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John W. Morris for the degree of Doctor of Philosophy in Forest Science

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Signature redacted for privacy.

Abstract approved: _____
Roy O. Morris

Signature redacted for privacy.

Abstract approved: _____
Steven H. Strauss

Agrobacterium tumefaciens and *Agrobacterium rhizogenes*, the causal agents of crown gall disease and hairy root disease, are known for their ability to transfer part of their DNA (the T-DNA) from the bacterial tumor-inducing (Ti) plasmid to the genome of the host plant. Interest has focused on these bacteria because they provide one of the more efficient means of introducing foreign DNA into susceptible plants. Although long recognized as a pathogen of dicotyledonous angiosperms, *Agrobacterium* strains have not generally been regarded as strongly tumorigenic on gymnosperms. Studies were undertaken which identified *Agrobacterium* strains which were both strongly tumorigenic and weakly tumorigenic on four pinaceous gymnosperms; *Abies procera*, *Pinus ponderosa*, *Pseudotsuga menziesii* and *Tsuga heterophylla*. Identification of opines in the tumor tissue confirmed host transformation.

Strongly tumorigenic bacterial strains were also isolated from native galls found on *P. menziesii*. Certain native bacterial isolates harbored Ti-plasmid sized plasmids and incited rapid tumor development.

Successful plant transformation by *Agrobacterium* requires the induction and expression of a cascade of bacterial virulence genes. This cascade is initiated when periplasmic or transmembrane regions of the bacterial virA protein interact with certain phenolic compounds originating from the host plant. Because it was not known whether the range in bacterial tumorigenicity observed on conifers was due to differences in strain sensitivity to host phenolic compounds, or was due to other causes, studies were undertaken to identify compounds in *P. menziesii* extracts capable of inducing expression of the *Agrobacterium* virulence genes.

Coniferin was identified as the major inducing compound in *P. menziesii* extracts. As a glucoside, coniferin has a novel structure in comparison to other *Agrobacterium* virulence gene inducers which are aglycones. In a representative set of *Agrobacterium* strains, interesting parallels exist between levels of bacterial *beta*-glucosidase, virulence gene induction by coniferin and tumorigenicity levels on conifer hosts. Further, *beta*-glucosidase activity is enhanced when strongly tumorigenic bacteria are cultured in the presence of coniferin, but not in the presence of coniferyl alcohol, the aglycone.

Efficient Transformation of Pinaceous Gymnosperm Cells by *Agrobacterium*

by

John W. Morris

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APPROVED:

Signature redacted for privacy.

Professor of Biochemistry, University of Missouri-Columbia, adjunct Professor of Forest Science, Oregon State University in charge of major.

Signature redacted for privacy.

Associate Professor of Forest Science, Oregon State University in charge of major.

Head of Department of Forest Science

Dean of Graduate School

Date thesis is presented June 5, 1990

by John W. Morris

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TABLE OF CONTENTS

INTRODUCTION 1
Review of Literature 4

AGROBACTERIUM INCITED GALL FORMATION ON CONIFERS 28
Introduction 28
Methods 29
Results 32
Discussion 42

BACTERIAL ISOLATES FROM *PSEUDOTSUGA MENZIESII* GALLS 46
Introduction 46
Methods 47
Results 50
Discussion 60

INDUCTION OF THE *AGROBACTERIUM VIR* CASCADE. 63
Introduction 63
Methods 65
Results 70
Discussion 83

CHARACTERIZATION OF INDUCING COMPOUNDS IN *PSEUDOTSUGA MENZIESII* EXTRACTS 86
Introduction 86
Methods 87
Results 93
Discussion 107
Project Summary 109

BIBLIOGRAPHY 111

APPENDICES 123
Agrobacterium strains 123
Host plants of origin and *Agrobacterium* strain suppliers 126
Suppliers of bacterial strains 128
Hexamer primer procedure for preparation of radiolabelled DNA probes 129
Cytokinin ELISA protocol 131
Tri-parental mating protocol 134
VirE::lacZ induction assay 135
Bacterial culture media 138

