WOUND INDUCED PLANT PHENOLIC COMPOUNDS AND VIRULENCE GENE EXPRESSION IN AGROBACTERIUM SPECIES

by

PAUL ANTHONY SPENCER

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Department of Botany

The University of British Columbia
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Abstract

Crown gall disease of plants is caused by introduction of foreign DNA into susceptible plant cells by strains of *Agrobacterium tumefaciens*. The expression of bacterial virulence genes is triggered by chemicals present in plant wound exudates. The exudates contain a number of phenolic compounds which act as chemical signals inducing expression of a number of genes directing the DNA transfer process. These are the virulence or *vir* genes, and *vir::lac* reporter gene fusions have been widely used to assay *vir* gene induction in *Agrobacterium tumefaciens* strains. Using such strains to monitor *vir* gene expression, Stachel *et al.* (1985) isolated from *Nicotiana tabacum* two active acetophenones: 3,5-dimethoxy-4-hydroxyacetophenone, ("acetosyringone" or AS), and α-hydroxy-3,5-dimethoxy-4-hydroxy-acetophenone, ("hydroxyacetosyringone" or HO-AS).

However, *in vitro* assay results suggested that other more common compounds also exhibited activity (Spencer and Towers, 1988). This analysis of structure-activity relationships of induced *vir* expression in *A. tumefaciens* was presented in a previous thesis (Paul Spencer, M.Sc. thesis). The results revealed that a variety of commonly occurring plant phenolic compounds were capable of activating *vir* genes. In addition to the acetophenones, a variety of benzoic and cinnamic acid derivatives, and even a few chalcones of appropriate ring substitution were active. This thesis reports the isolation and identification of a number of these compounds in plant wound exudates.

Some *Agrobacterium tumefaciens* strains are restricted in host range to certain grapevine cultivars. Subsequent to the development of a convenient and sensitive plate-bioassay method, a strongly active component in grapevine wound exudates was purified. A newly described *vir*-inducing phenolic compound was isolated from a number of *Vitis* cultivars using gel

filtration, thin layer and high pressure liquid chromatographies. This was identified as syringic acid methyl ester (3,5-dimethoxy-4-hydroxybenzoic acid, methyl ester), using mass spectrometry. However, the presence of this compound in grapevine wound exudates does not provide a simple explanation for host range limitation of grapevine strains since it induces *vir* gene expression in both limited and wide host range strains of *A. tumefaciens*. Interestingly, neither AS nor HO-AS were present in grapevine-derived extracts.

A convenient polyamide column chromatographic method was subsequently developed to permit rapid purification of plant-derived *vir* gene inducing mixtures, which were detected using the newly developed plate bioassay. Derivatized polyamide fractions were then analysed by combined gas chromatography-mass spectrometry (GC-MS). GC-MS proved to be an ideal means for the identification of the phenolic components in partially purified extracts. Examination of wound exudates from a range of host and non-host species revealed that the production of the acetophenones is restricted to members of the Solanaceae. Some experiments focussed on the biosynthetic precursors of the acetophenones in *Nicotiana* species. Wound exudates of the majority of species belonging to other plant families contained benzaldehydes and/or benzoic and cinnamic acid derivatives.

The induction of *vir*E gene expression was examined in the related *Agrobacterium* species, *A. rhizogenes*. To do this, the *vir*E::*lac*Z gene fusion plasmid pSM358cd was introduced into *A. rhizogenes* A4 by triparental mating and the strain "A4/pSM358cd" was used to analyze *vir* activation. Acetophenones, chalcones, benzaldehydes, and benzoic and cinnamic acid derivatives were found to activate *vir* genes in *A. rhizogenes*.

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List of abbreviations

Bacterial strains

A348	*A. tumefaciens* strain 348 (a C58 derivative with WHR pTiA6)
A348/pSM	*A. tumefaciens* strain 348 possessing *vir::lac* insertion plasmid
A856	*A. tumefaciens* strain 856 (a C58 derivative with LHR pTiAg162)
A856/pSM	*A. tumefaciens* strain 856 possessing *vir::lac* insertion plasmid
C58	WHR *A. tumefaciens* strain with nopaline-type Ti plasmid
JC2926	*E. coli* strain (provided by Dr. E.W. Nester, U.W.)
JC2926/pRK	*E. coli* strain possessing helper plasmid
JC2926/pSM	*E. coli* strain possessing *vir::lac* insertion plasmid
LHR	limited host range *A. tumefaciens* strain (eg. A348)
WHR	wide host range *A. tumefaciens* strain (eg. A856)

Plasmids

pRi	*A. rhizogenes* root inducing plasmid
pRiA4	*A. rhizogenes* strain A4 root inducing plasmid
pRK2013	triparental mating helper plasmid
pSM	*vir::lac* insertion plasmid
pTi	*A. tumefaciens* tumor inducing plasmid
pTiA6	*A. tumefaciens* strain A6 (WHR) tumor inducing plasmid
pTiAg162	*A. tumefaciens* strain Ag162 (LHR) tumor inducing plasmid
pVCK	cloning vehicle with portions of pTiA6 *vir* region

Genes and genetic elements

abg	cellobiase gene of *A. tumefaciens*
att	attachment gene of *A. tumefaciens*
cel	cellulose gene of *A. tumefaciens*
chv	chromosomal virulence gene of *A. tumefaciens*
lacZ	β-galactosidase gene of *E. coli*
nod	nodulation gene of *Rhizobium* species
psc	polysaccharide gene of *A. tumefaciens*
tzs	zeatin gene of *A. tumefaciens*
vir	virulence gene of *A. tumefaciens*
vir::lac	transcriptional and translational gene fusion between *vir* locus and *lacZ*
T-DNA	transferred DNA of Ti plasmids
Tn3::HoHo1	transposable genetic element for creation of transcriptional and translation gene fusions with *lacZ*

Chemicals and other abbreviations

^{1}H-NMR	proton nuclear magnetic resonance spectrum or spectrometry
4CL	4-coumarate CoA ligase
^{13}C-NMR	carbon nuclear magnetic resonance spectrum or spectrometry
ABA	abscisic acid
AS	3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone)
AV	4-hydroxy-3-methoxyacetophenone (acetovanillone)
BF_3	boron trifluoride
BSTFA	bis(trimethylsilyl)trifluoroacetamide
BuOAc	butyl acetate
C_6-C_1	benzoic acid derivative (ring plus one carbon)
C_6-C_2	acetophenone derivative (ring plus two carbons)
C_6-C_3	phenylpropanoid derivative (ring plus three carbons)
CA4H	cinnamate 4-hydoxylase
carb	carbenicillin
carb^r	carbenicillin resistant
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane
$ClCH_2CH_2Cl$	dichloroethane
$CHCl_3$	chloroform
DIBOA	2,4-dihydroxy-2H-1,4,-benzoxazin-3(4H)-one
DIM_2BOA	2,4-dihydroxy-6,7-dimethoxy-2H-1,4,-benzoxazin-3(4H)-one
DIMBOA	2,4-dihydroxy-7-metyhoxy-2H-1,4,-benzoxazin-3(4H)-one
DMSO	dimethylsulfoxide
EIMS	electron impact mass spectrum
EtOAc	ethyl acetate
FAXX	O-[5-0-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1-3)-O-ß-D-xylopyranosyl-(1-4)-D-xylanopyranose
GC-EIMS	gas chromatography-electron impact mass spectrometry
HO-AS	α-hydroxy-3,5-dimethoxy-4-hydroxyacetophenone, (α-hydroxyacetosyringone)
HO-AV	α-hydroxy-3-methoxy-4-hydroxyacetophenone, (α-hydroxyacetovanillone)
HOFm	formic acid
HPLC	high pressure liquid chromatography
HR-EIMS	high resolution electron impact mass spectrometry
kan	kanamycin
kan^r	kanamycin resistant
LB	Luria broth
LH-20	sephadex gel permeation chromatographic support
LiAlH	lithium aluminum hydride
M^+	molecular ion
M_2BOA	6,7-dimethoxy-2-(3H)-benzoxazolinone
m/z	mass over charge ratio

MBOA	6-methoxy-2-(3H)-benzoxazolinone
MeOH	methanol
MS	Murashige and Skoog plant cell culture medium
nal	nalidixic acid
nal^r	nalidixic acid resistant
NaOMe	sodium methoxide
n-BuOH	1-butanol
PAL	phenylalanine ammonia lyase
PD	potato dextrose
PDA	potato dextrose agar
R_f	retention factor used in TLC
R_t	retention time used in GC
SE-54	fused silica capillary column used in GC
spec	spectinomycin
$spec^r$	spectinomycin resistant
TLC	thin layer chromatography
TMS	trimethylsilyl group ($C_3H_{10}Si$, m.w. 73)
VLC	vacuum liquid chromatography
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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Chapter 1. General Introduction

1.1 The biology of *A. tumefaciens*

In recent times there have been significant advances in research concerning the interactions between plants and microorganisms. In part, this is because the techniques of molecular biology have allowed us to probe the genetic bases of such relationships. Perhaps nowhere else has this type of analysis progressed so rapidly as with the study of crown gall tumorigenesis. It turns out, however, that a complete understanding of the interaction of *Agrobacterium tumefaciens* with plants requires also an understanding of the biochemical reactions triggered at the site of plant wounds. In comparison with topics such as the biosynthesis of phytoalexins, this is a relatively uncharted domain.

Initiation of plant-microbe relationships often requires exchange of chemical signals (reviewed by Dixon and Lamb, 1990). The microbe detects host-derived chemicals that are produced under certain conditions (eg. wounding), or which the plant already possesses as part of its cell wall or membrane. The microbe may respond to these signals through spore germination (Fries *et al.*, 1987), directed growth or chemotaxis (Ashby *et al.*, 1987), and expression of genes necessary for subsequent stages of the interaction (Stachel *et al.*, 1985b). For example, expression of genes directing morphogenesis from saprophytic to parasitic stages in response to α-tocopherol is known in *Ustilago violacea* (Castle and Day, 1984). At present, there are only a few examples of cell-cell signalling between plants and microbes in which the chemicals that induce such responses have been identified. However, the signal molecules mediating early stages of certain

bacterial-plant interactions have been identified (Stachel *et al.*, 1985b; Peters *et al.*, 1986; Redmond *et al.*, 1986; Firmin *et al.*, 1986; Sadowsky *et al.*, 1988; Spencer *et al.*, 1990: Morris and Morris, 1990). These studies have focussed on compounds emanating from host plants and to which species of bacteria belonging to the Rhizobiaceae respond. This group of microbes includes the legume-nodulating *Rhizobium* species as well as the plant-transforming *Agrobacterium* species.

The study of signal compounds in relation to the biology of *Agrobacterium tumefaciens* is of considerable significance because successful gene manipulation in plants using this microbial vector is absolutely dependent on these chemicals. *A. tumefaciens* is a gram negative soil bacterium which causes crown gall disease of a wide variety of dicotyledonous plants (DeCleen and Deley, 1976). Smith and Townsend (1907) originally reported the isolation of the bacterium responsible for the disease. Since that time, and particularly recently, a phenomenal amount of research has been conducted on this subject.

By the beginning of the 1980's it was established that each virulent strain of *A. tumefaciens* possesses a large (ca. 200 kb) tumor-inducing plasmid (pTi). Also, it was established that the bacterium causes a neoplastic growth of the plant tissue by transferring part of this DNA (the T-DNA) into the host plant genome (Chilton *et al.*, 1977; Thomashow *et al.*, 1980; Yadav *et al.*, 1980, Chilton *et al.*, 1980; Willmitzer *et al.*, 1980; Zambryski *et al.*, 1980). No other procaryotic organism is known to be capable of this feat of natural genetic engineering, the transformation of eucaryotic cells. The T-DNA includes genes which encode enzymes of auxin (Schroder *et al.*, 1984; Thomashow *et al.*, 1984) and cytokinin biosynthesis (Akiyoshi *et al.*, 1983; Akiyoshi *et al.*, 1984), and these genes are expressed in the transformed plant cell (Hille *et al.*,

1984a; Willmitzer *et al.*, 1981). Also present in the T-DNA is a locus conferring on the transformed plant cells the ability to synthesize characteristic amino acids called opines (reviewed by Nester *et al.*, 1984). Opines produced by the transformed plant cells are catabolized by *A. tumefaciens* strains which possess specific loci for opine catabolism located on the Ti plasmid but not in the T-DNA. Opine production confers an advantage on the infecting strain of *A. tumefaciens* which has the ability to use the opines as a sole carbon and nitrogen source. In addition, it has been found that the opines stimulate conjugative pTi transfer in *A. tumefaciens* (Klapwijk *et al.*, 1978; Petit *et al.*, 1978).

It is unclear how such a system evolved, and the exact route by which the transforming DNA reaches the plant genome remains unknown. Useful vectors for genetic engineering in plants may be constructed by replacing T-DNA genes with new genes of interest. Plant cells are then infected with the bacterium containing the modified Ti-plasmid, transformants are selected, and finally plants are regenerated from the transformed cells. This system has been successful in a number of cases (Goodman *et al.*, 1987; Gaffer and Fraley, 1989, and references therein); however, it should be emphasized that it is ultimately dependent on the ability of the bacterium to detect susceptible cells and express a number of genes essential for successful transformation of the plant genome. It has therefore become of considerable importance that the molecular mechanism by which the bacterium accomplishes this feat be understood in greater detail.

Early studies indicated that attachment of the bacteria to the host plant cells occurs in a site-specific manner (Lippincott and Lippincott, 1969; Smith and Hindley, 1978). Attachment has been considered an essential stage in crown gall tumorogenesis (Lippincott and Lippincott, 1969). Attachment of *A.*

tumefaciens to the surfaces of a number of plant cell types has been studied by a variety of methods (Gee *et al.*, 1967; Bogers, 1972; Smith and Hindley, 1978; Douglas *et al.*, 1982; Matthysse and Gurlitz, 1982; Sigee *et al.*, 1982, Draper *et al.*, 1983; Douglas *et al.*, 1985a; Graves *et al.*, 1988). Hasezawa *et al.* (1983) reported the introduction by endocytosis of *A. tumefaciens* spheroplasts into *Vinca rosea* protoplasts. *A. tumefaciens* can attach to suspension culture cells of both monocotyledons and dicotyledons (Matthysse and Gurlitz, 1982; Douglas *et al.*, 1985a) and can attach either to intact cells or plant protoplasts (Matthysse *et al.*, 1982), but, interestingly, it cannot bind to cells of carrot suspension cultures which have been induced to form embryos (Matthysse and Gurlitz, 1982).

In general, the evidence from several labs suggests that young, developing cell walls (or damaged cell walls and those under repair at wound sites) are prime targets for binding by the bacteria. Both bacterial and plant components for this binding have been examined (Lippincott *et al.*, 1977; Lippincott and Lippincott, 1978; Gurlitz *et al.*, 1987). The plant component appears to be a plant cell wall pectin or protein to which a bacterial lipopolysaccharide binds.

A number of these studies show that the bacterial cells attached in a nonrandom manner; the cells attach to numerous sites on the cell surface first singly (by one end) and later in clusters. The elaboration of cellulose microfibrils is thought to bind the bacteria firmly to one another and onto the host cell surface (Matthysse *et al.*, 1981; Matthysse, 1983). In addition, numerous pili are produced (Lacey Samuels, unpublished observations). Electron microscopy by a number of research groups has revealed that a membranous structure subsequently envelopes the attached bacteria.

During the last decade, the genetics of *A. tumefaciens*, and especially of the early events of the infection process, became much better understood. In addition to the Ti plasmid virulence (*vir*) genes described below, chromosomal genes affecting virulence, eg. *chv*A and *chv*B (Douglas *et al.*, 1985b) and *chv*E (Huang *et al* ., 1990), cellulose synthesis (*cel*) (Mattysse, 1983; Robertson *et al.*, 1988), bacterial attachment (*psc*A and *att*) (Thomashow *et al.*, 1987; Robertson *et al.*, 1988) and cellobiase (*abg*) from *Agrobacterium* (sp. strain ATCC 21400) have been examined (Wakarchuk *et al.*, 1988). All of the nonattaching *Agrobacterium* mutants reported to date (*chv*AB, *psc*A and *att* mutants) are chromosomal mutants. *chv*E codes for a protein homologous to periplasmic receptor proteins involved in chemotaxis and uptake of sugars. *chv*E mutants display strongly attenuated virulence and restricted host range.

Detection of susceptible host cells and early stages of tumorigenesis are mainly controlled by a set of pTi genes known as the virulence (*vir*) genes (Garfinkel *et al.*, 1980; Klee *et al.*, 1983; Horsch *et al.*, 1986). Stachel and Nester (1986) investigated the genetic and transcriptional organization of the *vir* region of *Agrobacterium tumefaciens* by saturation mutagensis of cloned portions of the *vir* region with the modified transposon Tn3::HoHo1. This transposable element contains the ß-galactosidase structural gene (*lac*Z), and is engineered such that both transcriptional and translational gene fusions may be obtained. When *lac*Z is inserted in frame with a *vir* gene, ß-galactosidase activity can be measured quantitatively to monitor *vir* gene activation.

Reporter gene fusions were used to demonstrate that the *vir* genes are expressed upon cocultivation of the bacteria with host plant cells (Stachel *et al.*, 1985b; Stachel *et al.*, 1986), and that some diffusible, low molecular weight plant cell factor induced this response. This plant factor is required in the

early stages of tumorigenesis, and therefore is required in transformation of plant genomes. For these reasons, research has been directed towards (1) revealing the mechanism(s) involved in *vir* gene expression, (2) identifying the *vir* gene products, and (3) identifying the plant secondary metabolites which may induce or repress *vir* activity.

The genetic and transcriptional organization of the *vir* region of the wide host range *A. tumefaciens* Ti-plasmid pTiA6 (Figure 1.1) has been examined by a number of independent research groups. Two of the *vir* genes (A and G) are regulatory in nature (Winans *et al.*, 1986; Stachel and Zambryski, 1986-a). Apparently these act as a two component regulatory system to transmit to the bacterium information about external conditions; they trigger expression of the *vir* regulon if the external conditions are appropriate. The *vir*A gene product, the VirA protein, is also a host range determinant and is thought to be the environmental sensor of the plant derived inducer molecules (Leroux *et al.*, 1987). The VirA protein is a transmembrane protein homologous to the sensor component of other two component systems (Winans *et al.*, 1986) and it likely interacts with both plant derived phenolic compounds and monosaccharides (Shimoda *et al.*, 1990). It was recently determined that autophosphorylation of the VirA protein is required for induction of the *vir* regulon (Jin *et al.*, 1990a). The activated form of the VirG protein specifically binds to 5' nontranscribed sequences of the *vir* genes (Jin *et al* ., 1990b; Pazour and Das, 1990).

*vir*C mutants display attenuated virulence or altered host range (Hille *et al.*, 1984b; Hooykaas *et al.*, 1984; Yanofsky *et al.*, 1985; Yanofsky and Nester, 1986). The *vir*D operon is known to encode a site specific endonuclease which recognizes and cleaves the left and right border sequences of T-DNA (Yanofsky *et al.*, 1986; Stachel *et al.*, 1987). It was subsequently determined that

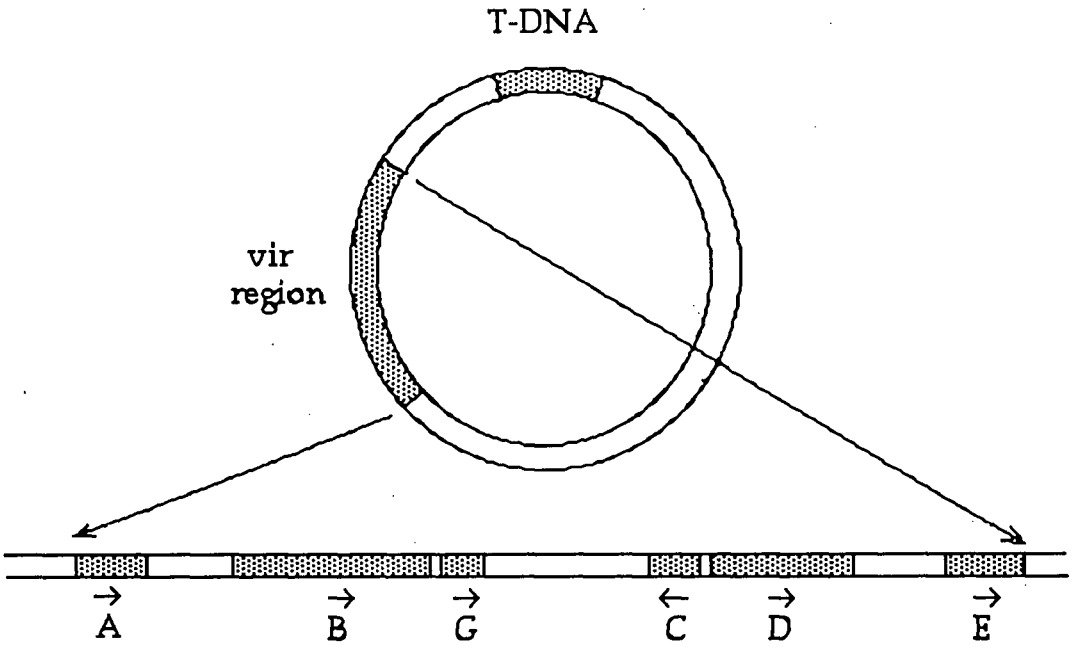


Figure 1.1 Genetic and transcriptional organization of the *vir* region of the Ti plasmid pTiA6. Arrows indicate the direction of transcription of *vir* loci.

the endonuclease remains associated with (perhaps covalently bound to) the 5' end of the T-DNA intermediate (Young and Nester, 1988). Perhaps the endonuclease also functions within the plant cell to create a site for insertion into the plant genome. The *vir*B operon encodes 11 different polypeptides (Engström *et al.*, 1987), thought to be homologous in function to pilus proteins, which are required for a presumed *A. tumefaciens*-plant cell conjugation event resulting in T-DNA transfer (Stachel and Zambryski, 1986b).

A number of factors are required for *vir* gene activity. For example, continued expression of *vir*D requires: (1) temperatures below 28 C, (2) acidic pH, (3) plant phenolic compounds (eg. AS), and (4) sucrose (Alt-Mörbe *et al.*, 1989). Interestingly, *vir*-induction can be enhanced with glycine betaine (Vernade *et al.*, 1988). This natural osmoprotectant apparently accelerates adaptation of the bacteria to the acidic induction medium.

Activation of *vir* gene expression is known to result in the production of multiple, linear, single-stranded T-DNA molecules (T-strands) within the bacterium (Stachel *et al.*, 1987). However, this is just one of a few potential intermediates in T-DNA transmission known to occur in *A. tumefaciens*. Circular forms of T-DNA have been reported by several groups (Koukolikova-Nicola *et al.*, 1985; Machida *et al.*, 1986; Alt-Moerbe *et al.*, 1986; Yamamoto *et al.*, 1987) and double-stranded cleavages, mediated by the *vir*D gene product, also occur within the T-DNA borders (Veluthambi *et al.*, 1987). Presumably, one or more of these T-DNA molecules are the elements which are transferred to the plant genome. They may be transported as a package along with a *vir* gene encoded protein(s). The *vir*E2 gene product was recently determined to be a single-stranded-DNA-binding protein that associates with T-DNA (Gietl *et al* ., 1987; Das 1988; Christie *et al.*, 1988). It

was proposed that the VirE2 protein is involved in the processing of T-DNA and in T-strand protection during transfer to the plant cell. In summary, the *vir* region comprises a set of genes that, when expressed, enable *A. tumefaciens* to detect susceptible plant tissue, that prepare the bacterium for its interaction with the plant cell, and that result in the production of the transforming DNA in a form ready for transmission to the host.

The *vir* loci were identified by random transposon mutagenesis of *A tumefaciens*. Insertions in these loci resulted in avirulence or altered virulence (Garfinkel and Nester, 1980). Expression of the *vir* genes was studied before their fuctions were known by inserting a "reporter" gene with easily measurable activity. As was noted above, Stachel *et al.* (1985b) prepared a Tn3::*lacZ* transposon for the random generation of ß-galactosidase gene fusions and used it to study gene expression in *A. tumefaciens*. This system is shown diagrammatically in Figure 1.2.

In a strain carrying a *vir* ::*lacZ* gene fusion plasmid, the degree of *vir*-induction can be determined simply by assaying ß-galactosidase activity. Using this system it was established that, with the exception of the regulatory locus *vir*A, expression of each of the *vir* genes can be induced by the presence of certain phenolic compounds (Bolton *et al.*, 1986). The regulation of the *vir* genes of pTiC58 has been examined with *vir*::*lux* gene fusions (Rogowsky *et al.*, 1987).

After it was established that cocultivation of the bacteria with the host plant cells resulted in *vir*-induction, it was determined that the inducing agent must have a molecular weight below 1000 (Stachel *et al.*, 1986). Bolton *et al.* (1986) found that a mixture of low molecular weight phenolic compounds (Figure 1.3) could be used to induce expression of most of the *vir* genes. However, quantitative analysis of *vir* gene induction by each

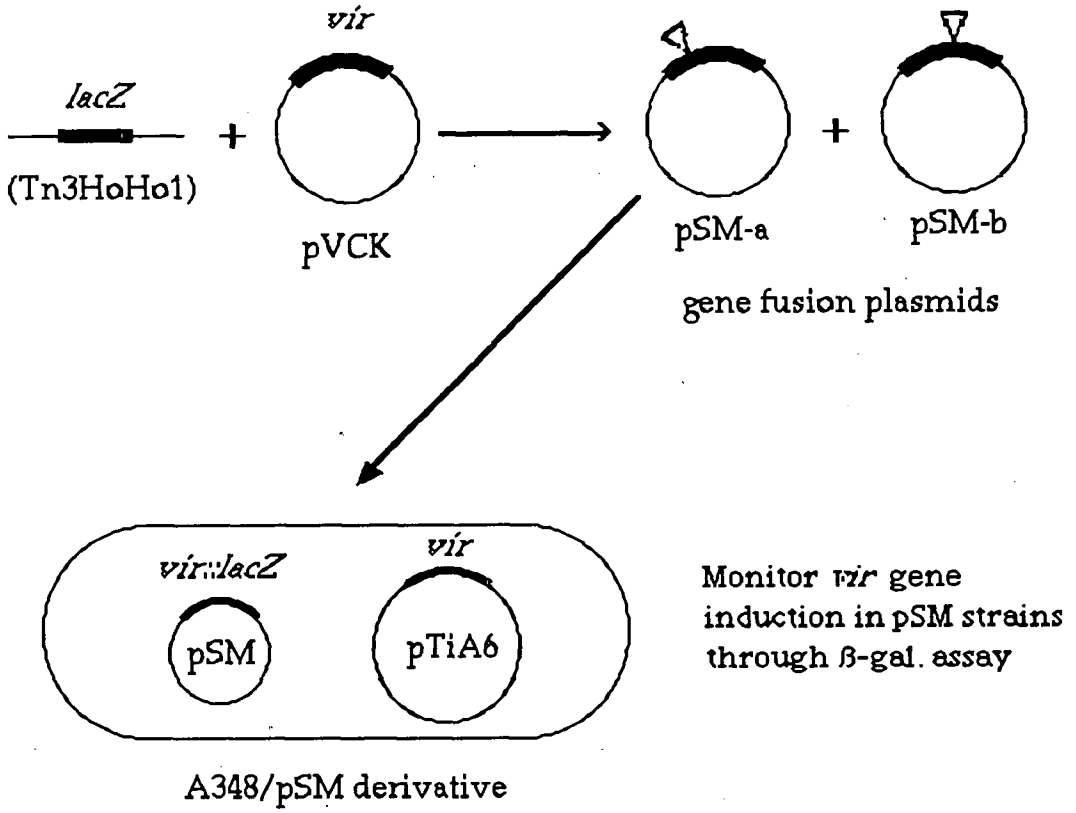


Figure 1.2 Strategy for analysis of signal compound inducible pTi genes. The modified transposon Tn3::HoHo1 carrying *lac*Z was inserted at numerous sites in the *vir* region on a cloning vehicle and pTi-carrying strains also carrying *vir*::*lac*Z gene fusion-containing (pSM) plasmids can be used in *vir*-induction bioassays.

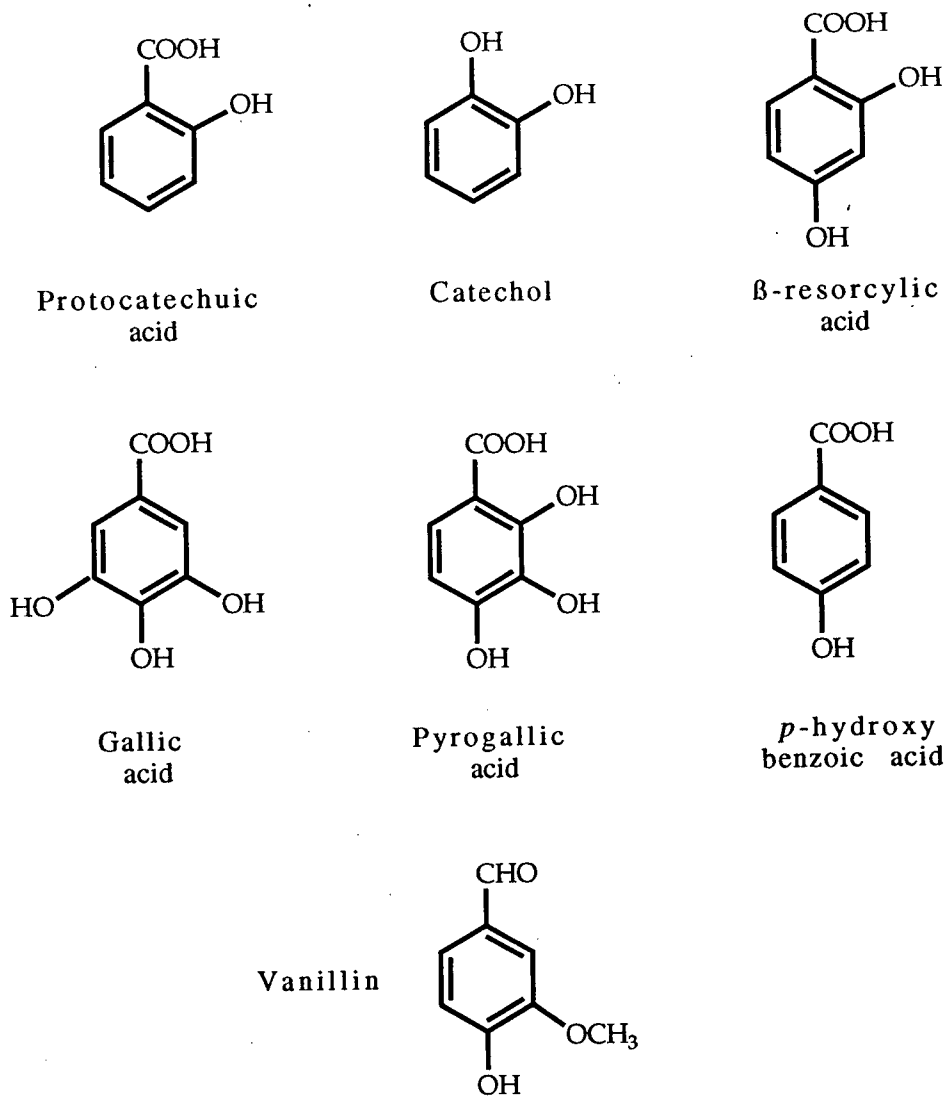
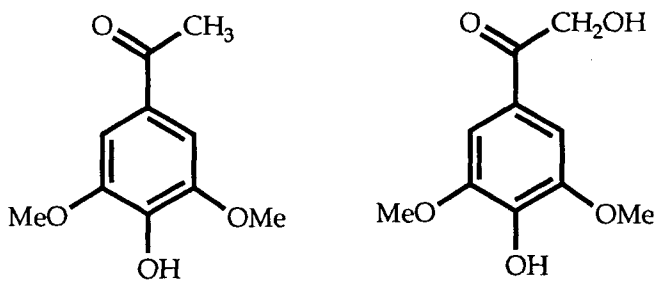


Figure 1.3. Structures of the seven phenolic compounds examined for *vir*-induction by Bolton *et al.* (1986).

component of the mixture was not reported. Stachel *et al.* (1985b) identified two active signal compounds, acetosyringone (AS) and α-hyroxyacetosyringone (HO-AS) (Figure 1.4), from a transformed tobacco root culture and from leaf discs. In that report a few other related compounds were assayed at one or more concentrations for their *vir*-inducing activity. This comprised a brief structure-activity study which yielded some information about the structural features required to confer activity. At the concentrations tested, none of the compounds examined by Stachel *et al.* (1985b) were as active as AS. Of the mixture of seven phenolics examined by Bolton *et al.* (1986) only vanillin (4-hydroxy-3-methoxybenzaldehyde) possesses a methoxy group adjacent to the phenolic hydroxyl, and indeed, when they were examined individually, only this compound was an effective *vir*-inducer (Spencer and Towers, 1988). The reported activity of a higher molecular weight and apparently proteinaceous inducer of the *vir*C locus (Okker *et al.*, 1984) remains at odds with the activity of the lower molecular weight phenolic compounds.

Stachel and Zambryski (1986b) have referred to the *A. tumefaciens*-plant cell interaction as "a novel adaptation of extracellular recognition and DNA conjugation". The presence of the plant cell wall and nuclear envelope present significant obstacles for the transfer of DNA. The exact mechanism by which T-DNA reaches the plant genome from the Ti plasmid remains unknown. It seems that aside from research into *vir* gene expression, a significant step in understanding crown gall tumorigenesis will be one of demonstrating the exact sites of bacterial cell attachment, involvement and production of pili, and transfer of the T-DNA package across the host cell wall and membrane.



AS HO-AS

Figure 1.4 The structures of acetosyringone (AS) and α-hydroxyacetosyringone (HO-AS). These acetophenone *vir*-inducers were isolated from transformed tobacco root culture conditioned medium by Stachel *et al.* (1985b).

In the Rhizobiaceae, chemotaxis towards aromatic and hydroaromatic compounds has been demonstrated (Parke *et al.*, 1985; 1987). It has been implicit in the reports concerning signal compounds for *A. tumefaciens*, that the bacteria are attracted to susceptible plant tissues by following a concentration gradient of the virulence inducing substances and some support for this idea was obtained by Ashby *et al.* (1987, 1988). Parke *et al.* (1987) have shown that AS is not a potent chemoattractant for *A. tumefaciens*, but that certain other phenolics (gallate, ß-resorcylate, protocatechuate, *p*-hydroxybenzoate, and vanillin (i.e. a number of the phenolics examined by Bolton *et al.*, 1986) induce chemotactic behaviour. They showed that chemotaxis towards *vir*-inducers is constitutively expressed in the absence of the Ti-plasmid. However, Ashby *et al.* (1988) subsequently demonstrated that chemotaxis in strain C58C1 is Ti plasmid-specified.

Another Ti plasmid gene is induced by plant phenolic compounds. The *tzs* locus, located within the nopaline-type Ti plasmid *vir* region, encodes a dimethylallyl transferase (an enzyme in cytokinin biosynthesis) whose expression is induced in a manner similar to that of the other *vir* loci (John and Amasino, 1988). Induced expression of the gene at this locus by AS was found to be pH-dependent in octopine strains and pH-independent in nopaline strains. Three possible roles (which need not be exclusive) were suggested for this plant-inducible cytokinin production. These are "(i) to condition plant cells to a state in which the transfer of DNA from the bacteria to the plant cell is optimal, (ii) to ensure that plant cells pass through stages of the cell cycle in which T-DNA integration can occur, or (iii) to stimulate high levels of postintegration T-DNA expression, thus leading to rapid tumor development".

Perhaps following detection of lignin precursors and other phenylpropanoid metabolites, the increased levels of cytokinins, resulting from the plant-induced expression of the *tzs* locus, induces cell divisions and such changes in the host cell wall as to expose binding sites to the bacteria. After binding, a bacterial cellulase may assist in creating a passageway for T-DNA transfer in the presumed conjugation-like process. Subsequent to passage of the T-DNA complex into the host cytoplasm, the DNA of a plant cell in the process of division would be more easily accessible to T-DNA integration.

Prior to my studies, the newly described compounds AS and HO-AS were generally regarded as unique chemicals which *A. tumefaciens* detects in nature and which trigger the initial events within the bacterium, resulting in tumor formation. Reports have appeared in the literature concerning the use of wound exudates from host plants or of AS to induce virulence of *A. tumefaciens* and thereby to extend the normal host range (Schäfer *et al .*, 1987) or to boost transformation efficiency (Sheikolleslam and Weeks, 1987; Mathews *et al .*, 1990). However, it has not been shown that AS is the signal molecule produced by any susceptible host other than *Nicotiana tabacum.*

Section 1.2.1, below, describes the analysis of structure-activity relationships in *vir* gene expression (Paul Spencer, M.Sc. Thesis; Spencer and Towers, 1988). The results of this work suggested other common plant phenolics may be involved in signalling *A. tumefaciens*, and serves as a point of reference in the search for novel *vir*-inducers in plant-derived exudates. Chapter 2 describes the isolation of a potent *vir*-inducer compound from grapevine cultivars, which are the hosts for limited host range ("LHR") *A. tumefaciens* strains. Chapter 3 describes new methods for, and the results of, a survey of naturally occurring wide host range ("WHR") signal molecules.

