1 PR1a level in wild type tobacco at 0 h

Lane 3 PR1a level in wild type tobacco at 6 h

Lane 5 PR1a level in wild type tobacco at 12 h

Lane 7 PR1a level in wild type tobacco at 24 h

Lane 9 PR1a level in wild type tobacco at 48 h

Lane 2 PR1a level in SH-L tobacco at 0 h

Lane 4 PR1a level in SH-L tobacco at 6 h

Lane 6 PR1a level in SH-L tobacco at 12 h

Lane 8 PR1a level in SH-L tobacco at 24 h

Lane 10 PR1a level in SH-L tobacco at 48 h

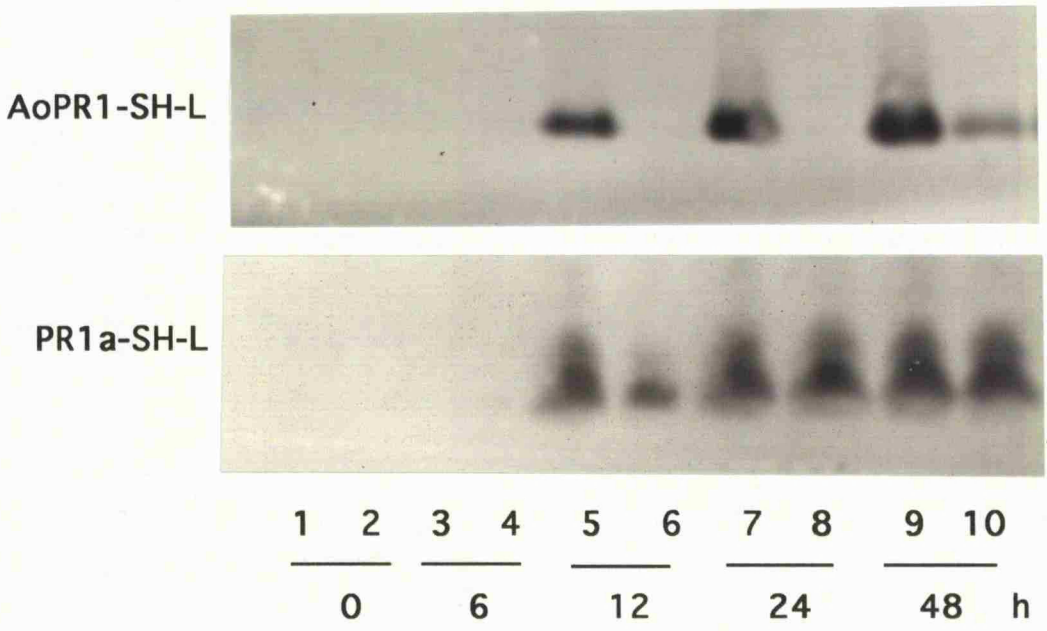
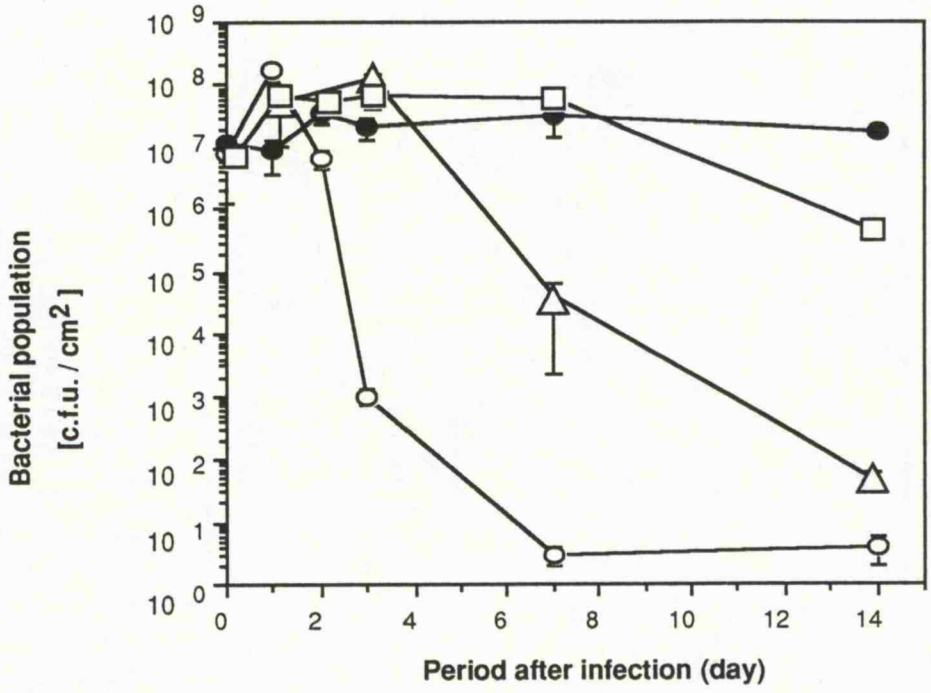

AoPR1-SH-L
PR1a-SH-L
1 2 3 4 5 6 7 8 9 10
0 6 12 24 48 h

Figure 5.12 Bacterial number in lesions arising in AoPR1-SH-L and PR1a-SH-L tobacco following infection with *P. s.* pv. *syringae*

P. s. pathovars were grown overnight in nutrient broth, centrifuged and then resuspended to an approximate concentration of 1 x 10^8 colony forming units (cfu) per ml in 10 mM phosphate buffer (pH 7.0). Tobacco leaves were infected with bacteria by injection of leaf intercellular spaces (see 2.28). Lesion materials were taken at different time points and the number of cfu determined.

Means ± SE shown, n = 3.



wild type infected with P. s. pv. syringae

wild type infected with P. s. pv. tabaci

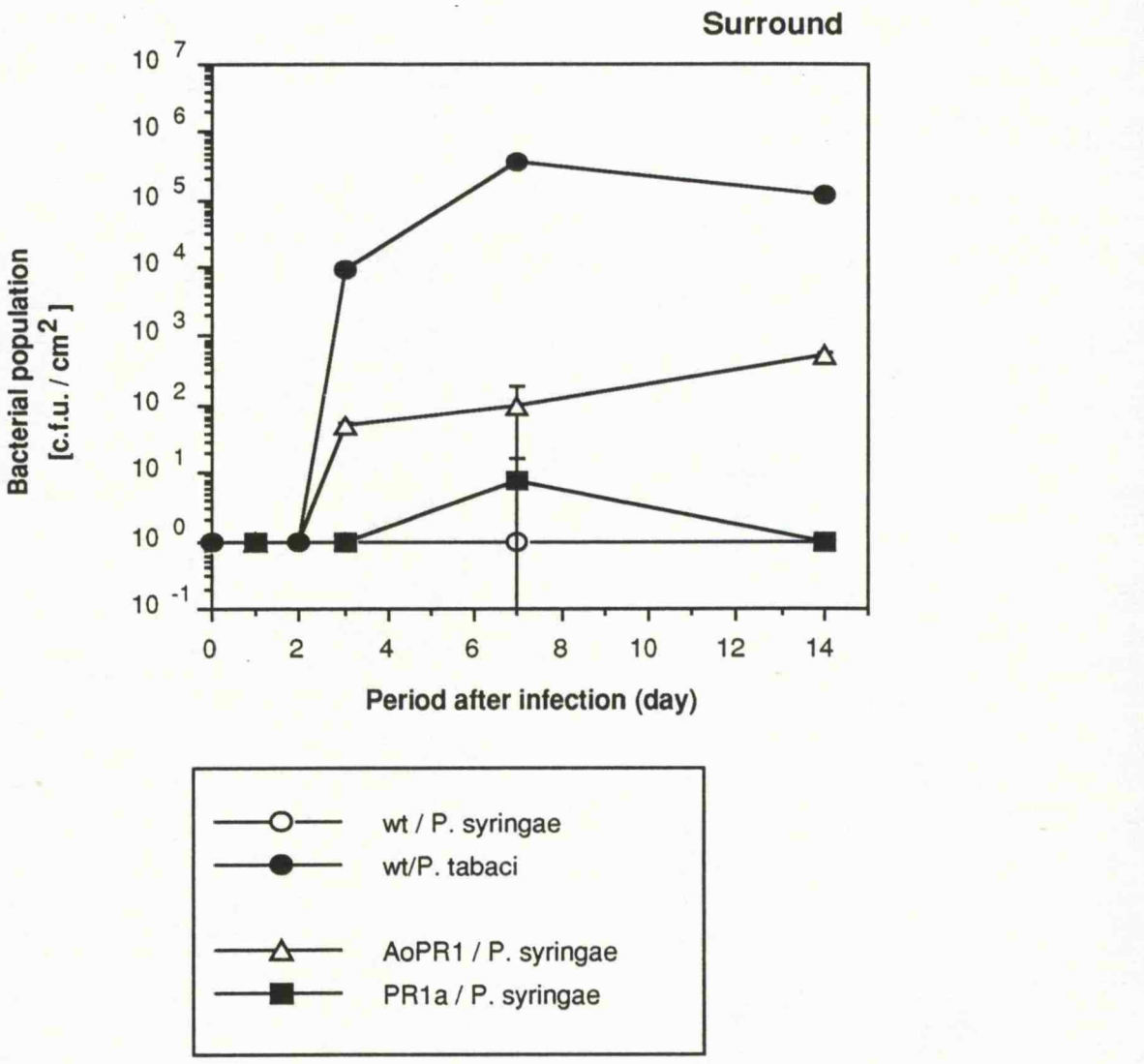
PR1a-SH-L infected with P.s. pv.syringae

AoPR1-SH-L infected with P.s. pv.syringae

Figure 5.13 Bacterial escape around lesions arising following infection with *P.s.* pathovars in AoPR1- and PR1a-SH-L tobacco

P. s. pathovars were grown overnight in nutrient broth, centrifuged and then resuspended to an approximate concentration of 1 x 10^8 colony forming units (cfu) per ml in 10 mM phosphate buffer (pH 7.0). Tobacco leaves were infected with bacteria by injection of leaf intercellular spaces (see 2.28). Surounding materials were taken at different time points and the number of cfu determined.

Means ± SE shown, n = 3.


Surround
Bacterial population
[c.f.u. / cm2]
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
10 -1
0
2
4
6
8
10
12
14
Period after infection (day)
wt / P. syringae
wt/P. tabaci
AoPR1 / P. syringae
PR1a / P. syringae

5.3 Conclusions and discussion

5.3.1 Early, local SA accumulation is essential for containment of invading pathogen

We have been investigating SA in defence responses by using transgenic tobacco constitutively expressing SH-L and we know that SA is required in SAR and maybe have a separate role in the local HR response (Chapter 4). We tried to separate the local and systemic effect of SA depletion by using two inducible promoters, one of which is strongly induced locally and the other systemically. It is worth noting that PR proteins are expressed during normal plant development (Fraser, 1981; Lotan and Fluhr, 1990). Thus, whether SA levels are elevated during some phases of plant development and what the side effects to plant development by blocking SA accumulation constitutively remains unknown. Therefore, it could be better to use inducible promoters to drive SH-L expression.

Because the AoPR1 promoter is only inducible locally, I expected that the results would be similar to those obtained with the 35S-SH-L plants in terms of local lesion formation but that we could possibly still get SAR.

As expected, TMV was not limited in the primary infected leaves (Fig.5.4) and cell-to-cell virus spreading was observed in AoPR1-SH-L plants (Fig.5.5). In bacterial infection, escape of normally incompatible pathogens was observed (Fig.5.10 & Fig.5.13) in AoPR1-SH-L plants, similar as in 35S-SH-L plants. Because the AoPR1 promoter is switched on within 3 hours after *P. s.* pv. *syringae* infection (Fig.5.2a), any early (4-6 h) SA accumulation locally would be destroyed by the early inducible expression of SH-L in the specific cell type as it is synthesized. This could be the reason why PR1a was not produced in AoPR1-SH-L tobacco as early as in wild type tobacco and why normally incompatible bacteria were surviving and escaping in AoPR1-SH-L tobacco (Fig.5.13). This gave us the information that at early time points, SA accumulation is very important in

mechanisms killing pathogens. So, apart from the conclusion in 4.3.3 which is "local SA accumulation is essential for containment of invading pathogen", it seems that the original conclusion should be revised to "early, local SA accumulation is essential for containment of invading pathogens". Although it has been shown that SA plays a role in slowing down the growth of incompatible bacterial populations in plants (Delaney *et al.*, 1994), this is the first demonstration that SA is essential for the function of defence processes resulting in subsequent bacterial death.

It seems that SAR is exhibited in AoPR1-SH-L plants as from Fig.5.9, it is clear that TMV lesion size was reduced in AoPR1-SH-L plants 3 days after secondary TMV infection. In the aims of this chapter, I was hoping that I could answer the question whether SA is a primary, mobile signal because local SA accumulation is blocked in AoPR1-SH-L plants and SAR should not be exhibited if SA is a transported signal. At the end of this study, no conclusion for this question has been achieved. Because the AoPR1 promoter is not switched on quickly enough (switched on 3 days after TMV infection, Fig.5.1a), SA could possibly already have built up at that time and this could be the reason why PR1a protein was detected in local TMV lesions (Fig.5.7). Thus, the question still remains whether SA is a primary signal transported from local infection.

5.3.2 Only a small increase in systemic SA level is required to induce SAR

For a start, I expected that SA accumulation around developing lesions should not be affected very much, but that systemic accumulation of SA could be blocked in PR1a-SH-L plants, so that SAR should possibly not be established. Surprisingly, PR1a was detected and SAR exhibited in PR1a-SH-L plants (Fig. 5.8 & Fig.5.9). Because there might be a very small amount of SA increase in systemic tissue before the PR1a promoter is switched on, therefore, it seems that this small increase in systemic SA levels is all that is required to induce SAR. Another unexpected result was that TMV was found to escape systemically in

PR1a-SH-L tobacco. Due to the fact that some mutant virus strains capable of overcoming the *N* gene-mediated HR have been isolated previously (Saito *et al.*, 1987; Knorr and Dawson, 1988; Meshi *et al.*, 1988,1989; Padgett and Beachy, 1993), experiments were performed to make sure that TMV U1 (applied in our experiments) was not mutated to some resistance-breaking strain. TMV particles reisolated from systemic lesions in PR1a-SH-L plants induced normal, restricted, local lesions on wild-type Samsum NN tobacco plants (5.2.3.3) showing that TMV is not mutated in SH-L plants. A major question then arises relating to why systemic TMV escape was not found in 35S-SH-L tobacco. The answer is yet to be found out.

Chapter 6
Hydrogen peroxide and salicylic acid in the induction of PR protein expression

6.1 Introduction

During the course of this study, Klessig and co-workers published a paper on the mechanism of action of SA (see 1.2.7). They purified a soluble, low-affinity binding protein which binds SA (K_D 14μM) and its active analogues *in vitro* and exhibited catalase activity which was inhibited by SA and its active analogues (Chen *et al.*, 1993a; 1993b). They suggested that SA may function in plants by inhibiting catalase thus allowing the accumulation of H_2O_2 which can then act as a second messenger to switch on defence gene expression and activate SAR. In support of this hypothesis it was demonstrated that both H_2O_2 and a catalase inhibitor, 3-amino, 1,2,4- triazole (3AT), can induce PR protein expression when injected directly into leaf tissue. Furthermore, they claimed to find that elevated levels of H_2O_2 (1-2 fold) were detected in leaf tissue fed with SA. However, there were no data suggesting that catalase activities were reduced and H_2O_2 levels elevated in systemic tissues following local pathogen infection. The hypothesis that H_2O_2 *in planta* may be a second messenger working downstream from SA in the signalling pathway responsible for establishing the SAR state is further explored in this chapter.

6.2 Results

6.2.1 Induction of PR1a by hydrogen peroxide and 3-AT

Treatment of wild-type tobacco with SA induced the expression of PR1a as determined by Western blotting (Fig.6.1). Lower levels of PR1a were detected following treatment of wild-type plants with H_2O_2 (5 mM) and the irreversible catalase inhibitor 3AT. Treatment with H_2O_2 levels of up to 0.5 M failed to achieve any increase in PR1a gene expression and caused substantial damage to leaf tissue (data not shown). As already noted, SA fails to induce PR1a expression in SH-L-transformed plants (see 3.2.6.1). Thus, it could be argued that if H_2O_2 lies downstream of SA in the SAR signalling pathway and if its accumulation is dependent upon catalase inhibition (Chen *et al.*, 1993b) then treatment with either H_2O_2 or 3AT should overcome the SA block. Figure 6.1 shows that H_2O_2 and 3AT are unable to induce PR1a expression in 35S-SH-L transformed plants suggesting that the mode of action of these compounds lies upstream of, and is dependent upon, SA accumulation.