LIST OF FIGURES

Figure		Page
1.	Structures of *Agrobacterium* virulence gene inducers	17
2.	Typical galls incited by *A. tumefaciens* strains on pinaceous hosts	36
3.	Relationship between tumorigenicity and tumor size	39
4.	Opine identification in gall extracts following paper electrophoresis	41
5.	Native galls of *P. menziesii*	51
6.	Colony hybridization of native bacterial isolates	56
7.	Large and small plasmids of native bacterial isolates	56
8.	Galls incited on conifers by native bacterial isolates	59
9.	Ti plasmid digests and *tzs* hybridization	75
10.	Effect of acetosyringone concentration on *vir* gene induction	77
11.	Effect of initial culture density on cell growth and *vir* gene induction	77
12.	Effect of incubation time on virulence gene induction	78
13.	Correlation of acetosyringone induced beta-galactosidase activity and cytokinin production	81
14.	*Agrobacterium* virulence gene induction by four phenolic compounds	82
15.	Induction of the *vir* gene cascade in B3/73(pSM358) and MFM83.4(pSM358) by compounds present in *P. menziesii* extracts	94
16.	Efficiency of polyvinylpolypyrolidone in enrichment of inducing compounds	94
17.	Presence of inducing compounds in solvent partition fractions	96
18.	Active compounds in *P. menziesii* shoot extract	97
19.	Effect of beta-glucosidase on purified PM1	98
20.	Mass spectra of PM1 and coniferin	98
21.	Nuclear magnetic resonance spectra of PM1 and coniferin	100
22.	Induction of virulence gene expression by PM1 and coniferin	101
23.	Strain specific induction by coniferin	101
24.	Induction of *Agrobacterium* beta-glucosidase expression	102
25.	Relationship between tumorigenicity and glucosidase expression	104

LIST OF TABLES

Table		Page
1.	Preliminary experiments on *Agrobacterium* tumorigenicity	33
2.	Tumor formation and opine identification in *Kalanchoe*	35
3.	Tumor frequency and tumor size on conifer hosts	38
4.	Native isolates and their sources	52
5.	Hybridization and plasmid status of native isolates	57
6.	Plasmid content and tumorigenicity of native isolates	58
7.	Effect of carbon source on growth of *A. tumefaciens* strain B3/73	71
8.	Growth optimization of *A. tumefaciens* strain B3/73	71
9.	Acetosyringone induced cytokinin production in *Agrobacterium* strains	73
10.	Acetosyringone induced *beta*-galactosidase activity and zeatin/zeatin riboside secretion	80
11.	Tumorigenicity and virulence gene induction of *Agrobacterium* strains	81
12.	Chromatography columns and gradients	88
13.	*Beta*-glucosidase activity of *Agrobacterium* strains	104
14.	Virulence gene induction by coniferyl alcohol and coniferin	105
15.	*Beta*-glucosidase activity in the presence of coniferyl alcohol and coniferin	106

PREFACE

Parts of this work have been previously published. Much of the information in Chapter II was published under the title "Efficacy of different *Agrobacterium tumefaciens* strains in transformation of pinaceous gymnosperms" by John W. Morris, Linda A. Castle and Roy O. Morris in the journal, Physiological and Molecular Plant Pathology (1989, volume 34, pages 451-461). Ms. Linda Castle was included as a author because of her significant efforts in planting, maintaining, inoculating and evaluating the seedlings. Those portions of the above article included in this work appear with the permission of the publisher, Academic Press, Ltd. Much of the information in Chapter V was published under the title "Identification of an *Agrobacterium tumefaciens* virulence gene inducer from the pinaceous gymnosperm *Pseudotsuga menziesii*" by John W. Morris and Roy O. Morris in the Proceedings of the National Academy of Science (1990, volume 87, pages 3614-3618).

EFFICIENT TRANSFORMATION OF PINACEOUS GYMNOSPERM CELLS BY *AGROBACTERIUM*

INTRODUCTION

The considerable interest in the plant pathogens *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* is based on their ability to stably transfer DNA to the plant host. It was recognized that genetic manipulation of *Agrobacterium* strains and subsequent plant transformation by such modified strains provides a mechanism to introduce heterologous genes into plants. At the initiation of this project most studies were undertaken using model angiosperm hosts such as tobacco. This project has sought to begin extending the potentials of *Agrobacterium* mediated gene transfer to conifers. As noted below, substantial progress has been made in understanding the mechanisms which underlie the transformation process.

Not all plants are highly susceptible hosts to *Agrobacterium* transformation (De Cleen and De Ley 1976). For a given *Agrobacterium* strain, the plant host range is typically restricted (Anderson and Moore 1979, Leroux *et al.* 1987). At the initiation of this project, reports of *A. tumefaciens* transformation of conifer hosts were limited to the work of Smith (1935, 1942). These investigations used relatively uncharacterized bacterial isolates from willow and peach crown galls. The bacteria were capable of inciting tumors on conifer stems after a period of 11 to 22 months and longer. Members of the *Taxaceae*, *Taxodiaceae*, and *Pinaceae* were examined including species from the genera, *Abies*, *Juniperus*, *Pinus*, *Picea*, *Psudotsuga*, *Thuja*, and others. Some species formed galls while others did not. No galls were reported on *Pinus*, and reports of tumor induction on

Psudotsuga were conflicting. It was therefore apparent that the establishment of host susceptibility was the first requirement of any project which sought to examine factors influencing *Agrobacterium* mediated transformation of conifers.

The conifers examined in the studies which follow, *Abies procera*, *Pinus ponderosa*, *Pseudotsuga menziesii*, and *Tsuga heterophylla*, were of interest because they represent a range of the genera within the *Pinaceae* and are commercially significant.