Data therein suggest that commonly occurring plant phenolics other than AS are important signal molecules, and indicate that the natural occurrence of acetophenone signal compounds may be restricted to the Solanaceae. In Chapter 4 an analysis of induced *vir* gene expression in the related species *A. rhizogenes* is presented. Chapter 5 describes the application of combined gas chromatography-mass spectrometry (GC-MS) to the analysis of *vir*-inducing phenolic mixtures.

1.2 Signal molecules inducing gene expression in the Rhizobiaceae

1.2.1 Structure-activity analysis of *A. tumefaciens vir* gene expression

This section describes the activity of a range of phenolic compounds which can induce *vir* gene expression in *A. tumefaciens*, and was reported in detail elsewhere (Paul Spencer, M.Sc. Thesis, Spencer and Towers, 1988). The results are summarized here to provide a point of reference in the search for novel *vir*-inducers in plant-derived exudates (Chapters 2 and 3). Analysis of structure-activity relationships of *vir* gene inducers indicated that a number of known phytochemicals are likely involved in inducing *vir* gene expression in *A. tumefaciens* (Spencer and Towers, 1988). As a part of my Masters Thesis, I reported the *vir*-inducing activity over a range of concentrations of a variety of plant-derived and synthetic phenolic compounds with structures related to that of AS, and presented new information regarding the structural features involved in the activation of *vir* genes. ß-galactosidase activity was assayed as a measure of *vir* gene induction in the *A. tumefaciens* strain A348/pSM358 (a C58 derivative harbouring pTiA6) which also carries the *vir*E::*lac*Z gene fusion plasmid pSM358 (Bolton *et al.*, 1986; Stachel *et al.*, 1985a and b). The activities of some cinnamic and benzoic acid derivatives, chalcones, and of the lignin precursors sinapyl alcohol and coniferyl alcohol were included. A number of these compounds were chosen for that study because they were known to be of considerably widespread occurrence, or because they are ubiquitous in plants, eg. monolignols.

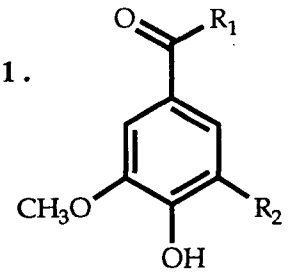
Of the mixture of seven phenolic compounds reported by Bolton *et al.* (1986), catechol, ß-resorcylic acid, and gallic acid exhibited very low, but detectable levels of induction; protocatechuic, pyrogallic, and *p*-

hydroxybenzoic acids did not induce *vir* expression significantly above background levels. Only vanillin greatly induced the expression of *vir* genes. Apparently, at least one ring methoxy substituent next to a para hydroxy group is required for activity. Consistent with this observation, Stachel *et al.* (1985b) reported negligible *vir*-induction in response to 50 μM *p*-hydroxyacetophenone. Further analysis was necessary in order to determine what other structural features (what other ring substitutions) conferred activity.

The structures of sixteen *vir*-inducing phenolic compounds were examined for the purpose of obtaining an understanding of the structure-activity relationships of *vir*-induction. These compounds may be assembled into four groups (Figure 1.5): **1.** acetophenones and benzoic acid derivatives, **2.** monolignols, **3.** cinnamic acid derivatives, and **4.** chalcone derivatives. Each structure contains a guaiacyl or syringyl nucleus, most possess a carbonyl group, and many are common plant-derived compounds of the phenylpropanoid pathway (Harborne, 1989). The *vir*-activation curves obtained for a number of these compounds are shown in Figures 1.6 and are discussed briefly below.

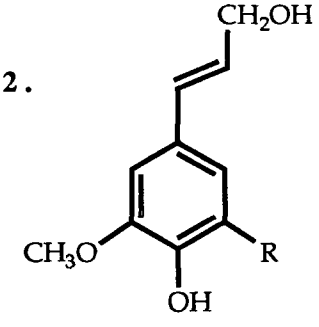
The lower activity of 4-hydroxy-3-methoxyacetophenone, or "acetovanillone" (of guaiacyl substitution) in comparison with that of acetosyringone (of syringyl substitution) indicates that for acetophenones a syringyl nucleus is more effective at *vir*-induction than is a guaiacyl nucleus. This supports the results of Stachel *et al.* (1985b) who assayed the relative activities of these two acetophenones at four concentrations using a *vir*B::*lac*Z strain of *A. tumefaciens*. They suggested that the acetyl substituent is important for activity because, in comparison, the carboxylic analogue of AS, syringic acid, was less active. I found that the methyl esters were roughly as

1.



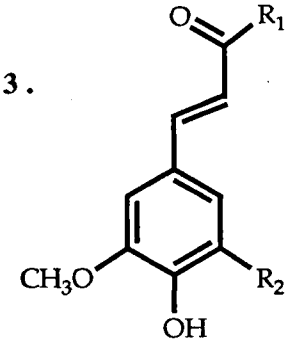
	R_1	R_2	
1a	H	H	vanillin
b	H	OMe	syringaldehyde
c	CH_3	H	acetovanillone
d	CH_3	OMe	acetosyringone
e	OH	OMe	syringic acid
f	OMe	OMe	Me-syringate

2.



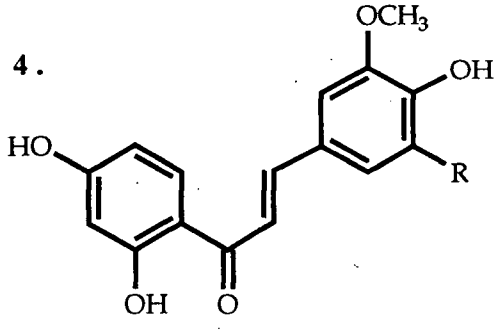
2a	R= H	Coniferyl alcohol
b	R= OMe	Sinapyl alcohol

3.



	R_1	R_2	
3 a	H	H	vanillalacetone
b	OH	H	ferulic acid
c	OH	H	sinapinic acid
d	OMe	H	Me-ferulate
e	OMe	OH	5-OH-Me-ferulate
f	OMe	OMe	Me-sinapinate

4.



Chalcones

4a	R= H	2',4',4-$(OH)_3$-3-OMe
b	R= OMe	2',4',4-$(OH)_3$-3,5-$(OMe)_2$

Figure 1.5 The 16 chemicals used in the study of structure-activity relationships, arranged into 4 classes.

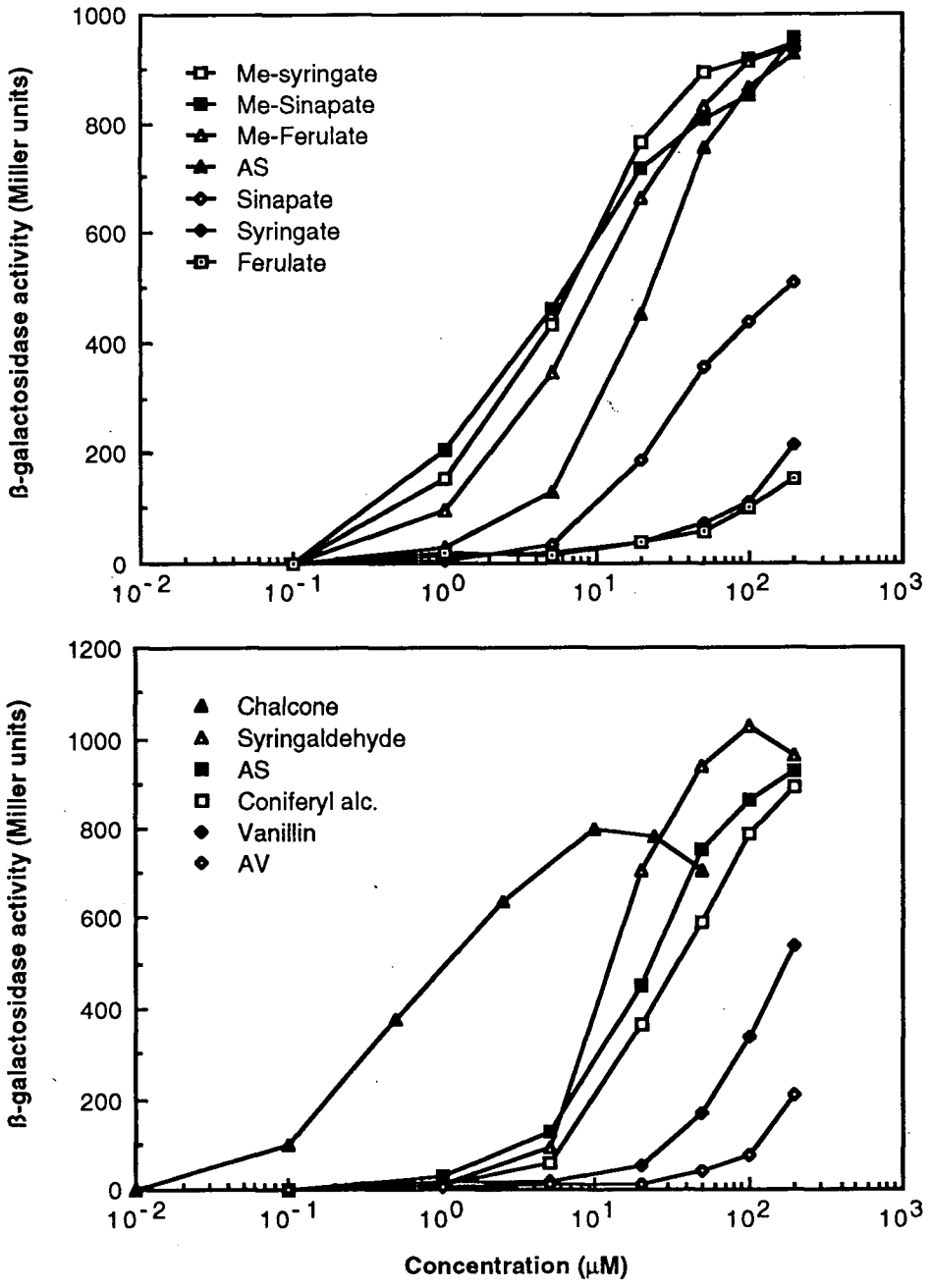


Figure 1.6 *vir*E expression in *A. tumefaciens* in response to phenolic compounds. Following 10 hrs. incubation with a compound in aqueous solution, ß-galactosidase activity was assayed in the strain *Agrobacterium* A348/pSM358, which carries a *vir*E::*lac*Z reporter gene fusion plasmid (data from Spencer and Towers, 1988).

active again as the acetophenones, suggesting that the acetyl group can be replaced by an ester group.

The curves of activity induced by the chalcones are somewhat different than those of any of the other compounds examined. 2',4',4-Trihydroxy-3-methoxy-chalcone displayed its greatest *vir* -inducing activity at 10 μM. The maximum levels of induction by all of the other compounds (except syringaldehyde) were obtained at the highest concentration tested, 200 μM. Unlike any of the other compounds, this chalcone was capable of low level *vir*-induction at 0.1 μM. The curve for 2',4',4-trihydroxy-3,5-dimethoxy-chalcone is shifted more to the right than the other chalcone, closer to that of acetosyringone, and its maximum activity is observed at 50 μM. Perhaps this is as a result of the syringyl substitution of its B-ring affording a structure more similar to that of acetosyringone.

Neither of these chalcones is very soluble in the buffer used, but they do exhibit significantly greater activity at lower concentrations than do any of the simpler phenolics tested. A number of chalcones are known to exhibit biological activity (Dhar, 1981) and it will be interesting to determine whether chalcones act as naturally occurring *vir*-inducers. Coincidentally, a new chalcone from alfalfa (4,4'-dihydroxy-2'-methoxychalcone) was recently found to induce nodulation (*nod*) gene expression in *Rhizobium meliloti* (Maxwell *et al.*, 1989; see section 1.2.2). Previously, this chalcone had been reported as a stress metabolite from *Pisum sativum* (Carlson and Dolphin, 1982). *nod*-inducers of *Rhizobium* species are discussed below, in section 1.2.2. At this point, I emphasize that Maxwell's report confirms that chalcones play a role in signalling between plants and microbes. Also, chalcone-mediated gene activation in these related genera is demonstrative of a close relationship in signalling systems between agrobacteria and rhizobia.

Other compounds tested, most of which possessed guaiacyl or syringyl substitution patterns but which exhibited little or no *vir*-inducing activity, include the following: phloridzin, chrysosplenol-6-C-glucoside, homoeridiodictyol, tricin, 3,5,7,4'-tetrahydroxy-3'-methoxy flavonol, plicatic acid, conidendron, substituted aurones, vanilloyl methyl ketone, 5-hydroxyvanillin, dihydrodiferulic acid, 5-hydroxyferulic acid, isoferulic acid, gluco-ferulaldehyde, and coniferin. Morris and Morris (1990) reported coniferin to be an active signal compound obtained from *Pseudotsuga menzesii* stem tissue. In this case, the *Agrobacterium* strain used also exhibited glucosidase activity and it is likely that in this case the bacteria generated the active aglycone coniferyl alcohol from the glucoside.

Apparently, 5-hydroxylation of any active compound possessing a guaiacyl nucleus decreases the compound's activity. Thus whereas ferulic acid is somewhat active, 5-hydroxyferulic acid is inactive. This decrease in activity appears to be true even of 5-hydroxy methyl ferulate, which retains activity similar to that of methyl ferulate, but reaches its maximum at a concentration of 50 μM. The inactivity of 5-hydroxyferulic acid is of interest because it occurs ester-bound in the cell walls of grasses (Ohashi *et al.*, 1987). Perhaps this is a factor in the resistance of certain monocots to infection by *A. tumefaciens*.

Repression of the expression of *vir* genes is another important subject requiring research, as it too may be an important factor in host range determination. Although inhibition of *vir* -induction by a phenolic compound was not demonstrated during the course of the structure-activity analysis, certain observations lead one to speculate about the possibility that phenolic *vir*-inhibitors exist which are common to all monocots. This would explain why the monocots are naturally resistant to infection by *A.*

tumefaciens, and also why preincubation of the bacteria with a known inducer (Schäfer *et al.*, 1987) can then allow transformation. In fact, it has now been established that inhibitory compounds are released from wounded *Zea mays* tissues (Sahi *et al.*, 1990). In this case, the well known hydroxamic acid DIMBOA and related compounds were found to inhibit growth and virulence of *A. tumefaciens*. Identification of DIMBOA and related compounds by HPLC is reported in Chapter 3.

The structures of the aglycones of gluco-ferulaldehyde and coniferin meet the putative requirements of an active signal compound (sumarized below) and yet the glycosides were found to be inactive. Although only two phenolic glycosides were tested, the results indicate that, during the exposure time of these assays, *Agrobacterium vir* genes are not induced by such compounds. If the vacuolar phenolics, which must be exuded upon wounding, are a source of *vir*-inducing phytochemical precursors, then it appears that plant glucosidases must act to yield the effective compound. Results presented in Chapter 3 suggest that glucosidases do indeed act on phenolic glucosides upon wounding.

Previously, I suggested that the role of glycosides such as coniferin [4-(3-hydroxy-1-propenyl)-2-methoxyphenyl-D-glucopyranoside] and isoconiferin [1-(4-hydroxy-3-methoxyphenyl)-propenyl-3-D-glucopyranoside] should be investigated (Paul Spencer, M.Sc. Thesis; Spencer and Towers, 1988). Coincidentally, coniferin is found in *Asparagus* (*Merck Index*, 10th edn. (1983) Merck Rahway), which appears to be one of the few monocots from which *Agrobacterium*-transformed tissue has been obtained by standard methods (Hernalsteens *et al.*, 1984). As noted above, for a certain *A. tumefaciens* strain, coniferin was recently reported to be the active compound

present in conifer extracts (Morris and Morris, 1990). Certain sugar esters of *vir*-inducing phenolics are also effective *vir*-inducers (see Chapter 3).

It is interesting to note that *Agrobacterium* species are known to degrade the lignin model compounds α-conidendron and veratrylglycerol-ß-coniferyl ether (Subba Rao *et al.*, 1971). In this study, *A. tumefaciens vir* genes were not activated by conidendron. However, this brings up an important point, namely, that *A. tumefaciens* may chemically alter the compounds which induce virulence. Tracer studies should be conducted in order to determine whether this is indeed the case, and the metabolites should be identified.

In addition to the inactive compounds listed above, most of the common phenolic compounds used by Bolton *et al.* (1986) were inactive. Only vanillin caused any significant *vir*-induction. Vanillin is not a lignin precursor, although it is a breakdown product from lignin (Freudenberg and Neish, 1968), as is syringaldehyde. This may indicate that both lignin precursors and lignin degradation products function in nature as *vir*-inducers, which in turn suggests that *A. tumefaciens* may be generally attuned to lignin metabolites. Interestingly, in comparison with the response induced by vanillin, the remaining compounds (gallic, ß-resorcylic, pyrogallic, *p*-hydroxybenzoic, and protocatechuic acids, and catechol) were essentially inactive. None of these inactive compounds possesses a guaiacyl or syringyl nucleus, which explains their lack of activity. It remains to be determined in what manner each of these compounds effects the activity of vanillin.

In summary, two basic structural features together are required to confer activity upon a compound. With the exception of the monolignols and the chalcones, these features are: 1) guaiacyl or (conferring enhanced

activity) syringyl substitution on a benzene ring, and with the exception of the monolignols, 2) a carbonyl group on a substituent para to the hydroxy substituent on the ring. There are restrictions on the nature of the carbonyl carbon. It may be one or three carbon atoms removed from the ring. However, to confer maximal activity, in the latter case there must be a double bond between the carbonyl carbon and the ring, as is present in the chalcones and cinnamic acid derivatives.

Furthermore, the carbonyl group of a free acid is less effective than that of the corresponding ester. The methyl esters of each of ferulic, syringic, and sinapic acids exhibited significantly greater activity than the corresponding free acids. Coincidentally, one of these esters, methylsyringate, was isolated from grapevine cultivars (described in Chapter 2). Perhaps the activity of esters of phenolic acids could be used to explain the early result (Okker *et al.*, 1984) which indicated the involvement of a proteinaceous inducer. The inducer obtained may have been a protein ester of some phenolic acid. Esterification alters the solubility of the compound. In addition, esterification prevents one oxygen of the carboxyl group from forming a partial double bond, thereby rendering the carbonyl group more reactive. In these cases, and the case of the aldehydes and chalcones, this carbonyl group forms the terminus of a conjugated double bond system running from the hydroxyl group and through the ring. Lastly, flavonoids other than the chalcones were inactive.

Cell-cell signalling must occur naturally in media of considerable chemical complexity, with both inducers and inhibitors present. Therefore, successful induction *vir* genes would require the correct balance between inducer and inhibitor molecules. These factors may be used to explain the artificial extension of the host range of *A. tumefaciens* (Schäfer *et al.*, 1987), as

well as the increased transformation efficiency obtained by pre-induction of *A. tumefaciens* with inducing exudates or AS (Sheikolleslam and Weeks, 1987).

A number of *vir*-inducing phenolic compounds are of widespread occurrence amongst dicotyledonous plants. The lignin precursors coniferyl and sinapyl alcohol must be ubiquitous amongst susceptible hosts. It would therefore be possible to conclude that, if present in the correct concentration, the presence of one or another of these compounds alone would determine whether a given plant is susceptible to infection by *Agrobacterium*. However, it is well known that monocots also produce phenolic compounds, even exuding phenolic acids such as syringic, sinapic and ferulic acid into the rhizosphere from intact roots (Tang and Young, 1982), and yet, with few exceptions (Hernalsteens *et al.*, 1984), monocots lie outside of the natural host range of any strain of *Agrobacterium*. Concentration effects, as well as the action of inhibitors of *vir*-induction, may be factors in the resistance of monocots to crown gall tumorigenesis.

Restrictions in host range remain a significant problem in the use of this organism as a vector for genetic engineering in plants. Factors other than inducer/inhibitor phytochemistry may well be involved in the immunity of monocots to transformation by *Agrobacterium*. Using the results of the structure-activity analysis as the foundation for subsequent research, this thesis reports the isolation and identification of phenolics affecting virulence from both monocotyledons and dicotyledons. Some methods and results presented in this thesis may facilitate research concerning factors determining host range and susceptbility to crown gall disease.

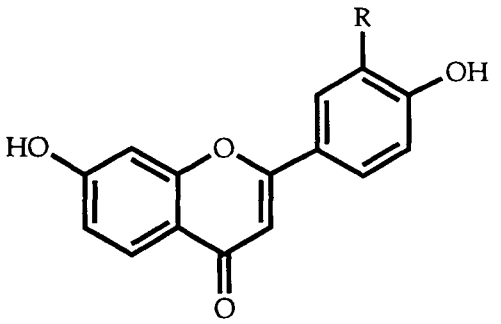
Important and unanswered questions concerning *vir*-inducers of *A. tumefaciens* are: (1) which phenolic compounds are naturally occurring

signal molecules, and (2) what are their immediate precursors in the unwounded plant? In section 1.3 a brief consideration of the possible sources of signal compounds from wounded plant cells is presented. Some answers to the question of biosynthetic precursors of *Agrobacterium* signal compounds are described in Chapter 3.

1.2.2 Flavonoid *nod* gene inducers of *Rhizobium* species

Comparisons may be made between the chemistry of *Agrobacterium* virulence-induction with that of nodulation (*nod*) gene induction in the closely related genus *Rhizobium*. The *nod* genes control early events in the formation of nitrogen fixing nodules on the roots of leguminous plants. Expression of the *nod* genes by *Rhizobium* species is essential for the successful nodulation of host plant species, and this gene expression is induced by host plant-derived flavonoids. The activity of the more typical flavonoids represents a significant difference in the range of structures inducing gene expression in the two genera. The structure-activity relationships reported in this thesis are clearly different from those reported for the activation of *nod* genes in *Rhizobium* species (Peters and Long, 1987; Zaat *et al.*, 1989). Hydroxylated flavones, isoflavones, dihydroflavonols, flavanones, and even a chalcone, in nM to μM concentrations induce expression of *nod* genes (Peters *et al.*, 1986; Redmond *et al.*, 1986; Firmin *et al.*, 1986; Sadowsky *et al.*, 1988; Maxwell, 1989; Zaat *et al.*, 1989).

The compounds involved in *nod* gene induction include the well known flavonoids luteolin, apigenin, inteolin, naringenin, eriodictyol, geraldone, and daidzein. The structures of a number of the active flavonoids which have been identified from host plants are shown in Figure 1.7. Firmin

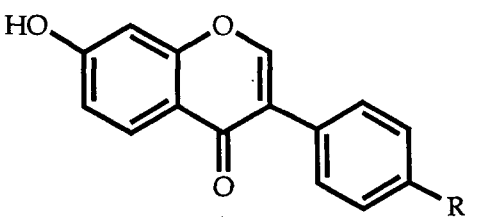


Rhizobium trifolii

Flavones

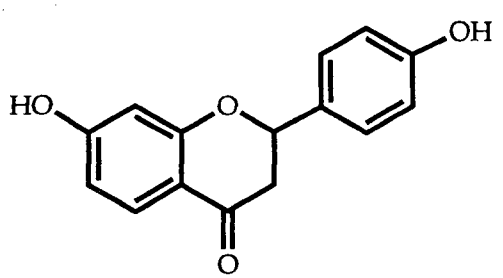
a. R=H, 7,4'-dihydroxyflavone (DHF)

b. R=OMe, 7,4'-dihydroxy-3'-methoxyflavone (Geraldone)



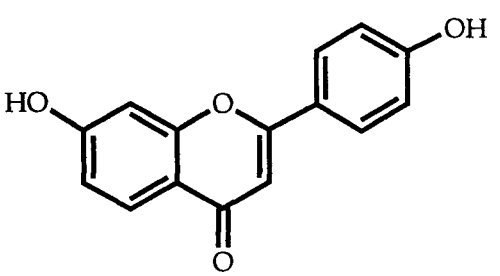
Rhizobium fredii

Isoflavone (Daidzein)
4',7-dihydroxyisoflavone

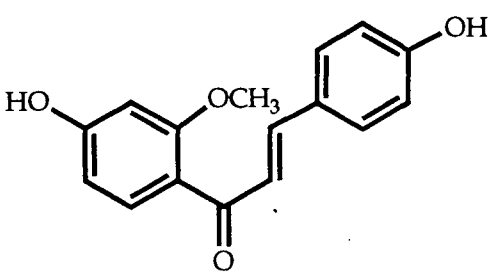


Rhizobium meliloti

Flavanone (weak inducer)
4',7-dihydroxyflavanone



Flavone (moderate inducer)
4',7-dihydroxyflavone



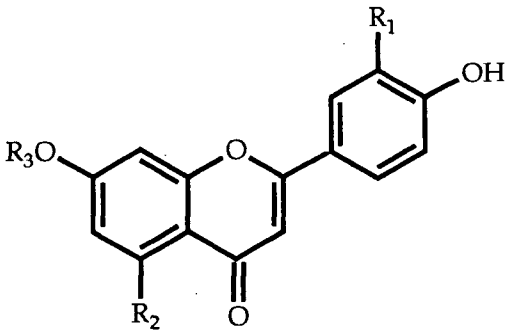
Chalcone (strong inducer)
4,4'-dihydroxy-2'-methoxychalcone

Figure 1.7 The structures *nod*-inducing flavonoids recently identified from host plants.

et al. (1986) did not isolate *nod*-inducing flavonoids from host exudates, but several commercially available flavonoids were examined (Figure 1.8) in order to outline the range of compounds which could induce *nod* gene expression in *R. leguminosarum*. Zaat *et al.* (1989) characterized 7 inducers for the *nod*A promoter of *R. leguminosarum* biovar *viciae* from the root exudate of *Vicia sativa*, and examined structure-activity correlations through the application of 34 authentic standard flavonoids.

Most *Rhizobium* species are relatively specific for their respective host plant species, and each *Rhizobium* species appears to exhibit a relatively high degree of specificity towards flavonoids from its host. Also, the compound which most strongly induces one species of *Rhizobium* can act as a potent inhibitor of *nod*-induction in another species of *Rhizobium*. By contrast, the original strain of *A. tumefaciens* from which the strain used in this study was derived exhibits a wide host range (WHR), and a comparatively much lower degree of signal compound specificity.

Firmin *et al.* (1986) also described a novel phenomenon: some of the very compounds which induce *vir* genes in *A. tumefaciens*, including AS, strongly inhibit *nod* gene activation by these flavonoids. At higher concentrations most of the *vir*-inducing phenolics are bacteriostatic even against *Agrobacterium* (data not shown), and presumably they act in this way against *Rhizobium* species, or they may act more directly by competitive inhibition of *nod*-induction.



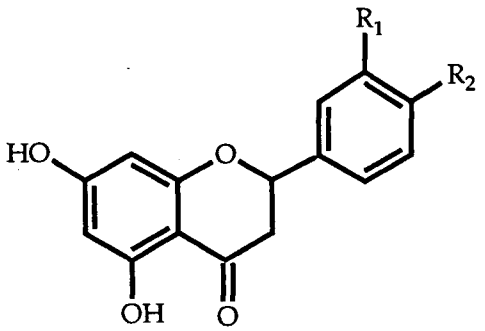
Flavones

a.R_1=H, R_2=OH, R_3=H (Apigenin)

b.R_1=OH, R_2=OH, R_3=H (Inteolin)

c.R_1=OH, R_2=H, R_3=H

d..R_1=H, R_2=OH, R_3=glc (Apigenin-7-O-glucoside)



Flavanones

a. R_1=H, R_2=OH (Naringenin)

b. R_1=OH, R_2=OMe (Hesperitin)

c. R_1=OH, R_2=OH (Eriodictyol)

Figure 1.8 Authentic flavonoids used in the analysis of *R. leguminosarum nod*-induction by Firmin *et al.* (1986).

1.3 Phenolic biochemistry of wounded plant tissues in relation to *vir* gene expression

In the course of their lives, many plants experience and overcome naturally inflicted wounds. They are surprisingly resilient, capable of healing reactions that are often successful processes. Crown gall disease requires of both pathogen and host a coordinated series of biochemical events, including wound-healing reactions by the host. The bacterial pathogen, *Agrobacteium tumefaciens*, causes tumors following infection at wound sites on a wide variety of host plants. This process is dependent on the bacteria sensing susceptible host cells by responding to the presence of signal compounds exuded specifically by wounded cells. Such compounds are also produced by suspension culture cells, presumably because the culture conditions mimic the wounded state. Signal compounds are rapidly elaborated by newly wounded tissues, but the source(s) of these interesting compounds has not been established. This section reviews information regarding wound biochemistry as it may relate to infection by *A. tumefaciens*.

The recent discoveries concerning signalling in plant-microbe interactions have opened up new areas of research in natural products chemistry. Important roles are being established for some classes of plant secondary metabolites, the functions of which, until now, were unknown. As described above, the virulence (*vir*) genes of this organism are switched on when it encounters plant phenolic compounds of guaiacyl or syringyl ring substitution (Stachel *et al.*, 1985; Spencer and Towers, 1988; Spencer *et al.*, 1990).

The known *Agrobacterium* signal compounds include the acetophenones AS and HO-AS (Stachel *et al.*, 1985), and the methyl ester of

syringic acid (Chapter 2, Spencer *et al.*, 1990). However, the range of effective phenolics may not be limited to C_6-C_2 and C_6-C_1 compounds. A number of cinnamic acid derivatives (phenylpropanoids), i.e. C_6-C_3 compounds, are also active (Spencer and Towers, 1988). The lignin precursors coniferyl and sinapyl alcohol, ferulic and sinapic acids and some of their esters are active *in vitro*, and may be naturally occurring signal compounds (described in Chapter 3). Until the completion of this thesis, however, these C_6-C_3 compounds had not been isolated from wounded plants on the basis of their biological activity.

Whereas a number of commonly occurring, potential signal compounds are known, only three previously unknown compounds mentioned above have been identified from wounded plant tissues. A growing number of research papers are appearing in the literature concerning the effects of these plant phenolics on *A. tumefaciens*. However, few of these reports have considered the biosynthetic source(s) of the inducers of virulence. It has been suggested by some authors that the acetophenones encountered by *A. tumefaciens* are intermediates in the biosynthesis of lignin (eg. Stachel *et al.*, 1985b). This seems to be an unrealistic view when one considers that only C_6-C_3 compounds have been identified as monomeric precursors or components of the lignin polymers. Certain cell wall bound phenolics may be liberated upon wounding or in connection with cell wall repair. These and phenolic glucosides appear to be more likely sources of signal compounds. In this section I shall review information available on the natural occurrence and possible routes for the production of signal compounds.

Extracts from fresh, nonwounded plant tissues apparently contain little or no *vir*-inducing substances (Stachel *et al.*, 1985). It was also shown that

cycloheximide treated wounded tissues also failed to produce the *vir*-inducers. This suggests that wound-induced protein synthesis and subsequent enzymatic activity are required for biosynthesis of these phenolics. The effects of cycloheximide on the biosynthesis of signal molecules are mentioned in sections 1.3.5 and 1.3.6, and some experimental results are described in Chapter 3.

Since it can be assumed that the plant does not synthesize such compounds solely for the purpose of signalling a pathogen, other reasons for their existence (*in planta* roles) must be found. So far, none has been established. Is it possible that these plant secondary compounds solely represent "metabolic chatter", which in *Agrobacterium* plays such an important role? Knowledge of the source of these compounds might aid our understanding of the raison d'etre of signal molecules. The sources of these compounds might include *de novo* biosynthesis via the phenylpropanoid pathway, synthesis from vacuolar precursors (eg. glycosides), liberation of cell wall-bound phenolics, including those of lignin or suberin (or precursors in the wound-induced biosynthesis thereof).

Wound-healing reactions have been the subject of microanatomical and biochemical analyses. Various biochemical events occur rapidly upon tissue damage. Cell contents are mixed, react with one another, and are exuded from the wound site. In addition, marked changes are induced in the secondary metabolism of neighboring cells. Infection by microorganisms can stimulate the production of plant defence compounds called phytoalexins, but this is not considered at length in this thesis. Indeed, this may occur when plants are inoculated with *Agrobacterium*. Perhaps phytoalexins are involved in the observed hypersensitive response of certain grapevine cultivars to incompatible *Agrobacterium* strains (Yanofsky *et al.*, 1985).

During the first week following wounding, some cell divisions occur and a layer of plant cells differentiates into wound periderm (Juniper and Jeffree, 1983). Lignification and suberization of these cells serves to wall off the injured area, preventing drying and infection. However, this is likely beyond the window of opportunity for infection by *Agrobacterium*. A number of subjects relating to the origin and nature of signal compounds are considered below.

1.3.1 Phytoalexins

An early definition of phytoalexins states that: "These are compounds (nonspecific toxins or antibiotics) which can inhibit the development of the pathogen but are only formed or activated when the latter comes into contact with the host cells" (Swain, 1977). A more contemporary definition of phytoalexins includes those compounds synthesized in response to infection or challenge of plant tissues with some pathogen or elicitor, for example a fungal cell wall fraction (reviewed by Friend, 1985). In the generally accepted sense of the term, therefore, the definition of phytoalexins is not sufficiently broad to include plant metabolites which act as *A. tumefaciens* signal compounds. Phytoalexins may be present at low levels in uninfected tissues, but they can accumulate to very high levels following elicitation. I found this to be true of acetophenones in the Solanaceae (described in Chapter 3). It is quite possible that host plants, or more likely non-host plants, when inoculated with *A. tumefaciens,* react by producing antibacterial compounds (anti-*Agrobacterium* phytoalexins). Perhaps this is a factor in *A. tumefaciens* host range phenomena, wherein a successful relationship is established only in those cases in which the bacteria do not elicit a response by the plant. To my knowledge this subject has not been investigated. Such research could be

important in revealing why the monocots, in general, are resistant to infection by *A. tumefaciens*, or why grapevine cultivars are susceptible mainly to LHR strains.

It is well established that signal compounds are synthesized solely in response to wounding of plant tissues, and that this does not require elicitation by any additional factors. I note that phytoalexins now include stress-related compounds, for example, compounds produced in response to wounding (eg. Basha *et al.*, 1990). A recent report (Threlfall and Whitehead, 1988), which focussed mainly on sesquiterpenoid phytoalexin metabolism in tobacco cultures, has complicated this issue. These workers studied the elicitation of antifungal sesquiterpenes in tobacco suspension cultures. Purified cellulase not only stimulated sequiterpenoid biosynthesis in these cultures, but apparently also the accumulation of acetosyringone. The cellulase used was found not to possess any β-glucosidase activity, so the acetosyringone was not simply a hydrolysis product of the corresponding glucoside.

Threlfall and Whitehead (1988) found that AS did not exhibit antifungal activity against their test organism, so the production of AS does not appear to represent a chemical defence against fungal infection. Also, in comparison with catechol, gallic, pyrogallic, or protocatechuic acids, AS is only weakly bacteriostatic even at relatively high concentrations (ca. 1 mM)(Paul Spencer, M.Sc. Thesis). In fact, to date no strong antimicrobial activity has been reported for AS, and one must conclude that AS is not a phytoalexin. Threlfall and Whiteheads' results are discussed further under cell wall-bound phenolics (section 1.3.2).

1.3.2 Cell wall-bound phenolics

Ester and ether-linkages of *p*-coumaric, ferulic, and dimeric acids to the cellulose or hemicellulose component of cell wall polysaccharides is of fairly widespread occurrence (El-Basyouni and Towers, 1964; Harris and Hartley, 1976; Hartley and Ford, 1989). These studies have examined graminaceous cell wall phenolics, but such linkages likely occur in the cell wall of dicotyledons as well.

Swain (1977) suggested that the presence of these compounds could be part of a primitive defence mechanism against attack by pathogens. These esterified phenolics also could act as anchors, or starting points for the synthesis of lignin or suberin, a means of crosslinking (Figure 1.9) between nonaromatic cell wall material (Markwalder and Neukom, 1976; Iyama *et al.*, 1990), or may limit binding of pathogens to cell walls (Hartley and Ford, 1989). This last idea is probably not a factor in the resistance of monocots to transformation by *A. tumefaciens*, because a number of studies have proven that binding of *A. tumefaciens* to various monocot cells occurs in a manner similar to that observed with dicot cells (Douglas *et al.*, 1985; Graves *et al.*, 1988). At least in certain cases, *p*-coumaric and ferulic acids are liberated from the cell walls during decay (Karunen and Kalviainen, 1988).