6.2.2 Inhibition of catalase by SA leaf extracts

Catalase can catalyze the conversion of H_2O_2 into H_2O and O_2. In recent work, instead of measuring H_2O_2 levels directly, Klessig and collegues measured catalase activity in crude extracts by monitoring oxygen evolution (Sanchez-Casas and Klessig, 1994). When catalase is inhibited, H_2O_2 driven O_2 evolution should be reduced. However, the ability to evolve oxygen was not definitely linked with catalase activity and peroxidases could act as competitors with catalase for H_2O_2 (thereby reducing oxygen evolution). Therefore, in all our experiments, 3AT-sensitive catalase activities (destruction of H_2O_2 by catalase) were monitored as described in 2.22. The catalase experiments were carried out in collaboration with Dr. Paul Kenton.

Figure 6.1 Accumulation of PR1a protein in response to SA, H_2O_2 and 3-amino -1, 2, 4-triazole (3AT)

Leaf discs of wild-type Samsun NN and 35S-SH-L Samsun NN tobacco plants were treated with 1mM SA, 5mM H_2O_2 and 4mM 3AT. After 2 days, PR1a accumulation was determined by probing 15μg of protein from infiltrated regions on western blots with PR1a anti-serum.

Lane 1 PR1a level in wild type tobacco treated with SA

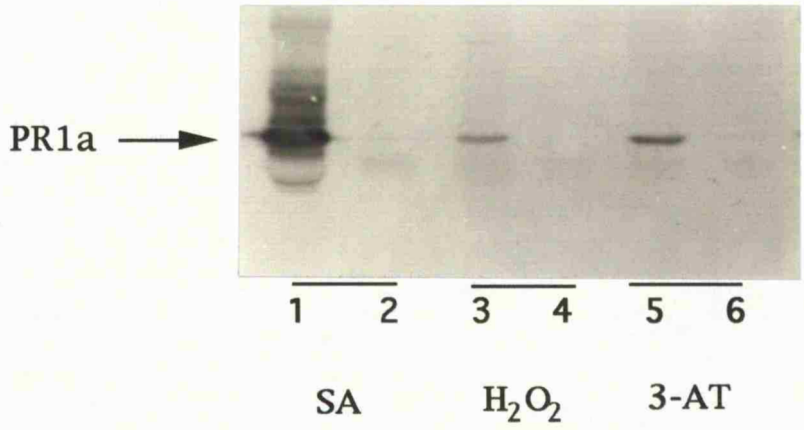
Lane 2 PR1a level in 35S-SH-L tobacco treated with SA

Lane 3 PR1a level in wild type tobacco treated with H_2O_2

Lane 4 PR1a level in 35S-SH-L tobacco treated with H_2O_2

Lane 5 PR1a level in wild type tobacco treated with 3AT

Lane 6 PR1a level in 35S-SH-L tobacco treated with 3AT


PR1a
1
2
3
4
5
6
SA
H_2O_2
3-AT

The ability of 3AT to induce local PR-protein expression in wild type tobacco plants is clear and the involvement of catalase in SAR is intriguing. To date SA at 1mM has been reported to reduce catalase activity *in vitro* using crude protein preparations from tobacco leaves (Chen *et al.*, 1993b). Therefore, experiments were designed to determine whether inhibition occurred at normal physiological levels simulated by externally-supplied SA (e.g. levels less than 100 μM SA) , or following pathogen challenge.

Catalase activity in wild-type Samsun NN tobacco leaf extracts was slightly depressed in the presence of SA compared to 4hBA and INA (Fig.6.2a). This inhibition was apparent only at 500 μM SA but not observed at lower concentrations. Inhibition of up to 30% was achieved at a concentration of 10mM SA (pers. comm. with Dr. Kenton). The degree of catalase inhibition was further increased by a 60 min preincubation with SA, but enzyme activity only really showed a significant reduction at SA levels greater than 250μM (Fig.6.2b). INA, a good inducer of PR protein gene expression (see 4.2.2.5), should inhibit catalase to a large extent according to Klessig's model. Surprisingly, it did not prove to be an inhibitor of catalase activity, even at high concentrations and with a 60 min preincubation (Fig.6.2b). In contrast, catechol was a potent *in vitro* inhibitor of catalase (89% inhibition at 1mM catechol compared to control; Fig.6.2a). In a recent paper by Conrath *et al.* (1995), it was shown that INA and SA could inhibit catalase activity. This is quite contradictory to what we obtained. Because O_2 evolution was monitored in their experiments as mentioned before and there was no definite link between O_2 evolution and catalase activity, it is possible that the different catalase assay method resulted in the different results.

6.2.3 Uptake of SA into leaf disks and effect on extractable catalase activity

If the mechanism of PR1a activation is dependent upon inhibition of catalase (Chen *et al.*, 1993b) then it follows that treatment of tobacco with SA should cause a decrease in

extracted catalase activity when injected into or applied to leaves *in planta*. Tobacco catalase has a K_D of 14 μM for SA and a half life for dissociation of around 40 min (Chen *et al.*, 1993a). In collaboration with Rob Darby and Dr. Kenton, experiments were designed to measure catalase activity in extracts from SA-treated leaf disks. SA (1mM) was taken up very efficiently into tobacco leaf disks, reaching concentrations of over 500 μM within 30min (Table 6.1). However, after just 6 hours incubation the levels of free SA in leaf tissue had dropped to around 100 μM (Table 6.1). The levels of SA in the leaf extracts used for catalase measurements prior to dilution for assay (Table 6.1) were all greater than the reported K_D of 14 μM for SA-binding to catalase. Inhibition of catalase activity was not observed in any of these leaf extracts.

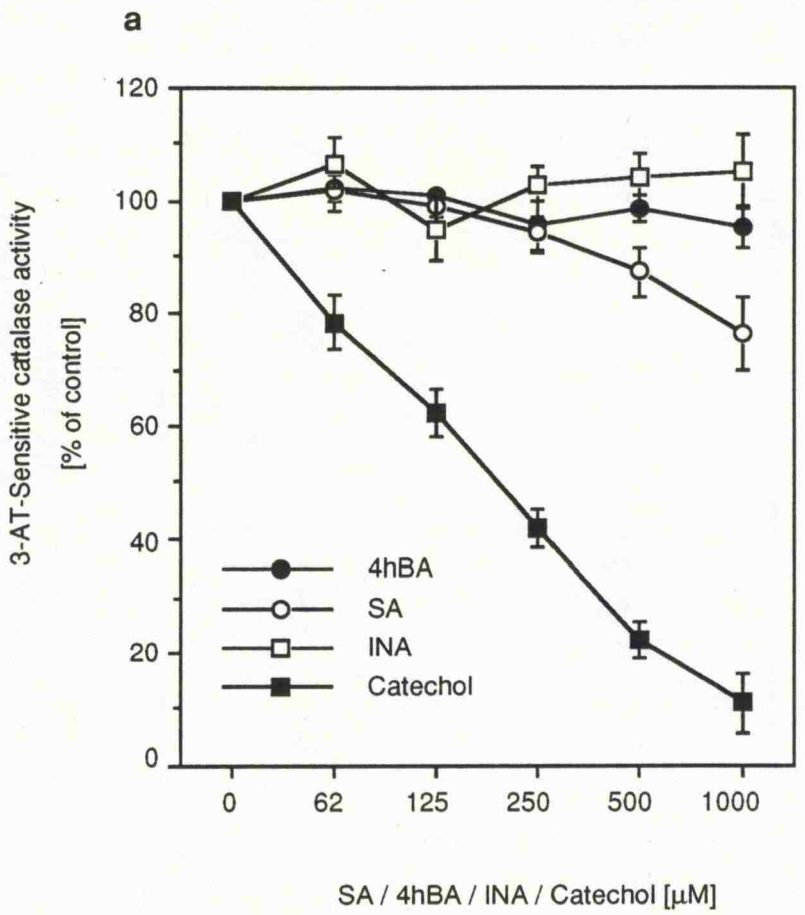
6.2.4 Catalase activity in leaves infected with *P. s.* pv. *syringae*

Intercellular injection of wild-type tobacco leaf tissue with *P. syringae* pv. *syringae* leads to visible tissue collapse within 6-8 hours and induces endogenous PR1a gene expression by 24 hours (Fig. 6.3 inset). Catalase was measured in samples taken from lesions (or mock-infiltrated areas) or adjacent areas of the same leaf. If Klessig's theory is right, catalase activity should be inhibited after this infection. In contrast, catalase activity was not altered significantly in either lesions or surrounding tissue of infected plants compared to mock-inoculated leaves (Fig.6.3). Actually, in these tissues there were higher levels of 3AT-insensitive H_2O_2-degrading activity following bacterial infection (Bi *et al.*, 1995), suggesting that the concentration of H_2O_2 in these tissues is low.

Figure 6.2 Effects of salicylic acid (SA), 4-hydroxybenzoic acid (4hBA), 2,6-dichloroisonicotinic acid (INA) and catechol on *in vitro* catalase activity of wild-type tobacco leaf extracts

(a) 50µl of Samsun NN leaf extract (0.2 mg protein.ml^{-1} in catalase extraction buffer) was incubated for 2 min at room temperature with 50µl of catalase extraction buffer containing increasing concentrations of SA, 4hBA, INA or catechol and 50µl of catalase assay buffer ± 10 mM 3-amino-1,2,4-triazole (3AT; final concentration). The reaction was stopped by addition of 200µl of catalase stop solution and the absorbance read at 405nm. Buffers described in method 2.22. Data expressed as percentage of 3AT-sensitive catalase activity compared to control. Means ± standard errors shown (where larger than symbols used). n = 3.

(b) Catalase activity in Samsun NN leaf extracts following 1 hour preincubation in the presence of SA, 4hBA or INA. Assays were carried out in the presence of SA, 4hBA and INA levels as indicated; preincubation conditions were thus 3.5 fold higher than those used in the assay. Data expressed as percentage of 3AT-sensitive catalase activity compared to control. Means ± standard errors shown. n = 3.


a
120
100
80
60
40
20
0
3-AT-Sensitive catalase activity
[% of control]
4hBA
SA
INA
Catechol
0
62
125
250
500
1000
SA / 4hBA / INA / Catechol [µM]

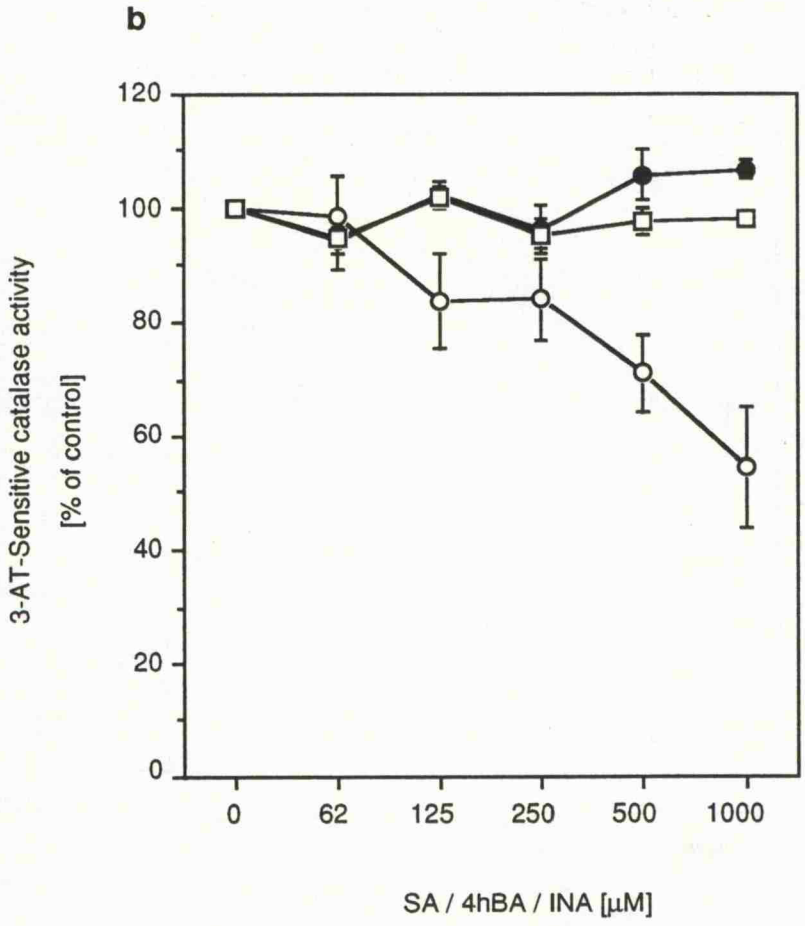

b
120
100
80
60
40
20
0
3-AT-Sensitive catalase activity
[% of control]
0
62
125
250
500
1000
SA / 4hBA / INA [µM]

Table 1

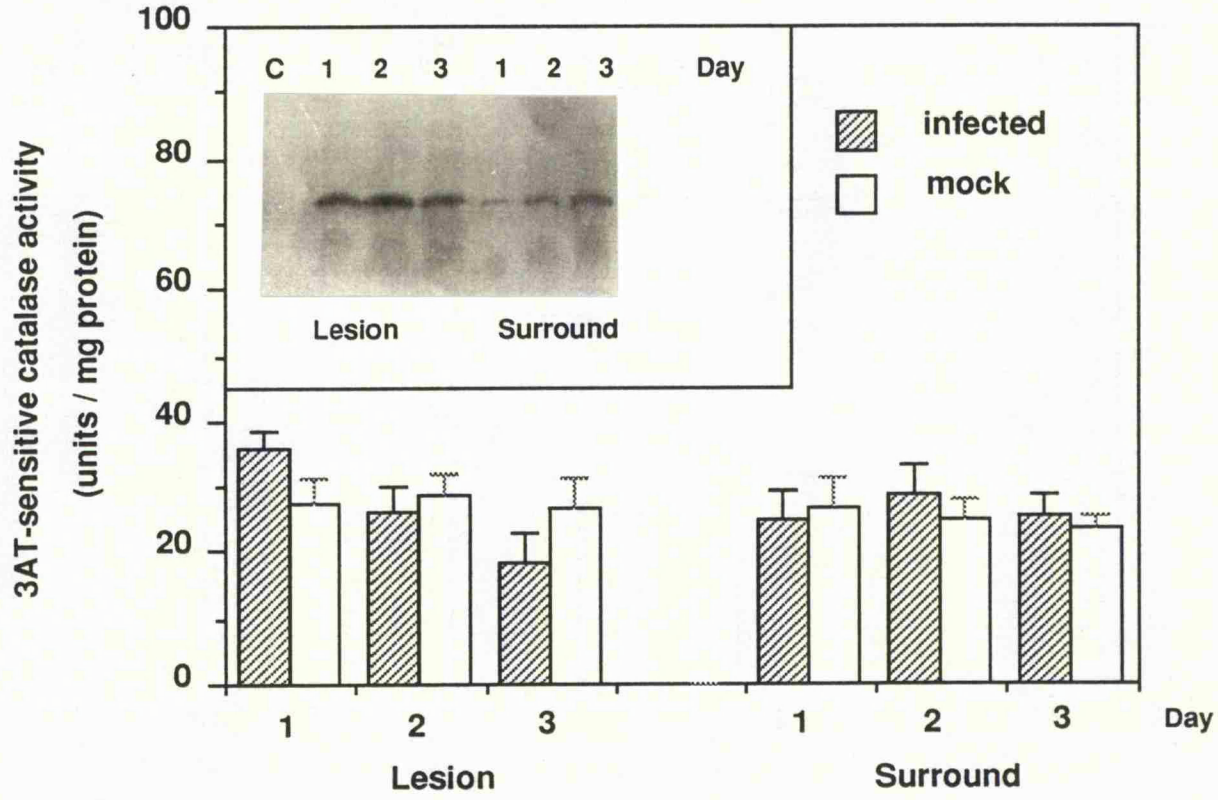
Salicylic acid uptake by tobacco leaf disks and catalase activity in soluble tissue extracts

Incubation time with 1mM SA (hr)	SA concentration in leaf disk (µg / g fresh wt)	(µM)	SA concentration in leaf extract (µM)	Catalase activity in leaf extract (% of 4hBA control)
0.5	47.3 ± 3.4	571	61.0 ± 3.1	121.9 ± 11.9
1.0	47.0 ± 2.1	567	73.0 ± 6.9	110.2 ± 3.8
3.0	24.3 ± 1.9	293	36.8 ± 4.0	122.8 ± 13.2
6.0	8.7 ± 2.9	105	14.7 ± 4.9	103.1 ± 3.7

1.5 cm leaf disks from Samsun NN tobacco (approximately 2 g per leaf) were floated for the times indicated in 1mM salicylic acid or 4-hBA and then rinsed in water and blotted dry with tissues. Disks were extracted as described in method 2.22 for catalase assays using 200µl per disk of extraction buffer. 200µl was removed and used for catalase assay, the remainder was processed for SA measurements (2.19 and 2.20). SA concentration is expressed as µg/gm fresh weight of leaf disk as well as an estimation of molarity in cell fluids based on the measurement that 61.3 ± 1.8% of tobacco leaf fresh weight is water content. The actual SA content in the leaf soluble protein extract prior to catalase assay is also presented. Means ± SE shown, n=3.