In simple terms this project sought to answer two questions.

First, are pinaceous species susceptible to *Agrobacterium* transformation?

Second, why are certain *Agrobacterium* strains more tumorigenic than others?

Objectives and key results of the project were as follows.

I. Identification of *Agrobacterium* strains which are strongly tumorigenic on conifer hosts.

Two approaches were taken. First, seedlings of the four hosts were inoculated with characterized *Agrobacterium* strains and the incidence of tumor formation measured. These characterizations of *Agrobacterium* strain tumorigenicity are described in Chapter II. *A. tumefaciens* strains K47 and B3/73 were notable for their high tumorigenicity levels.

II. Identification of *Agrobacterium* strains from naturally occurring *P. menziesii* galls.

The second approach, undertaken concurrently, was to identify highly tumorigenic *Agrobacterium* strains in galls found on *P. menziesii* growing in the wild. Because the successes of the *Agrobacterium* inoculations allowed the project to proceed to the question

as to why certain strains were more efficient, the characterizations of native gall bacteria remain incomplete. The initial characterization of the native gall isolates are described in Chapter III, and demonstrate that highly tumorigenic strains may be isolated from native galls.

III. Measurement of *vir* gene induction by strongly and weakly tumorigenic strains.

The second question of the project, that of the mechanisms defining strain efficiency, can only be partially addressed by this work. Strain to strain differences may exist which influence tumorigenicity at each step of the transformation process. Because induction of virulence (*vir*) gene expression is one of the earliest steps required in host transformation, it was possible measurement of *vir* gene induction in strongly and weakly tumorigenic *Agrobacterium* strains would provide insight into the basis for the tumorigenicity differences. Two approaches were used. First, acetosyringone induced expression of the Ti plasmid encoded gene *tzs* was examined. Second, a *vir* gene reporter fusion was transferred to a set of *Agrobacterium* strains, and acetosyringone induced expression of the fusion was examined. The fusion plasmid (pSM358) contained a *vir* gene promoter fused to the structural region of *lacZ*, which encodes *beta*-galactosidase. The results of these studies are described in Chapter IV, and indicate higher levels of *vir* induction by strongly tumorigenic strains than weakly tumorigenic strains.

IV. Identification of the major *vir* gene inducing compound found in *P. menziesii* extracts.

Acetosyringone, the inducer used in the virulence gene induction studies, had been isolated as the major active component in tobacco. However, was this the active inducer in conifers? To answer this question, the major inducing compound in P. *menziesii* extracts was identified. It is coniferin. Details of its isolation and identification are provided in Chapter V.

VI. Measurement of *beta*-glucosidase expression by strongly and weakly tumorigenic strains.

The *beta*-glucoside structure of coniferin suggested a further question. Is there bacterial expression of a *beta*-glucosidase, and if so is there strain specificity in glucosidase expression? Results described in Chapter V indicate bacterial expression may be important in strain tumorigenicity on conifers.

REVIEW OF LITERATURE

The review of literature presented below is, of necessity, selective. Current publications relating to *Agrobacterium* are in excess of 200 per year. The focus of the review is therefore only on components affecting *Agrobacterium* transformation, with emphasis on the bacterial side of the interaction (for additional reviews see Nester 1984, Zambryski 1988, Zambryski *et al.* 1989). Specific information on conifer transformation is included at the end of the review.

Members of the genus *Agrobacterium* are gram negative, rod-shaped, aerobic soil bacteria (Kersters and De Ley, 1984). *A. tumefaciens* is the causal agent of crown gall, a disease which results in neoplastic growth of infected tissue. Virulence is correlated with the presence of a large plasmid termed the tumor inducing plasmid (Ti plasmid). Tumor formation is the result of the transfer of a copy of a portion of the Ti plasmid (the T-DNA) to the host cell and expression of the encoded genes (Zambryski *et al.* 1989). Transfer of the T-DNA is mediated by Ti-plasmid encoded virulence genes. The T-DNA transfer complex is thought to consist of a copy of the T-DNA (the T-strand) and a set of associated proteins (Citovsky *et al.* 1989a).

Nomenclature.

The nomenclature for the genus is unsettled. Historically, the division of species followed the morphological characteristics of the disease. *A. tumefaciens* strains incited unorganized or shooty (crown gall) tumors. *A. rhizogenes* strains incited tumors which proliferated roots (hairy root). *A. radiobacter* was avirulent. With the advances in understanding of the molecular basis for *Agrobacterium* tumorigenesis, it became clear that species definition based on tumor morphology was inadequate. Bacterial virulence was found to be associated with the presence of large plasmids termed tumor inducing (Ti) plasmids for strains inciting crown gall, and root inducing (Ri) plasmids for strains inciting hairy root (Watson *et al.* 1975, White and Nester 1980a). Transfer of an Ri plasmid into a plasmidless derivative of *A. tumefaciens* resulted in a strain capable of inciting the massive root proliferation characteristic of hairy root disease (White and Nester 1980a). Defining the *Agrobacterium* species on the basis of the disease symptoms amounted to defining the presence of a Ti or Ri plasmid. These plasmid are typically on the order of 200 kilobasepairs (kb) in size (Sciaky *et al.* 1978) and therefore only a fraction of the bacterial genome. As defining a species based on this small fraction of the total genome was