Plate assays with cell wall feruloyl sugar esters (arabinose, xylose and glucose esters) indicate that these compounds are effective *vir* -inducers (see Chapter 3). Cellulase activity is known to cause the release of water soluble O-[5-0-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1-3)-O-ß-D-xylopyranosyl-(1-4)-D-xylanopyranose, or FAXX (Figure 1.10) (Hartley and Ford, 1989). It will be interesting to determine whether under any natural circumstances such cell wall fragments such as FAXX influence virulence of *A. tumefaciens* .

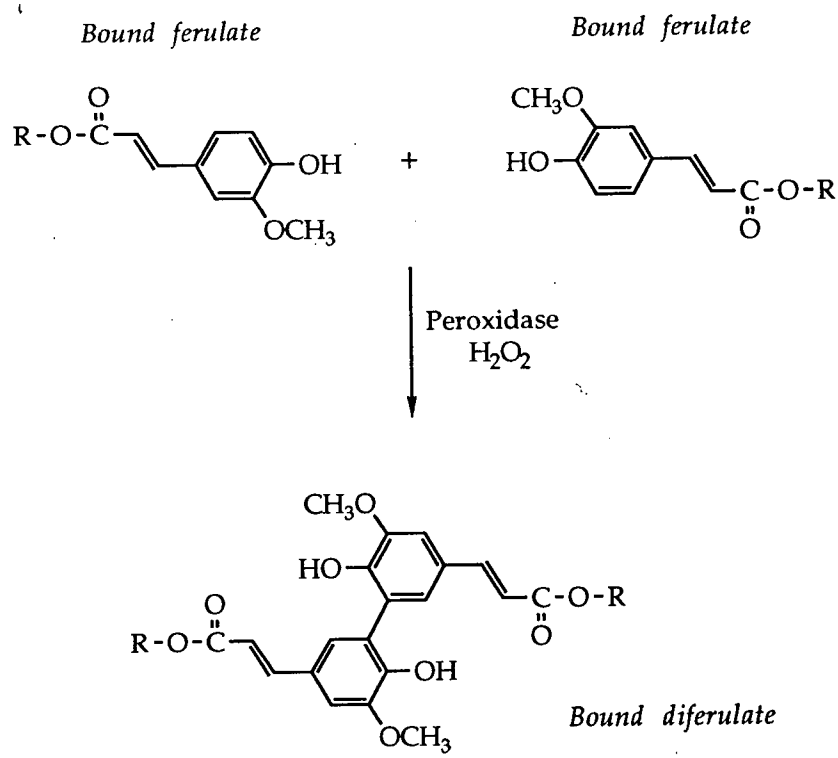


Figure 1.9 Formation of bound diferulate in cell walls. R=cell wall polymer.

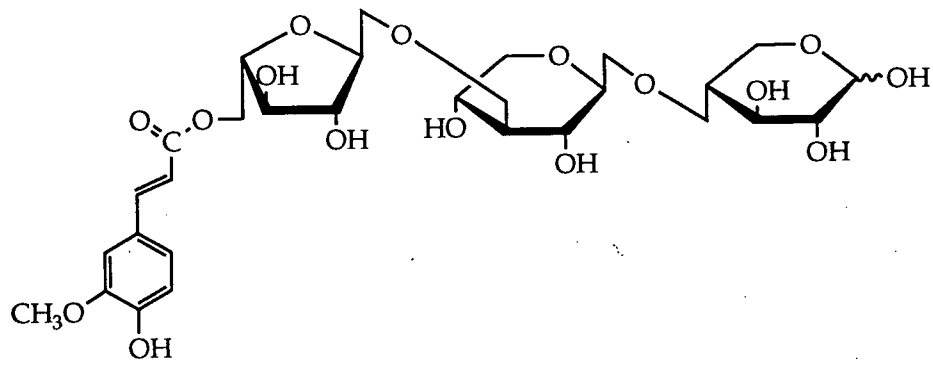


Figure 1.10 Chemical structure of FAXX.

As noted above, Threlfall and Whitehead (1988) reported that acetosyringone is produced in tobacco cell cultures following treatment with cellulase. It is not kown whether the compound is liberated from the cell wall or whether it is newly synthesized. The cellulase treatment may mimic the effects of wounding, and stimulate *de novo* biosynthesis of AS, not simply its release from cell walls. However there are data to suggest that compounds other than phenylpropanoids are important constituents of cell walls. Hartley and Ford (1989) noted the relationship between phenolic constituents of cell walls liberated by treatment with alkali (eg. 0.1 M NaOH, 20 °C) and biodegradability with cellulase. *p*-Hydroxybenzaldehyde, vanillin, syringaldehyde, *p*-coumaric acid, ferulic acid and dehydrodiferulic acid are released from graminaceous cell walls by mild alkali or cellulase treatment. The aldehydes are released as water soluble carbohydrate-aromatic compounds from which the aromatics are released by further alkali treatment. This suggests that the aldehydes are ether linked to the cell wall polysaccharides.

The ability of cellulase to release *vir*-inducers from cell wall material has not been established. It is known that treatment of certain plant tissues with cellulase prior to inoculation with *Agrobacterium* can permit transfomation of species that otherwise resist infection. In Chapter 3, the effect of cellulase on the production of *vir*-activating compounds is described.

1.3.3 Lignification

Unfortunately, many of the studies related to the production of lignin are based on plant responses to infection and not simply to wounding. However, phenylpropanoid biosynthesis likely related to lignification has been studied in potato tuber slices. An important enzyme, cinnamic acid 4-

hydroxylase is strongly stimulated in aging disks of potato, and roots and tubers of other species (Rhodes and Wooltorton, 1978). Increased peroxidase activities in sweet potato slices may be related to lignin biosynthesis since these enzymes are probably involved in oxidative polymerization of phenylpropanoid units (the monolignols) leading to lignin.

Spencer and Towers (1988) have shown the lignin precursors coniferyl alcohol and sinapyl alcohol to be relatively strong *vir*-inducers, but so far these compounds have not been detected in exudates from wounded plant material as *vir*- inducing chemicals (see, however, Chapter 3- *Taxus baccata*). The process of lignification is thought to require the phenylpropanoid alcohols (Freudenberg and Neish, 1968). These alcohols must be derived from the corresponding phenylpropanoid acids. In a detailed review of phenylpropanopid metabolism in cell walls, Yamamoto *et al.* (1989) discussed the biosynthesis of lignin and the turnover of monolignols (and their glucosides). Monolignols seldom accumulate in woody tissue, but are incorporated completely into the lignin framework. In angiosperms the accumulation of E-monolignol glucosides appear to be mainly restricted to the Magnoliaceae and Oleaceae families. In the bark of the American beech (*Fagus grandifolia*) only the Z-monolignols and their glucosides are present (Lewis *et al.*, 1988). It is thought that the E-monolignols may be used exclusively for lignin formation. Cell wall ß-glucosidases are thought to act on the monolignol glucosides to yield the aglycones. The efficient turnover of the aglycones, perhaps involving enzyme-bound intermediates, may preclude their presence in wound exudates and detection by *A. tumefaciens*. Spencer and Towers (1988) reported that the monolignol glucosides were inactive, but suggested that they were a logical precursor of coniferyl alcohol following wounding and subsequent hydrolysis. The monolignols were

included in the structure-activity study because they possess a propenol side chain, and this permitted comparison with the activity of the propenoic acid side chain of the corresponding position 3, 4, and 5-substituted rings. However, they were chosen primarily because as lignin precursors they would be common to all susceptible hosts.

Lignin composition has been studied by analysis of degradation products (reviewed by Gross, 1979). Nitrobenzene oxidation yields *p*-hydroxybenzaldehyde, vanillin and syringaldehyde, the latter two of which happen to be potent *vir*-inducers. Quantities of syringyl residues in herbaceous dicotyledon lignins ranges from 10-65%. Lignins of the Poaceae are characterized by a higher proportion of *p*-coumaryl residues. Perhaps this is a factor in the resistance of monocots to crown gall disease. Also, Gross also pointed out that lignin composition varies greatly between different tissues. Perhaps the crown region of plants are preferentially infected by *A. tumefaciens* partly on the basis of lignin composition. I have assayed the effect of *p*-coumaric acid and found that it had little suppressive effect on *vir*-induction by acetosyringone.

It is conceivable that signal compounds could be derived from lignin, following wounding, by enzymatic activity liberating C_6-C_2 or C_6-C_1 moieties. To my knowledge, no studies have focussed on the liberation, through natural means, of such low molecular weight units from lignin. In Chapter 3, an experiment is described in which the effect of cellulase on the production of acetosyringone from non-living, solvent extracted, tobacco cell wall material was examined.

1.3.4 Suberization

Wound-induced suberin is a complex, polymeric substance produced by wounded tissues to form a barrier to infection by disease organisms (Figure 1.11). Over the last two decades, Kolattukudy has contributed greatly to our knowledge of the composition of suberin. The aliphatic components of thesuberin structure have been particularly well characterized. Unfortunately, the phenolic components are less well known than are the aliphatic components. *p*-coumaroyl and feruloyl residues are thought to be the main phenolic residues to be found in suberin. Although pure suberin is impossible to prepare, suberin-enriched extracts can be prepared through treatment with cellulases and pectinases. Modern ^{13}C-NMR techniques have permitted analysis of suberin without extensive sample purification (Garbow *et al.*, 1989). This NMR analysis verified that suberin has phenylpropanoid groups characteristic of lignin.

Depolymerization of suberin involves cleavage of ester bonds. This is accomplished by hydrogenolysis with LiAlH , hydrolysis with alcoholic alkali, or transesterification with MeOH and BF_3 catalyst or NaOMe. Transesterification gives rise to four classes of monomers: fatty acid methyl esters, dicarboxylic acid methyl esters, fatty alcohols, and ω-hydroxy acid methyl esters. Combined GC-MS has been used extensively to characterize these components.

The phenolic fraction derived from suberin can also be examined by GC-MS. Hydrogenolysates of suberin samples from potato, sweet potato, turnip, rutabaga, carrot, and red beet gave dihydroconiferyl alcohol. Riley and Kolattukudy (1975) found ferulic and *p*-coumaric acids covalently attached to suberin. Apparently no systematic investigation of the phenolic components of suberin has been reported.

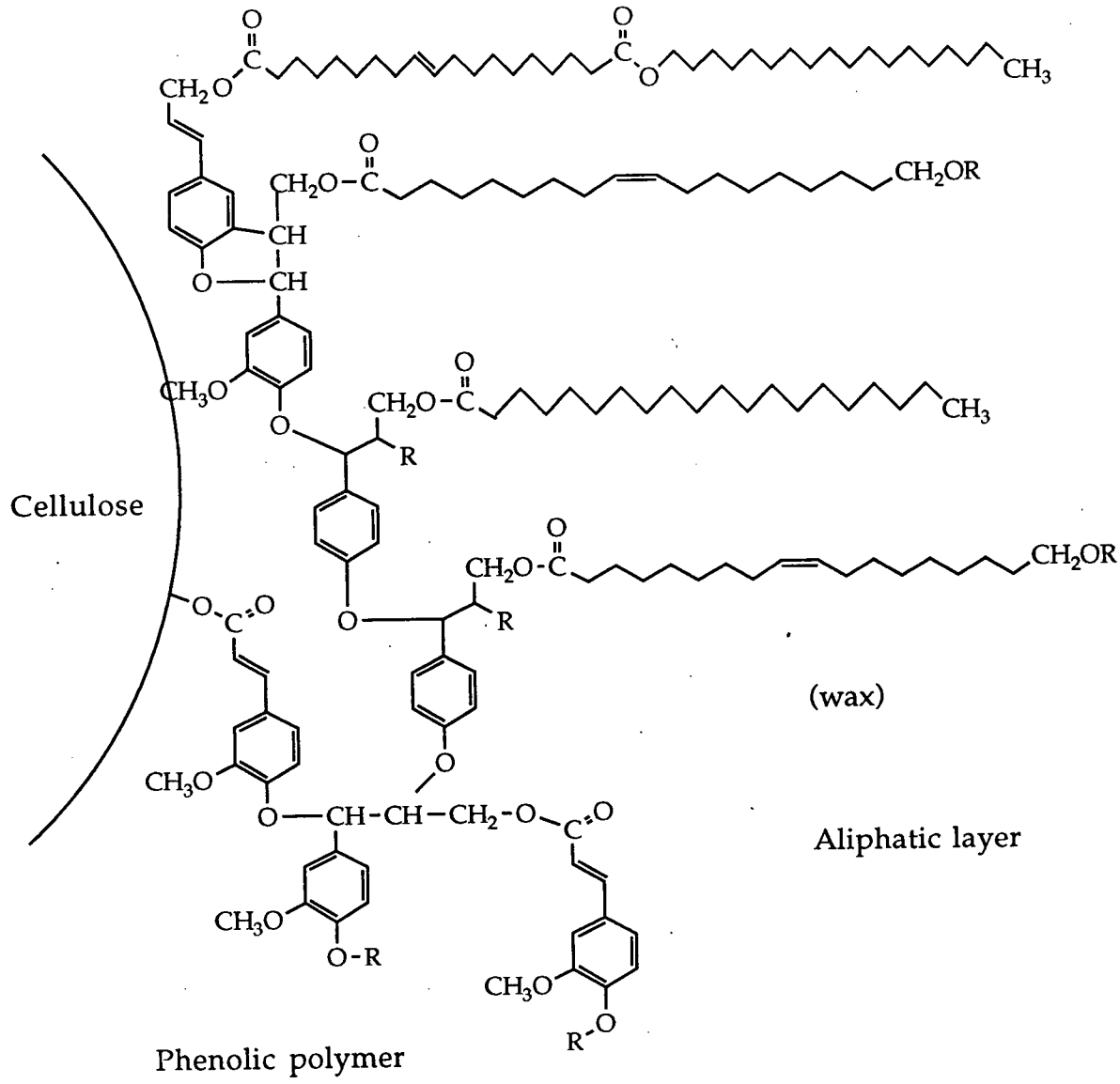


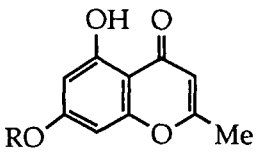
Figure 1.11 Generalized structure of suberin (after Kolattukudy, 1984).

All the experimental evidence available on the biosynthesis of the phenolic components strongly suggests that during wound healing the phenolic components are formed prior to the formation of the aliphatic components. It is noteworthy that, just as washing of explants can inhibit tumor formation by *A. tumefaciens*, thorough washing of potato discs immediately after preparation and up to three days following preparation severely inhibits suberization.

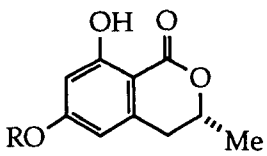
In addition to the presence of various phenolics (Chapter 3), GC-MS analysis of virulence inducing mixtures from wounded plant tissue revealed the presence of a number of fatty acids (see Chapter 4). Perhaps these acids are indicators of the process of suberization in the wounded plant tissue.

1.3.5 *De novo* biosynthesis of phenolic compounds

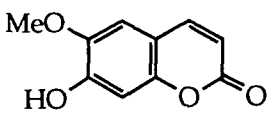
The synthesis of new phenylpropanoid units upon wounding and the induction of some of the required enzymes has been studied (reviewed by Rhodes and Wooltorton, 1978; Rhodes, 1985). Many of these studies have focussed on the production of chlorogenic acid or scopoletin in aging potato tuber discs. In carrot tissues, in addition to stimulation of chlorogenic acid and scopoletin biosynthesis, wounding results in the production of isocoumarins and chromones (Figure 1.12). 6-Hydroxymellein, 6-methoxymellein, 5-hydroxy-7-methoxy-2-methylchromone and 5,7-dihydroxy-2-methylchromone are produced following wounding or treatment with ethylene (Coxon *et al.*, 1973). From their structures one can surmise that these isocoumarins and chromones are likely not effective signal compounds, but they may effect growth of *A. tumefaciens*. Interest in these or related stress compounds stems from their toxic properties.



R=H, 5,7-dihydroxy-2-methylchromone
R=OMe, 5-hydroxy-7-methoxy-2methylchromone



R=H, 6-hydroxymellein
R=Me, 6-methoxymellein



Scopoletin

Figure 1.12 A coumarin, isocoumarins and chromones produced by carrot tuber tissue in response to wounding.

Typically, it seems that compounds produced in the greatest abundance, or those possessing striking biological activity are the first to be identified.

Acetophenones and hydroxybenzoic acids may be synthesized from phenylpropanoid precursors as indicated in Figure 1.13. Neish (1964) briefly discussed the likely biosynthetic routes to the acetophenones picein, pungenin, and androsin. A simple decarboxylation of a phenyl substituted ß-keto acid appears to be involved. C_6-C_1 aldehydes may be produced by reduction of the corresponding benzoic acids, or they may be synthesized before the acids, as has been shown in certain fungi (Wat and Towers, 1979). Very recently, Funk and Brodelius (1990) pointed out that the biosynthetic pathway to vanillin and other C_6-C_1 compounds in plants has not yet been established. Vanillin may be formed from feruloyl-CoA through a ß-oxidation-like process. *A. tumefaciens* is sensitive to most of the structures in the pathway following the production of ferulic acid from 3,4-dihydroxycinnamate.

The application of the metabolic inhibitor cycloheximide has shed some light on the biosynthesis of AS in wounded tobacco (Stachel *et al.*, 1985b). It was demonstrated that metabolically active plant cells (apparently directly adjacent to the wound site) were required for the production of large amounts of AS. On the basis of this result it seems certain that a detectable level of *de novo* synthesis occurs. In Chapter 3, GC-MS analyses reveal that medium extracts from cycloheximide-treated *Nicotiana* leaf sections were depressed in, but not devoid of acetophenones.

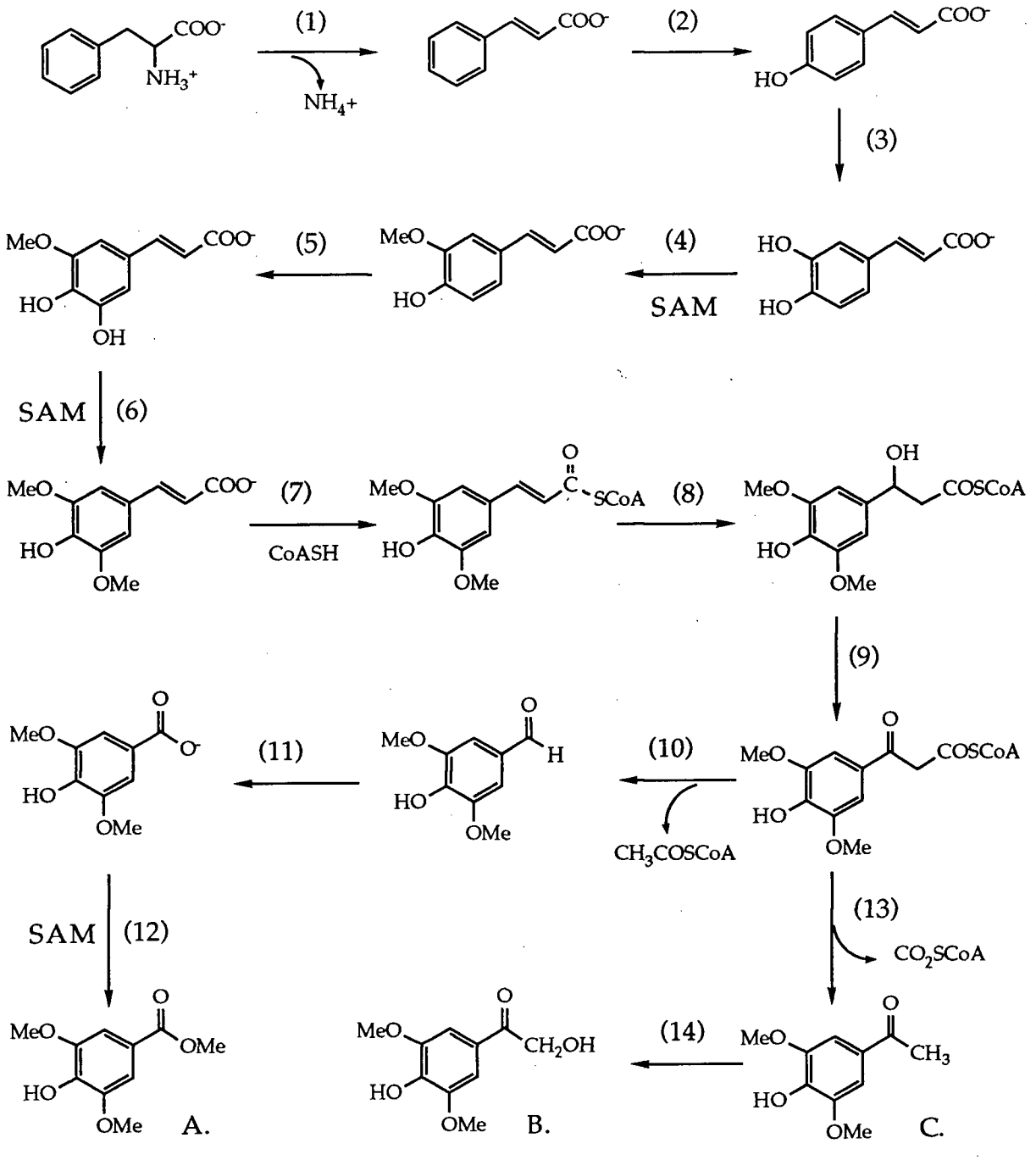


Figure 1.13 Possible biosynthetic routes from phenylalanine to methyl syringate (A), HO-AS (B), and AS (C). Enzymes: (1)=PAL,(2) cinnamate-4-hydroxylase, (3),(5) and (14)=hydroxylases, (4) and (6)=O-methyl transferases, (7)=CoA ligase, (13)=decarboxylase.

1.3.6 Vacuolar phenolics

A wide variety of plant phenolics are known and many of these are present as water soluble glycosides in vacuoles. It has long been considered that glycosylation serves a dual role in deactivating the phenolic nucleus and providing water solubility (Harborne, 1979). Glycosylation of *vir*- inducing phenolics renders the phenolic inactive by blocking the phenolic hydroxyl from interacting with the bacterial receptor or sensor. For example, for wide host range *Agrobacterium tumefaciens*, coniferin is inactive whereas the aglycone, coniferyl alcohol, is a strong inducer of *vir* gene expression (Spencer and Towers, 1988).

Cases are known in which phenolic glucosides released from wounded plant tissue are acted upon by plant ß-glucosidases to yield bioactive aglycones. A well known example of this is the occurrence of phloridzin and its fungitoxic aglycone phloretin (Overeem, 1976). Interestingly, the examples in the literature include compounds which readily oxidize to toxic quinones, e.g. juglone from *Juglans nigra* L., a classic example of an allelochemical stored as its inactive glucoside (Rhodes, 1985). The process of hydrolysis might apply equally well to both antimicrobial phenolics and to phenolics which act as signal compounds. Thus the signal compounds acetosyringone and methyl syringic acid may be present in the plant as the corresponding ß-D-glucosides. In fact, *n*BuOH extracts treated with TFA or ß-glucosidase did yield $CHCl_3$-soluble, *vir*-inducing compound(s) (see Chapter 3).

It has been reported that the production of acetosyringone from tobacco leaf discs can be greatly decreased by treatment with cycloheximide (Stachel *et al.*, 1985b). For cycloheximide to prevent production of active signal compounds from the corresponding glucosides, it would have to block the

synthesis of glucosidases following wounding. However, glucosidases are likely to be present before wounding, so some level of hydrolysis might be expected even in the presence of cycloheximide.

p-Hydroxybenzoic, salicylic, and vanillic acids are present in bound form, presumably as glycosides, in potato culture cells (Robertson *et al.*, 1969). More recently, Klick and Herrmann (1988) examined the distribution of hydroxybenzoic acid glucosides and hydroxybenzoylglucoses (glucose esters) in a number of plant families. The glucosides are more common than the esters. Salicylic, 4-hydroxybenzoic, vanillic, protocatechuic, and syringic acids are widespread, although in lower concentration than hydroxycinnamic acid derivatives.

Picien and androsin (Figure 1.14) are produced by spruce needles, and upon release are rapidly converted to the aglycones, *p*-hydroxyacetophenone and acetovanillone (Oswald and Benz, 1989). Acetovanillone is another virulence inducing phenolic compound (Stachel et al, 1985; Spencer and Towers, 1988), and so in inoculation of this species this compound must have an effect on the level of *vir* gene expression. In Chapter 3, the identification of the glucosides of acetosyringone, acetovanillone (ie. androsin), and their α-hydroxy derivatives in unwounded tobacco leaves is described.

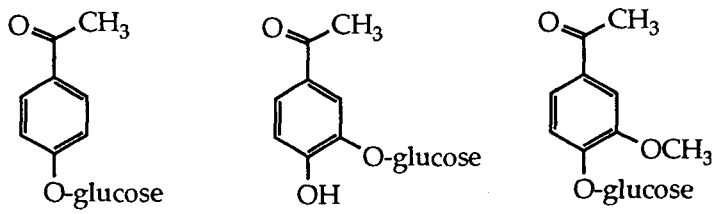
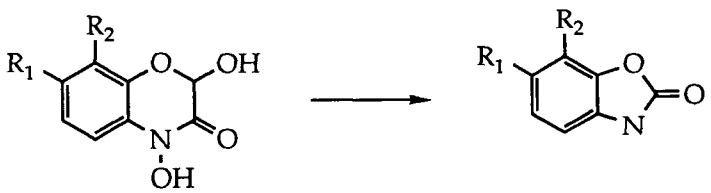


Figure 1.14 Structures of acetophenone glucosides picein, pungenin, and androsin. Androsin, or HO-AV-glucoside, was identified in intact tobacco leaves (see Chapter 3).

1.3.7 Repressors of *vir* expression

A recent report demonstrated that *vir* gene expression is repressed by nitrogenous compounds which are phenolic in character (Sahi *et al.*, 1990). The compound DIMBOA, 2,4-dihydroxy-7-methoxy-2H-1,4,-benzoxazin-3(4H)-one (Figure 1.15), from monocots such as maize and wheat, has long been known for its antifungal, bacteriostatic, and insect antifeedant properties. Sahi *et al.* (1990) isolated DIMBOA from maize homogenates on the basis of its suppresive effect on *vir*-induction. Also, they demonstrated that DIMBOA can prevent tumor formation on leaves of *Kalenchoë diagramontiana.*

DIMBOA is one of a number of hydroxamic acids present in monocots as its glucoside (Wahlroos and Virtanen, 1959). The glucosides are readily hydrolyzed when the structural integrity of the tissue is destroyed (Bailey and Larson, 1989). The aglycone inhibits *vir* gene induction by acetosyringone, and is likely a significant factor in the resistance of such monocots to infection by *A. tumefaciens*. An HPLC examination of hydroxamates from maize is described in Chapter 3.



$R_1=R_2=H$	**I**	DIBOA	→ **II**	BOA
$R_1=OCH_3$ $R_2=H$	**III**	DIMBOA	→ **IV**	MBOA
$R_1=R_2=OCH_3$	**V**	DIM_2BOA	→ **VI**	M_2BOA

Figure 1.15 Chemical structures of DIMBOA and related hydroxamic acids. DIBOA=2,4-dihydroxy-2H-1,4,-benzoxazin-3(4H)-one, DIMBOA=2,4-dihydroxy-7-metyhoxy-2H-1,4,-benzoxazin-3(4H)-one, DIM_2BOA=2,4-dihydroxy-6,7-dimethoxy-2H-1,4,-benzoxazin-3(4H)-one, BOA=2-(3H)-benzoxazolinone, MBOA=6-methoxy-2-(3H)-benzoxazolinone, M_2BOA=6,7-dimethoxy-2-(3H)-benzoxazolinone.

1.4 Concluding remarks

The *in planta* roles of wound-induced plant phenolic compounds, which *A. tumefaciens* detects as signal molecules liberated by host plants, remain unknown. Clearly, they do not function in the same way as do the dihydroxy-substituted ring compounds. Such compounds are extremely sensitive to oxidation and the products are often antibiotic. It must be assumed that *A. tumefaciens* has evolved the ability to detect compounds which are less toxic and which very many plants produce. It so happens that the known signal compounds (AS and HO-AS) had never previously been discovered, probably because we have had to await the means by which to observe the biological activity of compounds which are produced in such minute amounts.

At least in certain cases, the signal compounds are likely present in host plants as glucosides (see Chapter 3-hydrolysis of *Nicotiana* *n*-BuOH fractions). In these instances, wounding abolishes the compartmentalized state of the plant cells and brings together plant enzymes (glucosidases) and inactive conjugates (eg. the glucosides) to yield *vir*-activating compounds.

Chapter 2: Identification of an Agrobacterium signal compound from grapevine cultivars.

2.1 Foreword:

A major portion of this chapter has been published:

Spencer, P.A., Tanaka, A., and Towers, G.H.N. (1990) An *Agrobacterium* signal compound from grapevine cultivars. Phytochemistry **29**: 3785-3788.

The principle author and researcher was Paul A. Spencer, with some early bioassays and contribution of grapevine extracts from Dr. Akira Tanaka (Plantec Research Institute, Japan, supported by a National Science Foundation Grant (DMB-870-4292) to Dr. Eugene Nester, University of Washington). Otherwise, the research was conducted in the lab of Dr. G.H.N. Towers. Dr. Ed Neeland synthesized HO-AS, and Felipe Balza operated the GC-EIMS. Grapevine samples were provided by Dr. George Eaton (Plant Science, U.B.C.) and use was made of the U.B.C Chemistry Department Mass Spectrometry and NMR Facilities.

2.2 Introduction

A range of plant phenolic compounds have been demonstrated to be involved in induction of *Agrobacterium tumefaciens* virulence genes. Initially, acetosyringone, AS, and α–hydroxyacetosyringone, HO-AS, were identified as wide host range (WHR) signal compounds from transformed tobacco root cultures (Stachel *et al.*, 1985). Prior to this work, these acetophenones were not known as natural products. It was subsequently

demonstrated (Bolton *et al.*, 1986; Spencer and Towers, 1988) that the more common phenolics, such as certain phenylpropanoids (including monolignols) and benzoic acid derivatives, also showed significant *vir*-inducing activity. In this initial study, a WHR strain of *A. tumefaciens* was studied; this chapter examines *vir*-induction in a limited host range (LHR) *A. tumefaciens* strain.

Certain LHR strains of *Agrobacterium* (eg. A856, Thomashow *et al.*, 1981) are capable of inducing tumors on various grapevine cultivars, to the exclusion of typical hosts (eg. *Nicotiana tabacum*). Wide host range (WHR) strains (eg. A348, Thomashow *et al.*, 1981) can induce tumors on a wide variety of dicotyledons. However, WHR strains can cause a hypersensitive response on stems of certain grapevines and typically are unsuccessful at tumor induction on these cultivars (Yanofsky *et al.*, 1985). One cultivar, *Vitis* cv. Seyval, is a permissive host for both strains. Interestingly, *V. labruscana* cv. Steuben is a host only for LHR *A. tumefaciens*. The inability of WHR strains to form tumors on grapevines may be due to the hypersensitive response caused by WHR *Agrobacterium*.

Factors both within the T-DNA as well as the *vir* region have been shown to play a role in host range. Hoekema *et al.* (1984) analysed factors that confer limited host range on strain LBA649, which contains the octopine plasmid pTiAg57. A wide host range T-region gene was found to compliment the host range "defect" in pTiAg57, but a wide host range *vir* region failed to extend the host range of LBA649. However, this is not the case with all LHR strains studied. Determinants within both the T-DNA and *vir* region were found to contribute to the host specificity of LHR *Agrobacterium* possessing pTiAg162 (Yanofsky *et al.*, 1985). Experimental introduction of WHR *vir*A and *vir*C functions were required to confer upon the recipient LHR strain an

expanded host range. This was an important observation because the VirA gene product is considered to be the environmental sensor of plant derived signal compounds, and so artificial expansion of host range by introduction of a WHR *vir*A gene suggested that host range may be determined on the basis of signal molecule specificity. Thus, a factor that may influence host range specificity is the ability to detect the appropriate chemical signal by the bacterial receptor protein. For example, the limited host range *vir*-inducer may be some compound other than the previously mentioned acetophenones. Ma *et al.* (1987) examined thirteen strains of *A. tumefaciens* isolated from grapevine tumors in China. The DNA in these strains exhibited "little or no homolgy to a WHR *vir*A locus, but did show strong homology to a LHR *vir*A locus"; a WHR *vir*A probe failed to hybridize, and a LHR *vir*A probe strongly hybridized with *vir*A loci from the grapevine strains. This supports the concept of a specific *vir*A locus amongst *Agrobacterium* strains associated with grapevines. In addition, the putative sensors of the phenolic signal compound(s), the LHR and WHR *vir*A gene products, have been sequenced and compared (Leroux *et al.*, 1987). The gene products were determined to have diverged to the greatest degree in their assumed periplasmic domain, thus lending support to the concept that differing signal compounds may be required for *vir* induction in LHR and WHR strains.

For the purpose of understanding the mechanism(s) by which host ranges are established, investigations of the phytochemicals involved in *vir*-induction are necessary. In this Chapter is described the bioassay-directed isolation of a single *vir*-inducing substance present in various grapevine-derived wound exudates and its identification as the methyl ester of syringic acid. Neither HO-AS nor AS was found in these extracts.

2.3 Methods

2.3.1 Plant material

Samples of leaves, stems and wound eudates from *Vitis* cultivars Seyval, Foch, Pinot Noir, DeChaunac, Vidal256, Müller Thurgau, Leon Mallot, White Diamond, and Himrod were obtained from the U.B.C. Botanical Gardens and South Campus vineyard.

2.3.2 Extraction and Isolation

Fresh leaves and stems (0.5-1.0 kg per batch) were cut into small (ca. 1cm) pieces and incubated in 2.0 L filter-sterilized, pH 5.7 MS medium for 24-48 hrs. The filtered medium was partitioned with at least 3 volumes each of $CHCl_3$, CH_2Cl_2, EtOAc, and or *n*-BuOH. The organic solvents were removed with a rotory evaporator and the extracts resuspended in small volumes of MeOH. The conditioned tissues were extracted thoroughly with 70% MeOH, the MeOH removed with a rotory evaporator and partitioned against solvents as described above. The crude extracts were fractionated on Sephadex LH-20 columns using acetone, MeOH, or MeOH/$CHCl_3$. Active fractions were pooled, resuspended in HPLC grade MeOH, and separated further by semi-preparative HPLC (LiChrosorb RP-18, 3mL/min., 5% aq. HOAc/MeOH; 30-100% MeOH gradient/30min.). HPLC fractions were extracted with $CHCl_3$, rebioassayed, and the recovered materials in active HPLC fractions was further separated by TLC on silica [**1**. EtOAc, **2**. $CHCl_3$:MeOH, (9:1), **3**. $ClCH_2CH_2Cl$:CH_3COCH_3, (95:5), **4**. toluene:EtOAc:HOFm, (5:4:1)] or polyamide [EtOFm: cyclohexane: BuOAc: HOFm, (50:25:23:2)]. Where possible fractions were stored at or below 0 °C in HPLC grade MeOH or EtOAc, and reduced in volume under a stream of N_2.

2.3.4 *vir*-induction assays

The plate assay system was developed from the system reported by Bolton *et al.* (1986). In plate assays, a few µL of each test fraction or compound in MeOH, were spotted onto MeOH-washed 5 mm absorbent fiber discs and the solvent allowed to evaporate. Test discs were placed on separate sectors of an overnight lawn culture of either A856/pSM243cd (see below) or A348/pSM243cd (see below) grown on pH 5.5 AB medium (Chilton *et al.*, 1974) which contained 100 µg/mL carbenicillin and ca. 0.1% (600 µg/plate) X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside). This chromogenic substrate for the enzyme ß-galactosidase yields a blue colored product. The plates were incubated at 28 °C until blue zones of bacterial growth developed in the areas surronding discs with active compounds (24 hrs.).

Quantitative ß-galactosidase assays following induction (9 hrs./ 28 °C) in pH 5.5 AB medium (100 µg/mL carbenicillin) containing 0.1% DMSO, were conducted as described by Miller (1972).

2.3.5 Bacterial strains, media and plasmids

The LHR (pTiAg162) and WHR (pTiA6) plasmids and the strains containing them (A856 and A348) have been described previously (Thomashow *et al.*, 1981). A856/pSM243cd carries in addition to pTiAg162, a *vir*B::*lac*Z gene fusion, pSM243cd (Stachel and Zambryski, 1986; Winans *et al.*, 1986) in the absence of WHR *vir*A and is thus an indicator of LHR *vir*A-dependent *vir*-induction. Conversely, strain A348/pSM243cd carries a *vir* B::*lac* Z gene fusion (pSM243cd) in the presence of the WHR plasmid pTiA6 and is thus an indicator of WHR *vir*A-dependent *vir*-induction.