Figure 6.3 Catalase activity in and around lesions formed following infection of wild-type tobacco with *P.s.* pv. *syringae*.

Wild-type tobacco (Samsun) leaves were inoculated with 100µl per sample area of 10mM phosphate buffer (pH 7.0) - MOCK, or the same buffer containing 1 x 10^7 colony-forming units of *P. s.* pv. *syringae* - INFECTED. 1.5 cm discs were taken from infiltrated areas and adjacent uninfiltrated areas on the same leaf at 1, 2 and 3 days following infection. 3AT-sensitive catalase activity was measured as described in 2.22. Means ± standard errors shown. n = 9. **Inset** - PR1a expression, detected by Western blotting, in wild-type tobacco (Samsun) incubated as above with phosphate buffer or *P. s.* pv. *syringae*.


3AT-sensitive catalase activity
(units / mg protein)
100
80
60
40
20
0
C 1 2 3 1 2 3 Day
Lesion
Surround
infected
mock
1 2 3 1 2 3 Day
Lesion
Surround

6.3 Conclusion and Discussion

6.3.1 SA functions downstream of H_2O_2 in the induction of PR protein

The current data show that H_2O_2 is a relatively weak inducer of PR proteins in wild type tobacco and also demonstrate that treatment with either H_2O_2 or a catalase inhibitor cannot overcome the SH-L block to PR protein expression. In terms of systemic signalling Neuenschwander *et al.* (1995) concluded that systemic H_2O_2 levels were unaffected in wild type and *nah*G tobacco following local TMV infection. Neuenschwander *et al.* (1995) also showed that pretreatment of tobacco leaf tissue with H_2O_2 or 3AT did not induce resistance to TMV as would be evidenced in wild type plants by a reduction in lesion size, which is typical of an SAR response. These observations, coupled with the observation that PR1a expression in infected tissue is totally dependent on SA accumulation (see 4.2.2.3 & 4.2.2.4) suggest that H_2O_2 is probably not a second messenger working downstream of SA in the SAR response as suggested previously (Chen *et al.*, 1993b).

The hypothesis that catalase inhibition allowing H_2O_2 build up is responsible for PR protein induction and establishment of SAR is based on the observation that a specific SA-binding activity found in tobacco leaf extracts is a catalase (Chen *et al.*, 1993b). An earlier study (Chen *et al.*, 1993a) reported that catalase bound SA with a K_D of approximately 14μM. Using *in vitro* assays Klessig and co-workers have shown that the activity of purified tobacco catalase was inhibited in a specific fashion by 1mM SA. These studies have been extended recently to show that an SA binding activity is present in crude protein preparations purified from a range of plant species (Sanchez-Casas and Klessig, 1994). Our data confirmed that it is possible to reduce catalase activity in crude plant extracts using high concentrations of SA. However, SA was largely ineffective as a catalase inhibitor below 250μM, a fact which seems inconsistent with an apparent K_D of 14μM for

SA binding. Furthermore, INA, which is a good inducer of PR proteins in tobacco, was not able to reduce catalase activity *in vitro* significantly, even following a 60 min preincubation.

The maximum level of free SA in tissue adjacent to lesions in TMV infected tobacco leaves has been estimated to be in the region of 6μg/gm fresh weight (Enyedi *et al.*, 1992) which equates roughly to a SA concentration of around 70 μM if it is assumed that SA is not compartmentalised within plant cells. Thus, it would appear that SA concentrations required to inhibit catalase activity *in vitro* are much greater than the highest physiological levels reported around TMV lesions and therefore the significance of this inhibition to SAR is questionable. In addition, Chen *et al.* (1993b) reported that H_2O_2 levels were elevated for at least 24 hours in SA-treated tissue. However, the current data, demonstrated that, although 1mM SA gave full induction of PR1a expression in leaf disks, the level of free SA accumulating within such tissue was reduced to a level much lower than that required to inhibit catalase *in vitro* within just 6 hours.

The current data show clearly that 3AT is less active than SA at inducing PR proteins and thus there is a possibility that the irreversible inhibition of catalase by 3AT allows sufficient H_2O_2 to accumulate to trigger the SA-dependent induction of PR1a observed in wild-type tobacco but missing in SH-L plants. One potential explanation, that H_2O_2 induces an rise in endogenous SA, could be tested by intercellular infiltration of H_2O_2. However, these experiments may be hampered by the fact that externally-applied H_2O_2 is rapidly degraded (see Levine *et al.*, 1994) and thus very high levels of H_2O_2 have to be presented to mimic the oxidative burst that occurs following pathogen recognition. Indeed, in the paper by Neuenschwander *et al.* (1995), it is shown that leaf infiltration with H_2O_2 at concentrations approaching 0.5M induces a small increase in SA concentration, but under such conditions there is substantial tissue damage. Levine *et al.*, (1994) similarly reported considerable cell death when cell suspensions were treated with only 10mM H_2O_2.

If SA functioned *in vivo* by inactivating catalase, thus allowing H_2O_2 accumulation then several predictions might be made. Firstly, it would be expected that H_2O_2 acts downstream of SA in the SAR signalling pathway and thus addition of H_2O_2 or inhibition of catalase should induce PR protein expression, even in the absence of salicylate accumulation. It has been shown that this is not the case (Fig.6.1). Secondly, it may be possible to measure reductions in catalase activity in infected tissue following pathogen inoculation prior to induction of PR proteins. The present data suggested that catalase is not inhibited by direct application of SA, or inhibited following pathogen infection of tobacco leaves under conditions that induce PR protein expression and promote establishment of SAR (Fig.6.2 & 6.3). A recent study showed that UV and ozone treatments, which lead to the production of active oxygen species in plant tissues, increased BA2H activity (see 1.2.4.2), SA levels and PR protein content of tobacco leaves (Yalpani *et al.*, 1994). Experiments by Green and Fluhr (1995) also show that SA is able to elicit PR1 via a pathway which did not involve reactive oxygen species. Thus, data demonstrating that SA inhibits catalase activity *in vitro* may need to be interpreted cautiously before such observations are extrapolated to develop a hypothesis concerning the mechanism by which SA involves PR protein expression and SAR establishment in intact plants.

Chapter 7
General Conclusions and Discussion

7.1 SA is required for local PR protein induction

PR protein is not induced in the transgenic tobacco plants in the absence of SA. The results have clearly shown that SA is required for local PR protein induction (at least PR1a protein). This has actually given the answer to the first question in our first chapter which is whether SA is a local signal for some defence responses. In animal cells, SA and aspirin have been found to be able to inhibit the activation of the transcription factor ~~nuclear factor~~ kB (NF-kB), which is critical for the inducible expression of multiple genes involved in inflammation and infection (Kopp and Ghosh, 1994). It is still hard to say how it might work in plants but as only low levels of exogenous supplied SA (10 μM) are required to induce PR1a, it is possible that SA has a direct effect on transcription.

It is known that SA level can increase to 6 μg/gfw around TMV lesions (Malamy *et al.*, 1990) and this is equivalent to about 70 μM. Such a high concentration is not very common for a signal molecule of which is normally only around nM or even pM so that SA could have a separate role apart from local signalling.

7.2 SA is involved in local HR lesion development

The separate role for local SA could be its involvement in local lesion development. This is the conclusion for the second question in the aims of the project. We have been using viral and bacterial pathogens to investigate the importance of SA in the HR response. We have found that TMV is not well contained in primary infected leaves of transgenic tobacco plants expressing salicylate hydroxylase, as it would be in wild type *N*-tobacco. We have also found that incompatible bacterial pathogens invade to the adjacent tissue in SH-L plants, somewhat like a compatible pathogen would do. Thus, SA must be involved in the

processes which can limit virus spread and kill bacterial pathogens. How SA is involved in this process is a very interesting field to carry on research. One hypothesis is that SA may promote a more rapid oxidative burst which can cause rapid plant cell death and kill bacteria directly. Recent work by Kauss and Jeblick (1995) has shown that pretreatment of parsley suspension cultured cells with SA greatly enhances spontaneous and elicited production of H_2O_2. H_2O_2 has been demonstrated to have multiple physiological functions, such as directly as toxic defence agents against pathogens (Mehdy, 1994), or for the cross-linking of cell wall polymers (Bradley *et al.*, 1992; Brisson *et al.*, 1994). Chen's work (1993a, 1993b) has also indicated that SA may be able at high concentration to stimulate H_2O_2 production by inhibiting catalase activity, although this H_2O_2 production does not seem to be a second messenger in the induction of PR proteins and SAR (Bi *et al.*, 1995; Neuenschwander *et al.*, 1995). The increased level of H_2O_2 is harmful and therefore could be eliminated by antioxidants or detoxifying enzymes (Elstner and Osswald, 1994). Because compounds such as methyl 2,5-dihydroxycinnamic acid could act as H_2O_2 scavengers (Levine *et al.*, 1994) and because SA has a similar structure, it is possible that SA serves as a H_2O_2 scavenger at later stage. Indeed, both SA and benzoic acid have been reported to be scavengers of hydroxyl radicals (Yoshiki *et al.*, 1995). Recently Rob Darby (a collegue at Leicester) and Alan Crozier (Glasgow University) have reported the existance of new hydroxylated form of benzoic acid and SA following feeding which support their role as hydroxyl radical scavengers (pers. comm. with Rob Darby).

Some work still needs to be done in the near future, such as to determine the exact SA levels in different transgenic tobacco plants at different time points after pathogen attack to confirm the predicted effects of SH-L expression on SA accumulation. Preliminary data have shown that local SA level 3 day after TMV infection in AoPR1-SH-L plants is about the same as in wild type tobacco but after 7 days the level has dropped to about a quarter of that in wild type, which is in line with the expression profile.

7.3 SA - a mobile signal?

This question has been asked in the first chapter and I tried to use AoPR1-SH-L plants to test whether SA is a mobile signal. As mentioned in 5.3.1, no conclusion could be drawn using that material because of delayed SH-L expression in AoPR1-SH-L plants. Later, I intended to use a phloem-specific promoter to drive the expression of SH-L so as to block the accumulation of SA in the phloem. If the hypothesis that SA is a transported signal is right, I then expected that SAR would not be established in transgenic tobacco in which SA accumulation in phloem becomes impossible. Maize sucrose synthase (Sh) is specifically expressed in the phloem and the Sh promoter was obtained from Dr. Russell (Yang and Russell, 1990). A Sh-SH-L construct has been made and Sh-SH-L transgenic tobacco plants have been generated. At that time, Vernoiij *et al.* (1994) published a paper in which they drew the conclusion that SA is not a mobile signal transported from phloem (see 4.3.4). Preliminary data in Sh-SH-L tobacco showed that Sh-SH-L plants do exhibit SAR which is consistent with Vernoiij's result. However, the expression level of SH-L and the actual SA level in the phloem is unknown so the data is very preliminary. In the 35S-nahG tobacco plants which Vernoiij *et al.* used, there was always some SA present and their explanation was that SA could be synthesized in chloroplast and stored in that compartment. Whether there is a leakage of small amount SA from local infected leaf to systemic leaf in their nahG plants and how a small increase in systemic SA levels can affect SAR induction remians unknown.

7.4 Hydrogen peroxide does not function downstream of SA in the induction of PR protein expression

The fourth question in the introduction was whether SA really functions by affecting H_2O_2 levels, via inhibition of catalase in PR protein induction. The current work has shown that

SA actually acts upstream of H_2O_2 in SAR signaling. Recent papers have also demonstrated that H_2O_2 and ozone can actually induce SA synthesis (Yalpani *et al.*, 1994; Neuenschwander *et al.*, 1995). In summary, SA accumulation could be activated by H_2O_2 following an oxidative burst and accumulated SA could induce SAR gene expression and also promote further more rapid production of H_2O_2 and further SA synthesis following a second challenge which result in more rapid lesion formation. To test this hypothesis, experiments can be designed to measure H_2O_2 and SA production in secondary infected leaves. If the hypothesis is right, H_2O_2 and SA production in secondary infected leaves should be more rapid than in primary infected leaves.

7.5 Substrate ranges of SH-L and its effects on PR induction

Substrate ranges of SH-L and its effects on PR induction is another area worth working on. Methyl salicylic acid, a votatile SA ester, is found in many plants including tobacco and it has been found that large amount of volatile methyl SA are released from TMV-inoculated tobacco in parallel with tissue accumulation of SA (Lee *et al.*, 1995). Methyl SA is also a PR protein inducer (pers. comm. with Dr. L. Mur) and it is a substrate for SH-L (pers. comm. with Prof. Peter William). What the effects of these substrates in SH-L plants will be interesting to be found out.

7.6 Biosynthesis of SA

Another area that deserves further attention is the metabolism of SA. Although some of the steps in the pathway(s) are becoming clear and several of the enzymes are currently being characterized, much remains to be done. The enzymes have yet to be obtained in pure form and their respective genes have not been cloned. Relatively little is known concerning the regulation of these enzymes and their corresponding genes. Benzoic acid (BA) is a precusor of SA and a large conjugated pool of BA, but not SA, has been found in healthy tobacco plants (Leon *et al.*, 1993; Yalpani *et al.*, 1993). Preliminary experiments have

shown that BA can induce PR protein expression and can partially induce PR protein expression in SH-L background (pers. comm. with Una Higgins). Since SA is not suggested to be a primary transported signal, is it possible that BA, which is a poor substrate for SH-L, could have some functions of its own in SAR and might yet prove to be the mobile element?

7.7 Genetic approach to the defense signalling transduction pathway(s)

The complete signal transduction pathway(s) involving SA is likely to be highly complex. SA inhibits synthesis of ethylene in several suspension cell cultures (Leslie *et al.*, 1988; Romani *et al.*, 1989). SA also blocks JA biosynthesis (Pena-Cortes *et al.*, 1993). Signals from other pathways are likely to impact the SA pathway as well, such as ethylene appears to potentiate the SA-mediated induction of genes encoding acidic PR proteins in certain plants (Lawton *et al.*, 1994). As mentioned in the first chapter, the genetic approach to defining components in a signalling pathway(s) is very powerful and has been used in plants, particularly *Arabidopsis thaliana*. In our lab, mutants affecting the defense signalling transduction pathway are being identified and I have been involved in this work for a long time. The stratgy being used is to transform the PR1a-GUS construct into *Arabidopsis* (2.10) and mutagenize the resulting transgenic line by EMS treatment. The non-mutated plants should still exhibit normal inducible ability by SA while the mutants should either lose the ability to response to SA (GUS activity will not increase after SA treatment) or be upregulated constitutively (GUS activity is high even without SA treatment). So far, several upregulated and downregulated mutants have been identified. It is expected that the mutants showing constitutive expression of PR1a protein will increase the resistance to pathogen attack while the mutants with no PR1a protein expression will reduce the resistance. A number of mutants have been identified in other labs (Dietrich *et al.*, 1994; Greenberg *et al.*, 1993; Greenberg *et al.*, 1994; Cao *et al.*, 1994; Bowling *et al.*, 1994). Further identification, cloning and characterization of the regulatory genes

associated with these mutants will help to elucidate the molecular basis of SAR and this should be an area of fruitful research.

References

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E. and Ryals, J. (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. USA* **90**, 7327-7331

Anderson, J.M. (1985) Evidence for phloem transport of jasmonic acid. *Plant Physiol.* 77S, 75

Antoniw, J.F. and White, R.F. (1980) The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. *Phytopath. Z.* **98**, 331-341

Assinder, S.J. and Williams, P.A. (1988) Comparison of the meta pathway operons on NAH plasmid pWW60-22 and TOL plasmid pWW53-4 and its evolutionary significance. *J. Gen. Micro.* **134**, 2769-2778.

Bent, A., Kunkel, B., Dahlbeck, D., Brown, K., Schmidt, R., Giraudat, J., Leung, J. and Staskawicz, B. (1994) *RPS2* of *Arabidopsis thaliana*: A leucine-rich repeat class of plant disease resistance genes. *Science* **265**, 1856-1860

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acid. Res.* **12**, 8711-8721.

Bi, Y.M., Kenton, P., Mur, L., Darby, R. and Draper, J. (1995) Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. *Plant J.* **8**, 235-245

Bol, J., Linthorst, H.J.M. and Cornelissen, B.J.C. (1990) Plant pathogenesis-related proteins induced by virus infection. *Ann. Rev. Phytopathol.* **28**, 113-138.

Boller, T. (1991) Ethylene in pathogenesis and disease resistance. In The Plant Hormone Ethylene. A.K.Mattoo and J.C.Suttle eds. (Boca Raton, Florida: CRC press) pp. 293-313

Bonas, U., Stall, R.E. and Staskawicz, B. (1989) Genetic and structural characterization of the avirulence gene *avrBS3* from *Xanthomonas campestris* pv. *vesicatoria*. *Mol. Gen. Genet.* **218(1)**, 127-136

Bonas, U., Conrads-Strauch, J., and Balbo, I. (1993) Resistance in tomato to *Xanthomonas campestris* pv *vesicatoria* is determined by alleles of the pepper-specific avirulence gene *avrBs3*. *Mol. Gen. Genet.* **238**, 261-269

Bowles, D. (1990) Defense-related proteins in higher plants. *Ann. Rev. Biochem.* **59**, 873-907

Bowling, S.A., Guo, A., Cao, H., Gordon, S., Klessig, D.F. and Dong, X. (1994) A mutation in arabidopsis that leads to constitutive expression of systemic acquired resistance. *Plant Cell* **6**, 1845-1857

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities utilising the principle of protein dye binding. *Anal Biochem.* **72**, 248-254.