inappropriate, other classification criteria have been developed. Several investigators have identified metabolic indicators which allowed the classification of *Agrobacterium* strains into subgroups (Kersters and De Ley 1984, Knauf *et al.* 1982, Holmes and Roberts 1981). The nomenclature encouraged by Holmes and Roberts (1981) has been adopted by the Commonwealth Mycological Institute (CMI) (Bradbury 1986). This nomenclature relies on a set of microbiological tests and is independent of major pathological symptoms. The classification identifies three species which are approximately equivalent to the biotypes of Keane *et al.* (1970); *A. tumefaciens* for biotype I, *A. rhizogenes* for biotype II and *A. rubi* as the third species. Biotype III was not categorized in the CMI system. This leaves a gap in assigning a species name to isolates having biotype III characteristics. In this work, biotype III strains have been assigned to *A. tumefaciens*. Among the naturally occurring *Agrobacterium* strains, this nomenclature generally follows pathogenicity. Naturally occurring *A. tumefaciens* (biotype I strains) may be avirulent or incite tumors, but few strains which fall into this class are rhizogenic . Naturally occurring *A. rhizogenes* (biotype II strains) may be avirulent, but a large majority of the rhizogenic strains fall into this class (Bradbury 1986).

Several media formulations have been developed for the selective isolation of *Agrobacterium* from soil and plant tumor tissue samples (Schroth *et al.* 1965, Kado and Heskette 1970, Brisbane and Kerr 1983). In general they make use of metabolic characteristics to allow the differential growth of different biotypes. The three media developed by Brisbane and Kerr (1983) base selection on carbon and nitrogen sources. Growth of biotype I is supported by L-arabitol, biotype II by erythritol, and biotype III by tartrate and D-glutamate. Complete formulations are given in the appendix.

A common nomenclature used to distinguish among *A. tumefaciens* strains is to refer to the opine status of the strain. Opines are novel plant metabolites, nopaline, octopine, succinamopine, agropine and others (described below) formed by the activities of enzymes encoded on the T-DNA. Strains which encode nopaline synthase on their T-DNA are referred to as nopaline strains; octopine, succinamopine, and agropine strains are derived similarly.

Host Range.

Agrobacterium strains exhibit marked host specificity. Anderson and Moore (1979) examined the tumorigenicity of 176 strains on 11 host plants. Using a subset of 89 isolates they found 66 % were tumorigenic on 6, 7, or 8 hosts. A smaller fraction, 10 %, was tumorigenic on only 1 or 2 hosts. A few (2 %) exhibited a wide host range and were tumorigenic on 9 or 10 hosts. None of the 89 were tumorigenic on all 11 hosts. A range of host susceptibility was also demonstrated in this study. Tomato and *Datura* were most susceptible, 81 % of the strains were tumorigenic on each host. Least susceptible were apple (2% of strains tumorigenic) and sugar beet (7 % of strains tumorigenic). These same investigators found that host range differences among strains occur even among isolates from the same native gall. Certain isolates from a *Lippia canescens* gall were found to be tumorigenic on apple, radish or beet while others were not. Curiously, not all virulent isolates were tumorigenic on *Lippia*, the host of origin.

Host range is primarily determined by the Ti plasmid. Loper and Kado (1979) examined the effect of mobilizing an octopine wide host range Ti plasmid (15955) into a narrow host range grapevine isolate (1D1109) with the aid of the conjugative plasmid RK2. The host range of the resulting transconjugant, as assayed on 37 plant species, was identical to the donor strain 15955. The presence of plasmid RK2 had no effect on host range.

The host range specificities of wild-type grapevine isolates have been compared with the specificities of Ti plasmid transconjugants (Thomashow *et al.* 1980, Knauf *et al.* 1982). The Ti plasmids of the grapevine isolates were transferred to *A. tumefaciens* strain A136, a derivative of the wide host range nopaline strain C58 which lacks a Ti plasmid. In 20 of 22 cases the host range of the transconjugant was identical to the donor grapevine isolate. Interestingly, A136 harboring the wide host range octopine Ti plasmid B6806 was avirulent on grapevine. In view of the tumorigenicity associated with the grapevine Ti plasmids, the avirulence of strain A136(pTiB6806) must be an effect of the Ti plasmid (Knauf *et al.* 1982).