2.4 Results and Discussion

The plate/disc bioassay system described in section 2.3.3 was developed in order to follow *vir*-inducing activity following each stage of chromatographic fractionation. This assay system was sensitive to 0.5 μg of AS. Extracts of unwounded grapevine stem or leaf tissue did not exhibit any *vir*-inducing activity, confirming the requirement for wounding prior to tumor induction by *Agrobacterium*. However, the stem and leaf sections produced and exuded into the culture medium detectable amounts of *vir*-inducing substance, when incubated for 24-48 hrs. in MS medium. Interestingly, both A348/pSM243cd and A856/pSM243cd (see methods) responded positively to grapevine extracts in plate assays. The active, wound-induced compound was found to partition from the conditioned culture medium and methanolic tissue extracts into the CH_2Cl_2 or $CHCl_3$ phase.

Preliminary HPLC and TLC/bioassay experiments with extracts from each of the grapevine cultivars suggested that a single compound, common to all the grapevine cultivars, was likely responsible for the observed activity. Furthermore, the same compound was present in extracts from both the wounded stem tissue and the conditioned medium, although in much greater purity in the latter. Extracts from a number of cultivars were pooled in order to provide sufficient material for both bioassays and structure elucidation. No attempt was made to quantify the amounts of active compound produced by any of the grapevine cultivars. The active compound was usually well resolved by TLC on silica gel, although it was clear that only very small amounts were being eluted from the isolated bands. Following gel filtration on Sephadex LH-20, reversed phase HPLC, and multistep silica and polyamide TLC sufficient purified material remained to permit identification

by mass spectrometry (see Table 2.1). ^{1}H-NMR spectroscopy of the partially purified grapevine derived compound gave methoxy signals (singlets near 4 ppm) and a phenyl proton signal (singlet near 7.3 ppm).

Low resolution electron impact mass spectroscopy (EIMS) indicated the presence of a molecular ion (M^+) at *m/z* 212. High resolution mass spectroscopy (HR-EIMS) confirmed a molecular ion at *m/z* 212 (actually 212.0686), and indicated the molecular formula to be $C_{10}H_{12}O_5$. Two strong *vir* -inducers with this composition are α–hydroxyacetosyringone (Stachel *et al.*, 1985) and syringic acid methyl ester. The ester was included in the analysis of the structure-activity relationships of *vir*-induction (Spencer and Towers, 1988). Other fragments included *m/z* 181 ($C_9H_9O_4$), *m/z* 153 ($C_8H_9O_3$), *m/z* 149 ($C_8H_5O_3$).

GC-MS of the silylated derivative of the grapevine-derived *vir* -inducer, and comparison with that of authentic α–hydroxyacetosyringone and methyl syringate, unequivocally identified the inducer as the latter. Methyl syringate becomes monosilylated (M^+ 284), whereas HO-AS gains two TMS groups (M^+ 356). Since the same compound was obtained from a number of *Vitis* cultivars, including Seyval, and since no other highly active fractions were isolated, I concluded that the most significant naturally occurring limited host range signal compound from grapevines is syringic acid methyl ester. To my knowledge, this was the first report of the natural occurrence of this compound.

To investigate further the effects of syringic acid methyl ester on *vir*-induction in WHR and LHR *Agrobacterium*, I performed quantitative assays of β-galactosidase activity in bacteria possessing *vir::lac* gene fusions. The degree of *vir* gene induction with increasing concentration of authentic syringic acid methyl ester and AS in WHR and LHR strains is shown in

Table 2.1 Spectral and chromatographic data used in the identification of methylsyringate in grapevine wound exudates.

Active grapevine compound :

High resolution electron impact mass spectrometry (HR-EIMS) *m/z* : 212.0686 $[M]^+$ ($C_{10}H_{12}O_5$), 181.0501 $[M\text{-}OCH_3]^+$ ($C_9H_9O_4$), 153.0552 $[M\text{-}COOCH_3]^+$ ($C_8H_9O_3$); calculated for $C_{10}H_{12}O_5$, 212.0685; $C_9H_9O_4$; 181.0501, $C_8H_9O_3$; 153.0552.

Gas chromatography-electron impact mass spectrometry (GC-EIMS) of TMS derivative *m/z* : 284 $[M]^+$ (30), 269 $[M\text{-}Me]^+$ (44), 255 (14), 254 $[M\text{-}30]^+$ (100), 223 (22), 73 (31).

Thin layer chromatography (TLC): Si gel TLC (**1**) R_f= 0.52, (**2**) R_f= 0.63, (**3**) R_f= 0.28, (**4**) R_f= 0.46; polyamide TLC R_f= 0.78.

HPLC R_t= 17.0 min.

Authentic α-hydroxyacetosyringone :

Proton nuclear magnetic resonance (^1H NMR) ($CDCl_3$): 7.20 (s, 2H), 4.85 (s, 2H), 3.97 (s, 6H). ^{13}C NMR ($CDCl_3$): 196.7 (s, C=O), 147.1 (s), 140.8 (s), 124.8 (s), 105.0 (d), 65.0 (t, CH_2), 56.5 (q, OCH_3).

GC-MS of TMS derivative *m/z* (rel. int.): 356 $[M]^+$ (27), 341 $[M\text{-}Me]^+$ (44), 254 (45), 253 $[M\text{-}CH_2OTMS]^+$ (100), 223 (33), 103 $[CH_2OTMS]^+$ (27), 73 (88).

Authentic syringic acid methyl ester.

^1H NMR ($CDCl_3$): 7.33 (s, 2H), 5.94 (s, 1H), 3.95 (s, 6H), 3.91 (s, 3H).

EI-MS *m/z* (rel. int.): 212 (100), 197 (53.5),181 (89), 169 (19.5), 165 (20.6), 153 (45), 141 (72), 123 (40), 108 (32.3), 95 (30), 79 (28), 43 (50).

GC-MS of TMS derivative *m/z* (rel. int.): 284 $[M]^+$ (37), 269 $[M\text{-}Me]^+$ (59.5), 255 (23.5), 254 $[M\text{-}30]^+$ (100), 223 (32), 73 (20).

Thin layer chromatography (TLC): Si gel TLC (**1**) R_f= 0.52, (**2**) R_f= 0.63, (**3**) R_f= 0.28, (**4**) R_f= 0.46.

HPLC R_t= 17.0 min.

Figure 2.1. As was reported for the WHR strain A348/pSM358 (Spencer and Towers, 1988), the methyl ester is a more potent *vir*-inducer than is AS. LHR *vir* -induction does not reach its peak until the concentration rises to about 500 μM AS, or 200-500 μM methyl syringate. At concentrations that induce half-maximal WHR *vir* expression (about 20 μM methyl syringate), the LHR strain was not detectably activated. Also, the LHR strain had only reached half-maximal induction at concentrations that fully induced WHR *vir* gene expression.

The relevance of the results presented here to the phenomenon of host range specificity is obscure. Perhaps the LHR strains are simply limited to hosts that produce higher levels of signal compounds. Expression of *vir* genes of both WHR and LHR strains is inducible by both AS and methyl syringate, although the two strains appear to differ in their respective *vir*-inducing concentration optima. Previously, AS was identified from *N. tabacum*, a host for WHR *A. tumefaciens*. Methyl syringate is a very strong *vir*-inducer of both WHR and LHR strains, and yet grapevine cultivars are more susceptible to tumor formation by LHR strains. The concentrations of *vir*-inducing compounds produced by different host species must be determined. Additionally, an area yet to be explored regards the possible presence of grapevine-derived *vir*-inhibitors specific to WHR strains.

It should be emphasized that only syringic acid methyl ester was found in grapevine extracts; neither AS nor HO-AS, both acetophenones, was present. There is likely some significance to the elaboration of these different classes of *vir*-inducing phenolic compounds by the respective host groups. Coincidentally, HO-AS and syringic acid methyl ester are isomeric compounds, possessing the same molecular weight and formula, but subtly different structures. Production of AS can be elicited by treatment of *N.*

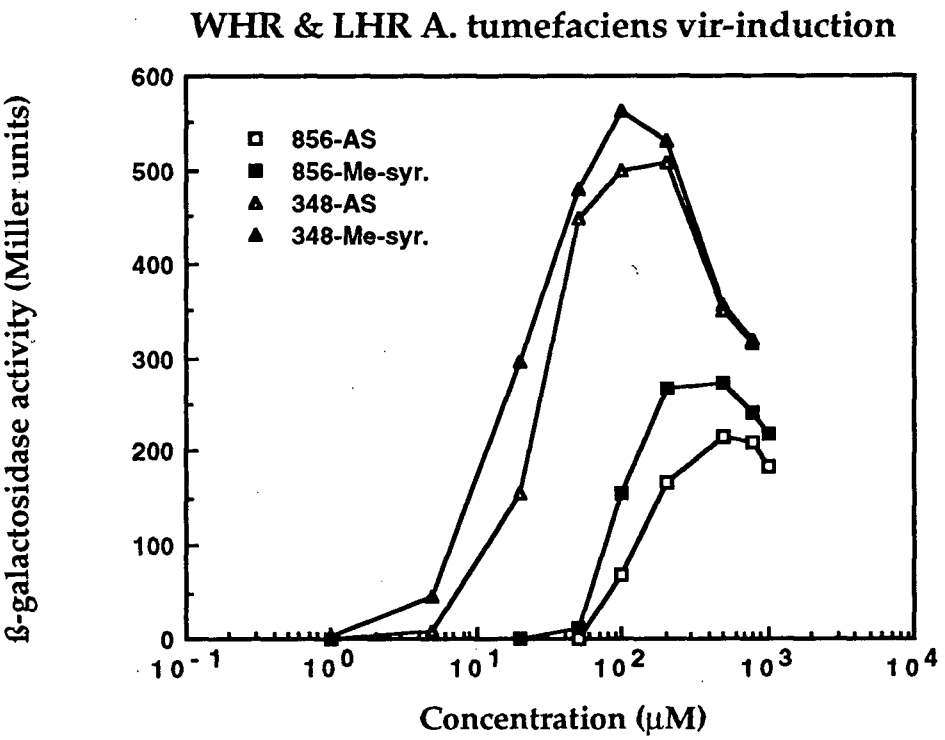


Figure 2.1. *vir*B-Induction in strains A348 (WHR) and A856 (LHR). Each of these strains possesses, in addition to its WHR or LHR-determining Ti plasmid, the *vir*B::*lac*Z plasmid pSM243cd. Following induction with either AS or syringic acid methyl ester (Me-syr.) ß-galactosidase activity was assayed according to Miller (1972).

tabacum cell suspension cultures with cellulase (Threlfall and Whitehead, 1988), although the significance of this compound, other than its role as a signal compound, is not known. The biosynthetic origins and roles for these wound response compounds must be investigated.

Chapter 3. Naturally Occurring WHR Signal Molecules

3.1 Foreword

A major portion of this chapter has been accepted for publication:

Spencer, P.A., and Towers, G.H.N. (1991) Restricted occurrence of acetophenone signal compounds. Phytochemistry (in press).

The research was conceived of and conducted by Paul A. Spencer in the laboratory of Dr. G.H.N. Towers.

This Chapter describes the application of combined GC-MS to the analysis of the phenolic components in partially purified WHR *A. tumefaciens vir* gene activating plant wound exudates. GC retention times and mass spectra were used to confirm the identity of the components in the active mixtures (refer to Chapter 5). Results confirmed that the production upon wounding of elevated levels of *vir*-activating acetophenones requires metabolically active tobacco plant cells. Additionally, this phenomenon appears restricted in distribution among plants to members of the Solanaceae. In contrast, species from other plant families were found to produce *vir*-inducing benzaldehydes, and benzoic and cinnamic acid derivatives but none of the acetophenones.

3.2 Introduction

Chapter 2 describes the multi-step isolation and the identification of methyl syringate from grapevine cultivars (Spencer *et al.*, 1990). The active component was found to have a molecular weight of 212, and the chemical composition $C_{10}H_{12}O_5$. On this information alone, it seemed likely that this

was the known inducing compound α-hydroxyacetosyringone. However, GC-mass spectrometry of derivatized grapevine samples and of the authentic acetophenone established that the unknown was not α-hydroxyacetosyringone, but an isomeric compound, syringic acid methyl ester. Coincidentally, this ester was an important compound included in the analysis of structure-activity relationship of *vir*-induction in wide host range (WHR) *Agrobacterium tumefaciens* (Spencer and Towers, 1988). Preparative reverse phase (RP-18) HPLC and normal phase silica gel TLC were used extensively to isolate methyl syringate.

Work on the LHR signal compound resulted in improved techniques for analysis of *vir*-inducing compounds from other host and non-host species. The plate bioassay (described in Spencer *et al.*, 1990) was subsequently used to screen for activity the numerous chromatographic fractions derived from other host plants. Also, whereas chromatography on silica gel may be convenient for TLC analysis of various extracts and fractions, it appears to irreversibly bind some phenolic compounds. Therefore, this was abandoned as a means to isolate *vir*-inducing fractions. Instead, as will be described in this chapter, short columns of polyamide were used to provide fractions greatly enriched with mixtures of *vir*-inducing compounds and without significant loss of material. Fractions prepared in this way were ready for derivatization and GC-mass spectrometry.

Prior to its discovery as a signal compound by Stachel *et al.* (1985), acetosyringone was not known as a natural product. It was conceivable that only Ri-transformed *N. tabacum* synthesized this novel compound - a quirk of secondary metabolism in an unusual cell culture environment. However, Threlfall and Whitehead (1988) have shown that acetosyringone is produced by *N. tabacum* suspension cultures treated with cellulase. This result

suggested either that the acetophenone is a natural cell wall constituent or that its production is otherwise elicited by the action of cellulase. Coincidentally, pretreatment of *Panax ginseng* callus tissue with cellulase has been used to acheive transformation with *Agrobacterium rhizogenes* (Yoshikawa and Furuya, 1987). In this case, however, the cellulase was part of a protoplasting treatment, and the protoplasts were well washed prior to inoculation. Such washing would likely remove any *vir*-inducing phenolic compounds released from cell walls by the cellulase.

GC-MS analysis of the "conditioned" culture medium from stem and leaf sections of *Nicotiana* species confirmed that acetosyringone and its α-hydroxy derivative are produced by nontransformed tissues. Moreover, in all tobacco extracts the corresponding pair of guaiacyl-substituted acetophenones (acetovanillone and α-hydroxyacetovanillone) were also detected. Additionally, the far more commonly occurring phenylpropanoid signal compounds were absent. In this chapter, wound-induced production of acetosyringone and other signal compounds from a variety of plant species was investigated.

The biosynthesis of signal compounds was also investigated by GC-mass spectrometry. Two experiments designed to establish the biosynthetic origin of the acetophenones were conducted. In one experiment, the glycoside fractions from non-wounded plants were prepared. The glycoside fraction exhibited no *vir*-inducing activity. These fractions were hydrolysed either with β-glucosidase or with 1M TFA (trifluoroacetic acid) and the hydrolysates tested for activity. In another experiment, the eucaryotic metabolic inhibitor cycloheximide was tested for its ability to inhibit wound-induced production of acetophenones from phenylpropanoid precursors. Under these conditions, the presence of the phenylpropanoids instead of the

acetophenones would lead one to suspect that the required enzymes are produced following wounding.

3.3 Methods

3.3.1. *vir*-Induction assays

Plate assays were conducted much as described in Chapter 2, except that here, care was taken to determine concentrations of the extracts. In these assays, 10-40 µL of 1 mg/mL solutions of each test fraction or compound in MeOH, were transferred to MeOH-washed 5 mm absorbent discs and the solvent allowed to evaporate. Test discs were placed on separated sectors of an overnight lawn culture of A348/pSM243cd grown on pH 5.5 AB medium which contained 100 µg/mL carbenicillin (AB/cb^{100}) and ca. 0.1% (600 µg/plate) X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside). The plates were incubated at 28 °C until blue zones developed in the areas surrounding discs with active compounds (24 hrs.). The plates were also examined after an additional 24 hrs. for late developing fractions.

Quantitative ß-galactosidase assays following induction (8-20 hrs./ 28°C) in MES buffered pH 5.5 AB medium containing 0.1% DMSO and 100 µg/mL carbenicillin, were conducted as described by Miller (1972). When quantitatively assaying the effect of pure compounds, DMSO was added to dissolve the crystals and filter sterilized AB/cb^{100} medium added to make stocks solutions (eg. 200 µM) containing 0.1% DMSO. Control experiments were conducted to check the effects of DMSO (and ethylene glycol monoethyl ether). This method works equally well for both *A. tumefaciens* and *A. rhizogenes* A4/pSM358cd (see Chapter 4).

3.3.2. Plant materials

At the outset of this project, the need for a "signal compound survey" was proposed. In other words, a variety of host and nonhost species should be examined for any of a number of biologically active phenolic compounds. At that time there was no established means by which such a survey could be pursued. No rapid bioassay or standardized chromatographic methods were known. Work on the isolation of the grapevine-derived signal compound (Chapter 2) resulted in standardized methods (notably polyamide VLC and GC-MS) which have been used here in accumulating data on a range of plant species. So far, the conditioned media from plant parts and cell cultures representing 41 species have been examined for $CHCl_3$-soluble *vir*-inducers (Table 3.1). Seedlings of *Nicotiana* species were kindly provided by the Agriculture Canada Research Station at U.B.C. These were brought to the Botany Department greenhouse and grown there until required. Most other species were available from the U.B.C. Botanical Gardens, endowment lands or South Campus area.

3.3.3. HPLC of maize hydroxamates

The EtOAc part from the aqueous medium conditioned by wounded corn stem sections was filtered, reduced to dryness under N_2 and resuspended in HPLC grade MeOH. A semipreparative LiChrosorb RP-18 (Merck) HPLC column was equilibrated with 15% MeOH/ 85% 10mM H_3PO_4 at a flow rate of 3 mL/min. and 100 µL aliquots were fractionated using gradient elution starting at 15 % for 4 min., increasing to 60% in 25 min., and then to 100% MeOH in 10 min. Compounds were detected by absorbance at 265 nm.

Table 3.1 Species examined for $CHCl_3$-soluble *vir*-inducers

Plant species	Family	Source of inducers
Ambrosia chamissonis	(Asteraceae)	root culture medium
Arabidopsis thaliana	(Brassicaceae)	stems
Asparagus officinale	(Liliaceae)	stems
Atropa belladonna	(Solanaceae)	leaves
Beta vulgaris	(Cheanapodiaceae)	tubers
Brassica sp.	(Brassicaceae)	stems
Capsella bursa-pastorus	(Brassicaceae)	stems
Chaenactis douglasii	(Asteraceae)	culture medium
Datura stramonium	(Solanaceae)	leaves
Daucus carota	(Apiaceae)	tubers
Discorea (?)	(Dioscoraceae)	tubers
Geranium richardsonii	(Geraniaceae)	stems
Hydrangea sp.	(Hydrangeaceae)	stems
Hyoscyamus niger	(Solanaceae)	leaves
Lolium perene	(Graminea)	crown region
Lycopersicon esculentum	(Solanaceae)	leaves
Kalanchoë diagramontiana	(Crassulaceae)	leaves
Mentha sp.	(Lamiaceae)	stems
Nepeta cataria	(Lamiaceae)	stems
Nicotiana tabacum	(Solanaceae)	stems+leaves
Nicotiana glauca	(Solanaceae)	stems+leaves
Nicotiana plumbaginofolia	(Solanaceae)	stems+leaves
Nicotiana rustica	(Solanaceae)	stems+leaves
Nicotiana silvestris	(Solanaceae)	leaves
Nicotiana clevelandii	(Solanaceae)	leaves
Nicotiana glutinosa	(Solanaceae)	leaves
Panax gensing	(Araliaceae)	culture medium
Picea sitchensis	(Pinaceae)	stems+needles
Polygonum aubertii	(Polygonaceae)	stems
Populus Hybrid H11	(Salicaceae)	stems+petioles
Pseudotsuga menzeisii	(Pinaceae)	stems
Rosa sp.	(Rosaceae)	stems
Solanum dulcimara	(Solanaceae)	leaves
Solanum tuberosum	(Solanaceae)	tubers
Taxus baccata	(Taxaceae)	needles
Taxus brevifolia	(Taxaceae)	culture medium
Thuja plicata	(Cupressaceae)	needles
Trifolium repens	(Fabaceae)	stolons
Vitis vinifera (9 cv.'s)	(Vitadaceae)	stems+leaves
Zea mays	(Poaceae)	crown region
Zingiber officinale	(Zingiberaceae)	rhizomes

3.3.4. Bioassay sample preparation

Stem, stolon, rhizome or tuber sections from fresh plant material approximately 0.5-1.0 cm in length (or about 1.0 cm^2) were prepared under semisterile conditions and immediately immersed in about 400 mL filter sterilized MS medium (15 g/L sucrose) at pH 5.7, without added buffer. In each case the wounded plant material was incubated at about 25 °C for 24 hrs. with continual shaking (100 rpm) in relative darkness. The conditioned medium from each plant species was filtered, and the final pH was recorded. The conditioned medium was partitioned with at least three volumes of distilled $CHCl_3$, CH_2Cl_2 or EtOAc. The organic phase was reduced to dryness with a rotory evaporator and resuspended in HPLC MeOH to give solutions of 20 mg/mL. 20-40 µL of each extract was assayed for *vir*-induction by disc-plate bioassay with *Agrobacterium* as described.

3.3.5. Polyamide VLC chromatography

The initial bioassays of crude $CHCl_3$ or CH_2Cl_2 fractions from conditioned media did not always give strongly positive responses and GC-MS revealed complex chemical compositions. Excellent purification of *vir*-inducers has been achieved using a variation of a technique intended for rapid isolation of plant alkaloids (Pelletier *et al.*, 1986). Vacuum-liquid chromatography (VLC) uses a gentle vacuum to draw solvent mixtures through columns of TLC grade sorbent. Small columns of TLC grade polyamide were prepared in 2.5 cm funnels with scintered glass filters. These columns were pre-washed with solvents (MeOH to $CHCl_3$:Hexane) using vacuum to draw the solvents through. A glass filter was applied to the top of each column to prevent disturbance of the polyamide as additional solvents were added. The extracts (usually only 10-20 mg) were applied in a minimal

amount of the first solvent and components eluted with the following solvent mixtures:

$CHCl_3$:hexane	(1:1)	2x~20mL
$CHCl_3$:hexane	(9:1)	2x~20mL
$CHCl_3$:MeOH	(9:1)	2x~20mL
MeOH	(neat)	2x~20mL

The fractions were reduced to dryness and resuspended (to 10 mg/mL) in HPLC MeOH for bioassay. At this stage only 10 µL, ie. only 100 µg, of each fraction was applied to a filter paper disc for plate assay. This system usually resolved inducing compounds into one or two fractions (usually the first two solvent mixtures).

Subsequent GC-MS analysis indicated that, compared with crude extracts, the complexity of these samples was greatly diminished, i.e. the number of GC peaks was significantly reduced. Further purification was unnecessary.

3.3.6. Hydrolysis of glycosides

The *n*-BuOH-soluble fraction of hot aqueous 60 % MeOH leaf extracts of *N. glauca* or *N. silvestris* were reduced to dryness and the residue was resuspended in pH 5.0 acetate buffer. This was incubated for 24hrs. with β-glucosidase (Sigma™). The hydrolyzed sample was diluted with distilled H_2O and partitioned with distilled $CHCl_3$. The organic phase was treated by polyamide VLC for aglycones as described above. The active fraction was examined by GC-MS.

Similarly, samples of the *n*-BuOH extracts were hydrolyzed with 1 M TFA (trifluoroacetic acid) and subsequently partitioned for aglycones with

distilled $CHCl_3$. Again, the organic phase was separated by polyamide VLC for aglycones as described above.

3.3.7 Cycloheximide treatment:

To examine the effects of cycloheximide on the production of signal molecules, 20 μM cycloheximide was added to the MS culture medium prior to the addition of *N. sylvestris* leaf sections. Otherwise, the sections were incubated for 24 hrs. under the same conditions as were non-cycloheximide treated leaf sections. The medium was then filtered, partitioned with $CHCl_3$ and analysed in the same way as were extracts from non-cycloheximide treated tissues.

3.3.8 Cellulase-induced release of inducers from *Nicotiana* cell walls

Solvent extracted cell wall material from *N. silvestris* (100 mg) was suspended in 50 mL pH 5.0 acetate buffer and incubated at 37 °C overnight with a mixture of cellulase and Drieselase. The supernatant was partitioned with $CHCl_3$, resuspended in MeOH to 1 mg/mL and tested for activity by plate-bioassay.

3.4 Results and Discussion

3.4.1 Signal compound mixtures

Wound induced plant phenolic compounds exhibiting biological activity in *Agrobacterium* strain A348/pSM243cd were successfully purified from leaf, petiole, stem, root, rhizome or tuber section-conditioned culture media by solvent partitioning and column chromatography. The polyamide VLC system usually resolved *vir* gene activating mixtures into one or two fractions (usually the first or second two solvent mixtures). In this way, for some samples, about 95% by mass of inactive material in the extracts could be removed prior to GC-MS. Because very small amounts of material were obtained, only rough estimates of the quantities of compounds were obtained. Based on plate assay responses equivalent to that of 0.1-10 µg AS, the range of concentrations of inducing substances produced by the *Nicotiana* species in 24 hrs. was approximately 1.0-10 µg AS eq./g.f.w. leaf tissue.

GC-MS data indicated the presence of a number of virulence inducing phenolic compounds in wounded plant conditioned media. GC-MS analysis of partially purified extracts is an ideal means by which a number of active compounds in mixtures can be identified. Individual components were not subjected to multiple chromatographic steps during their isolation, a procedure which leads to loss of material at each stage. The following example demonstrates the usefulness of GC-MS analysis in these studies. Following gel filtration, extensive silica gel TLC and HPLC, methylsyringate was isolated from wounded grapevine conditioned media (Chapter 2; Spencer and Towers, 1990). Using GC-MS of relatively crude grapevine extracts syringaldehyde and syringic acid were detected in addition to methyl

syringate. Apparently the aldehyde and the acid were lost during the isolation of the methyl ester. The trimethylsilyl (TMS) derivatives of these phenols typically give strong molecular M^+ ions, and fragmentation patterns often exhibited strong $[M-15]^+$, $[M-30]^+$, and $[M-45]^+$ ions. $[M-31]^+$ and $[M-61]^+$ ions were noted in mass spectra of benzoic acid methyl esters. $[M-89]^+$ and $[M-119]^+$ ions were observed in mass spectra of ferulic and sinapic acids. Ions of *m/z* 45, 59, 73, and 89 were very common.

Trends in the distribution of signal compounds elaborated from representative plant families were observed (Table 3.2). Differing groups of signal compounds were revealed by GC-MS analysis. The common phenolic acids vanillic, syringic, ferulic, and sinapic acids were present in many of the extracts from wounded plant tissue conditioned media. In certain media other known inducers were detected. These included the methyl esters of vanillic and syringic acids (eg. grapevine cultivars). Yet another group yeilded vanillin, and coniferyl alcohol (eg. conifers) upon wounding.

The differing arrays of virulence inducing compounds produced upon wounding may be used to arrange various plant species into chemotaxonomic groups. Certain conifers do produce some active phenolic compounds, but they include vanillin and in one case, coniferyl alcohol. Every solanaceous plant examined, including all species of tobacco, produced guaiacyl and syringyl substituted acetophenones and their α-hydroxy derivatives (section 3.4.2). Grapevine cultivars produced benzoic acids and their methyl esters. Many dicots and monocots produced active phenylpropanoids (cinnamic acid derivatives) as well as benzoic acid derivatives.

The active compounds present in a very active sample obtained from medium conditioned with *Phaseolus* sp. (Scarlet runner) leaf sections could

Table 3.2. The signalling phenolics detected by GC-MS of wound exudates from selected species of flowering plants. The signal molecules by number are: 1=AS, 2=HO-AS, 3=AV, 4=HO-AV, 5=vanillin, 6=syringaldehyde, 7=vanillic acid, 8=syringic acid, 9=ferulic acid, 10=sinapic acid, 11=methyl vanillate, 12=methyl syringate, 13=coniferyl alcohol. + = compound detected, +/- = compounds at detection limit, - = compound not detected.

Plant genus & family	Signal compound number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Monocotyledonae													
Asparagus.....Liliaceae	-	-	-	-	+/-	-	-	-	-	-	+	-	-
Dioscorea.....Dioscoraceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Lolium.....Poaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Zea.....Poaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Zingiber (rhizome) Zingiberaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Dicotyledonae													
Arabidopsis.....Brassicaceae	-	-	-	-	+/-	+/-	-	-	-	-	-	-	-
Brassica.....Brassicaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Capsella.....Brassicaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Daucus (tuber) .....Apiaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Geranium..... Geraniaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Helianthus.....Asteraceae	-	-	-	-	+/-	+/-	-	-	+/-	-	+/-	-	-
Hydrangea..... Hydrangeaceae	-	-	-	-	+/-	+/-	-	-	-	-	+/-	-	-
Mentha.....Lamiaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Phaseolus..... Fabaceae	-	-	-	-	-	-	-	-	-	-	-	-	-
Populus..... Salicaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Rosa.....Rosaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Vitis .....Vitidaceae	-	-	-	-	-	+	+	+	-	-	-	+	-
Atropa.....Solanaceae	+	+	+	+	+	-	-	-	-	-	+	-	-
Datura .....Solanaceae	+	+	+	+	+	-	-	-	-	-	+	-	-
Hyoscyamus .....Solanaceae	+	+	+	+	+	-	-	-	-	-	+	-	-
Lycopersicon .....Solanaceae	+	+	+	+	+	-	-	-	-	-	+	-	-
Nicotiana .....Solanaceae	+	+	+	+	+	-	-	-	-	-	+	-	-
Scopolia .....Solanaceae	+	+	+	+	+	-	-	-	-	-	+	-	-
Solanum .....Solanaceae	+	+	+	+	+	-	-	-	+/-	-	+	-	-
Solanum (tuber)....Solanaceae	-	-	-	-	-	-	+	+	+	+	-	-	-
Coniferae													
Picea.....Pinaceae	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudotsuga.....Pinaceae	-	-	-	-	+	+	-	-	-	-	-	-	-
Taxus..... Taxaceae	-	-	-	-	+	-	-	-	-	-	-	-	+
Thuja.....Cupressaceae	-	-	-	-	-	-	-	-	-	-	-	-	+/-

not be identified. None of the 13 compounds indicated in Table 3.2 were detected from bean leaves by GC-MS. The Fabaceae are well known for the variety of flavonoids they produce. Also, 4,4'-dihydroxy-2'-methoxychalcone is known both as a stress metabolite from *Pisum sativum* (Carlson and Dolphin, 1982) and as the *nod*-inducing signal molecule from alfalfa (Maxwell *et al.*, 1989). These facts lead me to suspect that the *Phaseolus* sample contained a *vir*-activating chalcone derivative(s). This species should be reexamined, and other members of the Fabaceae also, should be examined in particular for *vir*-inducing chalcones. Identification of legume-derived chalcone signal molecules would demonstrate homologous signalling systems in *A. tumefaciens* and *Rhizobium* species. Perhaps it will be found that chalcone signal molecules are restricted in occurrence to members of the Fabaceae, just as acetophenone signal molecules appear to be restricted in occurrence to members of the Solanaceae (described below).

3.4.2 Solanaceous *vir*-inducers

Following wounding, the *Nicotiana* species produced a pair of acetophenones and their α-hydroxy derivatives. The two known inducers acetosyringone (AS), and α-hydroxyacetosyringone (HO-AS), as well as acetovanillone (here called "AV") and a newly described compound, α-hydroxyacetovanillone (α-hydroxy-3-methoxy-4-hydroxyacetophenone, or "HO-AV") , were detected. Authentic standards were used to confirm the identities of AS, HO-AS and AV. HO-AV was identified by mass spectral similarity with authentic HO-AS (Spencer and Towers, 1990). The mass spectrum of the bis-TMSi derivative of HO-AS (*m/z* 356) exhibits low relative abundance M^+ and $[M-15]^+$ ions and a very strong $[M-103]^+$ fragment ion (base peak). This likely represents loss of $[CH_2O\text{-TMS}]$ from the molecular ion.

This pattern was also observed in the mass spectrum of HO-AV (*m/z* 326). Both leaf and stem sections produced these compounds. Leaf sections produced a greater amount of *vir*-inducers per g.f.w. *Nicotiana glauca*, a host for both wide and limited host range *Agrobacterium* strains (Yanofsky *et al.*, 1985), also produced the acetophenones. Wounded leaves of *Lycopersicon esculentum*, *Solanum dulcamara*, *S. tuberosum*, *Datura stramonium*, *Atropa belladonna*, *Hyoscyamus niger*, and *Scopolia japonicum* each produced the four acetophenones in varying proportions. From responses in plate bioassays the total activity in extracts could be estimated in terms of the equivalent activity of pure AS. Thus, *Lycopersicon esculentum* leaves produced at least 5.5 µg AS eq./g.f.w. *N. glutinosa* leaves produced about 9.5 µg AS eq./g.f.w. GC-MS revealed that sinapic and ferulic acids were not present in the active mixtures derived from these solanaceous plants. The benzoic acid derivatives, vanillic and syringic acids, were also generally absent. Vanillin and methyl vanillate were often present.

Ethyl acetate fractions from media conditioned with stems or leaves of *N. glauca* did not significantly induce virulence. In fact, 2 mm clearing zones were observed surrounding test discs, indicating the presence of a growth inhibitor. This was not further characterized. However, this result demonstrates that both activating and inhibitory substances may be released upon wounding.

The absence of the common cinnamic and benzoic acid derivatives suggests a general shunt of phenylpropanoid precursors towards C_6-C_2 compounds. It seems that the enzyme systems required for the synthesis of the acetophenones are switched on to high levels by wounding only in the Solanaceae (see Table 3.2). However, it should be stressed that some level of acetophenone synthesis occurs in the unwounded plant. The presence of the

acetophenone-glucosides in intact tobacco leaves (section 3.4.3) may simply represent a background level of biosynthesis in the absence of obvious wounding, or may reflect a small but important pool of glucosides ready for hydrolysis upon tissue damage.

3.4.3 Biosynthetic precursors of acetophenones in the *Nicotiana*

3.4.3.1 Glycosides as biosynthetic precursors

Many of the known plant phenolics are present in plant cells as water soluble glycosides contained within vacuoles. It has long been considered that glycosylation serves a dual role in deactivating the phenolic nucleus and providing water solubility (Harborne, 1979). Glycosylation of *vir*-inducing phenolics renders the phenolic inactive by blocking the phenolic hydroxyl from interacting with the bacterial receptor or sensor. For example, coniferin is inactive whereas the aglycone, coniferyl alcohol, actively induces *vir* gene expression (Spencer and Towers, 1988).

It seemed likely that signal compounds such as acetosyringone and methyl syringate are present in the plant as the corresponding glucosides. In fact, *n*-BuOH extracts from *Vitis* leaf-conditioned medium, when treated with TFA or β-glucosidase also yielded $CHCl_3$-soluble, *vir*-inducing compound(s) (data not shown). A search for pre-existing acetophenone glycosides was conducted by extraction of non-wounded tobacco leaves with hot 60 % methanol, hydrolysis with dilute TFA or emulsin (see methods), solvent partitioning for aglycones, polyamide VLC, trimethylsilyl derivitization and GC-MS.

For *N. glauca*, an estimated 100 ng AS eq./g.f.w. is present before wounding as glycosides. Following 24 hr. incubation of leaf sections, the level of *vir*-inducing aglycones recoverable from the culture medium increased to

about 2.5 μg AS eq./g.f.w. These data were estimated from plate assay responses, however we believe that it reflects a significant increase (ca. 25-fold) in the level of inducer compounds over glucoside precursor levels. The results suggest that a certain amount of the active aglycones AS and AV, as well as their α-hydroxy derivatives, may be derived from the corresponding glycosides by hydrolysis following wounding. However, the amounts present in intact leaves prior to wounding is not sufficient to account for the amount of aglycones recoverable from conditioned media. Thus, new acetophenone units must also be generated specifically in response to wounding (see also, section 3.4.3.2).

3.4.3.2 Effects of cycloheximide

Stachel *et al.* (1985) reported that 20 μM cycloheximide in the plant cell culture medium prevented production of *vir*-inducing compounds from tobacco leaf discs. In the present study, it was established by polyamide VLC and GC-MS, that 20 μM cycloheximide specifically inhibits production of large amounts of the acetophenones by incubated tobacco leaf sections. The results are in agreement with those of Stachel *et al.* (1985b). Apparently, *de novo* biosynthesis of signal molecules does occur.