Bradley, D.J., Kjellbm, P. & Lamb, C.J. (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* **70**, 21-30

Briggs, S.P. and Johal, G.S. (1994) Genetic patterns of plant host-parasite interactions. *Trens Genet.* **10**, 12-16

Brisson, L.F., Tenhaken, R. and Lamb, C.J. (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. *Plant Cell* **6**, 1703-1712

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J. and Broglie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254**, 1194-1197

Cameron, R.K., Dixon, R., and Lamb, C. (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. *Plant Journal* **5**, 715-725

Cane, P.A. and Williams, P.A. (1986) A restriction map of naphthalene catabolic plasmid pWW60-1 and the location of some of its catabolic genes. *J. Gen. Micro.* **132**, 2919-2929

Cao, H., Bowling, S.A., Gordon, S. and Dong, X. (1994) Characterization of an arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**,1583-1592

Carr, J.P. and Klessig, D.F. (1989) The pathogenesis-related proteins of plants. In: Setlow, J.K. (ed) *Genetic Engineering principles and methods*, vol 11 pp.65-109. Plenum Press, New York/London

Chadha, K.c. and Brown, S.A. (1974) Biosynthesis of phenolic acids in tomato plants infected with Agrobacterium tumefaciens. *Can. J. Bot.* **52,** 2041-2046

Chasan, R. (1994) Plant-pathogen encounters in Edinburgh. *Plant Cell* **6(10),** 1332-1341

Chen, Z. and Klessig, D.F. (1991) Identification of a soluble salicylic acid-binding protein that may function in signal transduction in the plant disease resistance response. *Proc Natl Acad Sci USA* **88,** 8179-8183

Chen, Z.X., Ricigliano, J.W. and Klessig, D.F. (1993a) Purification and characterization of a soluble salicylic acid-binding protein from tobacco. *Proc.Natl.Acad.Sci.* **90,** 9533-9537.

Chen, Z.X., Silva, H. and Klessig, D. (1993b) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* **262,** 1883-1886.

Chester, K.S. (1933) The problem of acquired physiological immunity in plants. *Q. Rev. Biol.* **8,** 275-324.

Clarke, M.C., Wei, W. and Lindsey, K. (1992) High-frequency transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*. *Plant Molecular Biology Reporter* **10 (2),** 178-189

Cleland, C.F. (1974a) Isolation of flower-inducing and flower-inhibiting factors from aphid honeydew. *Plant Physiol.* 54: 899-903

Cleland, C.F. and Ajami, A. (1974b) Identification of the flower-inducing factor isolated from aphid honeydew as salicylic acid. *Plant Physiol.* 54: 904-906

Collinge, D.B. and Slusarenko, A.J. (1987) Plant gene expression in response to pathogens. *Plant Mol. Biol.* **9,** 389-410

Conrath, U., Chen, Z., Ricigliano, J.R. and Klessig, D. (1995) Two inducers of plant defense responses, 2,6-dichloroisonicotinic acid and salicylic acid, inhibit catalase activity in tobacco. *Proc. Natl. Acad. Sci. USA* **92,** 7143-7147

Covey, S.N. and Hull, R (1981) Transcription of cauliflower mosaic virus DNA. detection of transcripts and location of the gene encoding the virus inclusion body protein. *Virology* **111,** 463-477

Culver, J.N., Lindbeck, A.G.C. & Dawson, W.O. (1991) Virus-host interactions - induction of chlorotic and necrotic responses in plants by tobamoviruses. *Ann. Rev. Phytopathol.* **29,** 193-217

Cutt, J.R. and Klessig, D.F. (1992) Pathogenesis-related proteins. In: Boller, T., Meins, Jr. F. (eds.) *Plant Gene Research: Genes involved in plant defense.* pp. 209-243. Springer-Verlag, Wien/New York

Dangl, J.L. (1995) Piece de resistance: Novel classes of plant disease resistance genes. *Cell* **80,** 363-366

Daniels, M.J., Collinge, D.B., Maxwell, D.J., Osbourn, A.E. and Roberts, I.N. (1987) Molecular biology of the interaction of *Xanthomonas campestris* with plants. *Plant Physiol. Biochem.* **25**, 353-359

Davis, J. I. and Evans, W. C. (1964) Oxidative metabolism of naphthalene by soil in *Pseudomonas putida Biochem. J.* **91**, 974-979.

Dawson, W.O. (1992) Tobamovirus-plant interactions. *Virology* **186**, 359-367

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1986) Data for biochemical research. Third edition, Clarendon Press, Oxford

Debleare, R., Bytebier, B., De Greve, H., Schell, J., Van Montagu, M., and Leemans, J. (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nuc. Acids Res.* **13**, 4777-4788

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994) A central role of salicylic acid in plant disease resistance. *Science* **266**, 1247-1250

Dempsey, D.A., Wobbe, K.K. and Klessig, D.F. (1993) Resistance and susceptible responses of *Arabidopsis thaliana* to turnip crinkle virus. *Phytopathol.* **83**, 1021-1029

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., and Dangl, J.L. (1994) Arabidopsis mutants simulating disease resistance response. *Cell* 77, 565-577

Dixon, R.A. (1986) the phytoalexin response: elicitation, signalling and the control of host gene expression. *Biol. Rev.* **61**, 239-291

Dixon, R.A. and Harrison, M.J. (1990) Activation, structure and organization of genes involved in microbial defense in plants. *Adv. Genet.* **28**, 165-234

Dixon, R.A., Harrison, M.J. and Lamb, C.J. (1994) Early events in the activation of plant defense responsses. *Anu. Rev. Phytopathol.* **32**, 479-501

Draper, J., Scott, R., Armitage, P. and Walden, R. (1988) *Plant Genetic Transformation and Gene Expression: A laboratory manual.* Blackwell Scientific Publications, Oxford, UK.

Dunn, N.W. and Gunsalus, I.C. (1973) Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida. J. Bacteriol.* **114**, 974-979

Ellingboe, A.H. (1981) Changes concepts in host-pathogen genetics. *Annu Rev Phytopathol* **19**, 125-143

Ellis, J.G., Lawrence, G.J., Finnegan, E.J., and Anderson, P.A. (1995) Contrasting complexity of 2 rust resistance loci in flax. *Proc. Natl. Acad. Sci. USA* **92(10)**, 4185-4188

Elstner, E.F. and Osswald, W. (1994) Mechanisms of oxygen activation during plant stress. *Proc. R. soc. Edinb.* **102**, 131-154

Enyedi, A.J., Yalpani, N., Silverman, P. and Raskin, I. (1992a) Signal molecules in systemic plant resistance to pathogens and pests. *Cell* **70**, 879-886

Enyedi, A.J., Yalpani, N., Silverman, P. and Raskin, I. (1992b) Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. USA* **89**, 2480-2484.

Firek, S., Ozcan, S.,Warner, S.A.J., & Draper, J. (1993) A wound-induced promoter driving *npt-II* expression limited to dedifferentiated cells at wound sites is sufficient to allow selection of transgenic shoots. *Plant Mol. Biol.* **22**, 129-142

Fraser, R.S.S. (1981) Evidence for the occurrence of the "Pathogenesis-related" proteins in leaves of healthy tobacco plants during flowering. *Physiol. Plant Path.* **19**, 69-76

Gaffney, T., Friedrich, L., Vernooji, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* **261**, 754-756.

Gianinazzi, S., Martin, C. and Vallee, J.C. (1970) Hypersensibilite aux virus, temperatures et proteins soluble chez le *Nicotiana tabacum* cv. Xanthi-nc. *CR Acad Sci Paris D* **270**, 2382-2386

Green, R. and Fluhr, R. (1995) UV-B-induced PR-1 accumulation is mediated by active oxygen species. *Plant Cell* **7**, 203-212

Greenberg, J.T. and Ausubel, F.M. (1993) *Arabidopsis* mutants compromised for the control of cellular damage during pathogenesis and aging. *Plant J.* 4, 327-341

Greenberg, J.T., Guo, A., Klessig, D.F. & Ausubel, F.M. Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense functions. *Cell* 77, 551-563 (1994).

Guerineau, F., Brooks, L. and Mullineaux, P (1991) Effect of deletions in the cauliflower mosaic virus polyadenylation sequence on the choice of the polyadenylation sites in tobacco protoplasts. *Mol. Gen. Genet.* **226**, 141-144

Harborne, J.B. (1980) Plant Phenolics. In *Secondary Plant Products*, ed. E.A.Bell, B.V.Charlwood, pp. 329-402. Berlin: Springer-Verlag. 674pp.

Haslam, E. (1974) The shikimate pathway. London: Butterworths. pp316

He, S.Y., Huang, H.C., and Collmer, A. (1993) *Pseudomonas syringae* pv *syringae* harpin$_{pss}$: a protein that is secreted via the hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**, 1255-66

Jefferson, R. A. Kavangh, T. A. & Bevan, M. W. (1987) GUS fusions : beta glucuronidase as a sensitive and versatile marker in plants. *EMBO J.* **6**, 3902-3907.

Johal, G.S. and Briggs, S.P. (1992) Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science* **258**, 985-987

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J., and Jones, J.D.G. (1994) Isolation of the tomato *cf9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* **266**, 789-793

Joosten, M.H.A.J., Cozijnsen, T.J., and De Wit, P.J.G.M. (1994) Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature* **367**, 384-386

Katagiri, M., Yamamoto, S., and Hayaishi, O. (1962) Flavin adenine dinucleotide requirement for the enzymic hydroxylation and decarboxylation of salicylic acid. *J. Biol. Chem.* **237**, 2413

Kauss, H. and Jeblick, W. (1995) Pretreatment of parsley suspension cultures with salicylic acid enhances spontaneous and elicited production of H_2O_2. *Plant Physiol.* **108**, 1171-1178

Keen, N.T., Ersek, T., Long, M., Bruegger, B., and Holliday, M. (1981) Inhibition of the hypersensitive reaction of soybean leaves to incompatible *Pseudomonas* spp. by blastocidin S., streptomycin or elevated temperature. *Physiol Plant Pathol.* **18**, 325-337

Keen, N.T. (1990) Gene-for-gene complementarity in plant-pathogen interactions. *Ann. Rev. Genet.* **24**, 447-463

Keen, N.T., and Buzzell, R.I. (1991) New disease resistance genes in soybean against *Pseudomonas syringae* pv. *glycinea* - evidence that one of them interacts with a bacterial elicitor. *Theor. Appl. Genet.* **81**, 133-138

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S. and Ryals, J. (1994) Induction of systemic acquired resistance in plants by chemicals. *Annu. Rev. Phytopathol.* **32,** 439-459

Klement, Z. (1963) Rapid detection of pathogenicity of phytopathogenic pseudomonas. *Nature* **199,** 299-300

Klement, Z., Farkas, G.L. and Lovrekovich, L. (1964) Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. *Phytopathology* **54,** 474-477

Klement, Z. and Goodman, R.N. (1967) The hypersensitive reaction to infection by bacterial plant pathogens. *Ann. Rev. Phytopathol.* **5,** 17-44

Knorr, D.A. and Dawson, W.O. (1988) A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*. *Proc. Natl. Acad. Sci. USA* **85,** 170-174

Kobe, B., and Deisenhofer, J. (1993) Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *Nature* **366,** 751-756

Koch, E. and Slusarenko, A. (1990) Arabidopsis is susceptible to infection by a downy mildew fungus. *Plant Cell* **2** (5), 437-445

Konieczny, A. and Ausubel, F. (1993) A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.* **4,** 403-410

Kopp, E. and Ghosh, S. (1994) Inhibition of NF-kB by sodium salicylate and aspirin. *Science* **265**, 956-959

Kuc, J. (1982) Induced immunity to plant disease. *BioScience* **32**, 854-860

Laemmli, U.K. (1976) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685

Lamb, C., Lawton, M.A., Dron, M. and Dixon, R.A. (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* **56**, 215-224

Lawton, K.A., Potter, S.L., Uknes, S. and Ryals, J. (1994) Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. *Plant Cell* **6**, 581-588

Lee, H., Leon, J. and Raskin, I. (1995) Biosynthesis and metabolism of salicylic acid. *Proc. Natl. Acad. Sci. USA* **92**, 4076-4079

Legrand, M. (1983) Phenylpropanoid metabolism and its regulation in disease. In: Callow (ed.) *Biochemical Plant Pathology*. Wiley, New York pp.367-384

Leon, J., Yalpani, N., Raskin, I. and Lawton, M.A. (1993) Induction of benzoic acid 2-hydroxylase in virus-inoculated tobacco. *Plant Physiol* **103**, 323-328

Leslie, C.A. and Romani, R.J. (1988) Inhibition of ethylene biosynthesis bt salicylic acid. *Plant Physiol.* **88**, 833-837

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 1-20.

Lindgren, P.B., Peet, R.C., and Panopoulos, N.J. (1986) Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* **168**, 512-522

Lindsay, W.P., Lamb, C.J., and Dixon, R.A. (1993) Microbial recognition and activation of plant defense systems. *Trends in Microbiology* **1 (5)**, 181-187

Linthorst, H.J.M. (1991) Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci.* **10**, 123-150

Loper, J.E. and Buyer, J.S. (1991) Siderophores in microbial interactions on plant surfaces. *Mol. Plant-Micro Intera.* **4(1)**, 5-13

Lotan, T. and Fluhr, R. (1990) Function and regulated accumulation of plant pathogenesis-related proteins. *Symbiosis* **8**, 33-46

Malamy, J., Carr, J.P. Klessig, D.F., and Raskin, I. (1990) Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**, 1001-1004.

Malamy, J., Hennig, J. and Klessig, D.F. (1992a) Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. *Plant Cell* **4**, 359-366

Malamy, J., and Klessig, D.F. (1992b) Salicylic acid and plant disease resistance. *Plant J.* 2, 643-654.

Marshall, B.J. and Ratledge, C. (1972) Salicylic acid biosynthesis and its control in *Mycobacterium smegmatis*. *Biochim. Biophys. Acta.* 264, 106-116

Martin, G.B., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D. and Tanksley, S.D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262, 1432-1436

Matsuta, C., van den Bulcke M., Bauw, G., van Montagu, M. and Caplan, A.G. (1991) Differential effects of elicitors om the viability of rice suspension cells. *Plant Physiol.* 97, 619-629

Mattoo, A.K. and Suttle, J.C. (1991) The Plant Hormone Ethylene. (Boca Raton, Florida: CRC press)

Mauch-Mani, B. and Slusarenko, A. (1994) Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. *Mol. Plant-Microbe Interact.* 7, 378-383

McGurl, B., Pearce, G., Orozca-Cardenas, M., and Ryan, C.A. (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. *Science* 255, 1570-1573

Mehdy, M. (1994) Active oxygen species in plant defence against pathogens. *Plant Physiol.* 105, 467-472.

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N. and Okada, Y. (1988) Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, *Tm-1*. *EMBO J.* 7, 1575-1581

Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, Y. and Okada, Y. (1989) Mutations in the tobacco mosaic virus 30-kD protein gene overcome *Tm-2* resistance in tomato. *Plant Cell* 1, 515-522

Metraux, J.P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M. and Ryals, J. (1989) Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. *Proc Natl Acad Sci USA* 86, 896-900

Metraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdoorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004-1006.

Metraux, J.P., Ahl Goy, P., Staub, T.H., Speich, J., Steinemann, A., Ryals, J., Ward, E. (1991) Induced systemic resistance in cucumber in response to 2,6-dichloro-isonicotinic acid and pathogens. *In* H Hennecke, DPS Verma, eds, *Advances in Molecular Genetics of Plant-Microbe Interactions*, Vol.1. Kluwer, Dordrecht, The Netherlands, 432-439.

Meuwly, Ph., Molders, W., Summermatter, K., Sticher, L. and Metraux, J.P. (1995) Salicylic acid and chitinase in infected cucumber plants. in press

Meyerowitz, E.M. (1989) *Arabidopsis*, a useful weed. *Cell* **56**, 263-269

Michiels, T. and Cornelis, G.R. (1991) Secretion of hybrid proteins by the yersinia yop export system. *J. Bacteriol.* **173**, 1677-1685

Mindrinos, M., Katagiri, F., Yu, G.-L., and Ausubel, F.M. (1994) The *A thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* **78**, 1089-1099

Mitchell, A.G. and Broadhead, J.F. (1967) Hydrolysis of solubilized aspirin. *J. Pharm. Sci.* **56**: 1261-1266

Neuenschwander, U., Vernooij, B., Friedrich, L., Uknes, S., Kessman, H. and Ryals, J. (1995) Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? *Plant J.* **8**, 227-233

Niepold, F., Anderson, D., and Mills, D. (1985) Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syringae*. *Proc, Natl. Acad. Sci. USA* **82**, 406-410

Ohshima, M., Itoh, H., Matsuoka, M., Murakami, T., and Ohashi, Y. (1990) Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein gene in transgenic tobacco. *Plant Cell* **2**, 95-106.