A limited host range was also found associated with an *A. tumefaciens* isolate from a native *Lippia* crown gall (Unger *et al.* 1985). When transferred to an avirulent strain, A702, the transconjugant acquired the same host range as the donor strain. In addition to its large size (500 kb) and association with a limited host range, the *Lippia* Ti plasmid was unusual in that it failed to specify catabolism of octopine, nopaline, agropine or mannopine. Also, it failed to show hybridization to T-DNA sequences from pTiA6. Homologous sequences were detected to pTiA6 virulence loci *virB*, *virC*, *virD*, and *virG*, but not to *virA* or *virE* (*vir* genes described below).

Several studies have attempted to determine exactly which Ti plasmid encoded factors influence host range. Knauf *et al.* (1984) investigated the narrow host range grapevine isolate Ag162, and found that pTiAg162 plasmid sequences were able to restore virulence to strains harboring mutagenized pTiA6. Complementation of virulence genes *virB*, *virD* and *virE* by pTiAg162 cloned fragments restored wide the host range of pTiA6. Therefore these genes were not responsible for the narrow host range of pTiAg162.

Further, complementation of pTiAg162 with *vir* region genes of pTiA6 did not extend the pTiAg162 host range.

Yanofsky *et al.* (1985) examined the T-DNA of this same strain, Ag162. Unlike the wide host range strains A6 or C58, the T-DNA encoded oncogenes *iaaM* and *iaaH* were located on a widely separated T-DNA segment from *ipt* (T-DNA gene loci described below). One segment, termed T_A-DNA contained sequences homologous to "transcript 5", "transcript 6", *ocs*, and *ipt*. The other T-DNA segment termed, T_B-DNA, showed regions homologous to *iaaH* and *iaaM*. Deletion of the T_A-DNA did not affect virulence on *Nicotiana rustica* or *Vitis labruscana* cv. Steuben. However, when inoculated onto *Nicotiana glauca* or *Vitis* sp. cv. Seyval, this deletion led to an avirulent phenotype. Reciprocal experiments were conducted in which T_A-DNA and T_B-DNA regions were introduced into the wide host range octopine strain ACH5 carrying a T-DNA deletion (derivative strain LBA4404). The strain containing the T_A-DNA was avirulent on *Nicotiana glauca*, *Nicotiana rustica* and *Kalanchoe*, but was tumorigenic on *Vitis labruscana* cv. Steuben. The strain containing the T_B-DNA was virulent on *Nicotiana glauca*, *Nicotiana rustica* and *Kalanchoe* as well as *Vitis* sp. cv. Seyval and *Vitis labruscana* cv. Steuben. The strain containing the T_B-DNA produced roots from tumors on *Nicotiana rustica* and *Kalanchoe*.

The oncogene *ipt* from wide host range plasmids was found to extend the host range of narrow host range grapevine isolates. The T-DNA genes *ipt*, 6a, and 6b contained on a segment of the pTiA6 T-DNA were inserted into the Ti plasmid of Ag63 (Buchholz and Thomashow 1984b). Strains which harbored pTiAg63 containing the insert were capable of inciting unorganized tumors on tomato, carrot and sunflower. These three hosts did not normally express a tumorous phenotype when inoculated with Ag63. Similarly, the *ipt* gene

of the wide host range strain Ach5 was able to extend the host range of strains harboring pTiAg57 (Hoekema *et al.* 1984).

Chromosomal effects have been noted on host range. Grapevine isolates Ag105 and Ag123 were capable of inciting tumors on tomato. Yet, when these Ti plasmids were transferred to A136, the resulting strains were avirulent on tomato while still virulent on *Nicotiana glauca* and grapevine (Knauf *et al.* 1982). Similarly, the host ranges associated with pTiAg57 or pTiAg63 were altered when the plasmids were transferred to A136. Small galls were incited on *Nicotiana tobacum* cv Turkish by parental strains, but transconjugants were avirulent. Virulence on *Vitis vinifera* cv. Sultanian and attenuated virulence on *Datura* and sunflower, however, were maintained (Thomashow *et al.* 1980).

Plant Growth Regulators and *Agrobacterium*.

Unlike tissue taken from normal plants, transformed tissues may be cultured in the absence of exogenously supplied auxin and cytokinin (Braun and White 1943, DeRopp 1947). In addition to cellular proliferation, other auxin-linked growth effects have been observed on tumorous plants (Brown 1936, DeRopp 1947). Following the discovery of zeatin, it was found that the addition of indoleacetic acid (IAA) and zeatin to the basal culture medium allowed normal tissue to match the growth of tumor tissue (Braun 1958). Subsequent work by many investigators has identified the presence of IAA (Amasino and Miller 1982, Nakajima *et al.* 1981), zeatin and zeatin-related cytokinins in tumors (Akiyoshi *et al.* 1983, Chen *et al.* 1976, Morris 1977, Peterson and Miller 1977, Weiler and Spanier 1981).