Despite the cycloheximide treatment, a small amount of each of the acetophenone aglycones were detected by GC-MS. For cycloheximide to completely prevent production of active signal compounds, plant glucosidases would have to be newly synthesized following wounding. However, glucosidase activity of cell walls is well established (see Fry, 1988), and this activity likely accounts for the production of some of the aglycones. Based on AS equivalents determined from plate assay responses for extracts from *N. silvestris*, an estimated 15-fold higher level of signal compound

production was found in the absence of cycloheximide. These results suggest that cycloheximide prevents formation of new acetophenones, but does not prevent formation of the aglycones from the small pool of glucosides by the action of endogenous β-glucosidase. Also, a small amount of *de novo* acetophenone biosynthesis may result from the action of a low level of the required enzymes present before the cycloheximide treatment.

The advantage, if any, to the plants producing these compounds upon wounding is not clear. The toxicity of the acetophenones in comparison with the phenolic acids towards other organisms should be investigated. AS is not strongly antifungal (Threlfall and Whitehead, 1988), and only at concentrations above 200 μM does it inhibit growth of *A. tumefaciens* (Spencer and Towers, 1988). It is interesting to note that addition of 50 μM AS and AV inhibit *nod* gene induction in *Rhizobium leguminosarum* by 46% and 85% respectively (Firmin *et al.*, 1986).

3.4.3.3 Cellulase treated cell wall material

The cellulase treatment released a quantity of *vir*-activating substances. It was of interest to establish whether acetophenones such as AS could be released from cell walls by this treatment, ie. whether AS is a component of cell walls in the *Nicotiana*. This result would explain the cellulase-induced production of AS in tobacco suspension cultures reported by Threlfall and Whitehead (1988). It would also have been the first report of cell wall bound acetophenones in plants.

GC-MS analysis did not confirm the release of acetophenones from cell wall material, however, both *cis* and *trans*-ferulic acid and small amounts of *cis* and *trans*-sinapic acid were detected. Perhaps these cinnamic acids are a

source of precursors in the production of the acetophenones following wounding.

3.4.4 Inducers and inhibitors from monocots

One line of research was to investigate whether there was any clear evidence for the elaboration of differing sets of signal compounds amongst hosts and nonhosts of *A. tumefaciens*. Since the monocots are generally resistant to infection by *Agrobacterium* strains, a few representatives were included in the survey. Surprisingly, monocot crown region, tuber and rhizome tissues were found to produce a number of the signal compounds elaborated by susceptible hosts. Cultured segments of the rhizome of *Zingiber officinale* exuded detectable amounts of *vir*-inducing phenolic acids into the medium. Transformation of *Asparagus officinalis*, without addition of exogenous virulence inducing compounds, has been demonstrated (Hernalsteens *et al.*, 1984). The active extract from stems of *Asparagus officinalis* contained methyl vanillate, but none of the other common signal compounds. *Zea mays* crown tissue produced the common *vir*-inducing phenolic acids mixture. Clearly, other factors, perhaps including *vir*-repressors, wound-induced monosaccharides and pH effects, must play a role in resistance of monocots to crown gall disease.

From wounded maize conditioned medium, *vir*-inducers were obtained in the $CHCl_3$ soluble fraction, and the *vir*-inhibitory hydroxamates DIMBOA and its breakdown products were tentatively identified in the EtOAc soluble fraction. Under the HPLC conditions described, the hydroxamates were eluted in the following order: DIM_2BOA, 22.69 min.; HMBOA, 23.76 min.; DIMBOA, 24.88 min.; MBOA, 25.79 min. These were tentatively identified by comparison with elution profiles obtained by Dr. Thor Arnason

(University of Ottawa, personal communication). Thus, upon inoculation of these monocot tissues with *A. tumefaciens*, the bacteria would be subject to both *vir*-activating and *vir*-inhibitory compounds. Another important bacterial growth and virulence factor, pH, is discussed in section 3.4.6.

3.4.5 Conifer extracts

Conifers produced other *vir*-activating compounds when wounded, but, clearing zones were also noted on assay plates. The active compounds produced by wounded *Taxus baccata* needles and stems included vanillin and coniferyl alcohol. The inhibitory compounds remain unidentified. Perhaps these compounds could be isolated by way of "*vir*-suppression" bioassays. A quinone (2,6-dimethoxyhydroquinone) may have been present in the active VLC fraction obtained from *Taxus baccata* (see Chapter 5). The effects of this and other quinones on *vir*-induction should be investigated. *Pseudotsuga menzeisii* produced very small quantities of vanillin and syringaldehyde. *Picea sitchensis* and *Thuja plicata* produced such small quantities of virulence inducers that GC-MS results were inconclusive. The presence of only low levels of *vir*-inducers from conifers is consistent with the fact that conifer species are resistant to crown gall disease.

Picien and androsin are produced by spruce needles, and upon damage they are rapidly converted to the aglycones, *p*-hydroxyacetophenone and acetovanillone (Oswald and Benz, 1989). Acetovanillone is another virulence inducing phenolic compound (Stachel *et al.*, 1985; Spencer and Towers, 1988), and so upon wounding this species one would suspect that this compound must have an effect on the level of *vir* gene expression. Curiously, this compound was not found in active VLC fractions from spruce.

3.4.6 pH effects

In most cases, the pH of the 24 hr. conditioned medium was recorded. Initially, the pH of the MS culture medium (15 g sucrose/L) was adjusted to pH 5.7. In the absence of a buffer system (as would be the case under natural circumstances), the ability of wounded plant tissues to acidify the culture medium may greatly effect the degree of *vir*-induction. The final pH after incubation of wounded plant tissues varied by 3 pH units between plant species (Table 3.3). This represents a fairly large range when compared with the optimum range for *vir*-induction (section 1.2).

Stem tissues and leaf tissues from the same plant species differed in their ability to reduce pH. This was further studied in the *Nicotina* species. For each species studied, wounded stem tissue conditioned media were of lower pH than wounded leaf conditioned media. Wounded *N. tabacum* stems lowered the pH to a level comparable to that by some monocots and conifer tissues. *N. rustica* leaves and *Solanum tuberosum* tuber tissues slightly raised the pH. Data concerning factors such as pH may be important in understanding crown gall host range phenomena. Wounded tissues that significantly reduce or increase the pH beyond the optimum for induction of virulence may completely prevent transformation.

A number of conifers and monocotyledons acidified the medium to a level that would lie outside the optimum pH for *vir*-induction. However, *Rosa* species are quite susceptible to crown gall and yet the collection reported here, a wild rose species, had a strongly acidifying effect on the culture medium. The lowest pH was recorded for media conditioned with wounded *A. officinale* stem tissue, and yet this is a monocot on which crown gall tumors can be produced. These last two observations do not support the view

that the ability to reduce the pH of the culture medium correlates with transformability.

TABLE 3.3 Final pH values.

Arabidopsis thaliana	stems	5.79
Asparagus officinale	stems	3.01
Beta vulgaris	tuber	5.17
Daucus carota	tuber	4.74
Dioscorea	tuber	4.19
Geranium richardsonii	stems	4.48
Hydrangea sp.	stems	3.86
Kalanchoë esculentum	leaves	4.64
Lycopersicon esculentum	leaves	4.13
N. glauca	leaves	5.59
N. glauca	stems	4.66
N. plumbaginafolia	stems	5.25
N. rustica	leaves	6.17
N. rustica	stems	4.49
N. silvestris	leaves	5.63
N. tabacum (Samsun)	stems	4.10
N. tabacum (White burley)	leaves	5.37
N. tabacum (White burley)	stems	4.10
Picea sitchensis	stems	3.52
Polygonum aubertii	stems	4.11
Populus , hybrid H11	stems	5.60
Pseudotsuga menzeisii	stems	3.87
Rosa sp.	stems	3.75
Solanum dulcimara	leaves	4.90
Solanum tuberosum	leaves	6.02
Solanum tuberosum	tuber	6.20
Taxus bacata	needles	4.47
Trifolium repens	stolons	4.78
Vitis sp. (cv. DeChaunac)	stem sap	5.30
Zea mays	crown	4.54
Zingiber officinale	rhizome	4.53

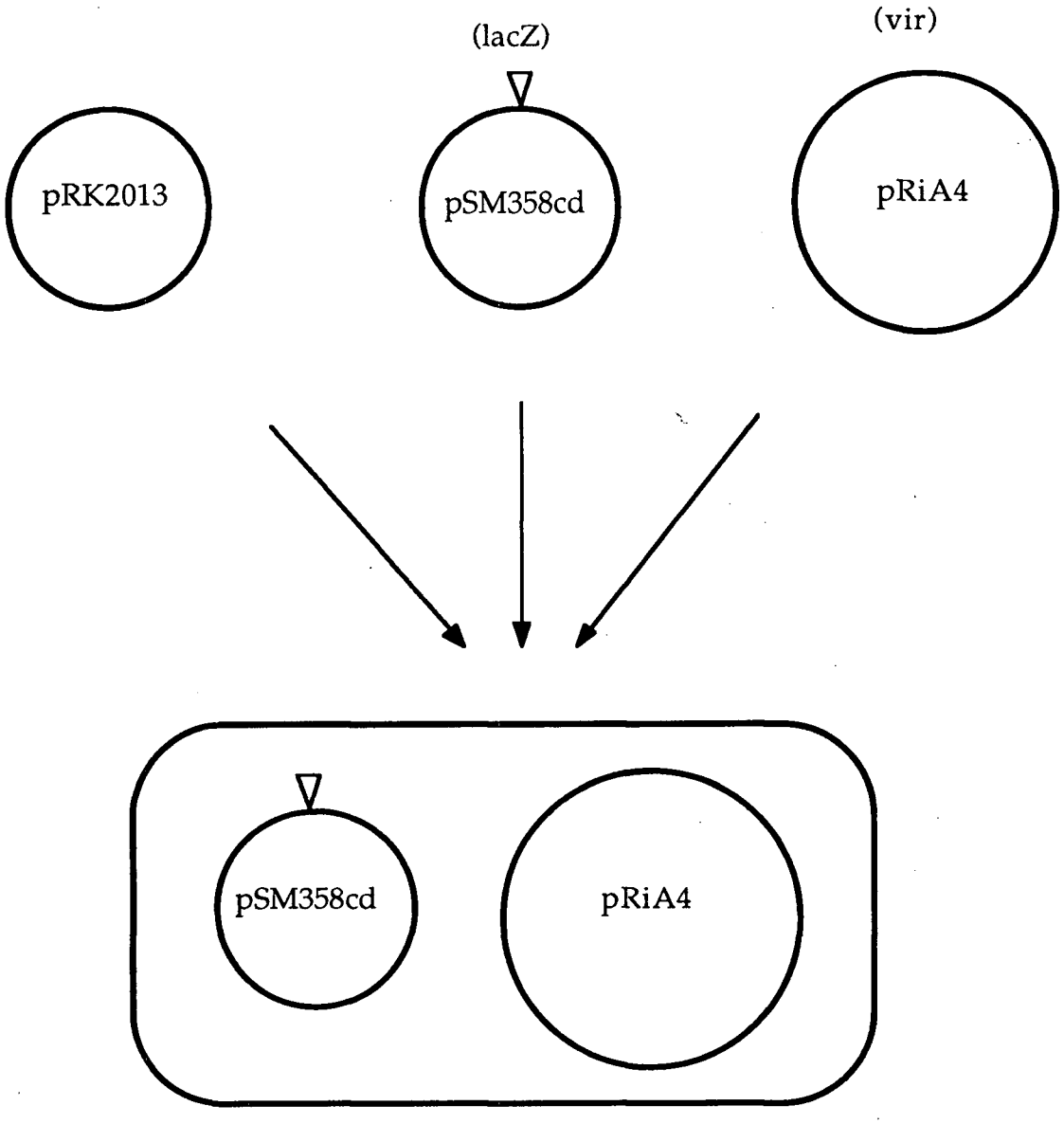
Chapter 4: *vir* expression in *A. rhizogenes*

4.1 Introduction

The analysis of *vir* gene expression in *Agrobacterium* species need not be restricted to wide and limited host range strains of *A. tumefaciens*. Another species of *Agrobacterium*, *A. rhizogenes*, is also capable of transforming plant cells, in this case causing hairy root disease. As in crown gall disease, DNA is transferred to susceptible plant cells (Chilton *et al.*, 1982). This very likely follows expression of a set of *vir* genes, although the analysis *vir* gene expression in this species has not been reported. The bacteria gain access to susceptible cells through wounds in plant roots, and presumably wound induced phenolics are responsible for *vir*-induction. The main objective of the experiments described in this chapter was to ascertain whether the *vir* genes of *A. rhizogenes* are induced by the phenolic compounds which induce *vir* genes of *A. tumefaciens*.

This chapter describes the results of triparental mating experiments and subsequent *vir*-induction assays with an *Agrobacterium rhizogenes* strain. The *A. rhizogenes* strain "A4" has been widely used to generate hairy root cultures of many plant species. The *vir* region of *A. rhizogenes* is known to be very similar to that of *A. tumefaciens* (Hirayama *et al.*, 1988), so it seemed that introduction of a *vir*::*lac* gene fusion ("reporter") plasmid into *A. rhizogenes* would likely permit analysis of *vir* gene expression in this species.

The *vir*E::*lac*Z fusion plasmid pSM358cd (Winans *et al.*, 1986), which contains the *A. tumefaciens vir*E promoter, was introduced into *A. rhizogenes* A4 by mating with *E. coli* strains pRK 2013 (helper) and pSM358cd



Agrobacterium rhizogenes A4/pSM358cd

(virE::lacZ)

Figure 4.1 Introduction of a *vir::lac* insertion plasmid into *A. rhizogenes* A4 by triparental mating.

(donor) to make the derivative A4/pSM358cd (outlined in Figure 4.1). The plasmid pSM358cd was chosen because the level of *vir* activity from its *vir*E promoter is greater than *vir*B reporter gene fusion pSM243cd (Winans *et al.*, 1986) and therefore *vir*-activation may be more accurately quantified. In this chapter, a brief structure-activity analysis of *vir* gene expression in the new strain A4/pSM358cd, like that reported by Spencer and Towers (1988), is described.

4.2 Methods

4.2.1 Triparental mating

Log phase cultures of donor, helper and recipient bacterial strains were prepared. The *E. coli* strains (kindly provided by Dr. Eugene Nester, U.W.) were grown at 37 °C in LB medium with the appropriate antibiotics. The helper strain (*E. coli* pRK 2013) was cultured in the presence of spectinomycin (30 μg/mL). The donor strain was cultured in the presence of carbenicillin and kanamycin (100 μg/mL). The recipient strain "A4" was first screened for resistance to antibiotics that could be used following mating to select for the recipient (transconjugant). *A. rhizogenes* A4 (nal^r), grown in PDB medium at 26° C required a longer culture period than did the *E. coli* strains (freshly prepared PDB from potatoes is recomended). Triparental mating was established between the *E. coli* strains JC 2926/pRK2013 ($spec^r$) and JC 2926/pSM358cd (kan^r, $carb^r$) and *A. rhizogenes* A4 (nal^r) by mixing together small swabs of each culture on an LB plate without antibiotics. After 24 hrs. at 28 °C, a sample was streaked out on a PDA selection plate containing kanamycin, carbenicillin and nalidixic acid. Colonies were selected and grown at 28° C in PD liquid medium with the same antibiotics. The

transconjugant (A4/pSM358cd) was grown in liquid culture and 0.5 mL 50% glycerol stocks stored at -80 °C in sterile Epindorf tubes.

4.2.2 *vir*-induction assays

For *vir*-induction assays, 10 mL log phase A4/pSM358cd cultures in freshly prepared PDB (potato dextrose broth) with 100 µg/mL carbenicillin were prepared from glycerol stocks and 100 µL of culture added to 10 mL of solutions of test compounds in MES buffered AB (Chilton *et al.*, 1974) medium at pH 5.5.

Quantitative ß-galactosidase assays following induction (10 hrs./ 26-28°C, 150 rpm) in filter sterilized pH 5.5 AB medium (100 µg/mL carbenicillin) containing 0.1% DMSO, were conducted essentially as described by Miller (1972). When quantitatively assaying the effect of pure compounds, sufficient DMSO was added to dissolve the crystals (roughly 40-60 µL) and sufficient AB medium added (roughly 40-60 mL) to make 50-400 µM stocks solutions containing 0.1% DMSO. As was previously reported (Spencer and Towers, 1988), difficulty was encountered in attempting to resuspend the chalcone derivative to concentrations greater than 50 µM.

4.3 Results and discussion

Preliminary ß-galactosidase assays indicated that triparental mating was successful. A 20 hour assay of A4/pSM358cd with and without acetosyringone in pH 5.5 AB medium supplemented with carbenicillin (100 µg/mL) was conducted at 21 and 28 °C. Some 8000 units of induced activity was recorded. This suggested that A4 is indeed induced by the same sort of

phenolics that induce *A. tumefaciens*. This level of activity is significantly greater than that observed with either WHR or LHR *A. tumefaciens* strains.

Activity curves for a collection of phenolic compounds are shown in Figures 4.2-4.7. A range of methoxyphenols were found to be capable of stimulating virulence in WHR *A. tumefaciens* (Spencer and Towers, 1988). Here, A4/pSM358cd was incubated with a number of these same compounds. These include acetosyringone, acetovanillone, vanillin, syringaldehyde, the acids vanillic, syringic, ferulic, sinapic acid and their methyl esters, coniferyl alcohol, and 2',4',4-trihydroxy-3-methoxychalcone. In this way the relative effectiveness of benzaldehydes, benzoic acids and their methyl esters, acetophenones, cinnamic acids and their methyl esters was assessed. In each case, phenolics of guaiacyl ring substitution were less effective than the corresponding syringyl substituted compounds. Also, in each case the methyl esters were more effective than the corresponding free acid.

The activity of the monolignol coniferyl alcohol and of the chalcone 2',4',4-trihydroxy-3-methoxychalcone were examined. The activity of the monolignol suggests that a carbonyl group is not an absolute requirement for *vir*-induction. The chalcone was tested to determine whether such a structure could activate *vir* genes in this species as it did in *A. tumefaciens* (Spencer and Towers, 1988). The curve of activity for the chalcone lies to the left of that of AS, indicating a more strongly active structure than that of the acetophenone.

The first general conclusion concerning *vir* gene expression in *A. rhizogenes* is that acetosyringone and other common plant phenolics are capable of inducing the sort of response observed with WHR and LHR *A. tumefaciens* strains. Second, there are significant differences in the absolute level of induction recorded in these three *Agrobacterium* strains. The units

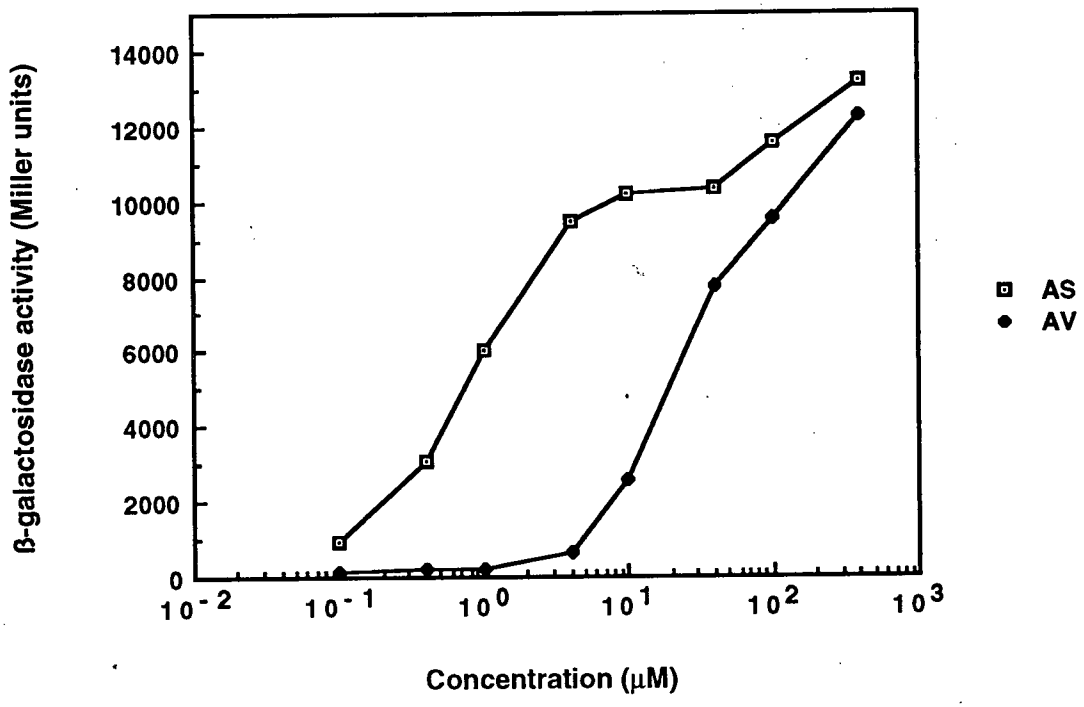


Figure 4.2 *vir* gene activation in A4/pSM358cd by acetophenones AS and AV

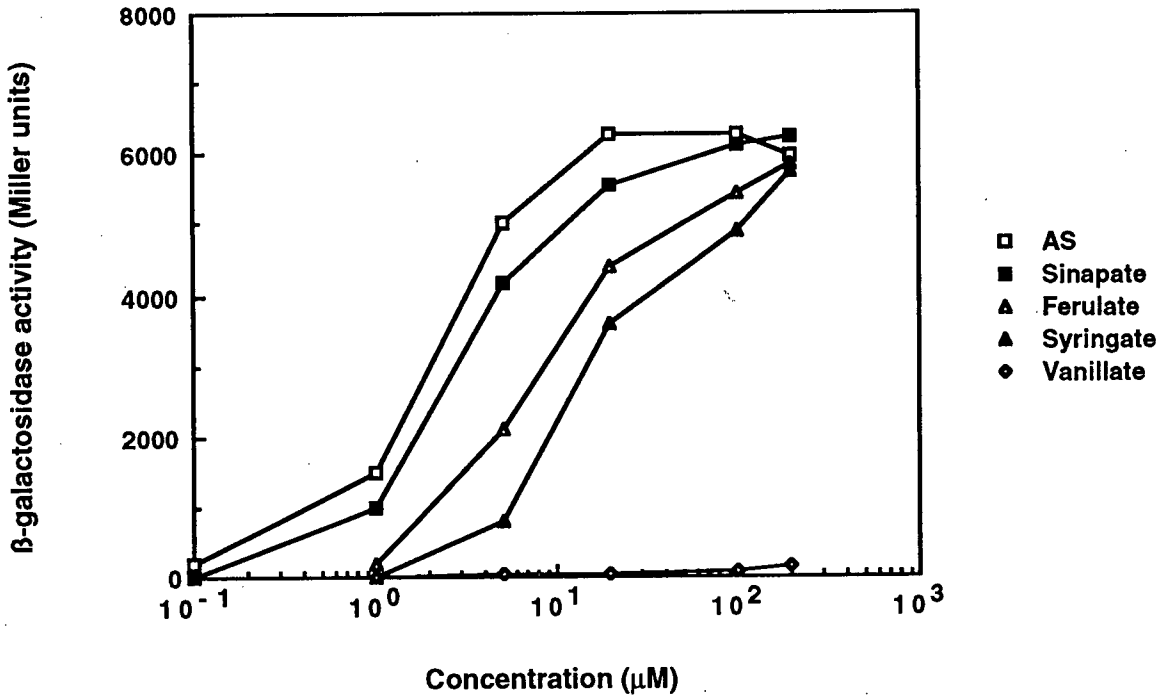


Figure 4.3 *vir* gene activation in A4/pSM358cd by methoxyphenolic acids

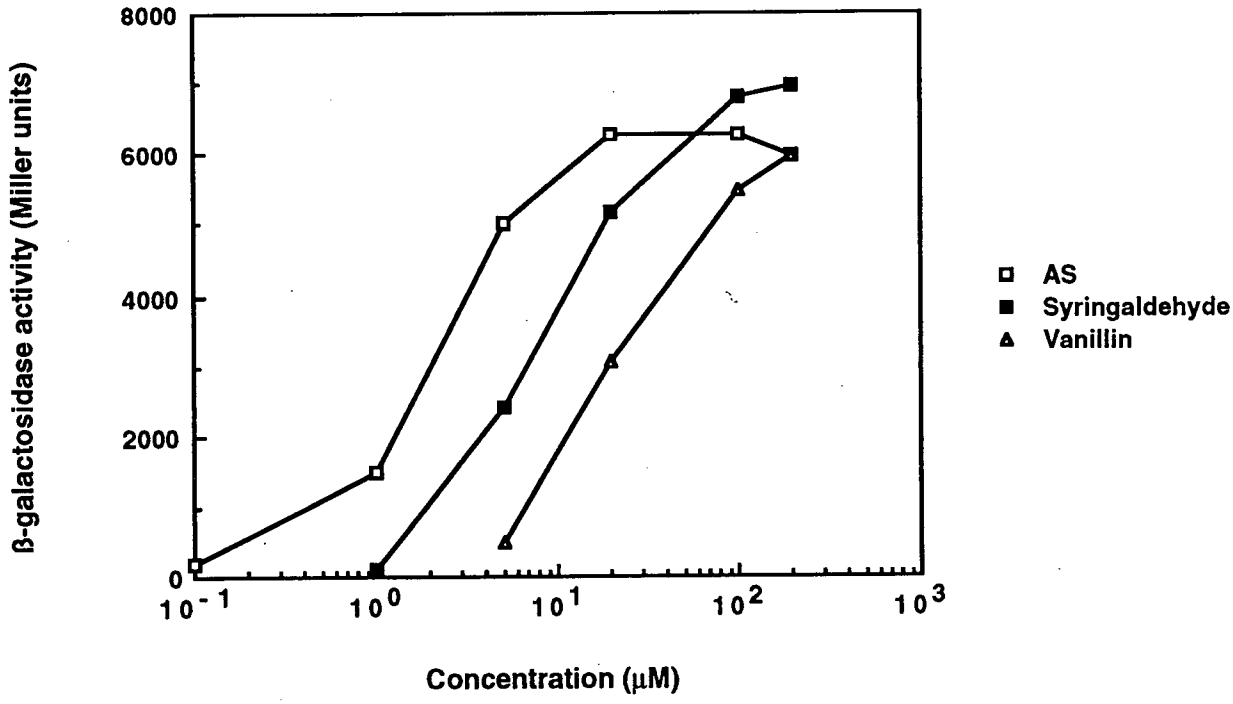


Figure 4.4 *vir* gene activation in A4/pSM358cd by methoxybenzaldehydes

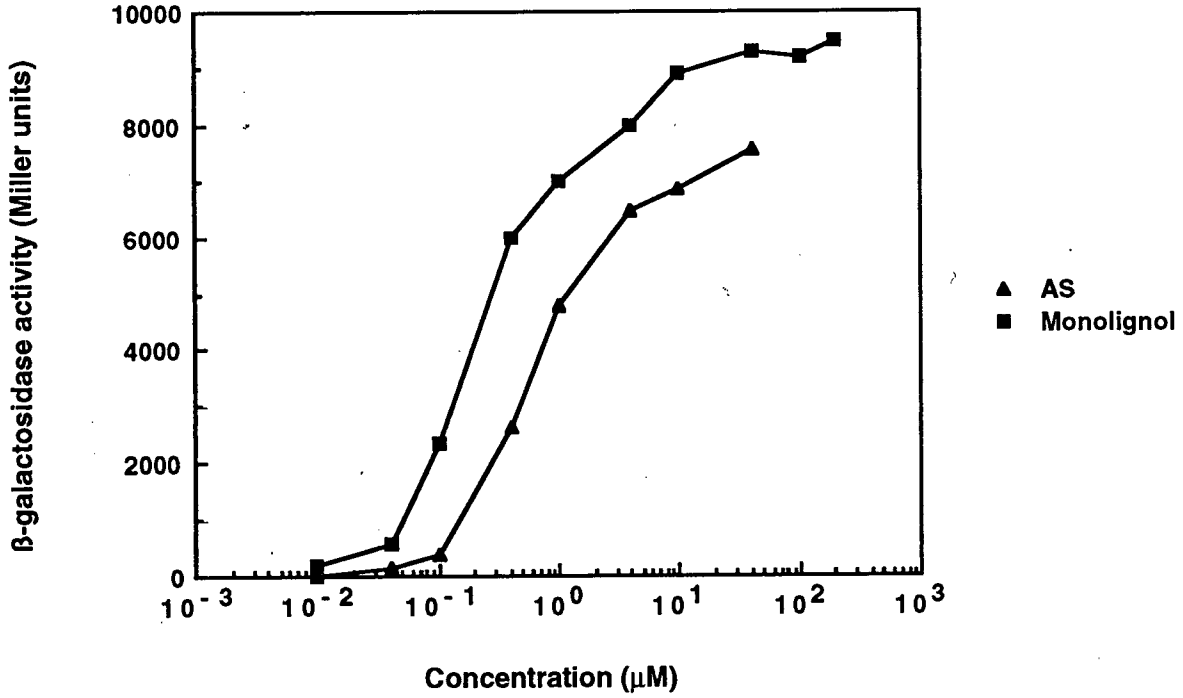


Figure 4.5 *vir* gene activation in A4/pSM358cd by coniferyl alcohol

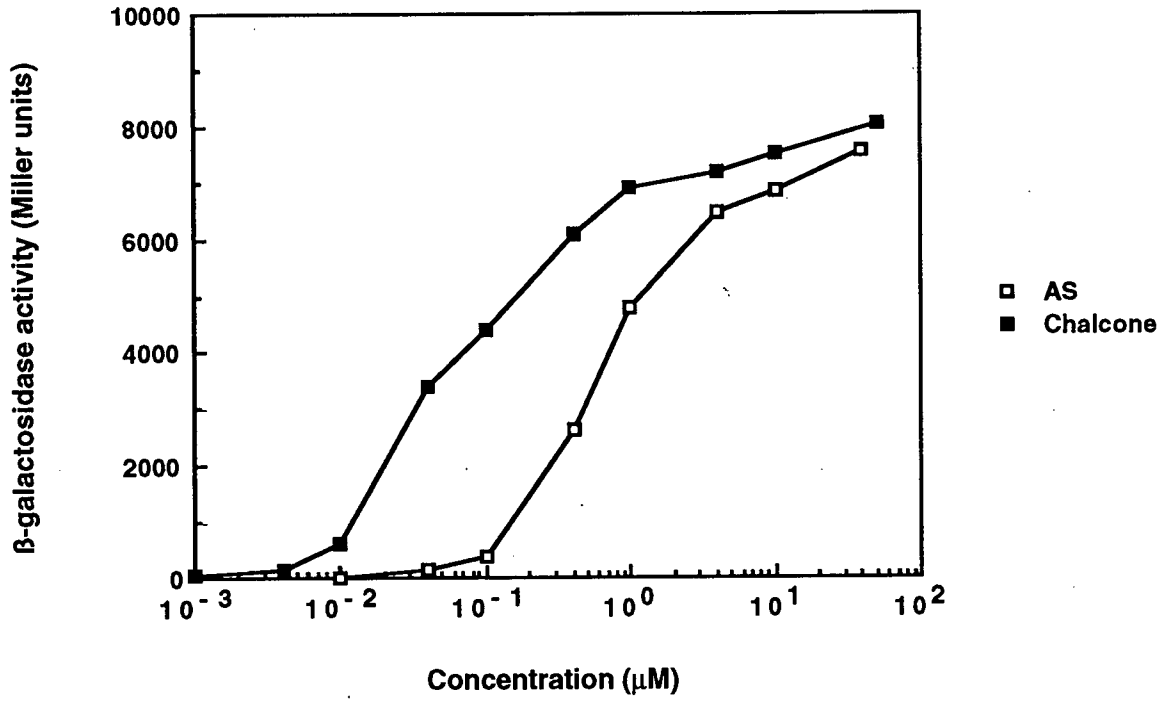


Figure 4.6 *vir* gene åctivation in A4/pSM358cd by 2',4',4-trihydroxy-3-methoxy chalcone.

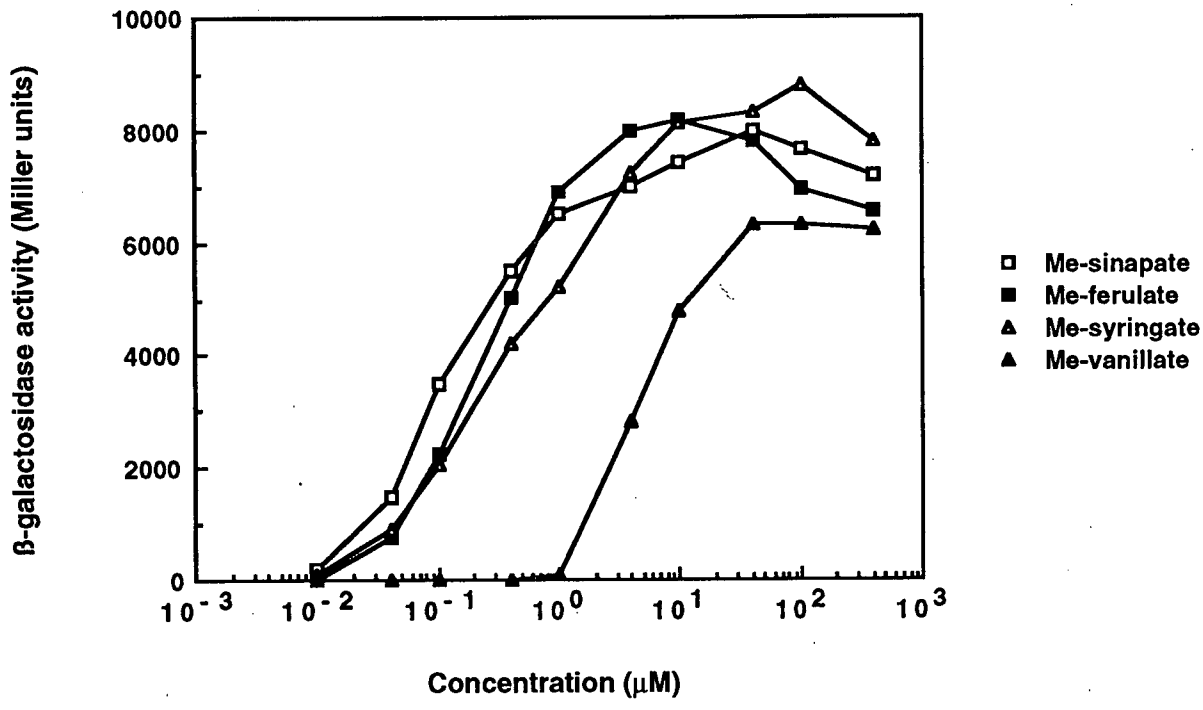


Figure 4.7 *vir* gene activation in A4/pSM358cd by phenolic methyl esters.

of activity recorded in the strain A4/pSM358cd greatly exceeded that observed in WHR strain A348. Similarly, at WHR and LHR *vir*B loci, the units of activity recorded in the strain A348/pSM243cd greatly exceeded that observed in LHR strain A856/pSM243cd (Spencer *et al.*, 1990). This may be connected with a somewhat longer culture period required by *A. rhizogenes* strain, or perhaps more stringent conditions of induction.

A comparison of the biological activity of various chemical structures was provided by application of authentic compounds. Phenolics of syringyl substitution are more active than the corresponding guiacyl substituted phenols. This applies to the benzoic acid derivatives vanillic and syringic acid, the cinnamic acid derivatives ferulic and sinapic acid (Figure 4.3), and to the aldehydes vanillin and syringaldehyde (Figure 4.4). The methyl esters of phenolic acids are more active than the free acids (Figure 4.7).

For compounds other than the chalcone, the optimum concentration for *vir*-induction was 10μM or greater. Concentrations as low as 0.1 μM were sufficient for half-maximal induction. The background level of induction, sometimes 1000 Miller units, was greater than that observed in *A. tumefaciens* strains (Spencer and Towers, 1988). Perhaps this is as a result of preculture of the bacteria in potato-dextrose broth, which may well contain active *vir*-inducing compounds. This background level of induction did not present a problem in analysis of *vir*-induction because these units of β-galactosidase activity was subtracted from the level of activity following induction, to give the induced units of activity.

Detectable levels of induction were observed with relatively low concentrations of inducer compounds compared with those required for *vir*-induction in *A. tumefaciens* strains. Thus, for a number of compounds, the

effective concentration of inducer extends into the nanomolar range. A low level of induction was noted by the chalcone at 10 nM.

These observations suggest that, while its sensitivity towards efective signal compounds may be diffierent, *A. rhizogenes* detects the same range of compounds as does *A. tumefaciens*. It was recently discovered that a naturally occurring chalcone (4,4'-dihydroxy-2'-methoxychalcone) acted along with two related flavonoids (4',7-dihydroxyflavone and 4',7-dihydroxyflavanone) in signalling *Rhizobium meliloti* (Maxwell *et al.*, 1989). Their studies showed that the chalcone was significantly more active than the other flavonoids. Assays with an authentic chalcone, 2',4',4-trihydroxychalcone, confirmed that chalcones were more active than the flavanone.