Padgett, H.S. & Beachy, R.N. (1993) Analysis of a tobacco mosaic-virus strain capable of overcoming N gene-mediated resistance. *Plant Cell* **5** (**5**), 577-586

Palva, T.K., Hurtig, M., Saindrenan, P. and Palva, E.T. (1994)

Salicylic acid-induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. *Mol. Plant-Microbe Interact.* 7, 356-363

Pearce, G., Strydom, D., Johnson, S., and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* **253**, 895-898

Pena-Cortes, H., Albrecht, T., Prat, S., Water, E.W. and Willmitzer, L. (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* **191**, 123-128

Qin, X.F., Holuigue, L., Horvath, D.M. and Chua, N.H. (1994) Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus As-1 element. *Plant Cell* **6(6)**, 863-874

Raskin, I., Ehmann, A. Melander W.R. Meeuse, B.J.D. (1987) Salicylic acid - a natural inducer of heat production in Arum lilies. *Science* **237**, 1601-1602

Raskin, I., Turner, I.M., Melander, W.R. (1989) Regulation of heat production in the inflorescences of an arum lily by endogenous salicylic acid. *Proc. Natl. Acad. Sci. USA* **86**, 2214-2218

Raskin, I. (1992) Role of salicylic acid in plants. *Ann. Rev. Plant Physiol.* **43**, 439-463.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N. (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae pv syringae* . *Plant Physiol.* **97**, 1342-1347.

Romani, R.J., Hess, B.M. and Leslie, C.A. (1989) Salicylic acid inhibition of ethylene production by apple discs and other plant tissues. *J. Plant Growth Regul.* **8**, 63-70

Ross, A.F. (1961a) Localized acquired resistance to plant virus infection in hypersensitive hosts. *Virology* **14**, 329-339

Ross, A.F. (1961b) Systemic acquired resistance induced by localized virus infections in plants. *Virology* **14**, 340-358.

Ryals, J., Uknes, S. and Ward, E. (1994) Systemic acquired resistance. *Plant Physiol.* **104**, 1109-1112.

Saito, T., Meshi, T., Takamatsu, N. and Okada, Y. (1987) Coat protein gene sequence of tobacco mosaic virus encodes a host response determint. *Proc. Natl. Acad. Sci. USA* **84**, 6074-6077

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY

Sanchez-Casas, P. and Klessig, D.F. (1994) A salicylic acid-binding activity and a salicylic acid-inhibitable catalase activity are present in a variety of plant species. *Plant Physiol.*, **106**, 1675-1679.

Schinelzer, E., Borner, H., Grisebach, H., Ebel, J. and Hahlbrok, K. (1984) Phytoalexin synthesis in soybean (*Glycine max*). Similar timecourse of mRNA induction in hypocotyls infected with a fungal pathogen and in cell cultures treated with fungal elicitor. *FEBS Lett* **172**, 59-63

Shen, W.J. and Forde, B.J. (1989) Efficient transformation of *Agrobacterium* spp by high-voltage electroporation. *Nucl. Acid. Res.* **17**, 8385.

Sigee, D.C. (1993) *Bacterial Plant Pathology: Cell and Molecular Aspects* Cambridge University Press, Cambridge, UK

Silverman, P., Nuckles, E., Ye, X.S., Kuc, J. and Raskin, I. (1993) Salicylic acid, ethylene, and pathogen resistance in tobacco. *Mol. Plant-Microbe Interact.* **6**, 775-781

Simmons, C.R., Litts, J.C., Huang, N., and Rodriguez, R.L. (1992) Structure of a rice beta-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors. *Plant Mol. Biol.* **18**, 33-45

Slusarenko, A.J. and Longland, A. (1986) Changes in gene activity during expression of the hypersensitive response in *Phaseolus vulgaris* cv. "Red Mexican" to an avirulent race 1 isolate of *Pseudomonas syringae* pv. *phaseolicola. Physiol Mol. Plant Pathol.* **29**, 79-94

Slusarenko, A.J., Croft, K.P. and Voisey, C.R. (1989) Biochemical and molecular events in the hypersensitive response of bean to *Pseudomonas syringae* pv. *phaseolicola.* In *Biochemistry and Molecular Biology of Plant--Pathogen Interactions.* Proceedings of the International Symposium of the European Phytochemical Society.

Smith, J.A., Hammerschmidt, R. and Fulbright, D.W. (1991) Rapid induction of systemic induction of systemic resistance in cucumber by *Pseudomonas syringae* pv. *syringae. Physiol. Mol. Plant Pathol.* **38**, 223-235

Somlyal, G., Solt, A., Hevesi, M., El-Kady, S., Klement, Z. and Kari, C. (1988) The relationship between the growth rate of *Pseudomonas syringae* pathovars and the hypersensitive reaction in tobacco. *Physiol. Molec. Plant Patho.* **33**, 473-482

Staswick, P.E. (1992) Jasmonate, genes and fragrant signals. *Plant Physiol.* **99**, 804-807

Staub, T. and Kuc, J. (1980) Systemic protection of cucumber plants against disease caused by *Cladosporium susumerinum* and *Colletotrichum lagenarium* by prior localized infection with either fungus. *Physiol. Plant Pathol.* **17**, 389-393

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M. and Fritig, B. (1993) Plant "pathogenesis-related" proteins and their role in defense against pathogens. *Biochimie* **75**, 687-706

Storrie, B. and Madden, E. A. (1990) Isolation of subcellular organelles. In : Methods in Enzymology, **182**, 2 03-225.

Summermatter, K., Meuwly, Ph., Molders, W., and Metraux, J.P. (1995) Systemic responses in *Arabidopsis thaliana* infected and challenged with *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* **108**, 1379-1385

Sutherland, M. (1991) The generation of oxygen radicals during host plant responses to infection. *Physiol. Mol. plant Pathol.* **39**, 79-93

Takemori, S., Hon-Nami, K., Kawahara, F., and Katagiri, M. (1974) Mechanism of the salicylate 1-monooxygenase reaction VI. The monomeric nature of the enzyme. *Biochem. Biophys. Acta* **342,** 137-144

Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C. (1995) Function of the oxidative burst in hypersensitive disease resistance. *Proc. Natl. Acad. Sci. USA* **92,** 4158-4163

Topping, J. F. , Wei, W. & Lindsey, K. (1991) Functional tagging of regulatory element in the plant genome. *Development,* **112,** 1009-1019.

Tu, S.C., Romero, F.A. and Wang, L.H. (1981) Uncoupling of the substrate monooxygenation and reduced pyridine nucleotide oxidation activities of salicylate hydroxylase by flavins. *Arch. Biochem. Biophys.* **209,** 423-432

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S.,Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. (1992) Acquired resistance in Arabidopsis. *Plant Cell* **4,** 645-655

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Thompson-Taylor, H., Potter, S., Ward, E. and Ryals, J. (1993a) Regulation of pathogenesis-related protein-1a gene expression in tobacco. *Plant Cell* **5,** 159-169.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E. and Ryals, J. (1993b) Biological induction of systemic acquired resistance in *Arabidopsis. Mol. Plant-Microbe Interact.* **6,** 692-698

vanden Ackerveken, G.F.J.M., vanKan, J.A.L. and DeWit, P.J.G.M. (1992) Molecular analysis of the avirulence gene *avr9* of the fungal tomato pathogen *C. fulvum* fully supports the gene-for-gene hypothesis. *Plant J.* **2**, 359-366

Van Loon, L.C. and Van Kammen, A. (1970) Polyacrylamide disc electrophoresis of the soluble proteins from Nicotiana tabacum var. "Samsun" and "Samsun NN". II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* **40**, 199-211

van Loon, L.C. (1983) The induction of pathogenesis-related proteins by pathogens and specific chemicals. *Neth. J. Plant Path.* **89**, 265-273

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* **6**, 959-965.

Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H., and Ryals, J. (1995) 2,6-Dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. *Mol. Plant-Micro Intera.* **8**, 228-234

Vick, B.A., and Zimmerman, D.C. (1984) Biosynthesis of jasmonic acid by several plant species. *Plant Physiol.* **75**, 458-461

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J-P., and Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* **3**, 1085-1094.

Warner, S.A.J., Scott, R. and Draper, J. (1992) Characterisation of a wound-induced transcript from the monocotyledon asparagus that shares homology with a new class of pathogenesis-related (PR) proteins. *Plant Mol. Biol.* **19**, 555-561.

Warner, S.A.J., Scott, R. and Draper, J. (1993) Isolation of an asparagus intracellular PR gene (AoPR1) wound-responsive promoter by the inverse polymerase chain reaction and its characterisation in transgenic tobacco. *Plant J.* **3**, 191-201.

Warner, S.A.J., Gill, A. and Draper, J. (1994) The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. *Plant J.* **6**, 31-43

Weiss, U. and Edwards, J.M. (1980) The biosynthesis of aromatic compounds New York/ Chichester: Wiley-Interscience. pp728

Weissmann, G. (1991) Aspirin. *Sci. Am.* **264**, 84-90

White, R.F. (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* **99**, 410-412

White, R.F. (1983) Serological detection of pathogenesis-related proteins. *Neth. J. Plant Path.* **89**, 311-317

White, R.F. and Antoniw, J.F. (1991) Virus-induced resistance responses in plants. *Crit. Rev. Plant Sci.* **9**, 443-455

White, R.F., Rybicki, E.P., von Wechmar, M.B., Dekker, J.L. and Antoniw, J.F. (1987) Detection of PR-1 type proteins in Amaranthaceae, Chemopodiaceae, Graminae and Solanaceae by immunoelectroblotting. *J. Gen. Virol.* **68**, 2043-2048

Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C. and Baker, B. (1994) The product of the tobacco mosaic virus resistance gene *N*: similarity to toll and the interleukin-1 receptor. *Cell* **78**, 1101-1115

Wills, D.K., Rich, J.J., and Hrabak, E.M. (1991) *hrp* genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* **4**, 132-138

Wilson, L.G. and Fry, J.C. (1986) Extensin - a major cell wall glycoprotein. *Plant Cell Env.* **9**, 239-260

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A. and Raskin, I. (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell* **3**, 809-818

Yalpani, N., Leon, J., Lawton, M.A., Raskin, I. (1993) Pathways of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. *Plant Physiol.* **103**, 315-321

Yalpani, N., Enyedi, A.J., Leon, J., and Raskin, I. (1994) Ultraviolet-light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. *Planta* **193**, 372-376

Yamamoto, S., Katagiri, M., Maeno, H. and Hayaishi, O. (1965) Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. I. Purification and general properties. *J. Biol. Chem.* **240**, 3408-3413.

Yang, N.S. and Russell, D. (1990) Maize sucrose synthase-1 promoter directs phloem cell-specific expression of *Gus* gene in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA* **87**, 4144-4148

Yoshiki, Y., Okubo, K., Onuma, M and Igarashi, K. (1995) Chemiluminescence of benzoic and cinnamic acids, and flavonoids in the presence of aldehyde and hydrogen-peroxide or hydroxyl radical by fenton reaction. *Phytochemistry* **39**, 225-229

You , I-S, Murray, R.I., Jollic, D. and Gunsalus I. C. (1990) Purification and characterization of salicylate hydroxylase from *Pseudomonas putida* PpG7. *Biochemical and biophysical research communications* **169**, 1049-1054

You , I-S, Ghosal , D. and Gunsalus I. C. (1991) Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene (*nah* G) and its 3'-flanking region. *Biochemistry* **30**, 1635-1641.

Yucel, I., Midlands, S.L., Sims, J.J. and Keen, N.T. (1994) Class I and Class II *avrD* alleles direct the production of different products in gram-negative bacteria. *Mol. Plant-Microbe Interact.* **7**, 148-150

Appendix

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Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression

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Summary

The roles of salicylic acid (SA) and H_2O_2 in the induction of PR proteins in tobacco have been examined. Studies were conducted on wild-type tobacco and plants engineered to express a bacterial salicylate hydroxylase capable of metabolizing SA to catechol (SH-L plants). Wild-type and PR-1a–GUS-transformed plants express PR-1a following challenge with *Pseudomonas syringae* pathovar *syringae*, SA or 2,6-dichloro-isonicotinic acid (INA). In contrast, SH-L plants failed to respond to SA but did express PR-1a following INA treatment. H_2O_2 and the irreversible catalase inhibitor 3-amino-1,2,4-triazole (3-AT) were found to be weak inducers of PR-1a expression (relative to SA) in wild-type tobacco but were unable to induce PR-1a in SH-L plants, suggesting that the action of these compounds depends upon the accumulation of SA. A model has been proposed suggesting that SA binds to and inhibits a catalase inducing an increase in H_2O_2 leading to PR protein expression. Catalase activity has been measured in tobacco and no significant changes in activity following infection with *P. syringae* pv. *syringae* were detected. Furthermore, inhibition of catalase activity *in vitro* in plant extracts requires pre-incubation and only occurs at SA concentrations above 250 μM. Leaf disks pre-incubated with 1 mM SA do accumulate SA to these levels and PR-1a is efficiently induced but there is no apparent inhibition of catalase activity. It is also shown that a SA-responsive gene, PR-1a, and a H_2O_2-sensitive gene, AoPR-1, are both relatively insensitive to 3-AT suggesting that induction of these genes is unlikely to be due entirely to inhibition of an endogenous catalase.

Introduction

Plants infected locally with a necrotizing pathogen often acquire broad-range systemic resistance to further pathogen challenge after a period of 5–7 days (Chester, 1933; Ross, 1961). The onset of systemic acquired resistance (SAR) is associated typically with the synthesis of a set of pathogenesis-related (PR) proteins (Bol *et al.*, 1990; Ward *et al.*, 1991). The regulation of expression of the acidic, secreted PR-1a protein is perhaps the most well studied (Ohshima *et al.*, 1990; Unkes *et al.*, 1993). Salicylic acid (SA; 2-hydroxybenzoic acid) has been shown to be intimately involved with the defence response in higher plants (see Malamy and Klessig, 1992; Raskin, 1992; Ryals *et al.*, 1994, for recent reviews). SA can switch on PR protein gene expression and induce the SAR state following exogenous application to tobacco plants. With only one or two exceptions, related phenolic acids such as 4-hydroxybenzoic acid (4hBA) cannot induce PR protein expression. Endogenous SA levels rise 20- to 40-fold locally around developing lesions in tobacco mosaic virus (TMV)-infected tobacco plants (Malamy *et al.*, 1990; Métraux *et al.*, 1990). In addition, SA levels rise approximately fivefold systemically in non-infected tissues distant from the inoculated leaves at the same time as the synthesis of PR proteins and exhibition of SAR is achieved.

The production of SA in TMV-infected tobacco is closely correlated with the induction of the hypersensitive response (HR). TMV-resistant plants inoculated with TMV at 32°C do not develop HR necrotic lesions and fail to restrict TMV replication and virus spread. Under these conditions SA remains at basal levels and PR proteins are not induced. On shifting the plant to a permissive temperature (<28°C) there is a synchronous development of large necrotic lesions and a dramatic rise in SA levels prior to PR protein gene transcription (Malamy *et al.*, 1992). It has been shown that endogenous levels of SA in cucumber phloem sap rise rapidly 8 h after infection with necrotic pathogens (Métraux *et al.*, 1990).

These data were consistent with the suggestion that SA may be a mobile systemic signal responsible for SAR induction. The importance of free SA in SAR has been investigated further by expressing a bacterial salicylate hydroxylase gene (*nah*G) in tobacco to convert SA to catechol which is not an inducer of SAR (Gaffney *et al.*, 1993). These experiments demonstrated that SA accumulation is essential for PR gene expression and SAR. Recent grafting experiments between *nah*G and wild-type tobacco have shown that although SA is probably not the primary mobile systemic signal, as suggested by the work of Rasmussen *et al.* (1991), its presence in systemic tissue is absolutely required for PR protein expression and establishment of SAR (Vernooij *et al.*, 1994).