Plant growth regulator production by *Agrobacterium* strains independent of plant association has also been characterized. Increased levels of IAA were reported to be

associated with the presence of the Ti plasmid (Liu *et al.* 1982). Zeatin was identified in culture filtrates of the nopaline strain C58 (Kaiss-Chapman and Morris 1977). The locus conferring the ability to secrete zeatin was shown to be Ti plasmid encoded (Regier and Morris 1982). This zeatin secretion locus, *tzs*, was found to be associated with nopaline Ti plasmids, and mannopine Ri plasmids (Akiyoshi *et al.* 1987). The cytokinin iso-pentenyladenine (iP) and its riboside are produce by *Agrobacterium* cultures, but zeatin is produced only by virulent strains (Kaiss-Chapman and Morris 1977).

Chromosomally encoded determinants of *Agrobacterium* virulence.

Genetic elements which are important in *Agrobacterium* virulence are encoded both chromosomally and on the Ti plasmid. In general, chromosomally encoded virulence genes influence cell surface composition and appear to influence bacterial attachment to the plant cell wall. The *cel* locus functions in the production of cellulose microfibrils by the bacterium (Matthysse 1983). *Cel*$^-$ mutants (generated by Tn*5* insertion) were found to be deficient in production of microfibrils and impaired in attachment to regenerating carrot protoplasts. However these *cel*$^-$ mutants were still virulent. The *att* locus influences cell surface protein composition and affects bacterial attachment (Matthysse 1987). *ChvA* and *chvB* are genetically linked chromosomal virulence loci which are involved in the production and secretion of *beta*-1,2-glucan (Douglas *et al.* 1985, Zorreguieta *et al.* 1988). *ChvB* codes for a membrane protein responsible for incorporation of UDP-glucose into the chain (Zorreguieta *et al.* 1988) while *chvA* functions in transport of the *beta*-1,2-glucan across the bacterial inner membrane (Cangelosi *et al.* 1989). An *Agrobacterium* homologue of the *Rhizobium exoC* gene, *pscA*, influences cyclic glucan and acidic succinoglycan synthesis (Cangelosi *et al.* 1987, Marks *et al.* 1987, Thomashow *et al.* 1987)

Ti plasmid encoded determinants of virulence.

The Ti plasmid of *A. tumefaciens* is approximately 200 kb in size and contains two regions which are important in transfer of genetic material to the host plant. The first region is the T-DNA, a copy of which is stably integrated into the host plant nuclear genome. The second region is the *vir* (virulence) region. Virulence gene expression is coordinately regulated and required for T-DNA transfer. A third set of genes present on the Ti plasmid, important in the biology of *Agrobacterium*, is involved in opine metabolism. These three regions are generally conserved among *Agrobacterium* strains as measured by DNA-DNA hybridization studies (Engler *et al.* 1981, Thomashow *et al.* 1981, White and Nester 1980a). Each of the three regions is described below.

The T-DNA.

The use of insertion and deletion mutagenisis has allowed the mapping of T-DNA loci involved in tumor morphology (Garfinkel *et al.* 1981, Ooms *et al.* 1981 Joos *et al.* 1983) mutations of the *tms* locus incited shooty tumors. Tumor formation by such mutated strains was stimulated by auxins. In contrast, strains carrying mutations of the *tmr* locus incited rooty tumors, and tumor formation was stimulated by cytokinin (Ooms *et al.* 1981, Joos *et al.* 1983). Mutations in a third locus, *tml* of pTiA6 produced large tumors on tobacco (Garfinkel *et al.* 1981). Mutation of the corresponding region of the pTiC58 T-DNA did not have any clear influence on tumor development in tobacco (Joos *et al.* 1983).

The oncogene loci, *tms* and *tmr*, have been sequenced and the encoded gene products characterized. *Tms* (now known to be *tms1* and *tms2*) has been shown to share homology with genes of the bacterial plant pathogen *Pseudomonas syringae* pathovar *savastanoi* (Yamada *et al.* 1985). *Tms1 (iaaM)* encodes tryptophan mono-oxygenase. *Tms2*

(*iaaH*) encodes indoylacetamide hydrolase. Together the enzymes provide a pathway for the biosynthesis of IAA from tryptophan (Yamada *et al.* 1985).

The *tmr* locus (now termed *ipt*) has been show to encode an iso-pentenyl transferase, which catalyzes the synthesis of the cytokinin iso-pentenyladenosine-5'-mono-phosphate from of dimethylallylpyrophosphate and adenosine 5' monophosphate (Akiyoshi *et al.* 1984, Barry *et al.* 1984, Buckman *et al.* 1985). The loci *iaaM*, *iaaH* and *ipt* are conserved among the well characterized Ti plasmids (Chilton *et al.* 1978, Depicker *et al.* 1978, Drummond and Chilton 1978).

Physical Organization of the T-DNA.