The activity of chalcones in signalling *A. tumefaciens*, and *A. rhizogenes* as well as *Rhizobium* species, and their relative activity compared with other structures, confirms the close genetic and biochemical relationship of these bacteria. These bacteria appear to have exploited different aspects of the same sort of signalling system. Generally speaking, rhizobia appear to have adapted to the presence of naturally occurring flavonoid root exudates, whereas agrobacteria have adapted to the presence of wound induced methoxy-benzoic and -cinnamic acid derivatives.

The experiments described in this chapter should be followed by identification of naturally occurring *vir*-inducers of *A. rhizogenes* in wounded root exudates. Since a number of *vir*-activating methoxyphenols have been identified from potato tubers and carrot roots (Chapter 3) it seems very likely that these compounds are naturally occurring *A. rhizogenes vir*-inducers.

Chapter 5: GC-mass spectrometry of *vir*-inducing mixtures

5.1 Introduction

GC-MS (combined gas chromatography-mass spectrometry) is an ideal method for the rapid identification of the phenolic components present in partially purified *vir*-inducing mixtures. It offers important advantages over HPLC analysis in that determinations of chemical structures are not dependent on UV absorption spectra, which are not diagnostic for specific phenolic compounds even with a photodiode array detector. In comparison, GC retention time and mass spectra, especially of TMS derivatives, provide rigorous proof of a compounds identity. In addition, only very small amounts of sample mixtures are required, and a number of components can be identified in a single GC-MS run.

A significant limitation in GC-MS is the requirement for sample vaporization (Vouros, 1980). Chemical derivitization is widely used to increase volatility, and can greatly assist mass spectral analysis by generating abundant molecular ions and fragments of diagnostic value. Treatment of samples with the derivatizing agent BSTFA (bis-(trimethylsilyl)trifluoroacetamide) in dry pyridine yields the trimethylsilyl (TMS) derivatives of active hydrogens. Concerning signal compound mixture analysis, each *vir*-activating structure forms a TMS ether at the phenolic hydroxyl group. In addition, benzoic and cinnamic acids also form TMS esters.

The derivatized mixture of compounds is then separated by gas chromatography (GC) on a fused silica capillary column. As the TMS derivatives are eluted, they are fed into the mass spectrometer, ionized by

electron impact (70 eV), and the mass and abundance of the resultant molecular and fragment ions are recorded. The GC retention times and the mass spectra of the compounds are characteristic.

5.2 Methods

5.2.1 Derivatization

Aliquots (ca. 400 μg, i.e. 40 μL of 10 mg/mL solutions) of active extracts were prepared for BSTFA treatment by first drying small amounts in micro-"reactivials" on a freeze drying apparatus for at least 24 hrs. With oven dried syringes, 5 μL of redistilled, dry pyridine, followed by 5 μL of BSTFA (Sigma Co.) was added to each sample. Periodically pyridine was fractionally distilled, collected over molecular sieves, and stored in darkness under N_2 to ensure dryness. Heating was not required to achieve derivitization and 1 μL samples were injected directly onto the GC.

5.2.2 GC conditons

The GC was fitted with a fused-silica capillary column (30m x 0.25 mm) of SE-54 with He as the carrier gas. The column was held at 150°C for 2 min, programmed at 10°C min^{-1} to 300°C and held for 10 min. The injector and zone temp. was 250°C. 1000 scans were accumulated (in 17.30 min.) over each GC run. Compounds of interest were eluted from about 200 to 800 scans (R_t 3.46-13.84 min.).

5.2.3 Mass spectrometer

The mass spectrometer component of the combined GC-MS was a Finnigan automated MS model 1020 operated in the electron-ionization

mode (70 eV) with an ion source temperature of 95°. An on line library of mass spectra (National Bureau of Standards) was used, in conjunction with authentic standards, to assist in identification of plant-derived compounds.

5.3 Results and Discussion

The technique of combined GC-mass spectroscopy was found to be ideal for the identification of phenolic compounds present in partially purified *vir* -inducing mixtures. Only very small quantities of the active fractions were required (although this sometimes required the entire sample), and the components were well separated by gas chromatography on the fused-silica capillary column. With few exceptions, the mass spectrum and retention time of each compound were unique and often these were readily compared with those of an authentic standard.

Spectral data accumulated over each GC-MS run were temporarily stored on hard disks (10 MB capacity). Data processing entailed examination of the mass spectrum of each major GC peak, and computer-assisted searches for specified molecular or fragment ions. Because the derivatized phenolics were eluted at predictable times in each GC run, the number of mass spectra that required examination was greatly reduced. A typical spectrum consisted of a strong M^+ ion, plus major fragment ions of $[M-73]^+$, $[M-15]^+$, $[M-30]^+$, and often $[M-45]^+$.

Mass spectra of interest were compared either with those stored in an on-line library (National Bureau of Standards), or with spectra of authentic compounds. Mass spectra of 11 of the *vir*-activating methoxyphenols observed during the course of this study are shown in Figures 5.1-5.11. These are presented in order of acetophenones, α-hydroxyacetophenones, cinnamic

acids, benzoic acids, methyl esters, and the monolignol coniferyl alcohol. Table 5.1 lists the compounds observed during the course of this survey. These were observed either in active extracts or were isolated directly from wounded plant tissues. A number of these are very well known ("common") monocyclic phenolic compounds. Characteristic molecular and fragment ions for a number of key compounds are listed in Table 5.2. This table also lists the GC R_t (by scan number). Table 5.3 lists the molecular weights and molecular ions of various TMS derivatives, and their approximate retention time (by scan number in a 1000 scan run).

The most commonly observed *vir*-inducing compounds included vanillic, syringic, ferulic, sinapic acids (Figures 5.5-5.8). However, a variety of other phenolics were detected in active mixtures. Traces of vanillin and/or syringaldehyde were detected in many samples. The known virulence inducers, AS and HO-AS were identified as their TMS ethers (Figure 5.1 and 5.2) only in an extracts from species in the Solanaceae (discussed further in Chapter 3). Also identified were the TMS derivatives of AV and HO-AV (Figure 5.3 and 5.4). The TMS ether of AS produces a very strong M^+ at *m/z* 268 , $[M-15]^+$ at *m/z* 253, $[M-30]^+$ at *m/z* 238, $[M-45]^+$ at *m/z* 223, whereas the TMS ether of AV (30 mass units less than AS) produces a very strong M^+ at *m/z* 238 , $[M-15]^+$ at *m/z* 223, $[M-30]^+$ at *m/z* 208, $[M-45]^+$ at *m/z* 193. The two

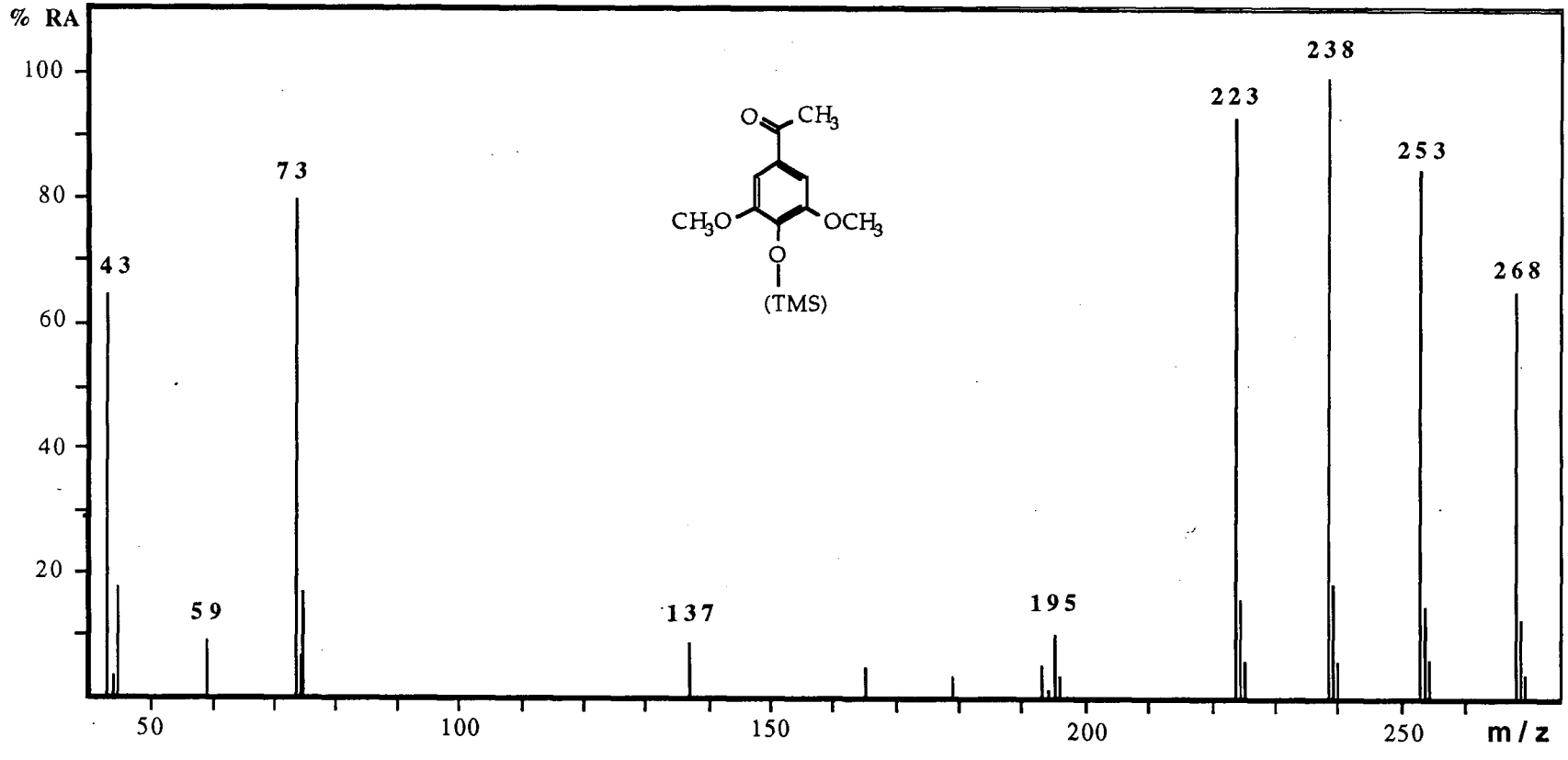


Figure 5.1 Mass spectrum of 3,5-dimethoxy-4-hydroxyacetophenone (TMS derivative)

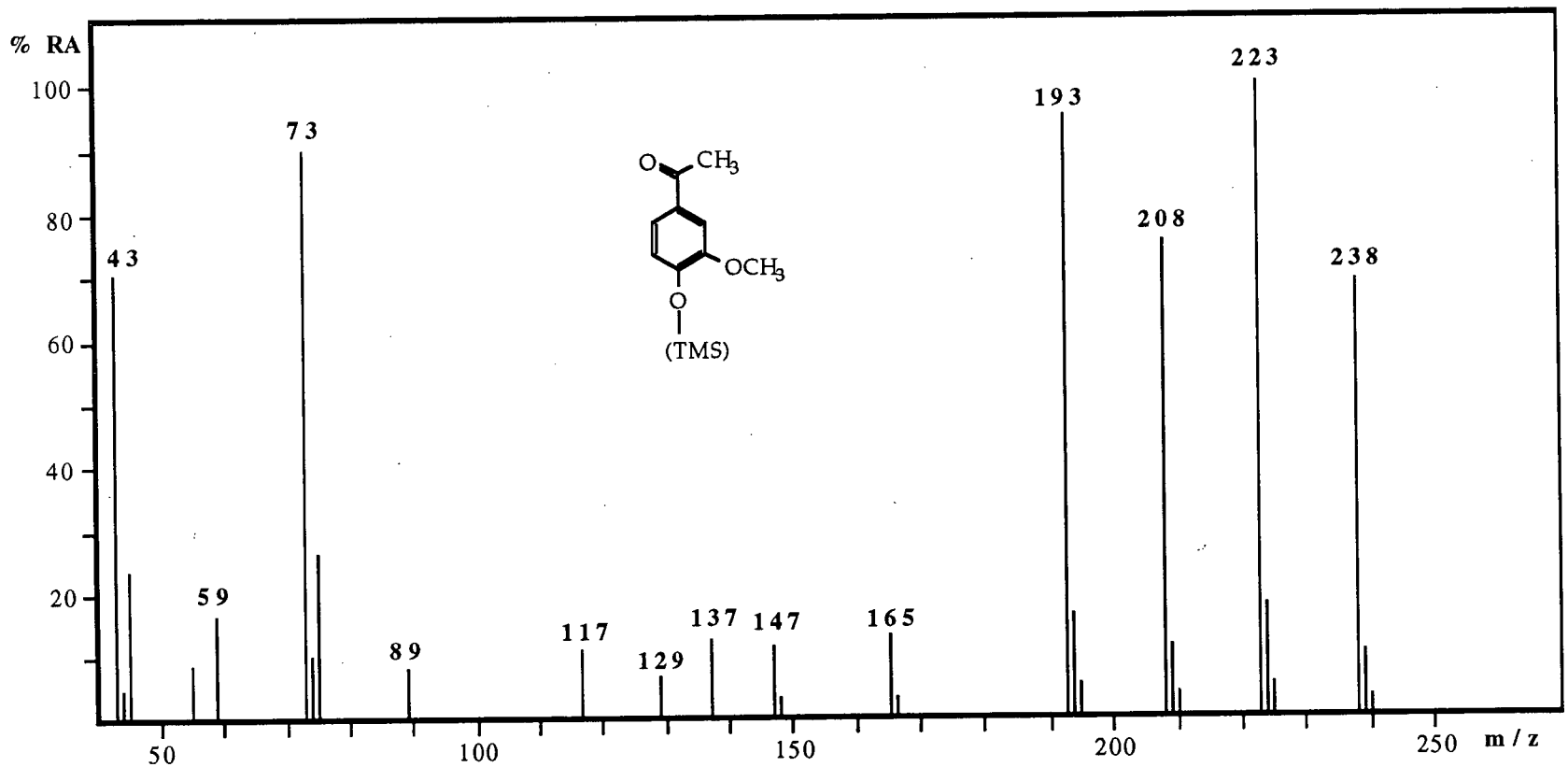


Figure 5.2 Mass spectrum of 3-methoxy-4-hydroxyacetophenone (TMS derivative)

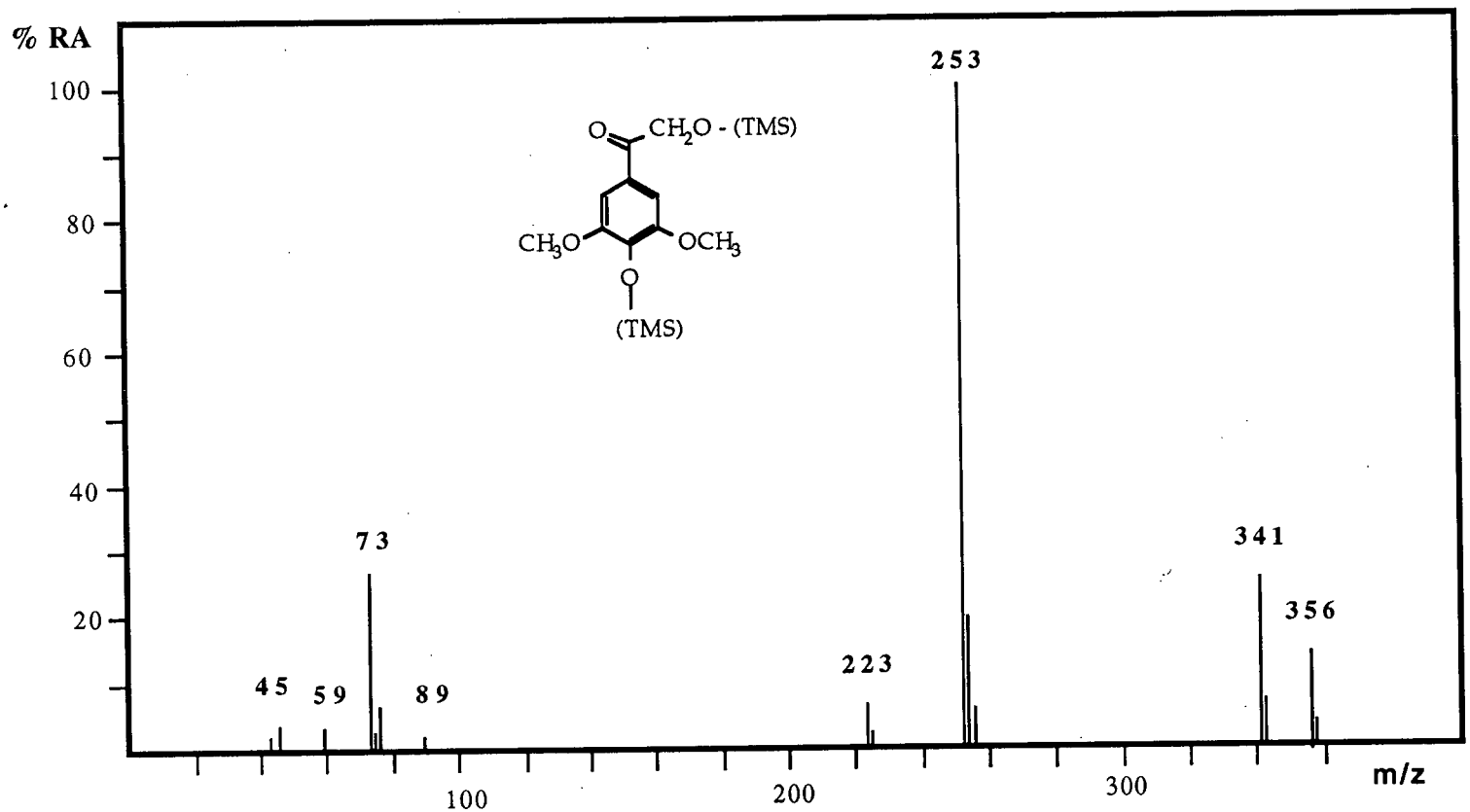


Figure 5.3 Mass spectrum of α-hydroxy-3,5-dimethoxy-4-hydroxyacetophenone (TMS derivative)

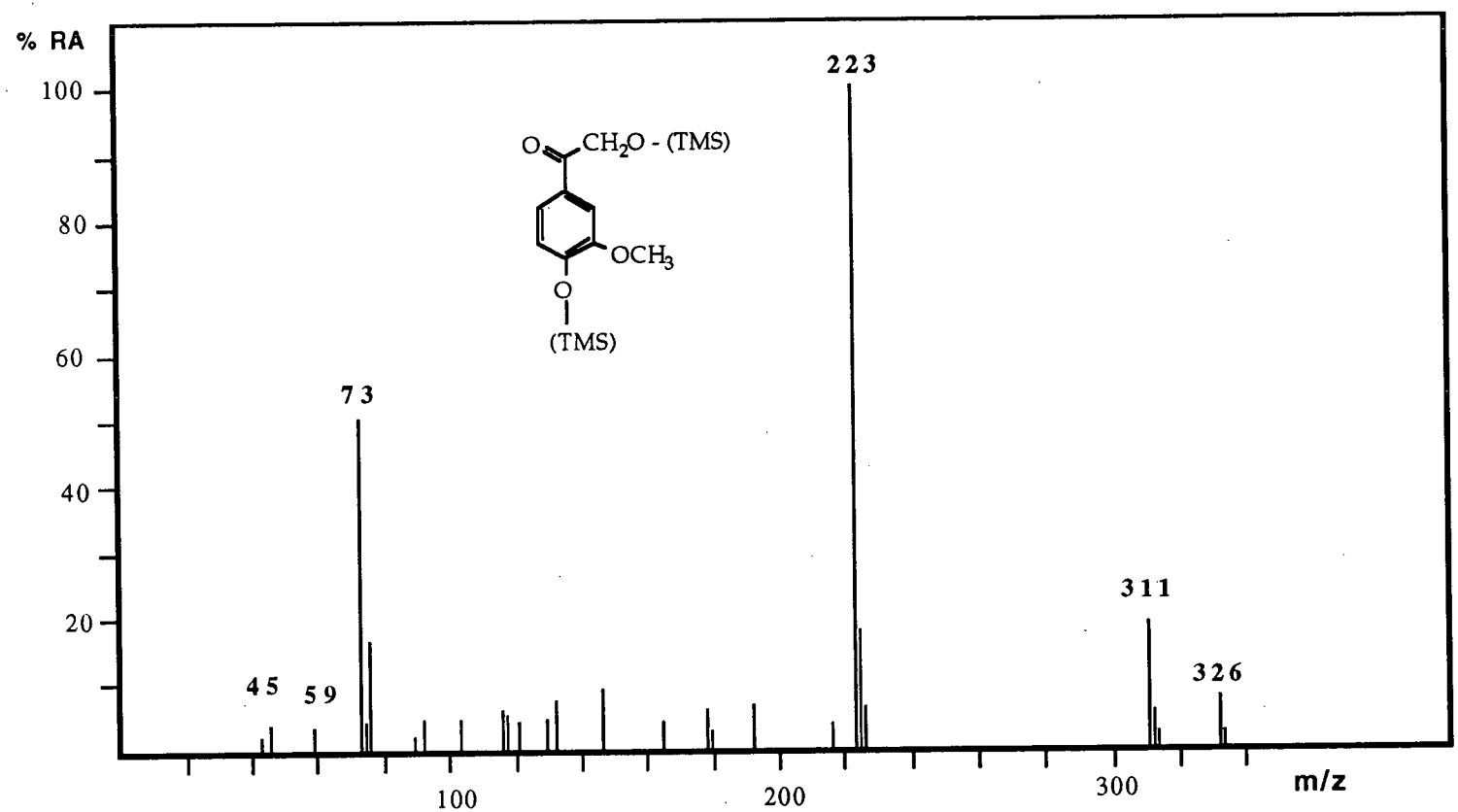


Figure 5.4 Mass spectrum of α-hydroxy-3-methoxy-4-hydroxyacetophenone (TMS derivative)

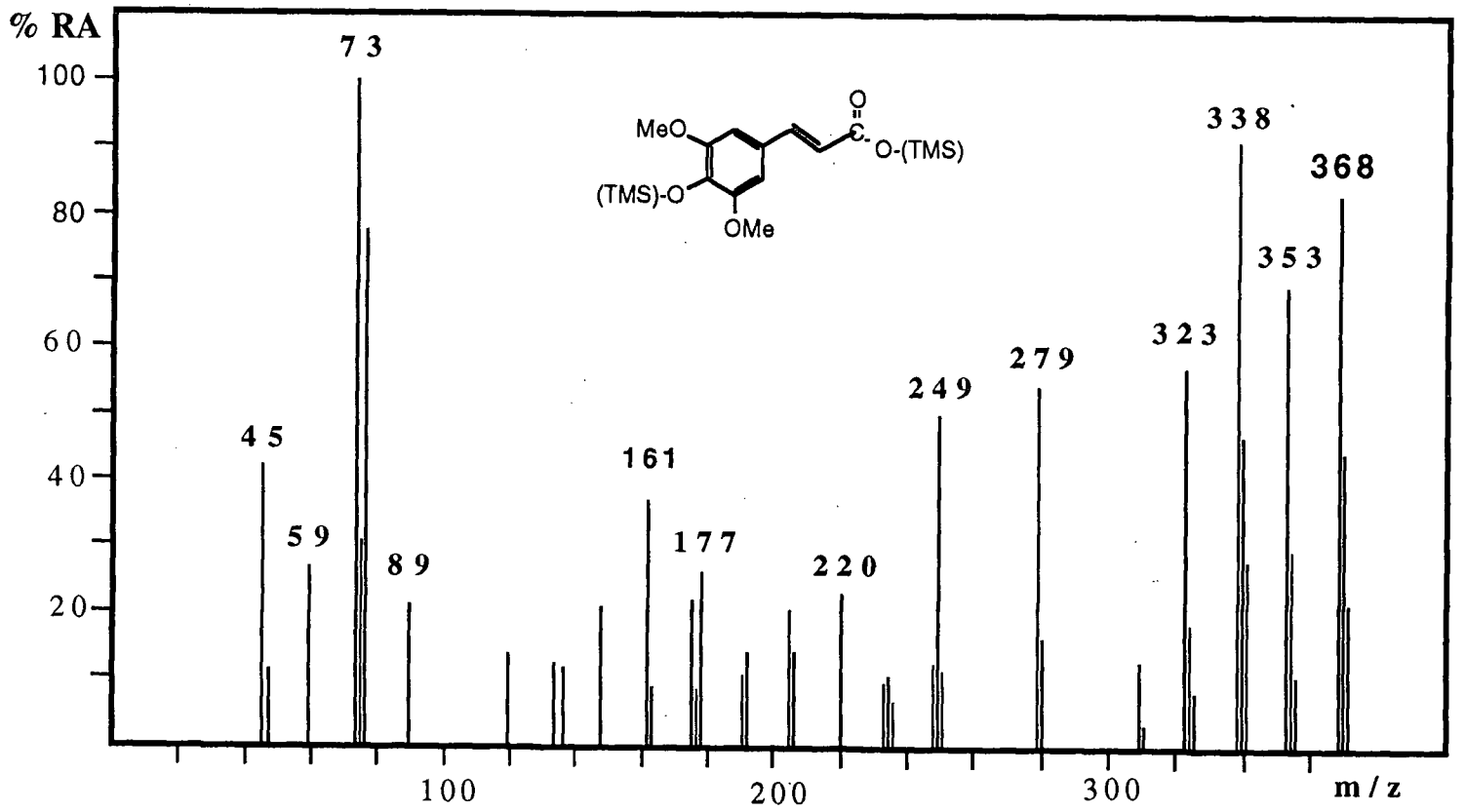


Figure 5.5 Mass spectrum of 3,5-dimethoxy-4-hydroxycinnamic acid (TMS derivative)

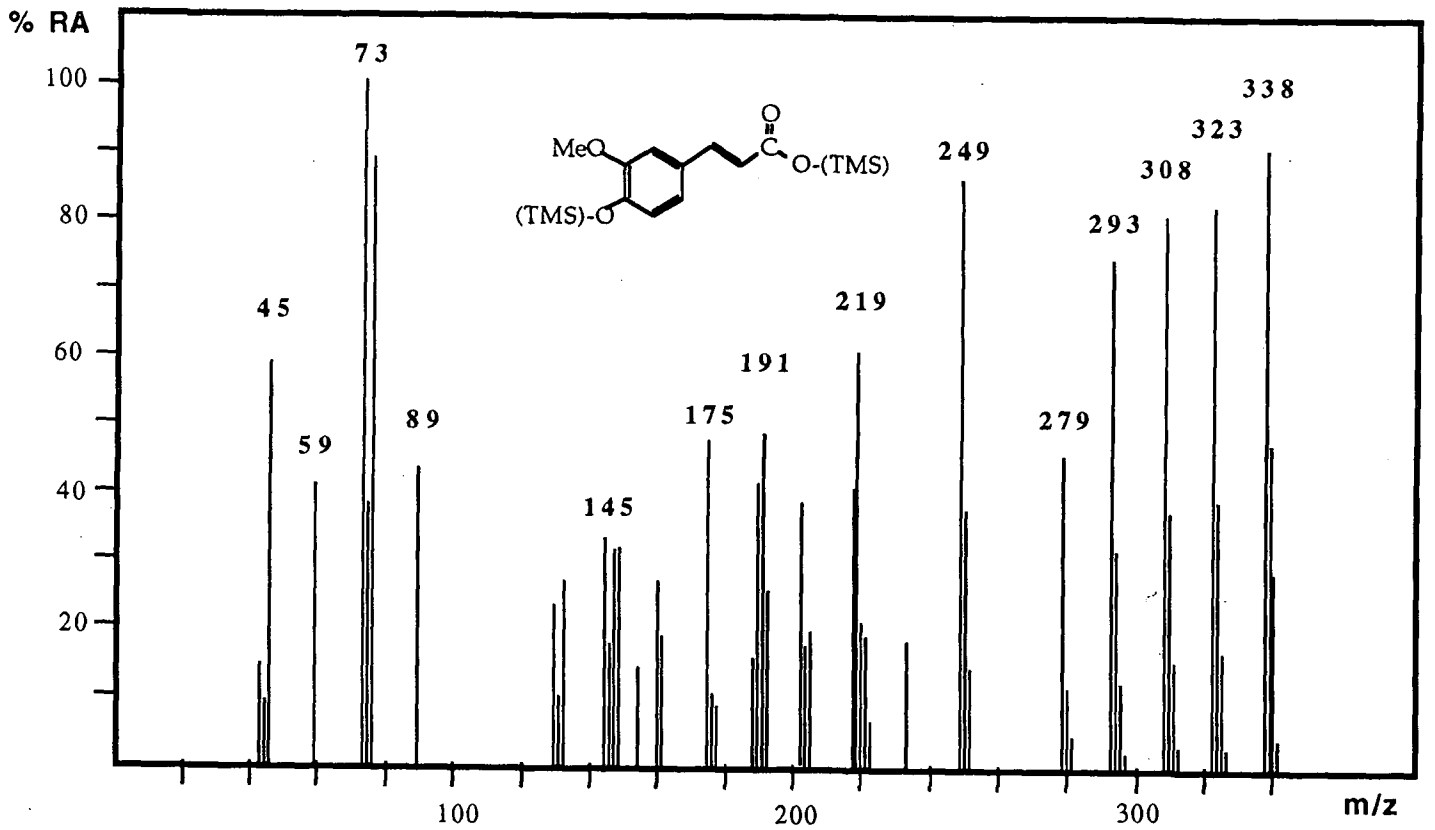


Figure 5.6 Mass spectrum of 3-methoxy-4-hydroxycinnamic acid (TMS derivative)

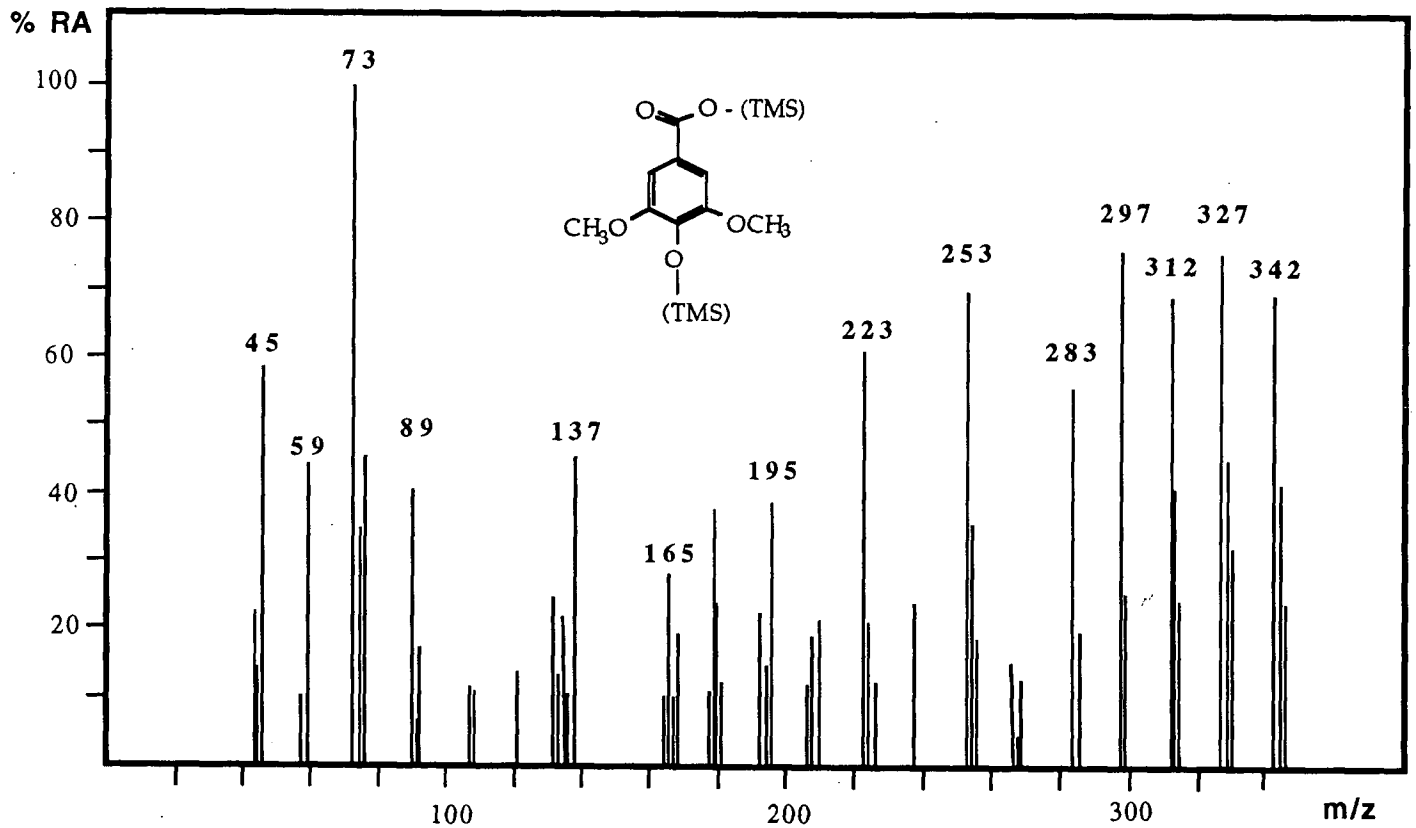


Figure 5.7 Mass spectrum of 3,5-dimethoxy-4-hydroxybenzoic acid (TMS derivative)

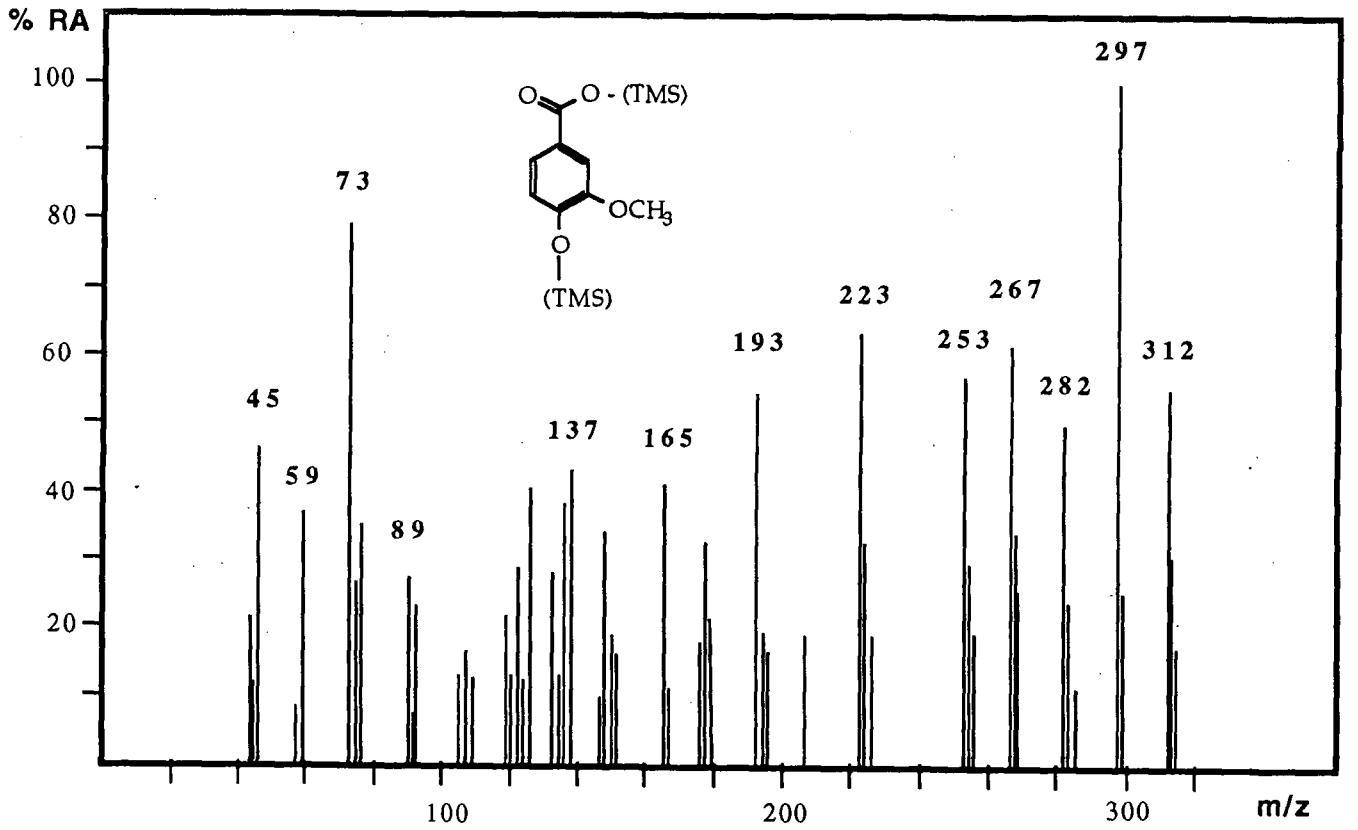


Figure 5.8 Mass spectrum of 3-methoxy-4-hydroxybenzoic acid (TMS derivative)