Klessig and co-workers have purified a soluble low-affinity binding protein which binds SA (K_D 14 μM) and its active analogues and exhibits SA-inhibitable catalase activity (Chen *et al.*, 1993a, 1993b). These authors have

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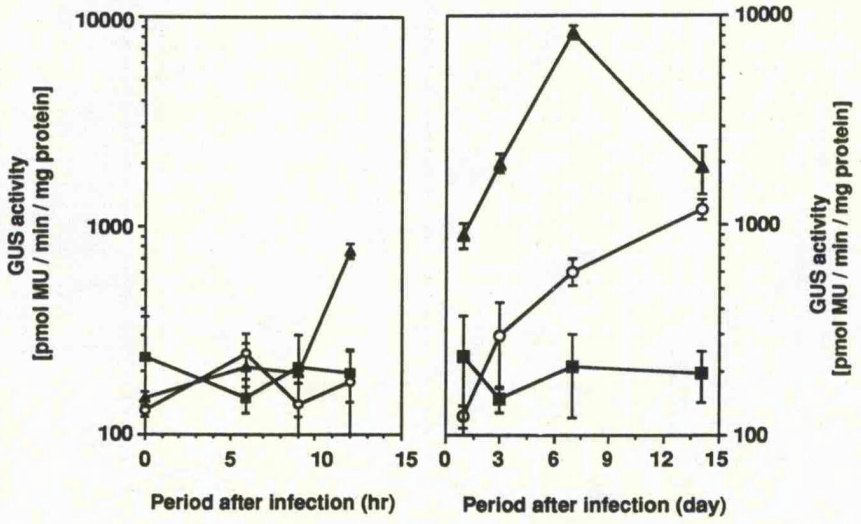

Figure 1. Local and systemic GUS activity in PR-1a–GUS Samsun NN tobacco following challenge with *P. s.* pv. *syringae*.
Leaves of PR-1a-GUS Samsun NN tobacco were infiltrated with *P. s.* pv. *syringae*. Samples were periodically taken from necrotic lesions (–▲–) and from upper uninfected (–○–) leaves and compared with mock-inoculated PR-1a-GUS tobacco (–■–). Means ± SE. $n = 3$.

suggested that SA may function by allowing the accumulation of H_2O_2 which can then act as a second messenger to switch on defence gene expression and activate SAR. In support of this hypothesis it was demonstrated that both H_2O_2 and a catalase inhibitor, 3-amino-1,2,4- triazole (3-AT), can induce PR protein expression when injected directly into leaf tissue. Furthermore, elevated levels of H_2O_2 were detected in leaf tissue fed with SA. However, there are no data suggesting that catalase activities are reduced and H_2O_2 levels elevated in systemic tissues following local pathogen infection. In the present paper and accompanying article (Neuenschwander *et al.*, 1995) the hypothesis that H_2O_2 *in planta* may be a secondary messenger working downstream from SA in the signalling pathway responsible for establishing the SAR state is further explored.

Results

Leaf infiltration with the bacterial pathogen Pseudomonas syringae *pv.* syringae *induces PR protein gene expression*

The expression of the tobacco PR-1a protein has been shown to be induced following infection with TMV or treatment with SA (Malamy *et al.*, 1990; Ward *et al.*, 1991). Likewise, plants transformed with a construct consisting of the PR-1a promoter linked to β-glucuronidase (PR-1a-GUS) exhibit elevated GUS levels following challenge with TMV or SA (Ohshima *et al.*, 1990; Uknes *et al.*, 1993). In this study we have used *Pseudomonas syringae* pathovar *syringae* (*P. s.* pv. *syringae*) as well as TMV as a pathogenic model since it rapidly produces large necrotic lesions in a defined location. Infection of Samsun NN PR-1a–GUS-transformed tobacco with *P. s.* pv. *syringae* induced a rise in GUS activity compared with mock inoculated plants (Figure 1). GUS activity was detectable at the infection site after 10–12 h, rising approximately 50-fold above initial levels by day eight and declining thereafter. This local increase was attended by a more delayed rise in GUS activity in upper uninfected leaves. The systemic expression of the PR-1a–GUS gene was detectable 3 days after infection and rose approximately five-fold above baseline by day 14.

The induction of PR proteins around lesions is dependent on SA

Genes coding for salicylate hydroxylase (SH) are carried on several different plasmids found in naphthalene degrading *Pseudomonas putida* strains (Davies and Evans, 1964). Transgenic tobacco plants were generated containing a transcriptional fusion between the CaMV 35S promoter and an *nah*G gene from strain NCIB9816 which codes for a salicylate hydroxylase enzyme (SH-L). When tobacco leaves were infiltrated with SA, those taken from wild-type plants responded with the appearance of a protein recognized by rabbit antiserum to PR-1a (Figure 2a). Infiltration with 4hBA was ineffective as an inducer of PR proteins. The PR-1a protein was not present in SA-treated leaves from transgenic tobacco plants expressing the SH-L protein. Externally supplied 2,6-dichloro-isonicotinic acid (INA) has been shown to induce SAR and elevate expression of SAR-associated genes (Métraux *et al.*, 1991; Ward *et al.*, 1991). INA was able to induce PR-1a expression in both wild-type and 35S–SH-L-transformed plants indicating clearly that some mechanisms activating PR gene expression are still functional in the absence of salicylate accumulation. As expected, infection of wild-type tobacco with TMV resulted in the expression of endogenous PR-1a in infected tissue and in systemic leaves (Figure 2b). In contrast, local or systemic PR-1a induction was not detected at 7 days in 35S–SH-L transformants challenged with TMV. Collectively, these data provide clear evidence that SA is

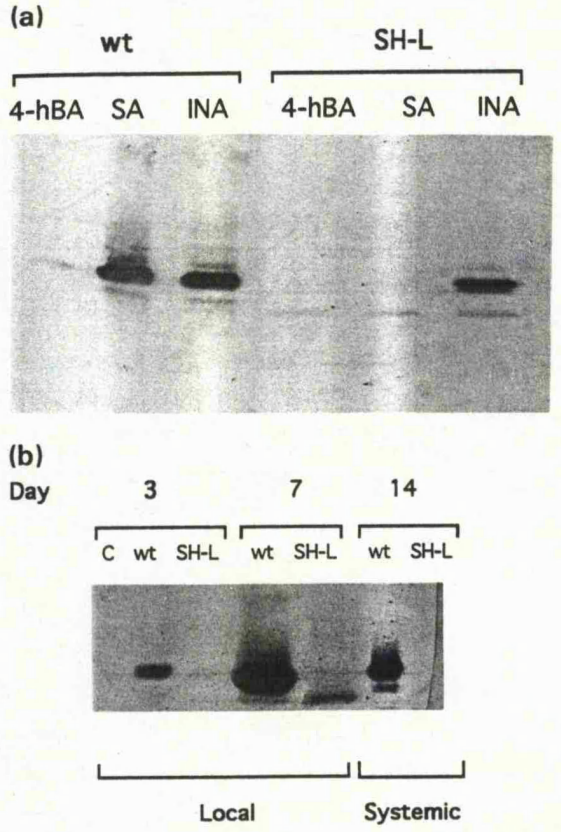

Figure 2. Accumulation of PR-1a protein in Samsun NN and 35S–SH-L Samsun NN tobacco plants following application of systemic acquired resistance-inducing chemicals or tobacco mosaic virus (TMV) infection.
(a) Wild-type Samsun NN (wt) and Samsun NN 35S–SH-L (SH-L) tobacco plants were infiltrated with 1 mM 4-hBA, 1 mM SA or 0.5 mM INA, by injection. After 3 days, PR-1a protein accumulation was determined by PR-1a antiserum.
(b) Accumulation of PR-1a protein following infection of Samsun NN and 35S–SH-L Samsun NN tobacco plants with TMV. Wild-type Samsun NN (wt) and Samsun NN 35S–SH-L (SH-L) tobacco plants were infected with TMV. Mock-inoculated wild-type Samsun NN plants were used as a control (C). After 3 and 7 days (local tissue) and 14 days (systemic tissue), PR-1a protein accumulation was determined by PR-1a anti-serum.

essential in the activation of PR protein gene transcription at sites of necrotic lesion formation.

The induction of PR-1a by hydrogen peroxide or catalase inhibitor is dependent on SA accumulation

Treatment of wild-type tobacco with SA induced the expression of PR-1a as determined by Western blotting (Figure 3a). Lower levels of PR-1a were detected following treatment of wild-type plants with H_2O_2 (5 mM) and the irreversible catalase inhibitor 3-AT. Treatment with H_2O_2 levels of up to 0.5 M failed to achieve any further increase in PR-1a gene expression and caused substantial damage to leaf tissue (data not shown). As already noted, SA fails to induce PR-1a expression in SH-L-transformed plants. Thus,

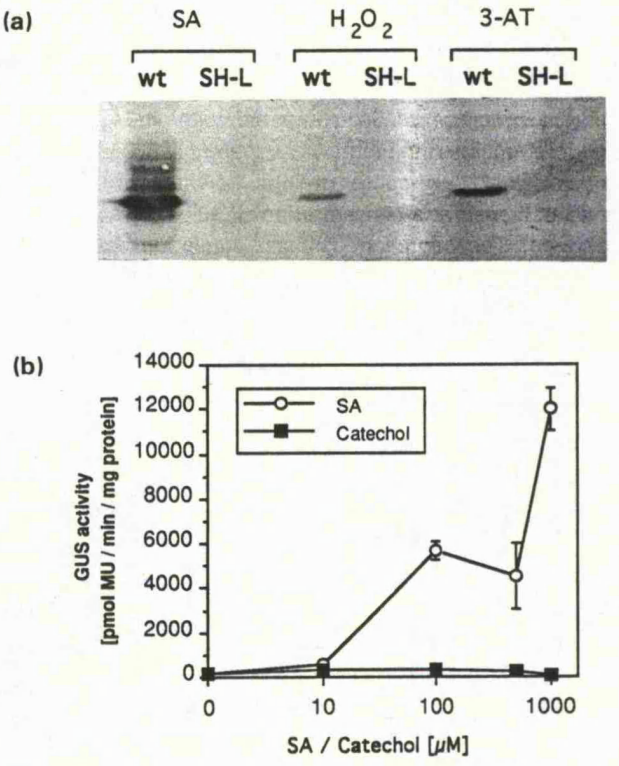

Figure 3. Accumulation of PR-1a protein in response to SA, H_2O_2 and 3-AT and effect of catechol on PR-1a–GUS expression.
(a) Leaf discs of wild-type Samsun NN (wt) and 35S–SH-L Samsun NN (SH-L) tobacco plants were treated with 1 mM SA, 5 mM H_2O_2 and with 4 mM 3-AT. After 2 days, PR-1a accumulation was determined by probing 15 μg of protein from infiltrated regions on Western blots with PR-1a anti-serum.
(b) Leaf intercellular spaces of PR-1a–GUS Samsun NN tobacco plants were infiltrated with increasing levels of catechol or SA and GUS activity measured after 2 days. Means ± SE shown (where larger than symbols used), $n = 3$.

it could be argued that if H_2O_2 lies downstream of SA in the SAR signalling pathway and if its accumulation is dependent upon catalase inhibition (Chen *et al.*, 1993b) then treatment with either H_2O_2 or 3-AT should overcome the SA block. Figure 3(a) shows that H_2O_2 and 3-AT are unable to induce PR-1a expression in 35S–SH-L-transformed plants suggesting that the mode of action of these compounds lies upstream of, and is dependent upon, SA accumulation. Catechol, the product of SA degradation by salicylate hydroxylase, is unable to induce PR-1a expression (Figure 3b).

SA is a weak in vitro *inhibitor of endogenous catalase activity in leaf extracts*

The ability of 3-AT to induce local PR-protein expression in wild-type tobacco plants is undeniable and the involvement of catalase in SAR is intriguing. To date SA at 1 mM has been reported to reduce catalase activity *in vitro* using crude protein preparations from tobacco leaves (Chen *et al.*, 1993b). We were interested to determine whether

inhibition occurred at physiological levels of SA, or following pathogen challenge. Catalase activity in wild-type Samsun NN tobacco leaf extracts was slightly depressed in the presence of SA compared with 4hBA and INA (Figure 4a). This inhibition was apparent at 500 μM but not observed at lower concentrations. Inhibition of up to 30% was achieved at a concentration of 10 mM SA (data not shown). The degree of catalase inhibition was further increased by a 60 min pre-incubation with SA, but enzyme activity only really showed a significant reduction at SA levels greater than 250 μM (Figure 4b). Inhibition by high levels of SA may be a general feature of catalases since 1 mM SA inhibited bovine liver catalase by 24% compared with 1 mM 4hBA (data not shown). Surprisingly, INA, a good inducer of PR protein gene expression, did not prove to be an inhibitor of catalase activity, even at high concentrations and with a 60 min pre-incubation (Figure 4b). In contrast, catechol was a potent *in vitro* inhibitor of catalase (89% inhibition at 1 mM catechol compared with control; Figure 4a). Catechol also inhibited bovine liver catalase by 62% (data not shown).

Catalase activity is not reduced in leaves infected with P. s. *pv.* syringae

Intercellular injection of wild-type tobacco leaf tissue with *P. s.* pv. *syringae* leads to visible tissue collapse within 6–8 h and induces endogenous PR-1a gene expression by 24 h (Figure 5a, inset). Catalase was measured in samples taken from lesions (or mock-infiltrated areas) or adjacent areas of the same leaf. Catalase activity was not significantly altered in either lesions or surrounding tissue of infected plants compared with mock-inoculated leaves (Figure 5a). However, levels of 3-AT-insensitive H_2O_2-degrading activity in lesions appeared to be double those in either mock-inoculated tissue or in surrounding tissue (Figure 5b). The nature and origin of this 3-AT-insensitive H_2O_2-degrading activity remains to be determined. Nevertheless, this suggests that peroxide levels are reduced in pathogen-induced lesions within 24 h post-infection.

SA is taken up efficiently into leaf discs but fails to inhibit endogenous catalase activity

If the mechanism of PR-1a activation is dependent upon inhibition of catalase (Chen *et al.*, 1993b) then it follows that treatment of tobacco with SA should cause a decrease in extracted catalase activity when injected into or applied to leaves *in planta*. Tobacco catalase has a K_D of 14 μM for SA and a half-life for dissociation of around 40 min (Chen *et al.*, 1993a). Experiments were designed to measure catalase activity in extracts from SA-treated leaf discs. Salicylic acid (1 mM) was taken up very efficiently into tobacco leaf discs, reaching concentrations of over 500 μM

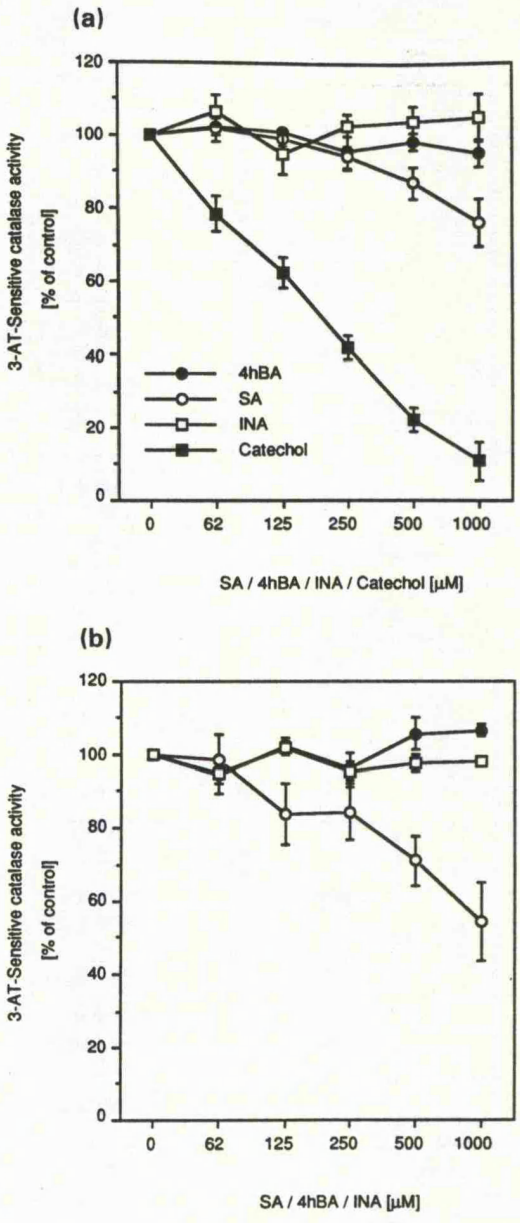

Figure 4. Effects of SA, 4hBA, INA and catechol on *in vitro* catalase activity of wild-type tobacco leaf extracts.
(a) Fifty microlitres of Samsun NN leaf extract (0.2 mg protein ml^{-1} in catalase extraction buffer) were incubated for 2 min at room temperature with 50 μl of catalase extraction buffer containing increasing concentrations of SA, 4hBA, INA or catechol and 50 μl of catalase assay buffer ± 10 mM 3-AT (final concentration). The reaction was stopped by addition of 200 μl of catalase stop solution and the absorbance read at 405 nm. Buffers are described in Experimental procedures. Data are expressed as percentage of 3-AT-sensitive catalase activity compared with control. Means ± SE shown (where larger than symbols used), $n = 3$.
(b) Catalase activity in Samsun NN leaf extracts following 1 h pre-incubation in the presence of SA, 4hBA or INA. Assays were carried out in the presence of SA, 4hBA and INA levels as indicated; pre-incubation conditions were thus 3.5-fold higher that those used in the assay. Data are expressed as percentage of 3-AT-sensitive catalase activity compared with control. Means ± SE shown, $n = 3$.