The T-DNA of octopine plasmids is split into T_L-DNA or T_R-DNA (Thomashow *et al.* 1980a). Further characterization of the T-DNA sequence (Barker *et al.* 1983) and the transfer process identified T-strands containing the middle segment (T_c-DNA) (Stachel *et al.* 1987). The loci *iaaM*, *iaaH* and *ipt* of the A6 octopine Ti plasmid have been localized to the T_R-DNA (Garfinkel *et al.* 1981) and transfer of the T_R-DNA alone is sufficient for tumor formation (Ooms *et al.* 1982). Similarly, the Ri plasmids of the *A. rhizogenes* wide host range strain A4 and the narrow host range strain Ag162 were shown to have a split T-DNA (Huffman *et al.* 1984, White *et al.* 1985, Yanofsky *et al.* 1985b). In contrast to the divided T-DNAs of octopine and *A. rhizogenes* plasmids, the well characterized nopaline Ti plasmids of strains C58 and T37 have T-DNA which is transferred as a single segment (Chilton 1977, Lemmers 1980, Yang 1980).

***A. tumefaciens* T-DNA transcription.**

Willmitzer *et al.* (1983) identified thirteen T-DNA encoded, poly-adenylated transcripts from tobacco teratoma cell lines incited by *A. tumefaciens* nopaline strain T37.

At least five of the transcripts; *iaaM* (transcript 1), *iaaH* (transcript 2), *ipt* (transcript 4), and *tml* (transcripts 6a and 6b) cross hybridized with transcripts from tobacco tumor lines incited by *A. tumefaciens* octopine strain A6. In addition to the oncogenes, opine biosynthetic gene transcripts have been identified; nopaline synthase (*nos*), agrocinopine synthesis (*acs*) and octopine synthase (*ocs*) (Willmitzer *et al.* 1983, Schröder *et al.* 1981).

Transcription of parts of the T-DNA is found in the bacterium as well as in transformed plants (Janssens *et al.* 1984). At least twelve T-DNA transcripts were present in *A. tumefaciens* strain C58. Six of the transcripts appeared to correspond to T-DNA transcripts found in plants; d, 5, 2 (*iaaH*), 1 (*iaaM*), 6a, 6b (*tml*). In addition, a likely correlation exited between transcript 3 (nos) and a very weak bacterial transcription from *Hin*dIII fragment 23.

The entire T-DNA of the *A. tumefaciens* octopine strain 15955 has been sequenced (Barker *et al.* 1983). Within the 22,874 nucleotides there were 26 open reading frames longer than 300 bases which started with an ATG initiation codon. Fourteen of the open reading frames were bounded by putative eukaryotic promoters and poly(A) addition sites. Two 24 base-pair repeats bordered the T-DNA, and another two similar repeats were found internally which divided the T-DNA into three segments; T_L-DNA, T_C-DNA and T_R-DNA. All of the open reading frames having eukaryotic transcription characters were found in the T_R-DNA or T_L-DNA.

T-DNA of Ri plasmids.

The T-DNA of Ri plasmids show homology to the auxin biosynthetic genes, but *ipt* is absent (Huffman *et al.* 1984). In addition to *iaaM* and *iaaH*, the T-DNA of Ri plasmids contain four additional *rol* loci which are involved in the production of the hairy root

phenotype. Inoculation of strains of *A. rhizogenes* A4 carrying transposon mutations in the T_L-DNA onto *Kalanchoe* exhibited different tumor phenotypes. Mutations of the *rolA* locus produced straighter roots than the wild-type strain. *RolB* mutants were avirulent. *RolC* mutants were attenuated in root growth. *RolD* mutants produced more callus and root growth was retarded (White *et al.* 1985). Mutagenisis and complementation of the *rol* loci identified *rolA* as responsible for the wrinkle leaf phenotype which is characteristic of regenerated, transformed plants carrying Ri T-DNA (Sinkar *et al.* 1988).

Opines.

Tumor formation is accompanied by the synthesis of novel plant metabolites termed opines (coined by Shell and Van Montagu 1977). Seven opine families are presently identified: nopaline, octopine, agropine, succinamopine, cucumopine, leucinopine, and agrocinopine. Multiple family members may be synthesize by tumor tissue incited by a single strain. Thus agrocinopines A and B commonly occur together with nopaline, while agrocinopines C and D commonly are present with agropine (Ellis *et al.* 1982). Nopaline, octopine, succinamopine, leucinopine and cucumopine are amino acid conjugates to keto acids, while agropine is a cyclic conjugate of mannose and glutamine (Petit *et al.* 1983, Chilton *et al.* 1984, Chilton *et al.* 1985, Davioud *et al.* 1988). Agrocinopines are phosphorylated sugar derivatives (Ellis and Murphy 1981, Ryder *et al.* 1984).