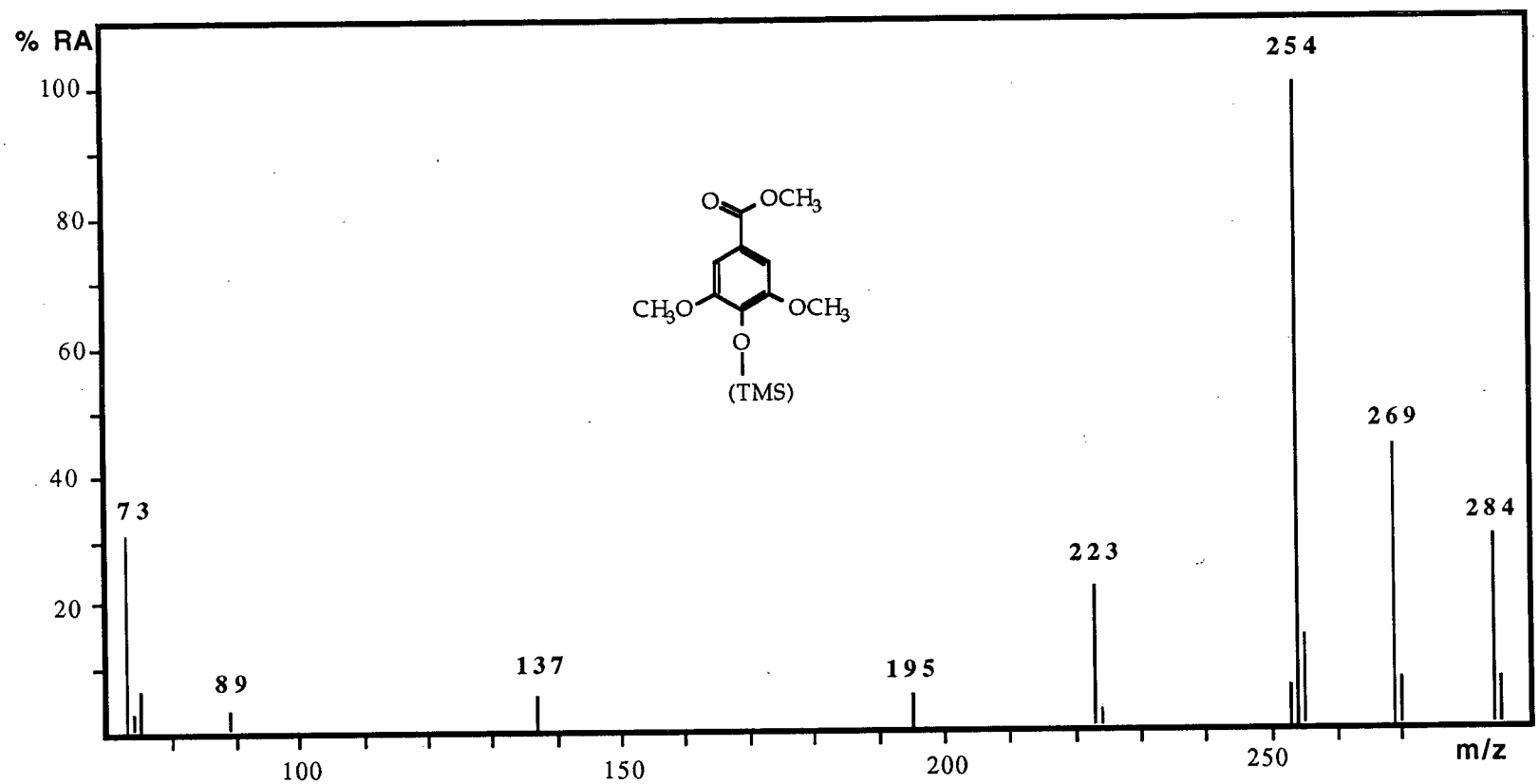


Figure 5.9 Mass spectrum of methyl-3,5-dimethoxy-4-hydroxybenzoic acid (TMS derivative)

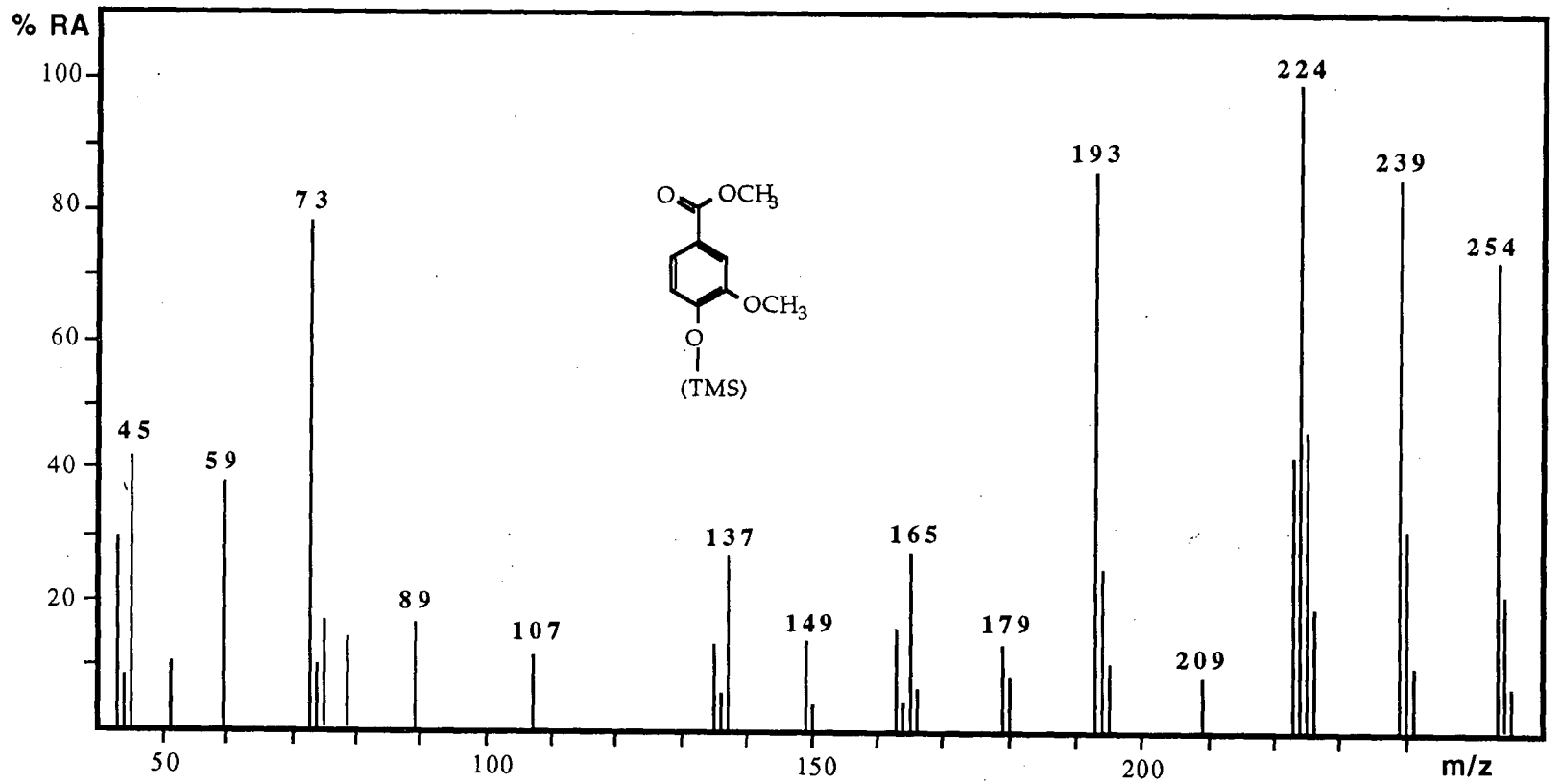


Figure 5.10 Mass spectrum of methyl-3-methoxy-4-hydroxybenzoic acid (TMS derivative)

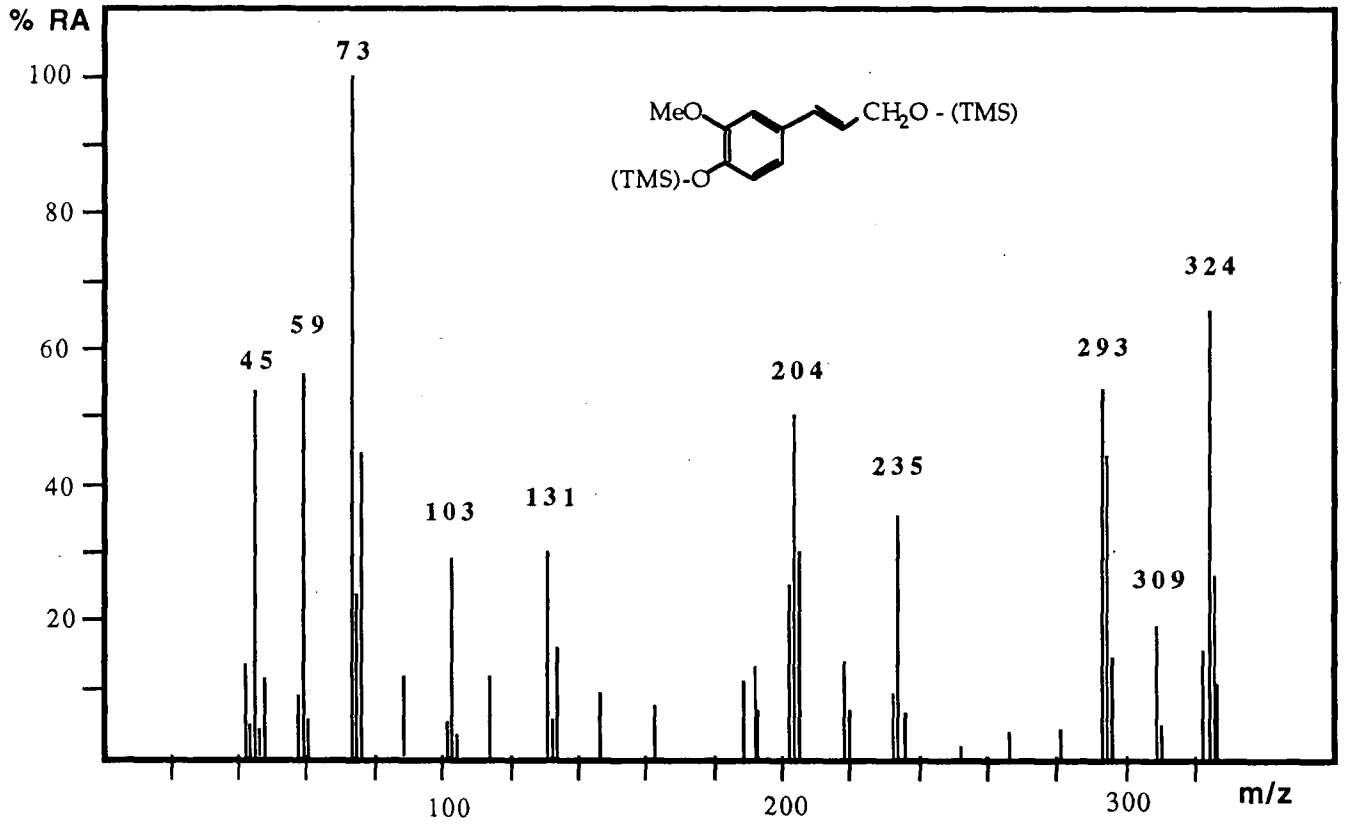
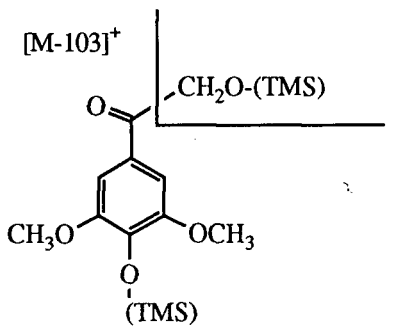


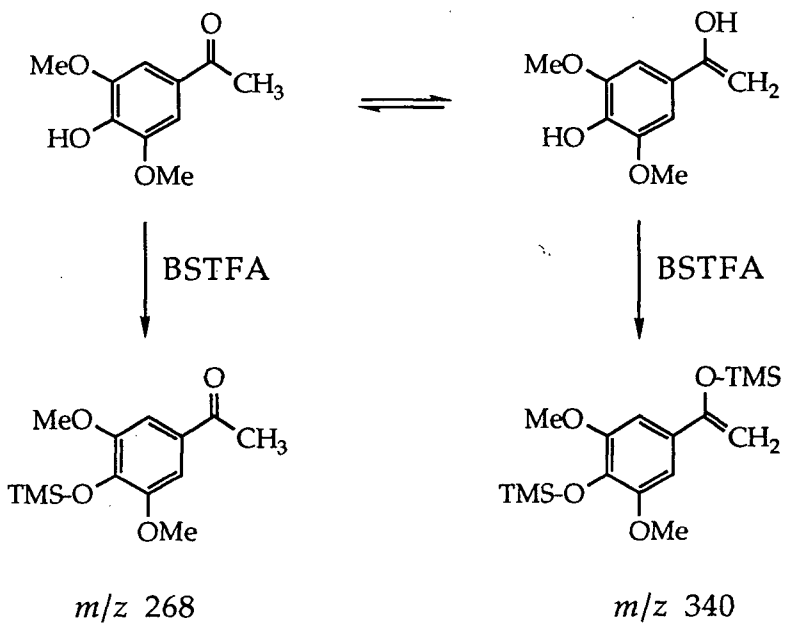
Figure 5.11 Mass spectrum of coniferyl alcohol (TMS derivative)

α-hydroxy derivatives, HO-AS and HO-AV (Figure 5.4) each exhibited a strong $[M-103]^+$ fragment (base peak) which likely arose as follows:



In fact, HO-AV was identified by mass spectral similarity with HO-AS. Both compounds exhibit the $[M-103]^+$ ion as their base peak, as well as relatively small M^+ and $[M-15]^+$ ions (10-20% RA).

These mass spectral data are in agreement with those reported very recently by Klaus Niemelä (1990). The kraft pulping process generates in spent (black) liquor a diverse range of substituted phenols and other compounds, which he sought to identify. With and without derivatization, Niemelä identified a total of 358 organic compounds in birch kraft black liquor, and presented 156 mass spectra, including those of AS, AV, HO-AS, and HO-AV. In addition, he identified the enol tautamers of AS, M^+ 340, and AV, M^+ 310 (Figure 5.12-14). Both Niemelä (1990), and Klaus and Spiteller (1989) noted the generation of characteristic $[M-1]^+$ and $[M-31]^+$ ions in the mass spectra of acetophenone enol tautomers. Perhaps these enol tautomers were present in the GC-MS samples I examined, but they were overlooked, since the spectra did not match any reference spectrum in the Library used. It would be interesting to determine the conditions effecting keto-enol



m/z 268 *m/z* 340

Figure 5.12 Keto-enol tautomerism of acetophenones. TMS derivatives may be used to distinguish these tautomers: the acetophenone is monotrimethylsilylated, whereas the enol forms a bis(TMS) ether.

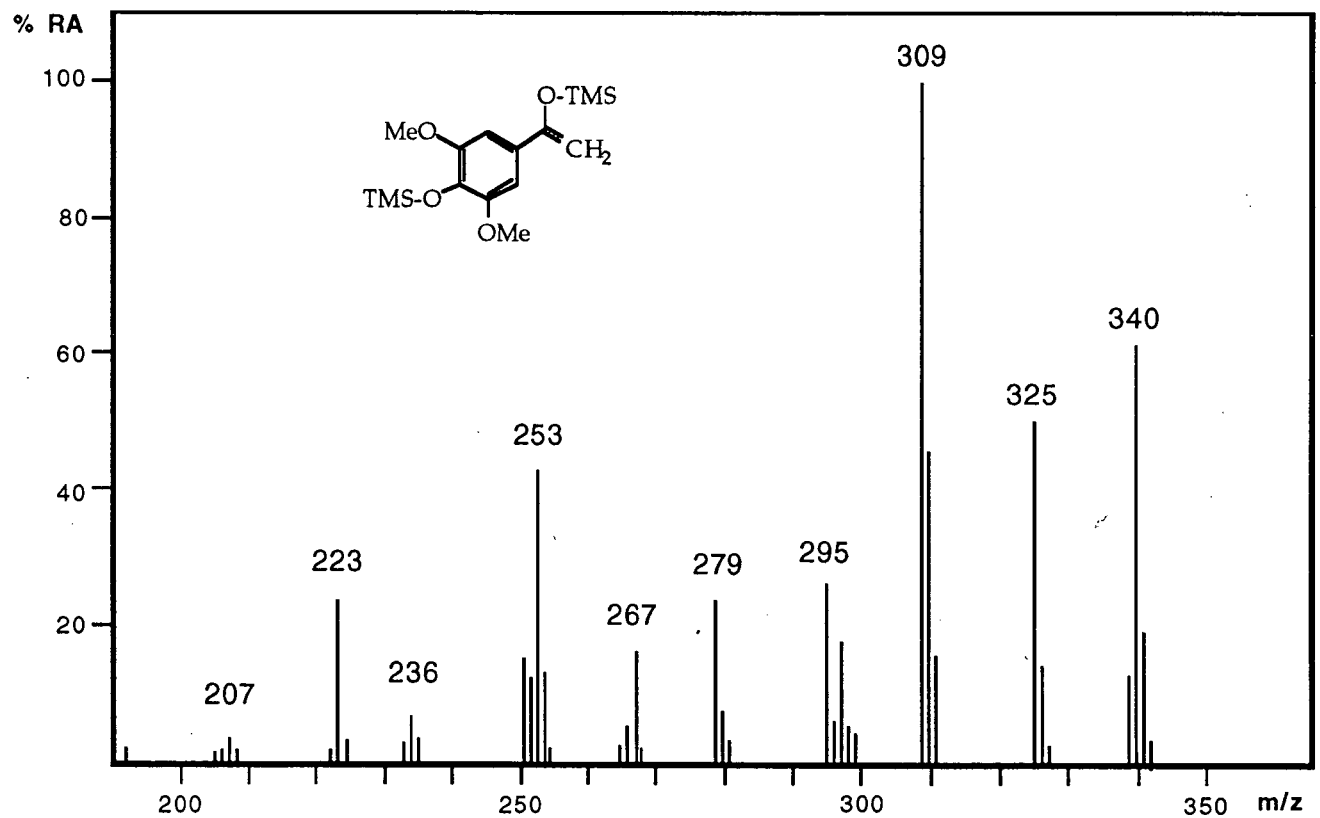


Figure 5.13 Partial mass spectrum of 3,5-dimethoxy-4-hydroxyphenethylenol (TMS) derivative. This AS-enol tautomer was identified in birch kraft black liquor by GC-MS (Niemelä, 1990).

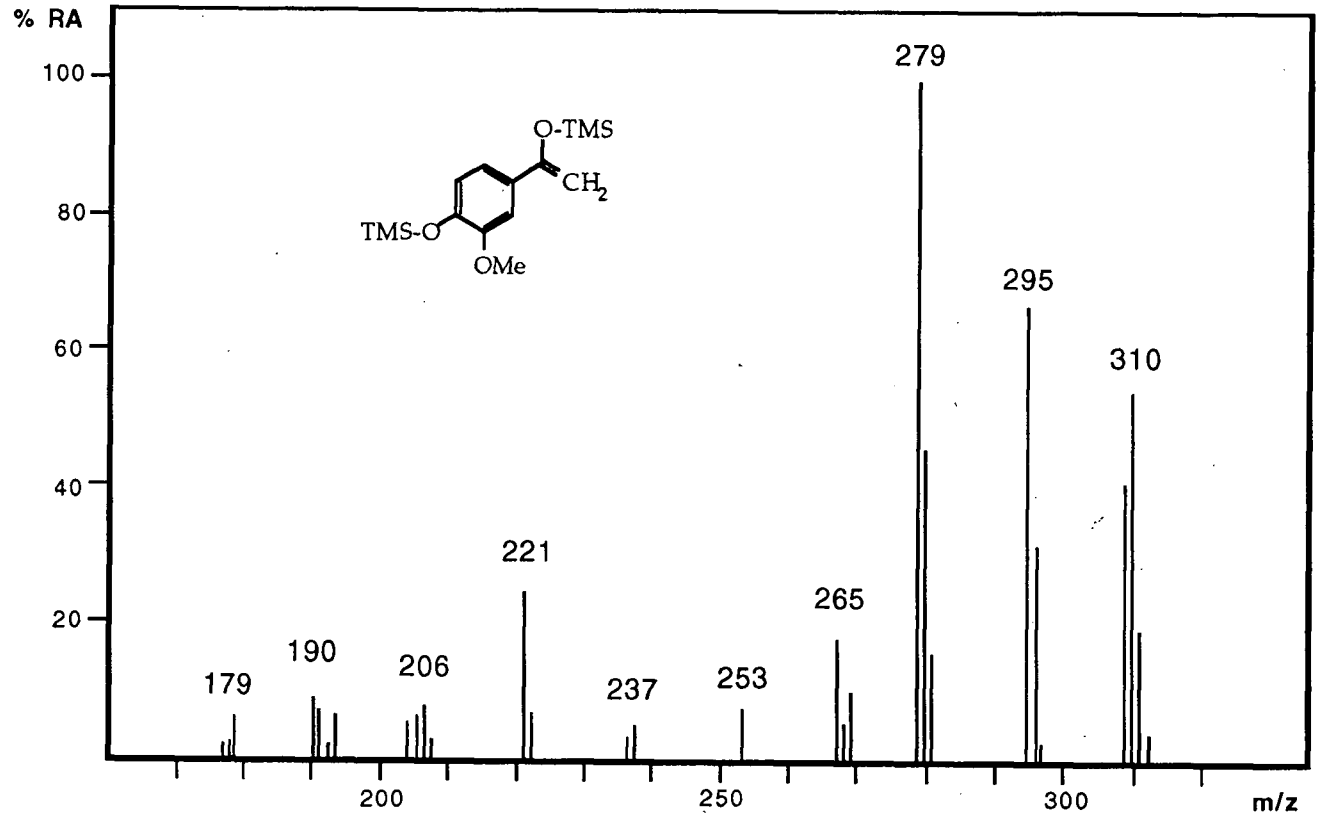


Figure 5.14 Partial mass spectrum of 4-hydroxy-3-methoxyphenethylenol (TMS) derivative. This AV-enol tautomer was identified in birch kraft black liquor by GC-MS (Niemelä, 1990).

tautomerism of these acetophenones, and the stability and biological activity of the enol tautomers.

Niemelä also identified 2,6-dimethoxyhydroquinone as its bis-TMS ether (M^+ 314, $[M-15]^+$ 299, $[M-30]^+$ 284-base peak). Coincidentally, the mass spectrum of an unknown, in the active VLC fraction from *Taxus* needles happens to closely match the reported mass spectrum of the bis-TMS ether of this quinone (Niemelä, 1990). Quinones should be examined for their effect on growth and virulence induction in *A. tumefaciens*.

Virtually every compound had a unique M^+ ion, with the important exceptions of syringaldehyde, methyl vanillate, and an unknown isomer, all of M^+ 254. Fortunately, these can be distinguished on the basis of GC elution profile and mass spectral fragmentation pattern. Authentic syringaldehyde exhibits a strong M^+, $[M-15]^+$, $[M-30]^+$, and $[M-31]^+$, but a very small $[M-61]^+$. In contrast, the mass spectrum of methylvanillate (and that of methylisovanillate) exhibits a strong $[M-61]^+$ (*m/z* 193). Hence the unknown (Table 5.2, scan #269) may have been methylisovanillate. Two pairs of phenolic compounds, each exhibiting the same M^+ ion, were *cis* and *trans* isomers of ferulic and sinapic acids (M^+ 338 and 368, respectively). These isomers were well separated by GC, with each *cis* isomer eluting first (Table 5.3).

Fatty acids were also observed in extracts from virtually every plant species. A few of these fatty acids are listed, with M^+ and chemical formulae, in Table 5.4. The fatty acid mass spectra generally exhibited weak M^+ ions, and $[M-15]^+$ ions roughly 5 times more abundant. Saturated fatty acid-TMS derivatives had in common a number of characteristic fragment ions including those at *m/z* 145, 131, 129, and 117. These observations are in agreement with those of Niemelä (1990).

Based on the known requirements for *vir* -induction, it is unlikely that the saturated acids were involved in signalling *Agrobacterium*. Nevertheless, their presence in crude and VLC-purified samples is at least of passing interest. Ferulate esters of higher fatty alcohols are known from roots of *Kalanchoë diagremontiana* (Nair *et al.*, 1988). Through growth inhibition bioassays these esters of C_{22}-C_{30} alcohols were evaluated as allelopathic agents. Interestingly, aside from trace quantities of benzaldehydes, no rigorous identification of the active components could be made from GC-MS of a *vir*-inducing extract from *K. diagremontiana*-conditioned medium. It is conceivable that in certain cases such esters could be effective signal molecules detected by *Agrobacterium*, but none were detected by GC-MS of active extracts.

In summary, the differing compositions of signal compound mixtures detected by GC-MS suggest that the acetophenones are not generally responsible for signalling *Agrobacterium* (see Chapter 3). Since acetosyringone had not been reported from any plant source prior to its identification as a signal compound from *N. tabacum*, and since I found a variety of common plant phenolics were capable of inducing the same response in *Agrobacterium*, I hypothesized that acetosyringone was not likely the only naturally occurring signal compound. GC-MS analyses of active samples extracted from media conditioned with wounded plant tissues have demonstrated that this is indeed the case. Also, as demonstrated in Chapter 3, the technique of GC-MS can be employed in signal molecule biosynthetic studies. It is anticipated that future analysis of *vir*-activating mixtures will rely, at least in part, on integrated GC-MS.

Table 5.1 Phenolic compounds identified by GC-MS.

Chemical name	Common name or abbrevaiation
α-hydroxy-3,5-dimethoxy-4-hydroxyacetophenone	HO-AS
3,5-dimethoxy-4-hydroxyacetophenone	Acetosyringone (AS)
4-hydroxy-3-methoxyacetophenone	Acetovanillone (AV)
α-hydroxy-4-hydroxy-3-methoxyacetophenone	HO-AV
4-hydroxyacetophenone	
3,5-dimethoxy-4-hydroxybenzaldehyde	Syringaldehyde
3-methoxy-4-hydroxybenzaldehyde	Vanillin
2-hydroxybenzoic acid	Salicylic acid
3-hydroxybenzoic acid	
4-hydroxybenzoic acid	*p* -hydroxybenzoic
4-hydroxy-3-methoxybenzoic acid	vanillic acid
4-hydroxy-3-methoxybenzoic acid, methyl ester	vanillic acid methyl ester
3,5-dimethoxy-4-hydroxybenzoic acid	syringic acid
3,5-dimethoxy-4-hydroxybenzoic acid, methyl ester	syringic acid methyl ester
Z-4-hydroxycinnamic acid	*cis-p* -coumaric acid
E-4-hydroxycinnamic acid	*trans-p* -coumaric acid
3,4-dihydroxycinnamic acid	caffeic acid
Z-3,4-dihydroxycinnamic acid, methyl ester	*cis* -methylcaffeiate
E-3,4-dihydroxycinnamic acid, methyl ester	*trans* -methylcaffeiate
Z-4-hydroxy-3-methoxycinnamic acid	*cis* -ferulic acid
E-4-hydroxy-3-methoxycinnamic acid	*trans* -ferulic acid
E-3,5-dimethoxy-4-hydroxycinnamic acid	*trans* -sinapic acid
3,5-dimethoxy-4-hydroxycinnamyl alcohol	Coniferyl alcohol

Table 5.2 Mass spectral data for *vir*-inducing phenolics.

Compound	mass	% RA	
vanillin	224	(29.18)	scan # 276
	209	(49.64)	
	194	(100.0)	
	193	(51.09)	
syringaldehyde	254	(33.95)	scan # 400
	239	(45.04)	
	224	(100.0)	
	223	(23.70)	
acetosyringone	268	(42.30)	scan # 417
	253	(56.64)	
	238	(70.88)	
	223	(72.81)	
HO-AS	356	(14.51)	scan # 587
	341	(25.37)	
	253	(100.0)	
	223	(6.55)	
	73	(26.87)	
acetovanillone	238	(50.52)	scan # 323
	223	(97.00)	
	208	(65.70)	
	193	(100.0)	
	73	(93.29)	
HO-AV	326	(6.92)	scan # 513
	311	(16.96)	
	223	(84.4)	
	218	(21.27)	
	203	(22.07)	
	175	(18.32)	
	73	(100.0)	
syringic acid	342	(69.72)	scan # 510
	327	(75.60)	
	312	(68.97)	
	297	(75.74)	
	283	(55.28)	

	253	(69.94)	
	223	(60.57)	
	73	(100.0)	
ferulic acid	338	(91.22)	scan # 622
	323	(82.43)	
	308	(81.37)	
	293	(74.81)	
	279	(45.95)	
	249	(86.39)	
	219	(61.10)	
	73	(100.0)	
sinapic acid	368	(83.32)	scan # 698
	353	(69.57)	
	338	(91.30)	
	323	(57.43)	
	309	(13.25)	
	279	(54.40)	
	249	(50.15)	
	73	(100.0)	
vanillic acid	312	(54.74)	scan # 426
	297	(100.0)	
	282	(50.94)	
	267	(61.71)	
	253	(57.14)	
	223	(63.47)	
	193	(54.86)	
	73	(79.86)	
methyl syringate	284	(30.38)	scan # 446
	269	(43.65)	
	254	(100.0)	
	223	(22.36)	
	73	(31.31)	
methyl vanillate	254	(73.08)	scan # 346
	239	(85.41)	
	224	(100)	
	193	(86.09)	
	73	(77.94)	
unknown	254	(14.74)	scan # 269
	239	(1.50)	
	224	(48.71)	

209	(67.59)
194	(100.0)
193	(64.22)
73	(40.69)

Table 5.3 *vir*-inducing phenolics listed by m.w., M^+ , and scan #

Compound	MW	TMSi m/z	(~) scan #
AS	196	268	(422)
HO-AS	212	356	(587)
acetovanillone	166	248	(323)
HO-AV	182	326	(513)
Methylsyringate	212	284	(446)
Vanillin	152	224	(276)
Syringaldehyde	182	254	(400)
Vanillic acid	168	312	(426)
Methylvanillate	182	254	(346)
Syringic acid	198	342	(510)
Cinnamic acid	158	230	(327)
Caffeic acid	180	252	(576)
Ferulic acid	194	338	(619)E (516)Z
Sinapic acid	224	368	(698)E (572)Z
Methylcaffeate	194	338	(505)E
Methylferulate	208	280	(n.d.)
Methylsinapate	238	310	(n.d.)
Coniferyl alcohol	180	324	(528)
Sinapyl alcohol	210	354	(n.d.)
p -OH benzoic acid	138	282	(322)
o -OH benzoic acid	138	282	(265)
p -coumaric acid	164	308	(n.d.)
p -OMe-cinnamic	178	250	(438)
Avenalumic acid	190	334	(n.d.)
OMe-avenalumic acid	220	364	(n.d.)
Zingiberone	194	266	(n.d.)

n.d.=not determined

Table 5.4 Commonly observed saturated fatty acids in *vir*-inducing mixtures.

Compound	M^+	formula
hexadecanoic acid	328	$C_{19}H_{40}O_2Si$
heptadecanoic acid	342	$C_{20}H_{42}O_2Si$
octadecanoic acid	356	$C_{21}H_{44}O_2Si$
dodecanoic acid	272	$C_{15}H_{32}O_2Si$
oleic acid	354	$C_{21}H_{42}O_2Si$
tetradecanoic acid	300	$C_{19}H_{36}O_2Si$

Chapter 6: Conclusions

6.1 Survey of *Agrobacterium* signal compounds

Agrobacterium vir-inducers are to be found in, and can be recovered from the organic phase (CH_2Cl_2 or $CHCl_3$ part) of virtually any wounded plant conditioned cell culture medium. Interestingly, the range of plant species which produce these compounds is not limited to the normal dicotyledonous hosts. Some hosts exude into the medium only the common cinnamic or benzoic acid derivatives. Certain others produce benzaldehydes or *vir*-inducing methyl esters. Interestingly, the occurrence of *vir*-activating acetophenone derivatives appears to be restricted to members of the Solanaceae. In addition, glucosides of active phenolic compounds are present in unwounded plant tissues. Thus *Nicotiana* species have small amounts of each of the four acetophenones, as glucosides (see Chapter 3), in leaf tissue prior to wounding.

It was established also that monocots exude into the culture medium a number of the same *vir*-inducing phenolic aglycones as do dicot hosts. The benzoxazinone DIMBOA produced by *Zea mays*, inhibits growth and virulence of *A. tumefaciens* and its activity is therefore likely responsible for the immunity of monocots (such as wheat or maize) to agroinfection. High and low DIMBOA strains could be examined for *vir*-inducers and also for DIMBOA content. This may yield important information regarding the susceptibilty at least of certain monocots to transformation by *Agrobacterium* Other research groups are likely already studying these subjects. Repressors of virulence were noted also in gymnosperm-conditioned media, and in the

EtOAc soluble fractions from media conditioned with maize crown tissue and *N. glauca* stem sections, but these compounds were not identified.

Correlations between phenol substitution patterns and induced *vir* gene activity were previously examined (Spencer and Towers, 1988). Those early results suggested that commonly occurring phenolic compounds may be involved in signalling *Agrobacterium*. In the course of the present research, and with an understanding of the secondary plant compounds likely involved, bioassay-directed purifications and GC-MS searches were conducted. GC-MS data confirmed that the suspected phenolics are the active compounds in extracts. These compounds include the common phenylpropanoid acids, benzoic acids, methyl esters, and the acetophenones.

Interestingly, one of the first reported signal compounds, AS, was found only in the active extracts from species within the Solanaceae. In these active mixtures where acetophenones were found, the common phenylpropanoid acids were absent. This could be interpreted as meaning that the C_6-C_3 acids were the biosynthetic precursors in the pathway to the C_6-C_2 compounds. AS, HO-AS, AV and HO-AV were produced by a number of species of *Nicotiana*. This is the first report of the occurrence of AV and HO-AV as signal molecules. HO-AV was tentatively identified by comparison of its mass spectrum with that of HO-AS (described in Chapter 5), previously synthesized in our lab (Spencer *et al.*, 1990). The presence of these compounds suggests that the enzyme systems may not be specific to structures with syringyl substitution (sinapic acid), but may also use ferulic acid as a substrate.

At least two enzymes would be required to produce the acetophenones from methoxycinnamic acid precursors (Figure 6.1). First, a hydrolyase would add an equivalent of water to the propenyl double bond. Second, a carbonyl

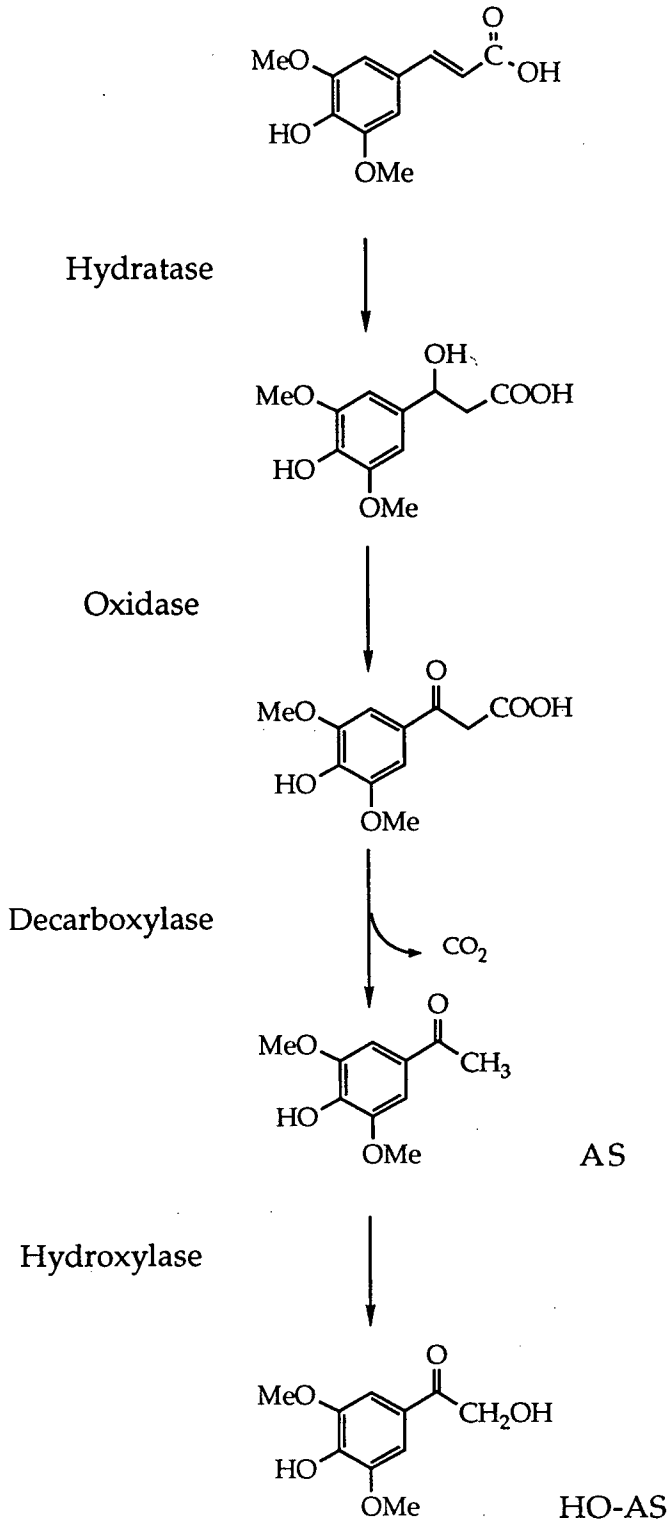


Figure 6.1 Hypothesized pathway to the acetophenones in Solanaceous plants.

function would be formed at the new ß-hydroxy group to yield the ß-keto acid. Decarboxylation could occur spontaneously, or by the action of a third enzyme, to yield the acetophenone. α-Hydroxylation would require an additional enzyme. Thus three or four enzymes, which direct the phenylpropanoid pool towards acetophenone derivatives, may be rapidly synthesized upon wounding. Cycloheximide inhibition of acetophenone production may occur by inhibition of synthesis of these enzymes.