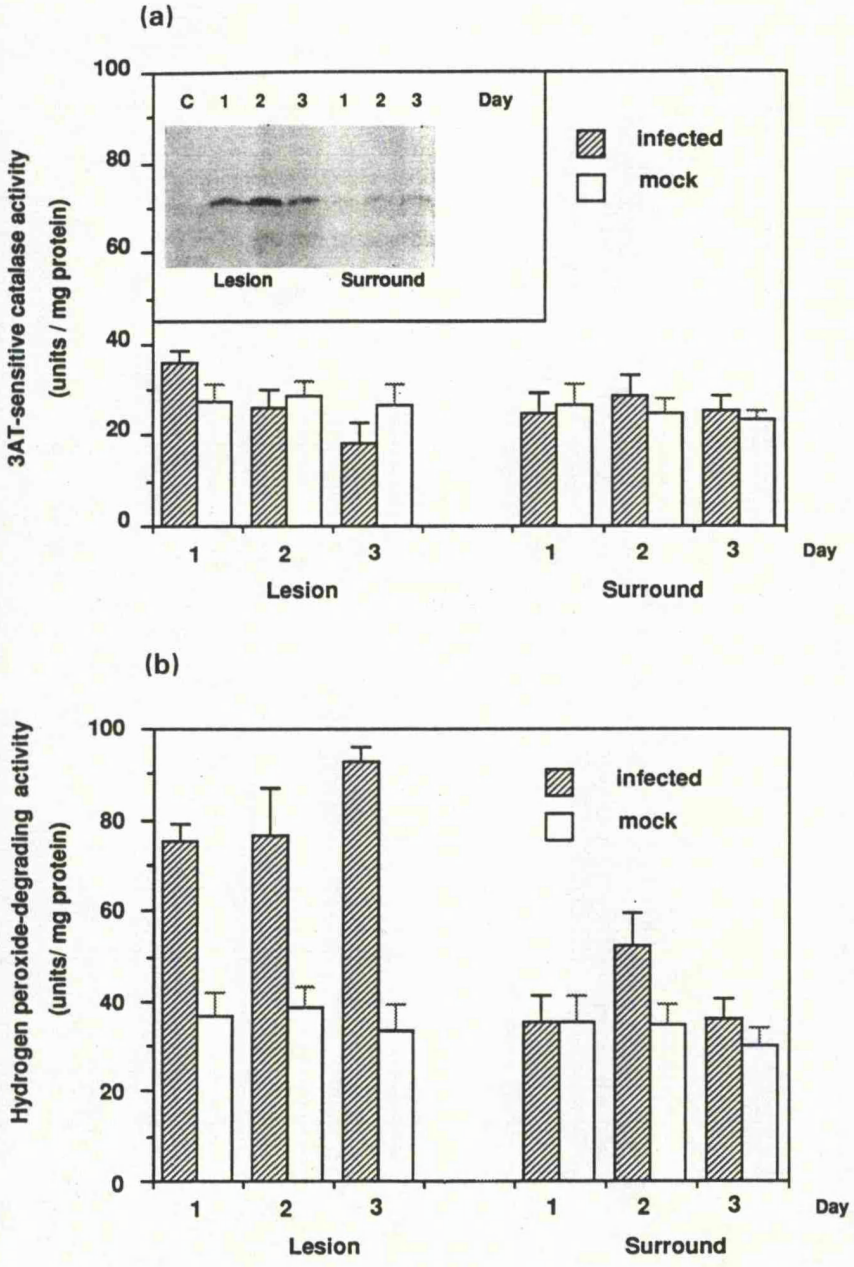

Figure 5. Catalase activity in and around lesions formed following infection of wild-type tobacco with *P. s.* pathovar *syringae*.
(a) Wild-type tobacco (Samsun) leaves were inoculated with 100 µl per sample area of 10 mM phosphate buffer (pH 7.0)—mock, or the same buffer containing 1×10^8 colony-forming units per ml of *P. s.* pv. *syringae*—infected. Discs (1.5 cm) were taken from infiltrated areas and adjacent uninfiltrated areas on the same leaf at 1, 2 and 3 days following infection. 3-AT-sensitive catalase activity was measured as described in Experimental procedures. Means ± SE shown, n = 9. Inset—PR-1a expression, detected by Western blotting, in wild-type tobacco (Samsun) incubated as above with phosphate buffer or *P. s.* pv. *syringae*.
(b) Total H_2O_2-degrading activity in the same samples assayed in (a). In this case the total apparent destruction of H_2O_2 was measured rather than 3-AT-sensitive peroxide degradation.

within 30 min (Table 1). However, after just 6 h incubation the levels of free SA in leaf tissue had dropped to around 100 µM (Table 1). The levels of SA in the leaf extracts used for catalase measurements prior to dilution for assay (Table 1) were all greater than the reported K_D of 14 µM for SA-binding to catalase. Inhibition of catalase activity was not observed in any of these leaf extracts. Leaf pieces incubated for up 2 days in the presence of 1 mM SA expressed high levels of PR proteins but still exhibited normal levels of catalase activity (data not shown). Similar results were obtained in experiments where intact leaves were infiltrated with 1 mM SA or fed for 3 days with 1 mM SA via the petiole.

Local expression of PR proteins is induced to suboptimal levels by local application of H_2O_2 or the catalase inhibitor 3-AT

Recent work in this laboratory on AoPR-1, an intracellular PR protein of *Asparagus officinalis* (Warner *et al.*, 1992, 1993) has shown that it is possible to separate the effects of H_2O_2 and SA on the induction of defence responses (Mur *et al.*, manuscript submitted). As noted previously (Figure 3a), H_2O_2 is a relatively weak inducer of endogenous PR-1a expression. Figure 6 shows that following injection into leaf tissue 1 mM H_2O_2 and 1 mM 3-AT induce approximately eight-fold lower expression of PR-1a–GUS than

Table 1 Salicylic acid uptake by tobacco leaf discs and catalase activity in soluble tissue extracts

Incubation time with 1 mM SA (h)	SA concentration in leaf disc		SA concentration in leaf extract (μM)	Catalase activity in leaf extract (% of 4hBA control)
	(μg g^{-1} fresh wt)	(μM)		
0.5	47.3 ± 3.4	571	61.0 ± 3.1	121.9 ± 11.9
1.0	47.0 ± 2.1	567	73.0 ± 6.9	110.2 ± 3.8
3.0	24.3 ± 1.9	293	36.8 ± 4.0	122.8 ± 13.2
6.0	8.7 ± 2.9	105	14.7 ± 4.9	103.1 ± 3.7

Leaf discs (1.5 cm) from Samsun NN tobacco (approximately 2 g per leaf) were floated for the times indicated in 1 mM salicylic acid or 4hBA and then rinsed in water and blotted dry with tissues. Discs were extracted as described in Experimental procedures for catalase assays using 200 μl per disc of extraction buffer. Two hundred microlitres were removed and used for catalase assay, the remainder were processed for SA measurements. Salicylic acid concentration is expressed as μg gm^{-1} fresh weight of leaf disc as well as an estimation of molarity in cell fluids based on the measurement that 61.3 ± 1.8% of tobacco leaf fresh weight is water content. The actual SA content in the leaf soluble protein extract prior to catalase assay is also presented. Means ± SE shown, $n = 3$.

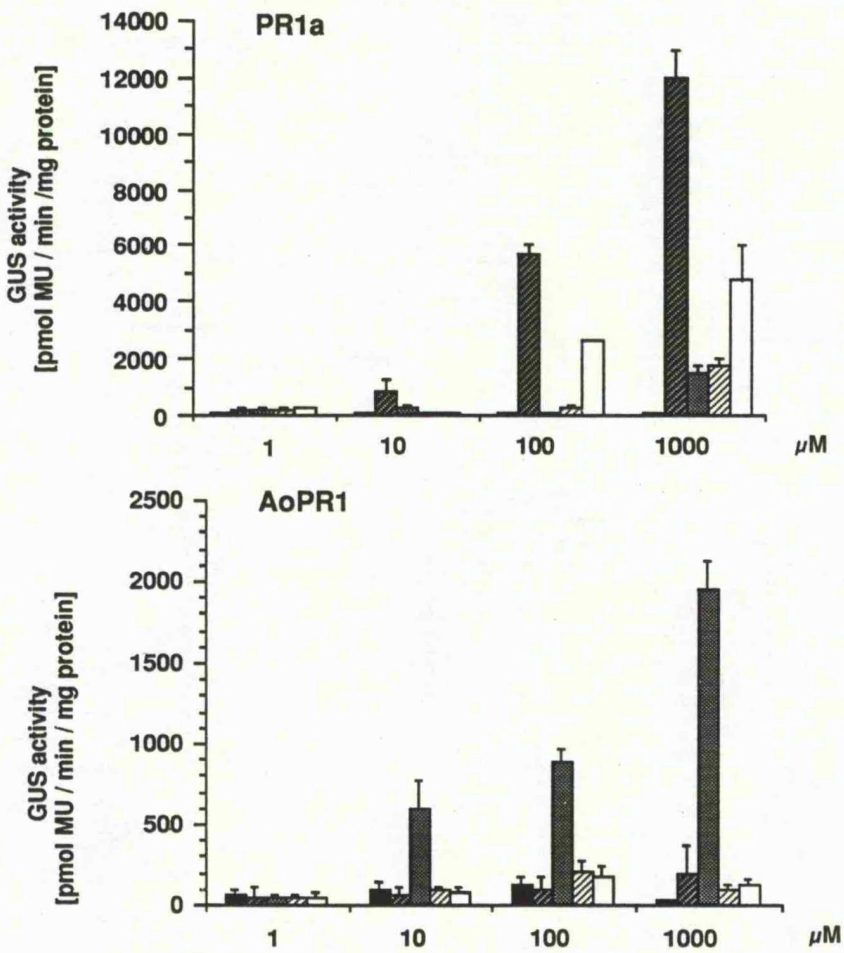

Figure 6. Comparison of classical and intracellular PR gene induction using chemical elicitors.
Varying concentrations (μM) of 4hBA (■), SA (■), H_2O_2 (▨), 3-AT (▨) and INA (□) were injected into leaf intercellular spaces of PR-1a–GUS tobacco variety Samsun NN and AoPR-1–GUS tobacco variety Samsun NN plants. GUS activity of 1.5 cm diameter cores of the infiltrated region were assayed after 3 days. Means ± SE shown, $n = 3$.

1 mM SA. However, when tobacco plants transformed with an AoPR-1 promoter–GUS construct were treated with SA or H_2O_2, the opposite effect was noted. Thus, H_2O_2 proved to be a good inducer of AoPR-1–GUS but SA was incapable of altering the expression of this construct (Figure 6). Interestingly, 3-AT was similarly unable to stimulate AoPR-1–GUS expression despite the induction shown by H_2O_2. This suggests that the effects of H_2O_2, in some cases at least, may be distinguished from those of SA and that inhibition of endogenous catalase activity by 3-AT is relatively ineffective in the induction of at least some classes of either H_2O_2- or SA- sensitive genes.

Discussion

Local induction of the PR-1a gene is dependent on salicylate-mediated signalling

A major aim of the present study was to examine the role of SA in defensive signalling in tobacco (specifically PR-1a expression) by preventing its accumulation via the constitutive expression of a salicylate hydroxylase enzyme (SH-L). Catechol, the initial product of SA degradation, could not induce the expression of PR-1a and would not therefore interfere with interpretation of the results. Indeed, HPLC analysis, using a UV monitor, indicated that catechol did not accumulate in TMV-infected CaMV-35S–SH-L plants and, in more sensitive radioactive HPLC assays, uniformly ring-labelled ^{14}C SA fed to transgenic CaMV-35S–SH-L plants was found to be entirely degraded to highly polar, non-aromatic compounds within 4 h (Darby and Bi, unpublished observations).

In previous experiments, using *nah*G to stop SA accumulation in transgenic tobacco, PR-1a expression was inhibited in systemic, non-infected tissue, but only slightly reduced in pathogen-challenged leaves (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994). In contrast, by expressing the *nah*G isoenzyme SH-L in tobacco we demonstrated for the first time that PR-1a expression in infected tissues is dependent on SA accumulation, which supports the suggestion that at least local induction of this PR protein operates via salicylate signalling. In comparative studies with transgenic line *nah*G10 (Gaffney *et al.*, 1993) we have shown that SH-L is expressed more efficiently in transgenic plants which perhaps explains this difference (Bi and Mur, unpublished observations). It was interesting to note that INA (which is not a substrate for SH-L) could still induce PR protein expression in plants expressing the SH-L gene. It is further demonstrated in the accompanying paper by Neuenschwander *et al.*, (1995) that SA treatment of *nah*G plants does not induce PR-1a mRNA accumulation, whilst INA is effective in this respect, thus confirming that the effect of INA is at the level of transcription. These data suggest that PR protein genes in a *nah*G background are still capable of responding to signals that lie downstream of SA, or are responding to signals that activate a separate signalling pathway. A similar observation has been published recently (Vernooij *et al.*, 1995).

SA acts upstream of H_2O_2 in SAR signalling

The current data show that H_2O_2 is a relatively weak inducer of PR proteins in wild-type tobacco and also demonstrate that treatment with either H_2O_2 or a catalase inhibitor cannot overcome the SH-L block to PR protein expression. In terms of systemic signalling Neuenschwander *et al.* (1995) concluded that systemic H_2O_2 levels were unaffected in wild-type and *nah*G tobacco following local TMV infection. Neuenschwander *et al.* (1995) also showed that pretreatment of tobacco leaf tissue with H_2O_2 or 3-AT did not induce resistance to TMV as evidenced in wild-type plants by a reduction in lesion size which is typical of an SAR response. These observations, coupled with the observation that PR-1a expression in infected tissue is totally dependent on SA accumulation, suggest that H_2O_2 is probably not a secondary messenger working downstream of SA in the SAR response as suggested previously (Chen *et al.*, 1993b). Indeed, in previous publications the effect of SA on catalase activity was only demonstrated *in vitro* using high concentrations of SA and thus the current data represent the first testing of this hypothesis in whole plants *during pathogen attack*.

The hypothesis that catalase inhibition allowing H_2O_2 build up is responsible for PR protein induction and establishment of SAR is based on the observation that a specific SA-binding activity found in tobacco leaf extracts is a catalase (Chen *et al.*, 1993b). An earlier study (Chen *et al.*, 1993a) reported that catalase bound SA with a K_D of approximately 14 μM. Using *in vitro* assays Klessig and co-workers have shown that the activity of purified tobacco catalase was inhibited in a specific fashion by 1 mM SA, but lower concentrations were not tested. These studies have been extended recently to show that an SA-binding activity is present in crude protein preparations purified from a range of plant species (Sanchez-Casas and Klessig, 1994). In the same paper it was shown that 1 mM SA would reduce oxygen evolution from crude leaf protein preparations spiked with H_2O_2. However, the ability to evolve oxygen was not definitely linked with catalase activity by demonstrating 3-AT sensitivity as in the present study. In addition, since peroxidases can act as competitors with catalase for H_2O_2 (and thereby reduce O_2 evolution), it is essential that 3-AT-sensitive activity is monitored in order to exclude effects due to variations or changes in the peroxidase content of the tissue examined. Further, SA binding could not always be correlated with oxygen evolution. Our data confirm that it is possible to reduce catalase activity in crude plant extracts using high concentrations of SA. However, SA was largely ineffective as a catalase inhibitor below 250 μM, a fact that seems inconsistent with an apparent K_D of 14 μM for SA binding. Furthermore, INA, which is a good inducer of PR proteins in tobacco, was not able to reduce catalase activity *in vitro* significantly, even following a 60 min preincubation.

The level of free SA in tissue adjacent to lesions in TMV-infected tobacco leaves has been estimated to be in the region of 6 μg g^{-1} fresh weight (Enyedi *et al.*, 1992) which equates roughly to a SA concentration of around 70 μM if it is assumed that SA is not compartmentalized within plant cells. Thus, it would appear that SA concentrations

required to inhibit catalase activity *in vitro* are much greater than the highest physiological levels reported around TMV lesions and therefore the significance of this inhibition to SAR is questionable. In addition, Chen *et al.* (1993b) reported that H_2O_2 levels were elevated for at least 24 h in SA-treated tissue. However, the current data demonstrate that although 1 mM SA gave full induction of PR-1a expression in leaf discs, the level of free SA accumulating within such tissue was reduced to a level much lower than that required to inhibit catalase *in vitro* within just 3 h.

If SA functioned *in vivo* by inactivating catalase, thus allowing H_2O_2 accumulation, then several predictions might be made. First, it would be expected that H_2O_2 acts downstream of SA in the SAR signalling pathway and thus addition of H_2O_2 or inhibition of catalase should induce PR protein expression, even in the absence of salicylate accumulation. Secondly, it may be possible to measure reductions in catalase activity in infected tissue following pathogen inoculation prior to induction of PR proteins. The present data suggest that catalase is not inhibited by direct application of SA, or inhibited following pathogen infection of tobacco leaves under conditions that induce PR protein expression and promote establishment of SAR. Indeed, the total amount of H_2O_2-degrading activity was found to rise rather than fall over a period of 3 days in pathogen-infected tissue. This activity is mostly 3-AT-insensitive and unlikely to be catalase, therefore it may reflect increased levels of peroxidases or non-enzymatic anti-oxidants. Thus, data demonstrating that SA inhibits catalase activity *in vitro* may need to be interpreted cautiously before such observations are extrapolated to develop a hypothesis concerning the function of SA in SAR signalling in intact plants.

The current data show clearly that 3-AT is less active than SA at inducing PR proteins and thus there is a possibility that the irreversible inhibition of catalase by 3-AT allows sufficient H_2O_2 to accumulate to trigger the SA-dependent induction of PR-1a observed in wild-type tobacco but missing in SH-L plants. One potential explanation, that H_2O_2 induces a rise in endogenous SA, could be tested by intercellular infiltration of hydrogen peroxide. However, these experiments may be hampered by the fact that externally applied H_2O_2 is rapidly degraded (see Levine *et al.*, 1994) and thus very high levels of hydrogen peroxide have to be presented to mimic the oxidative burst that occurs following pathogen recognition. Indeed, in the accompanying paper by Neuenschwander *et al.* (1995) it is shown that leaf infiltration with H_2O_2 at concentrations approaching 0.5 M induces a small increase in SA concentration, but under such conditions there is substantial tissue damage. Levine *et al.* (1994) similarly reported considerable cell death when cell suspensions were treated with only 10 mM H_2O_2.

Salicylate and H_2O_2-mediated defence gene expression can be separated

Using a GUS reporter gene fused to the PR-1a gene promoter to monitor SAR signalling we have shown that inoculation of tobacco leaf lamina with a bacterial pathogen is a useful method of inducing PR proteins. Using this technique, defence gene expression in and around inoculated zones can be measured prior to the appearance of necrotic lesions. Our data show that measurable PR-1a induction only occurs after 8–10 h which is consistent with the time required for the synthesis of SA following plant inoculation with a bacterial pathogen (Métraux *et al.*, 1990; Rasmussen *et al.*, 1991). Using the same inoculation procedure, GUS expression driven by the AoPR-1 promoter begins to rise within 3 h (Mur *et al.*, 1995; submitted). Neither PR-1a nor AoPR-1 are strongly induced by 3-AT (when compared with SA or H_2O_2 respectively). Thus, in conclusion, if H_2O_2 plays a role in SA-induced PR protein expression, or is able to act as an SA-independent inducer, then, as suggested recently by Levine *et al.* (1994), it is unlikely to arise from the inhibition of endogenous catalase.

Experimental procedures

Chemicals

3-Amino-1,2,4-triazole (3-AT), catechol, 3% H_2O_2, 4-hydroxybenzoic acid (4hBA), salicylic acid (SA) and bovine catalase were obtained from Sigma Ltd (Poole, UK). Stock solutions of SA, 4hBA and catechol were made up in water and the pH adjusted to 7.0. These solutions were stored at 4°C. 2,6-dichloro-isonicotinic acid (INA) was a gift of Dr Helmut Kessmann (Ciba-Geigy Ltd, Basel, Switzerland). Rabbit antiserum to PR-1a was a gift of Dr R.F. White (Rothamsted Experimental Research Station, UK).

Plant growth conditions and treatments

Transgenic tobacco lines used were all T_2 generation derived as described by Warner *et al.* (1993). Chemical treatment of tobacco leaf material involved either injection of intercellular leaf spaces *in planta* with a 2 ml syringe fitted with a 27G needle, or utilized 1.5 cm diameter leaf discs cored with a cork-borer and treated in Multi-Well dishes (Nunc). Injected samples were isolated from leaves as 1.5 cm diameter discs prior to assay.

Pathogens and infection techniques

Tobacco mosaic virus strain U1 was the gift of R.F. White (Rothamsted Experimental Research Station, UK). TMV infections involved rubbing viral suspensions (1 μg ml^{-1}) mixed with 20 mg ml^{-1} carborundum carmine in water on to the leaf lamina. After abrasion, carborundum carmine was removed by spraying with water. *Pseudomonas syringae* pathovar *syringae* strain 2774 was the gift of John Taylor, HRI, Wellesbourne, UK. *P. s.* pv. *syringae* was grown overnight in nutrient broth (Oxoid), centrifuged and then resuspended to an approximate concentration of 1×10^8 colony forming units per ml in 10 mM phosphate buffer (pH 7.0).

Tobacco leaves were infected with *P. s.* pv. *syringae* by injection of leaf intercellular spaces using a 2 ml syringe fitted with a 27G needle. Each infiltrated region covered an area of approximately 2 cm^2 and typically resulted in the application of approximately 100 µl bacterial suspension.

Cloning of the nah*G gene coding for the salicylate hydroxylase (SH-L) enzyme of* P. putida *strain NCIB9816*

The plasmid pWW60-3022 carrying a 4.7 kb *Xho*I fragment containing *nah*R, G and H genes, components of the naphthalene (*nah*) catabolism operon of *Pseudomonas putida* strain NCIB9816 (Assinder and Williams, 1988) was a gift of Professor Peter Williams (University of Wales, Bangor, UK). Two PCR primers, 5'-CG GGATCC AGCATGAAAAACAATAAA CTTGG (containing a *Bam*HI site) and 3'-CG GAATTC CGTTGTCACCCTTGACG (containing an *Eco*RI site) were designed from the published sequence of a *nah*G gene coding for a related salicylate hydroxylase protein (You *et al.*, 1991) and used to amplify the entire *nah*G open reading frame. Following a standard polymerase chain reaction (PCR) using these primers and pWW60-3022 as the template, the resulting roughly 1300 bp PCR product was digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI/*Eco*RI-digested pBluescript to produce pSK-*nah*G. *E. coli* strains harbouring pSK-*nah*G produced a brown discoloration around colonies when grown on media containing 1 mM IPTG and 2 mM SA, due to conversion of SA to catechol by SH-L and its subsequent oxidation, hence demonstrating that the SH-L enzyme was functional. The *nah*G gene was cloned into pROK2, derived from pBin19 (Bevan, 1984) to create a transcriptional fusion with the CaMV-35S promoter to produce p35S–SH-L. The p35S–SH-L binary vector was electroporated (Shen and Forde, 1989) into *Agrobacterium tumefaciens* strain pGV2260.

Generation of transformed tobacco lines

A binary vector containing a PR-1a promoter–GUS fusion was the gift of Dr Scott Uknes (Ciba-Geigy, North Carolina). Transgenic tobacco (*Nicotiana tabacum*) Samsun NN lines harbouring PR-1a–GUS or 35S–SH-L transgenes were generated using standard procedures (Draper *et al.*, 1988). The activity of SH-L was measured in crude leaf extracts of several transgenic lines according to Yamamoto *et al.* (1965). Three lines (1, 4 and 7) showed high SH-L specific activity (> 0.3 U mg^{-1} of total protein) and were selected for further analysis. Two lines (4 and 7) were selected for further study as in preliminary experiments these plants did not accumulate PR-1a protein (as determined by Western blotting) after 2 days treatment with 1 mM SA.

GUS assays

Analyses of changes in gene expression using chimeric promoter GUS fusions were carried out in three plant lines for each transgene. PR-1a–GUS tobacco variety Samsun NN lines 3, 4 and 13 were selected as having 'high', 'medium' and 'low' GUS expression, respectively, in response to treatment with 1 mM SA. AoPR-1-GUS tobacco variety SR1 lines 1 and 14 (Warner *et al.*, 1993) and Samsun NN line L with varying wound-inducible GUS activities were selected as representative of AoPR-1. Fluorimetric determination of GUS activities followed the Jefferson *et al.* (1987) protocol with the modifications of Topping *et al.* (1991). Assays were carried out on soluble-protein extracts from 1.5 cm diameter discs of sample tissue, removed from leaves using a cork-borer.

Western blotting

Western blotting was carried out as previously described (Warner *et al.*, 1993). Ten micrograms of protein were loaded per lane. Rabbit antiserum to PR-1a was used at a dilution of 1:5000 and secondary antiserum at 1: 1000.

Catalase assay

The assay method used was based on that of Storrie and Madden (1990). Leaf discs (1.5 cm) were ground in 200 µl of ice-cold 20 mM imidazole buffer (pH 7.0) containing 0.2% Triton X-100 (catalase extraction buffer). Whole leaves were ground using a pestle and mortar containing sand in ice-cold catalase extraction buffer. Samples were then centrifuged for 1 min at 11 600 ***g***. Protein content was determined by the method of Bradford (1976). Assays were performed in 96 well microtitre plates. Unless stated otherwise in figure legends, standard assay conditions were 20 µl of sample (diluted to 0.2 mg ml^{-1} in catalase extraction buffer) to which were added 50 µl of catalase extraction buffer containing 1 mg ml^{-1} bovine serum albumin and 0.06% H_2O_2 (catalase assay buffer). In all experiments less than 12 min elapsed from extract dilution to end of assay period. Three reactions per sample were set up containing H_2O_2, H_2O_2 plus 10 mM 3-AT and a peroxide-free background incubation. The reaction was allowed to proceed for 2 min after which 200 µl of catalase stop solution (2.25 g l^{-1} $TiOSO_4$ in 0.1M H_2SO_4) were added. Absorbance was then read at 405 nm. Following subtraction of background, absorbance units were converted to H_2O_2 content by reference to a H_2O_2 standard curve and the difference between 3-AT-containing and 3-AT-free incubations taken as 3-AT-sensitive catalase activity. The unit definition used is: 1 unit = 1 µmol H_2O_2 degraded per min. The standard curve was linear up to 0.82 mmol H_2O_2 , had a limit of detection of at least 13 nmol H_2O_2 and a sensitivity of 1.44 absorbance units per mmol H_2O_2. Under standard conditions, the assay was linear for at least 3 min. In some cases involving samples taken from infected tissue, the protein concentration of the sample assayed was below 0.2 mg ml^{-1}.

Extraction of SA

The method used was modified from that reported in Malamy *et al.* (1992). Leaf discs were treated as stated in the section referring to catalase assays. The supernatant remaining, following that removed for catalase assays, was made up to 20 ml with 100 mM sodium phosphate pH 8.0, and thoroughly mixed. The mixture was then acidified to pH 2.5 by the addition of HCl, and partitioned three times against an equal volume of ECI (ethyl acetate:cyclopentane:isopropanol/50:50:1). The combined organic phases were reduced to dryness in a Speedvac (Savant). Samples were resuspended in 500 µl methanol:acetonitrile:water (10%:4.5%:85.5%) and 50 µl were subjected to HPLC analysis. Recovery of SA was estimated by addition of 50 000 d.p.m. of 7-^{14}C SA (American Radio Chemicals) to the starting supernatant, and then calculating the amount of radioactivity remaining following the extraction.

HPLC analysis

The method used was based on that reported in Malamy *et al.* (1992). Samples were analysed using an ATI Unicam HPLC system. Samples were separated by reverse phase analysis utilizing a gradient of acetonitrile/water increasing from 5–40% acetonitrile

in 30 min. An ODS Techsphere 5 μm 250 mm × 4.5 mm column was used in all assays. SA was determined by fluorescence with an excitation of 304 nm and emission of 440 nm using a Thermo Separations FL2000 fluorescence detector.

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References

Assinder, S.J. and Williams, P.A. (1988) Comparison of the meta pathway operons on NAH plasmid pWW60-22 and TOL plasmid pWW53-4 and its evolutionary significance. *J. Gen. Microbiol.* **134**, 2769–2778.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**, 8711–8721.

Bol, J., Linthorst, H.J.M. and Cornelissen, B.J.C. (1990) Plant pathogenesis-related proteins induced by virus infection. *Ann. Rev. Phytopathol.* **28**, 113–138.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities utilising the principle of protein dye binding. *Anal Biochem.* **72**, 248–254.

Chen, Z.X., Ricigliano, J.W. and Klessig, D.F. (1993a) Purification and characterization of a soluble salicylic acid-binding protein from tobacco. *Proc. Natl Acad. Sci. USA*, **90**, 9533–9537.

Chen, Z.X., Silva, H. and Klessig, D. (1993b) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science*, **262**, 1883–1886.

Chester, K.S. (1933) The problem of acquired physiological immunity in plants. *Q. Rev. Biol.* **8**, 275–324.

Davis, J. I. and Evans, W. C. (1964) Oxidative metabolism of naphthalene by soil in *Pseudomonas putida*. *Biochem. J.* **91**, 974–979.

Draper, J., Scott, R., Armitage, P. and Walden, R. (1988) *Plant Genetic Transformation and Gene Expression: A Laboratory Manual.* Oxford: Blackwell Scientific Publications.

Enyedi, A.J., Yalpani, N., Silverman, P. and Raskin, I. (1992) Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl Acad. Sci. USA*, **89**, 2480–2484.

Gaffney, T., Friedrich, L., Vernooji, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic aquired resistance. *Science*, **261**, 754–756.

Jefferson, R.A., Kavangh, T.A. and Bevan, M.W. (1987) GUS fusions: beta glucuronidase as a sensitive and versatile marker in plants. *EMBO J.* **6**, 3902–3907.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, **79**, 1–20.

Malamy, J. and Klessig, D.F. (1992) Salicylic acid and plant disease resistance. *Plant J.* **2**, 643–654.

Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990) Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**, 1002–1004.

Malamy, J., Hennig, J. and Klessig, D.F. (1992) Temperature-dependent induction of salicylic acid and its conjugates during the resistance to tobacco mosaic virus. *Plant Cell*, **4**, 359–366.

Métraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdoorf, K., Schmid, E., Blum, W. and Inverardi, B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science*, **250**, 1004–1006.

Métraux, J.P., Ahl Goy, P., Staub, T.H., Speich, J., Steinemann, A., Ryals, J. and Ward, E. (1991) Induced systemic resistance in cucumber in response to 2,6-dichloro-isonicotinic acid and pathogens. In *Advances in Molecular Genetics of Plant–Microbe Interactions*, Volume 1 (Hennecke, H. and Verma, D.P.S. eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 432–439.

Neuenschwander, U., Vernooij, B., Friedrich, L., Uknes, S., Kessmann, H. and Ryals, J. (1995) Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? *Plant J.* **8**, 227–233.

Ohshima, M., Itoh, H., Matsuoka, M., Murakami, T. and Ohashi, Y. (1990) Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein gene in transgenic tobacco. *Plant Cell*, **2**, 95–106.

Raskin, I. (1992) Role of salicylic acid in plants. *Ann. Rev. Plant Physiol.* **43**, 439–463.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N. (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. *Plant Physiol.* **97**, 1342–1347.

Ross, A.F. (1961) Systemic acquired resistance induced by localized virus infections in plants. *Virology*, **14**, 340–358.

Ryals, J., Uknes, S. and Ward, E. (1994) Systemic acquired resistance. *Plant Physiol.* **104**, 1109–1112.

Sanchez-Casas, P. and Klessig, D.F. (1994) A salicylic acid-binding activity and a salicylic acid-inhibitable catalase activity are present in a variety of plant species. *Plant Physiol.* **106**, 1675–1679.

Shen, W.J. and Forde, B.J. (1989) Efficient transformation of *Agrobacterium* spp by high-voltage electroporation. *Nucl. Acids Res.* **17**, 8385.

Storrie, B. and Madden, E. A. (1990) Isolation of subcellular organelles. *Methods Enzymol.*, **182**, 203–225.

Topping, J.F., Wei, W. and Lindsey, K. (1991) Functional tagging of regulatory element in the plant genome. *Development*, **112**, 1009–1019.

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Thompson-Taylor, H., Potter, S., Ward, E. and Ryals, J. (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. *Plant Cell*, **5**, 159–169.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell*, **6**, 959–965.

Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H. and Ryals, J. (1995) 2,6-Dichloroisonicotinic acid-induced resistance to pathogens does not require the accumulation of salicylic acid. *Mol. Plant–Microbe Interact.* **8**, 228–234.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold,

D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P. and Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell*, **3**, 1085–1094.

Warner, S.A.J., Scott, R. and Draper, J. (1992) Characterisation of a wound-induced transcript from the monocotyledon asparagus that shares homology with a new class of pathogenesis-related (PR) proteins. *Plant Mol. Biol.* **19**, 555–561.

Warner, S.A.J., Scott, R. and Draper, J. (1993) Isolation of an asparagus intracellular PR gene (AoPR-1) wound-responsive promoter by the inverse polymerase chain reaction and its characterisation in transgenic tobacco. *Plant J.* **3**, 191–201.

Yamamoto, S., Katagiri, M., Maeno, H. and Hayaishi, O. (1965) Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. I. Purification and general properties. *J. Biol. Chem.* **240**, 3408–3413.

You, I.-S., Ghosal, D. and Gunsalus, I. C. (1991) Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene (*nah* G) and its 3′-flanking region. *Biochemistry*, **30**, 1635–1641.