Since phenolic compounds other than the acetophenones were identified from non-solanaceous dicot species, it can be concluded that there is no single chemical signal to *Agrobacterium*. Indeed, the compound syringic acid methyl ester was isolated a significantly active chemical from a number of grapevine cultivars (Chapter 2; Spencer *et al.*, 1990). The limited host range (LHR) strain A856 responds to methyl syringate in a similar manner to, but with a different concentration optimum than, the wide host range (WHR) strain A348. As was discussed in Chapter 2, the presence of the ester in active mixtures obtained from grapevine cultivars does not provide a simple explanation for host range determination.

Other factors, for example differences in T-DNA, *vir*C functions, the pH of the conditioned medium, occurence of binding sites or types of saccharides produced, must play a role in limitation of host range. Perhaps it will be found that the majority of monocot species elaborate the incorrect monosaccharides following wounding.

6.2 Glucoside precursors and the effects of cycloheximide

The biosynthesis of signal compounds was briefly examined. The effects of glucosidase and cycloheximide on production of these compounds

were assessed. Glucosides were identified in intact leaf tissue, but not in concentrations sufficient to account for the amounts of aglycones recoverable from conditioned media. Thus the production of the acetophenones upon wounding cannot be explained solely by the action of β-glucosidase activity. Stachel *et al.* (1985) reported that cycloheximide inhibited production of acetosyringone in *N. tabacum*. As described in Chapter 3, 20μM cycloheximide was added to determine its effect on the biosynthesis of inducer compounds from *Nicotiana* species. Subsequent GC-MS analysis of the active fraction still indicated the presence of small amounts of the acetophenone mixture. We interpret this to mean that acetophenone aglycones can be liberated from the glucosides by the action of pre-existing glucosidases, but that synthesis of enzymes is required for production of greater levels of signal compounds from phenylpropanoid precursors.

Threlfall and Whitehead (1988) reported that cellulase treatment induced production of acetosyringone. It remains unclear whether acetophenones can be liberated by cellulase from cell walls. It seems more likely that cellulase treatment may elicit *de novo* synthesis of acetosyringone by mimicking wounding.

6.3 pH changes in conditioned media

The initial pH of the MS culture medium was adjusted to 5.70. No additional buffer was added to this medium. Immediately following 24 hours incubation at ca. 26 C and 100 rpm, the pH was recorded with a pH meter. Table 3.1 lists the final pH values for a range of species. It is not possible to make generalized conclusions regarding hosts and nonhosts on the basis of pH from the data. Nonhost tissues, such as those from conifers and

monocots, can significantly lower the pH of the medium. However, so can certain wounded host tissues such as *Nicotiana* stem tissue versus leaf tissue.

6.4 *vir* expression in *Agrobacterium rhizogenes* A4/pSM358cd

Positive results from ß-galactosidase assays with induced cultures of the recipient strain indicated that triparental mating between donor (*E. coli* pSM358cd), helper (*E. coli* pRK 2013) and recipient (*A. rhizogenes* A4) was successful. It was found that under appropriate conditions, an *Agrobacterium rhizogenes* A4 background was capable of inducing ß-galactosidase activity from the *vir*E::*lac*Z gene fusion reporter plasmid pSM358cd. A brief structure-activity analysis of methoxyphenol-induced virulence in *A. rhizogenes* was conducted.

A. rhizogenes A4 responds to µM quantities of acetosyringone and to other structurally related phenolics. Fortunately, the derivative A4/pSM358cd grows and is induced very well under the conditions used for analysis of *vir* gene expression in *A. tumefaciens.* However, *Agrobacterium rhizogenes* A4/pSM358cd does not respond to the phenolics in exactly the same way as does *A. tumefaciens.* A structure-activity analysis of *vir*-induction was presented and discussed in Chapter 4. The background level of A4/pSM358cd and its sensitivity to the phenolic compounds is different than in *A. tumefaciens.* Compared with *A. tumefaciens,* a somewhat lower concentration of inducer compound is required to stimulate a significant response.

A4/pSM358cd responds to the presence of acetophenones, benzoic and cinnamic acid derivatives, and their methyl esters, coniferyl alcohol and a chalcone derivative. The activity of these compounds in the A4/pSM358cd

system illustrates the close relationship between *A. rhizogenes* and *A. tumefaciens*. Furthermore, the finding that a chalcone can activate *vir* gene expression in this species links signalling systems in the agrobacteria and rhizobia.

References

Akiyoshi, P.E., Klee, H., Amasino, R.M., Nester, E.W., and Gordon, M.P. (1984) T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. (USA) **81**: 5994-5998.

Akiyoshi, P.E., Morris, R.O., Hing, R., Mischke, B.S., Kosuge, T., Garfinkel, D.J., Gordon, M.P., and Nester, E.W. (1983) Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. Proc. Natl. Acad. Sci. (USA) **80**: 407-411.

Alt-Mörbe, J., Bak, B., and Schroder, J. (1986) A 3.6-kbp segment from the *vir* region of Ti plasmids contains genes responsible for border sequence-directed production of T-region circles in *E. coli.* EMBO J. **5**: 1129-1135.

Alt-Mörbe, J., Kühlmann, H., and Schröder, J. (1989) Differences in induction of Ti plasmid virulence genes *vir*G and *vir*D, and continued control of *vir* D expression by four external factors. Mol. Plant-Microbe Int. **2**: 301-308.

Ashby, A.M., Watson, M.D., and Shaw, C.H. (1987) A Ti-plasmid function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. Fed. Eur. Micro. Soc. **41**: 189-192.

Ashby, A.M., Watson, M.D., Loake, G.J., and Shaw, C.H. (1988) Ti plasmid specified chemotaxis of *Agrobacterium tumefaciens* C58C^1 toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. J. Bacteriol. **170**: 4181-4187.

Bailey, B.A., and Larson, R.L. (1989) Hydroxamic acid glucosyltransferase from maize seedlings. Plant Physiol. **90**: 1071-1076.

Basha, S.M., Musingo, M.N., Mohauty, B., Dorner, J.W., and Cole, R.J. (1990) Phytoalexins are produced in peanut seed in response to wounding. J. Plant Physiol. **136**: 143-148.

Bercetche, J., Chriqui, D., Adam, S., and David, C. (1987) Morphogenetic and cellular reorienations induced by *Agrobacterium rhizogenes* (strains 1855 and 8196) on carrot, pea and tobacco. Plant Sci. **52**: 195-210.

Bogers, R.J. (1972) On the interaction of *Agrobacterium tumefaciens* with cells of *Kalanchoë diagramontiana*, In (H.P. Maas Geesteranus, ed.): Proceedings of the third international conference on plant pathogenic bacteria, Center for Agricultural Publishing and Documentation, Wageningen, pp. 356.

Bolton, G., Nester, E.W., and Gordon, M. (1986) Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science **232**: 983-985.

Carlson, R.E., and Dolphin, D.H. (1982) *Pisum sativum* stress metabolites: two cinnamylphenols and a 2'-methoxychalcone. Phytochemistry **21**: 1733-1736.

Castle, A.J., and Day, A.W. (1984) Isolation and identification of α-tocopherol as an inducer of the parasitic phase of *Ustilago violacea*. Phytopathology **74**: 1184-1200.

Chilton, M.-D., Montoya, A.L., Merlo, D.J., Drummond, M.H., Nutter, R., Gordon, M.P., and Nester, E.W. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorogenesis. Cell **11**: 263-271.

Chilton, M.-D., Saiki, R.K., Yadav, N., Gordon, M.P., and Quetier, F. (1980) T-DNA from *Agrobacterium* Ti-plasmid is in the nuclear DNA fraction of the crown gall tumor cells. Proc. Natl. Acad. Sci. (USA) **77**: 4060-4064.

Chilton, M.-D., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F., and Tempe, J. (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genome of host plant root cells. Nature **295**: 432-434.

Christie, P.J., Ward, J.E., Winans, S.C., and Nester, E.W. (1988) The *Agrobacterium tumefaciens vir*E2 gene product is a single-stranded-DNA-binding protein that associates with T-DNA. J. Bacteriol. **170**: 2659-2667.

Coxon, D.T., Curtis, R.F., Price, K., and G. Levett. (1973) Abnormal metabolites produced by carrot roots. Phytochemistry **12**: 1881-1885.

Das, A. (1988) *Agrobacterium tumefaciens vir*E operon encodes a single-stranded DNA-binding protein. Proc. Natl. Acad. Sci. (USA) **85**: 2909-2913.

DeCleen, M., and Deley, J. (1976) The host range of crown gall. Bot. Rev. **42**: 389-466.

Dhar, D.N. (1981) *The Chemistry of Chalcones and Related Compounds.* John Wiley and Sons, New York. pp. 285.

Dixon, R.A., and Lamb, C.J. (1990) Molecular communication in interactions between plants and microbial pathogens. Ann. Rev. Plant Physiol. Plant Mol. Biol. **41**: 339-367.

Douglas, C.J., Halperin, W., and Nester, E.W. (1982) *Agrobacterium tumefaciens* mutants affected in attachment to plant cells. J. Bacteriol. **152**: 1265-1275.

Douglas, C.J., Halperin, W., Gordon, M.P., and Nester, E.W. (1985-a) Specific attachment of *Agrobacterium tumefaciens* to bamboo cells in suspension cultures. J. Bacteriol. **161**: 764-766.

Douglas, C.J., Staneloni, R.J., Rubin, R.A., and Nester, E.W. (1985-b) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. J. Bacteriol. **161**: 850-860.

Draper, J., MacKenzie, I.A., Davy, M.R., and Freeman, J.P. (1983) Attachment of *Agrobacterium tumefaciens* to mechanically isolated *Asparagus* cells. Plant Sci. Lett. **29**: 227-236.

El-Basyouni, S., and Towers, G.H.N. (1964) The phenolic acids in wheat I. Changes during growth and development. Can. J. Biochem. **42**: 203-210.

Engström, P., Zambryski, P., Van Montagu, M., and Stachel, S.E. (1987) Characterization of *Agrobacterium* virulence proteins induced by the plant factor acetosyringone. J. Mol. Biol. **197**: 635-645.

Firmin, J.I., Wilson, K.E., Rossen, I., and Johnston, A.W.B. (1986) Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature **324**: 90-92.

Freudenberg, K., and Neish, A.C. (1968) *Constitution and Biosynthesis of Lignin. Molecular Biology Biochemistry and Biophysics* 2. pp. 129. Springer-Verlag, New York.

Friend, J. (1985) Phenolic substances and plant disease. Ann. Proc. Phytochem. Soc. Eur. **25**: 367-392

Fries, N., Serck-Hassen, K., and Dimberg, L.H. (1987) Abietic acid, an activator of spore germination in ectomycorrhizal species of the genus *Suillus* (Boletaceae). Exp. Mycol. **11**: 360-363.

Funk, C., and Brodelius, P. (1990) Influence of growth regulators and an elicitor on phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia*. Phytochemistry **29**: 845-848.

Gaffer, C., and Fraley, R. (1989) Genetic engineering plants for crop improvement. Science **244**: 1293-1299.

Garfinkel, D.J., and Nester, E.W. (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumorogenesis and octopine catabolism. J. Bacteriol. **144**: 732-743.

Gee, M.M., Sun, C.N., and Dwyer, J.D. (1966) An electron microscope study of sunflower crown gall tumor. Protoplasma **64**: 195-200.

Gietl, C., Koukolíková-Nicola, Z., and Hohn, B. (1987) Mobilization of T-DNA from Agrobacterium to plant cells involves a protein that binds single-stranded DNA. Proc. Natl. Acad. Sci. (USA) **84**: 9006-9010.

Goodman, R.M., Hauptli, H., Crossway, A., and Knauf, V.C. (1987) Gene transfer in crop improvement. Science **236**: 48-54.

Gross, G.G. (1979) Chemistry and biochemistry of lignin. In (T. Swain, J.B. Harborne, and C.F. Van Sumere, eds.): Biochemistry of plant phenolics, Recent advances in phytochemistry, Vol. 12. Plenum Press, New York, pp. 651.

Gurlitz, R.H.G., Lamb, P.W., and Matthysse, A.G. (1987) Involvement of carrot cell surface proteins in attachment of *Agrobacterium tumefaciens*. Plant Physiol. **83**: 564-568.

Harborne, J.B. (1979) Variation in and functional significance of phenolic conjugation in plants. In (T. Swain, J.B. Harborne, C.F. Van Sumere, eds.): Biochemistry of Plant Phenolics, Recent Advances in Phytochemistry, Vol. 12. Plenum Press, New York, pp. 651.

Harborne, J.B. (1989) General procedures and measurement of total phenolics. In (P.M. Dey, J.B. Harborne, eds.): Methods in Plant Biochemistry, Volume 1, Plant Phenolics. Academic Press, Toronto, pp. 552.

Harris, P.J., and Hartley, R.D. (1976) Detection of bound ferulic acid in cell walls of the Gramineae by ultraviolet fluorescence microscopy. Nature **259**: 508

Hartley, R.D., and Ford, C.W. (1989) Phenolic constituents of plant cell walls, In (N.G. Lewis and M.G. Paice, eds.): ACS Symposium Series 399, Plant Cell Wall polymers. ACS, Washington, pp. 676.

Hasezawa, S., Matsui, C., Nagata, T., and Syono, K. (1983) Cytological study of the introduction of *Agrobacterium tumefaciens* spheroplasts into *Vinca rosea* protoplasts. Can. J. Bot. **61**: 1052-1057.

Hernalsteens, J.P., Thia-Toong, L., Schell J, and van Montagu, M. (1984) An *Agrobacterium* -transformed cell culture from the monocot *Asparagus officinalis*. EMBO J. **3**: 3039-3041.

Hille, J., Hoekema, P., Hoojkaas, P., and Shilperoort, R. (1984a) Gene organization of the Ti plasmid. In (D.P.S. Verma and T Hohn eds.): Plant gene research. Genes involved in plant-microbe interacations. Spinger-Verlag, New York, pp. 393.

Hille, J., van Kan, J., and Shilperoort, R. (1984b) *trans*-acting virulence functions of the octopine Ti-plasmid from *Agrobacterium tumefaciens*. J. Bacteriol. **158**: 754-756.

Hirayama, T., Muranaka, T., Ohkawa, H., and Oka, A. (1988) Organization and characterization of the *vir*CD genes from *Agrobacterium rhizogenes*. Mol. Gen. Genet. **213**: 229-237.

Hoekema, A., de Pater, B.S., Fellinger, A.J., Hooykaas, P.J.J., and Schilperoort, R.A. (1984) The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region. EMBO J. **3**: 3043-3047.

Hooykaas, P.J.J., Hofker, M., Den Dulk-Ras, H., and Shilperoort, R.A. (1984) A comparison of virulence determinants in an octopine Ti-plasmid, a nopaline Ti-plasmid and an Ri-plasmid by complimentation analysis of *Agrobacterium tumefaciens* mutants. Plasmid **11**: 195-205.

Horsch, R.B., Klee, H.J., Stachel, S., Winans, S.C., Nester, E.W., Rogers, S.G., and Fraley, R.T. (1986) Analysis of *Agrobacterium tumefaciens* virulence mutants in leaf discs. Proc. Natl. Acad. Sci. (USA) **83**: 2571-2575.

Huang, M.W., Cangelosi, G.A., Halperin, W., and Nester, E.W. (1990) A chromosomal *Agrobacterium tumefaciens* gene required for effective plant signal transduction. J. Bacteriol. **172**: 1814-1822.

Iyama, K., Lam, T.B.T., and Stone, B.A. (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry **29** (3): 733-737.

Jin, S., Roitsch, T., Ankenbauer, R.G., Gordon, M.P., and Nester, E.W. (1990a) The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene regulation. J. Bacteriol. **172**: 525-530.

Jin, S., Roitsch, T., Christie, P.J., and Nester, E.W. (1990b) The regulatory VirG protein specifically binds to a *cis*-acting regulatory sequence involved in transcriptional activation of *Agrobacterium tumefaciens* virulence genes. J. Bacteriol. **172**: 531-537.

John, M., and Amasino, R.M. (1988) Expression of an *Agrobacterium* Ti plasmid gene involved in cytokinin biosynthesis is regulated by virulence loci and induced by plant phenolic compounds. J. Bacteriol. **170**: 790-795.

Juniper, B.E., and Jeffree, C.E. (1983) Plant Surfaces. Edward Arnold Ltd., London, pp. 93.

Kahl, Gunter (ed.) (1978) Biochemistry of Wounded Plant Tissues. Walter de Gruyter, Berlin, pp. 680.

Karunen, P. and Kalviainen, E. (1988) Lipids and hydroxycinnamic acids in cell walls of *Eriophorum vaginatum*. Phytochemistry **27** : 2045-2048.

Klapwijk, P.M., Scheuldermon, T., and Schilperoort, R.A. (1978) Coordinated regulation of octopine degradation and conjugative transfer of Ti-plasmids in*Agrobacterium tumefaciens*: Evidence for a common regulatory gene and separate operons. J. Bacteriol. **136**: 775-785.

Klee, H.J., White, F.F., Iyer, V.N., Gordon, M.P., Nester, E.W. (1983) Mutational analysis of the virulence region of an *Agrobacterium tumefaciens* Ti plasmid. J. Bacteriol. **153**: 878-883.

Klick, S., and Herrmann, K. (1988) Glucosides and glucose esters of hydroxybenzoic acids in plants. Phytochemistry **27** : 2177-2180.

Kolattukudy, P.E. (1981) Structure, Biosynthesis and Biodegradation of Cutin and Suberin. Ann. Rev. Plant Physiol. 32: 539-567.

Kolattukudy, P.E. (1984) Fungal penetration of defensive barriers of plants. In (W.M. Dugger and S. Bartnicki-garcia, eds.) Structure, function, and biosynthesis of plant cell walls, Proceedings of the Seventh Annual Symposium in Botany. Waverley Press, Baltimore, pp. 507.

Koukolikova-Nicola, Z., Shillito, R.D., Hohn, B., Wang, K., Van Montagu, M. and Zambryski, P. (1985) Involvement of circular intermediates in the transfer of T-DNA from *Agrobacterium tumefaciens* to plant cells. Nature **313**: 191-196.

Kraus, R., and Spiteller, G. (1989) Unusual fragmentation of trimethylsilylated enols derived from *m*- and *p*-hydroxyacetophenones. Org. Mass Spectrom. **24**: 861-865.

Leroux, B., Yanofsky, M.F., Winans, S.C., Ward, J.E., Ziegler, S.F., and Nester, E.W. (1987) Characterization of the *vir*A locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. EMBO J. **6**: 849-856.

Lewis, N.G., Inciong, Ma. E.J., Ohashi, H., Towers, G.H.N., and Yamamoto, E. (1988) Exclusive accumulation of Z-isomers of monolignols and their glucosides in bark of *Fagus grandifolia.* Phytochemistry **27**: 2119-2121.

Lippincott, B.B., and Lippincott, J.A. (1969) Bacterial attachment to a specific wound site as an essential stage in tumor formation by *Agrobacterium tumefaciens*. J. Bacteriol. **97**: 620-628.

Lippincott, B.B., Whatley, M.H., and Lippincott, J.A. (1977) Tumor induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. Plant Physiol. **59**: 388-390.

Lippincott, J.A., and Lippincott, B.B. (1978) Cell walls of crown gall tumors and embryonic plant tissues lack *Agrobacterium* adherence sites. Science **199**: 1075-1078.

Ma, D., Yanofsky, M.F., Gordon, M.P., and Nester, E.W. (1987) Characterization of *Agrobacterium tumefaciens* strains isolated from grapevine tumors in China. Appl. Env. Microbiol. **53**: 1338-1343.

Machida, Y., Usami, S., Yamamoto, A., Niwa, Y., and Takebe,I. (1986) Plant-inducible recombination between the 25bp border sequences of T-DNA in *Agrobacterium tumefaciens*. Mol. Gen. Genet. **204**: 374-382.

Markwalder, S., and Neukom, H. (1976) Diferulic acid as a possible crosslink in hemicellulose in wheat endosperm. Phytochemistry **15**: 836-837.

Mathews, H., Bharathan, N., Litz, R.E., Narayanan, K.R., Rao, P.S., and Bhatia, C.R. (1990) The promotion of *Agrobacterium* mediated transformation in *Atropa belladona* L. by acetosyringone. J. Plant Physiol. **136**: 404-409.

Matthysse, A.G. (1983) Role of bacterial cellulose microfibrils in *Agrobacterium tumefaciens* infection. J. Bacteriol. **154**: 906-915.

Matthysse, A.G., and Gurlitz, R.H. (1982) Plant cell range for attachment of *Agrobacterium tumefaciens* to tissue culture cells. Physiol. Plant Pathol. **21**: 381-387.

Matthysse, A.G., Holmes, K.V., and Gurlitz, R.H.G. (1981) Elaboration of cellulose microfibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. J. Bacteriol. **145**: 583-595.

Matthysse, A.G., Holmes, K.V., and Gurlitz, R.H.G. (1982) Binding of *Agrobacterium tumefaciens* to carrot protoplasts. Physiol. Plant Pathol. **20**: 27-33.

Maxwell, C.A., Hartwig, U.A., Joseph, C.M., and Phillips, D.A. (1989) A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti.* Plant Physiol. **91**: 842-847.

Miller, J.H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, New York, p.433.

Morris, J.W. and R.O. Morris (1990) Identification of an *Agrobacterium tumefaciens* virulence gene inducer from the pinaceous gymnosperm *Pseudotsuga menziesii* . Proc. Natl. Acad. Sci. (USA) **87**: 3614-3618.

Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Planta **15**: 473-497.

Nair, M.G., Epp, M.D., and Burke, B.A. (1988) Ferulate esters of higher fatty alcohols and allelopathy in *Kalanchöe diagramontiana.* J. Chem. Ecol. **14**: 589-603.

Neish, A.C. (1964) Major pathways of biosynthesis of phenols. In (J.B. Harborne, ed.): Biochemistry of phenolic compounds. Academic Press, London, pp. 618.

Nester, E.W., Gordon, M.P., Amasino, R.M., and Yanofsky, M.F. (1984) Crown gall: a molecular and physiological analysis. Ann. Rev. Plant Physiol. **35**: 387-413.

Niemelä, K. (1990) Low-molecular-weight organic compounds in birch kraft black liquor. Ph.D. Thesis. University of Technology, Helsinki, Finland.

Ohashi, H., Yamamamoto, E., Lewis, N., and Towers, G.H.N. (1987) 5-Hydroxyferulic acid in *Zea mays* and *Hordeum vulgare* cell walls. Phytochemistry **26**: 915-916.

Okker, R.J.H., Spaink, H., Hille, J., van Brussel, T.A.N., Lugtenberg, B., and Schilperoort, R.A. (1984) Plant-inducible virulence promoter of the *Agrobacterium tumefaciens* Ti plasmid. Nature **312**: 564-566.

Oswald, W.F., and B. Benz (1989) *p*-Hydroxyacetophenone and picein contents of healthy and damaged spruce needles from different locations in Bavaria. Eur. J. For. Path. **19**: 323-334.

Overeem, J.C. (1976). In (J. Friend and D.R. Threllfall, eds.): Biochemical aspects of Plant-Parasite Relationships. Academic Press, London, pp. 195.

Parke, D., Ornston, L.N., and Nester, E.W. (1987) Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. J. Bacteriol. **169**: 5336-5338.

Parke, D., Rivelli, M., and Ornston, L.N. (1985) Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. J. Bacteriol. **163**: 417-422.

Pazour, G.J., and Das, A. (1990) *virG*, an *Agrobacterium tumefaciens* transcriptional activator, initiates translation at a UUG codon and is a sequence-specific DNA-binding protein. J. Bacteriol. **172**: 1241-1249.

Pelletier, S.W., Chokshi, H.P., and Desai, H.K. (1986) Separation of alkaloid mixtures using vacuum liquid chromatography. J. Natural Products **49**: 892-900.

Peters, K. and S. Long (1987) The role of plant compounds in the regulation of *Rhizobium* nodulation genes. Symposium #7. 27th Annual Meeting of the Phytochemical Society of North America. Tampa, Florida. June 21-26, 1987.

Peters, N.K., Frost, J.W., and Long, S.R. (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233**: 977-980.

Petit, A., Tempé, J., Kerr, A., Holsters, M., Van Montagu, M., and Schell, J. (1978) Substrate induction of conjugative activity of *Agrobacterium tumefaciens*. Nature **271**: 570-572.

Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L., and Rolfe, B.G. (1986) Flavones induce expression of nodulation genes in *Rhizobium*. Nature **323**: 632-635.

Rhodes, M.J.C. (1985) The physiological significance of plant phenolic compounds. In (C.F. Van Sumere and P.J. Lea, eds.): Annual Proceedings of the Phytochemical Society of Europe, Vol. 25. Oxford University Press, Oxford, pp. 483.

Rhodes, M.J.C., and Wooltorton, L.S.C. (1978) The biosynthesis of phenolic compounds in wounded plant storage tissues. In (Gunter Kahl, ed.): Biochemistry of Wounded Plant Tissues. de Gruyter, Berlin and New York, pp. 680.

Riley, R., and Kolattukudy, P.E. (1975). Evidence for covalently attached *p*-coumaric acid and ferulic acid in cutins and suberins. Plant Physiol. **56**: 650-654.

Robertson, J.L., Holliday, T., and Matthysse, A.G. (1988) Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose sinthesis and bacterial attachment to host cells. J. Bacteriol. **170**: 1408-1411.

Robertson, N.F., Friend, J., and Aveyard, M.A. (1969) Production of phenolic acids by potato tissue culture after infection with *Phytophthora infestans* Phytochemistry **8**: 7.

Rogowsky, P.M., Close, T.J., Chimera, J.A., Shaw, J.J., and Kado, C.I. (1987) Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. J. Bacteriol. **169**: 5101-5112.

Sadowsky, M.J., Olson, E.R., Foster, V.E., Kosslak, R.M., and Verma D.P.S. (1988) Two host-inducible genes of *Rhizobium fredii* and characterization of the inducing compound. J. Bacteriol. **170**: 171-178.

Sahi, S.V., Chilton, M-D., and W.S. Chilton. (1990) Corn metabolites affect growth and virulence of *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. (USA) **87**: 3879-3883.

Schäfer, W., Görz, A., and Kahl, G. (1987) T-DNA integration and expression in a monocot crop plant after induction of *Agrobacterium*. Nature **327**: 529-532.

Schroder, G., Waffenschmidt, S., Weiler, F.W., and Schroder, J. (1984) The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. Eur. J. Biochem. **138**: 387-391.

Sheikholleslam, S.N., and Weeks, D.P. (1987) Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. Plant Mol. Biol. 8: 291-298.

Shimoda, N., Toyoda-Yamamoto, A., Nagamine, J., Usami, S., Katayama, M., Sakagami, Y., and Machida, Y. (1990) Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. Proc. Natl. Acad. Sci. (USA) **87**: 6684-6688.

Sigee, D.C., Smith, V.A., and Hindley, J. (1982) Passage of bacterial DNA into host cells during *in vitro* transformation of *Nicotiana tabacum* by *Agrobacterium tumefaciens*. Microbios **34**: 113-132.

Smith, E.F., and Townsend, C.O. (1907) A plant tumor of bacterial origin. Science **25**: 671-673.

Smith, V.A. and Hindley, J. (1978) Effect of agrocin 84 on attachment of *Agrobacterium tumefaciens* to cultured tobacco cells. Nature **276**: 498-500.

Spencer, P.A., and G.H.N. Towers (1988). Specificity of virulence inducing phenolics detected by *Agrobacterium tumefaciens*. Phytochemistry **27**: 2781-2785.

Spencer, P.A., Tanaka, A., and Towers, G.H.N. (1990) An *Agrobacterium* signal compound from grapevine cultivars. Phytochemistry **29**: 3785-3788.

Stachel, S.E., An, G., Flores, C., and Nester, E.W. (1985a) A Tn3 *lac* Z transposon for the random generation of ß-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. **4**: 891-898.

Stachel, S.E., Messens, E., Van Montagu, M., and Zambryski, P. (1985b) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature **318**: 624-629.

Stachel, S.E., Nester, E.W., and Zambryski, P.C. (1986) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc. Natl. Acad. Sci. (USA) **83**: 379-383.

Stachel, S.E., Timmerman, B., and Zambryski, P. (1987) Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5' *vir*D gene products. EMBO J. **6**: 857-863.

Stachel, S.E., and Zambryski, P.C. (1986a) *vir*A and *vir*G control the plant-induced activation of the T-DNA transfer process of *Agrobacterium tumefaciens*. Cell **46**: 325-333.

Stachel, S.E. and Zambryski, P.C. (1986b) *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. Cell **47**: 155-157.

Subba Rao, P.V., Nambudiri, A.M.D., and Bhat, J.V. (1971) Microbial degradation of phenylpropanoid compounds. J. Sci. Ind. Res. **30**: 663-679.

Swain, T. (1977) Secondary compounds as protective agents. Ann. Rev. Plant Physiol. **28**: 479-501.

Tang, C., and Young, C. (1982) Collection and identification of allelopathic compounds from the undisturbed root system of bigalta limpograss (*Hemarthria altissima*). Plant Physiol. **69**: 155-160.

Thomashow, L.S., Reeves, S., and Thomashow, M.F. (1984) Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indole acetic acid. Proc. Natl. Acad. Sci. (USA) **81**: 5071-5075.

Thomashow, M.F., Karlinsey, J.E., Marks, J.R., and Hurlbert, R.E. (1987) Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects ploysaccharide composition and plant cell attachment. J. Bacteriol. **169**: 3209-3216.

Thomashow, M.F., Knauf, V.C., and Nester, E.W. (1981) Relationship between the limited and wide host range octopine-type Ti-plasmids of *Agrobacterium tumefaciens*. J. Bacteriol. **146**: 484-493.

Thomashow, M.F., Nuter,R., Montoya, A.L., Gordon, M.P., and Nester, E.W. (1980) Integration and organization of Ti-plasmid sequences in crown gall tumors. Cell **19**: 729-739.

Threlfall, D.R., and Whitehead, I.M. (1988) Co-ordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum* Phytochemistry **27**: 2567-2580.

Veluthambi, K., Jayaswal, R.K., and Gelvin, S.B. (1987) Virulence genes A, G, and D mediates the double-stranded border cleavage of T-DNA from the *Agrobacterium* Ti plasmid. Proc. Natl. Acad. Sci. (USA) **84**: 1881-1885.

Vernade, D., Herrera-Estrella, A., Wang, K., and Van Montagu, M. (1988) Glycine betaine allows enhanced induction of the *Agrobacterium tumefaciens vir* genes by acetosyringone at low pH. J. Bacteriol. **170**: 5822-5829.

Vouros, P. (1979) Chemical derivatization in gas chromatography-mass spectrometry. In (Charles Merritt, Jr. and Charles N. McEwen, eds.): Mass Spectrometry, Part B. Marcel Dekker, Inc. New York and Basel, pp. 186.

Wahlroos, Ö., and Virtanen, A.I. (1959) The precursors of 6-methoxybenzoxazolinone in maize and wheat plants, their isolation and some of their properties. Acta. Chem. Scand. **13**: 1906-1908.

Wakarchuk, W.W., Greenberg, N.M., Kilburn, D.G., Miller, R.C.Jr., and Warren, R.A.J. (1988) Structure and transcriptional analysis of the gene encoding a cellobiase from *Agrobacterium* sp. strain ATCC 21400. J. Bacteriol. **170**: 301-307.

Wat, C.-K., and Towers, G.H.N. (1979) Metabolism of aromatic amino acids. In (T. Swain, J.B. Harborne, and C.F. Van Sumere, eds.): Biochemistry of Plant Phenolics, Recent Advances in Phytochemistry, Vol 12. Plenum Press, New York, pp. 651.

Willmitzer, L., De Beuckeleer, M., Lemmers, M., Van Montagu, M., and Schell, J. (1980) The Ti-plasmid derived T-DNA is present in the nucleus and absent from the plastids of plant crown gall cells. Nature **287**: 359-361.

Willmitzer, L., Schmalenbach, W., and Schell, J. (1981) Transcription of T-DNA in octopine and nopaline crown gall tumors is inhibited by low concentrations of α-aminitin. Nuc. Acid Res. **9**: 4801-4812.

Winans, S.C., Ebert,P.R:, Stachel, S.E., Gordon, M.P., and Nester, E.W. (1986) A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. Proc. Natl. Acad. Sci. (USA) **83**: 8278-8282.

Woodward, M.D., Corcuera, L.J., Helgeson, J.P., and Upper, C.D. (1978) Decomposition of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one in aqueous solutions. Plant Physiol. **61**: 796-802.

Yadav, N.S., Postle, K., Saiki, R.K., Thomashow, M.F., and Chilton, M. -D. (1980) T-DNA of a crown gall teratoma is covalently joined to host plant DNA. Nature **287**: 458-461.

Yamamoto, A., Iwahashi, M., Yanofsky, M.F., Nester, E.W., Takebe, I., and Machida, Y. (1987) The promoter proximal region in the *vir*D locus of *Agrobacterium tumefaciens* is necessary for the plant-inducible circularization of T-DNA. Mol. Gen. Genet. **206**: 174-177.

Yamamoto, E., Bokelman, G.H., and Lewis, N.G. (1989) Phenylpropanoid metabolism in cell walls. In (N.G. Lewis and M.G. Paice, eds.): Plant cell wall polymers, ACS Symposium series 399, ACS, Washington, pp. 676.

Yanofsky, M., Lowe, B., Montoya, A., Rubin, R., Krul, W., Gordon, M., and Nester, E.W. (1985) Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*. Mol. Gen. Genet. **201**: 237-246.

Yanofsky, M.F., and Nester, E.W. (1986) Molecular characterization of a host-range-determining locus from *Agrobacterium tumefaciens*. J. Bacteriol. **168**: 244-250.

Yanofsky, M.F., Porter, S.G., Young, C., Albright, L.M., Gordon, M.P., and Nester, E.W. (1986) The *vir*D operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. Cell **47**: 471-477.

Yoshikawa, T., and Furuya, T. (1987) Saponin production by cultures of *Panax ginseng* transformed with *Agrobacterium rhizogenes*. Plant Cell Report **6**: 449-453.

Young, C., and Nester, E.W. (1988) Association of the VirD2 protein with the 5' end of T strands in *Agrobacterium tumefaciens*. J. Bacteriol. **170**: 3367-3374.

Zaat, S.A.J., Schripsema, J., Wijffelman, C.A., van Brussel, A.A.N., and Lugtenberg, B.J.J. (1989) Analysis of the major inducers of the *Rhizobium nod*A promoter from *Vicia sativa* root exudate and their activity with different *nod*D genes. Plant Mol. Biol. **13**: 175-188.

Zambryski, P., Holsters, M., Kruger, K., Depicker, A., Schell, J., Van Montagu, M., and Goodman, H.M. (1980) Tumor DNA structure in plant cells transformed by *A. tumefaciens*. Science **209**: 1385-1391.