Enzymes required for opine biosynthesis are T-DNA encoded (Garfinkel *et al.* 1981, Bevan *et al.* 1983, Joos *et al.* 1983, DeGreve *et al.* 1983). Enzymes for opine catabolism are encoded by Ti plasmid sequences which map to positions outside the T-DNA and correspond to the opine biosynthetic enzymes encoded by T-DNA (Holsters *et al.* 1982). Opines may serve the bacteria as carbon, nitrogen, and energy sources (Bomhof *et al.* 1976, Montoya *et al.* 1977, Garfinkel *et al.* 1980). Octopine and agrocinopines also serve

as signals for conjugative transfer of the Ti plasmids which encode their respective biosynthetic genes (Kerr *et al.* 1977, Petit *et al.* 1978, Ellis *et al.* 1982).

Induction of Virulence Region Gene Expression.

Transposon mutagenisis and complementation of pTiA6 identified six complementation groups (*virA* to *virE* and *virG*) which were required for normal virulence (Klee *et al.* 1982, Klee *et al.* 1983). These loci have been shown to function in *trans* for T-DNA transfer when located on separate plasmids (Barton 1983, Hoekema 1983, Holsters 1980, Klee *et al.* 1982).

The regulation of expression of the *vir* genes was examined using transposon mutagenisis (Stachel and Nester 1986). The transposon Tn*3*-HoHo1 carries a promoterless *E. coli lacZ* gene (which encodes *beta*-galactosidase) and its insertion into an open reading frame produces either a transcriptional or translational gene fusion (Stachel *et al.* 1985a). Estimation of *vir* promoter activity from *vir:lacZ* genes can readily be undertaken by measurement of *beta*-galactosidase activity.

Induction of *beta*-galactosidase expression was found when *A. tumefaciens* strains containing *vir:lacZ* inserts were incubated with tobacco suspension cultures. *VirB*, *virC*, *virD* and *virE* were inducible. *VirA* was found to be constitutively expressed, while *virG* was both constitutively expressed and inducible (Stachel and Nester 1986).

Using *vir:lacZ* induction as the basis for assay, acetosyringone and hydroxyacetosyringone were identified as the major native inducers in tobacco suspension cultures and root cultures (Stachel *et al.* 1985b). Other native inducers have recently been identified from petunia pollen and stigma. They are flavonol glycosides kaempferol 3-

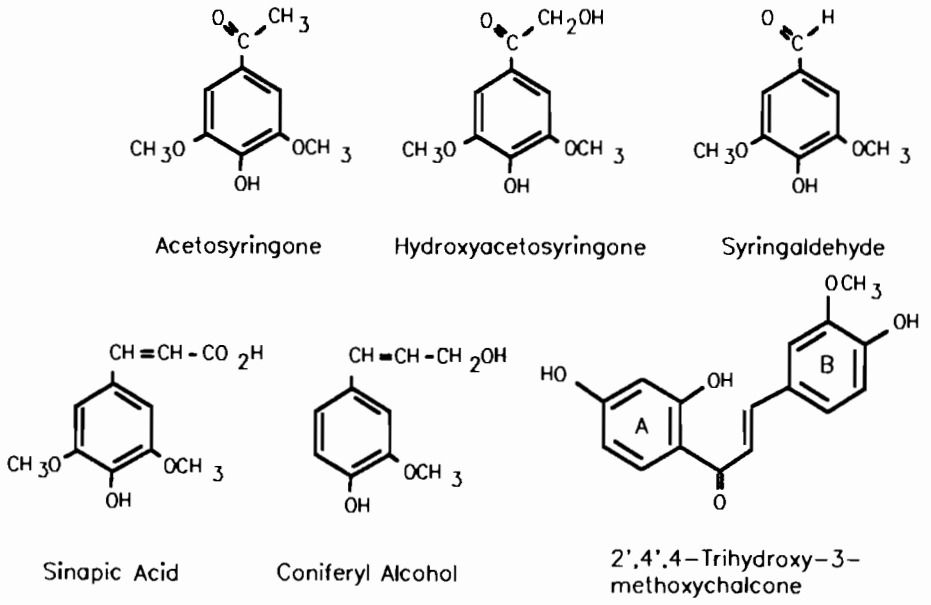

Figure 1. Structures of *Agrobacterium* virulence gene inducers. The list is not complete, other compounds of related structure act as inducers.

glucosylgalactoside and quercetin 3-glucosylgalactoside. Induction activities of strain A348 by both flavonol compounds were approximately 500 fold less than acetosyringone on a molar basis (Zerback *et al.* 1989).

In addition to compounds isolated as native inducers, many structurally related compounds were found to be effective in *vir* gene activation. Coniferyl alcohol, sinapic acid, ferulic acid, syringic acid, syringaldehyde and others are all active (Stachel *et al.* 1985b, Bolton *et al.* 1986, Spencer and Towers 1988, Melchers *et al.* 1989). The structures of several of these compounds are shown in Figure 1. In general, compounds active at micromolar concentrations share certain structural features. These include a hydroxyl group in the four position on the aromatic ring, a methoxy group at the three position (frequently a second methoxy group at position five), and a side chain at the one position with an electron withdrawing group. However, limited but significant induction was found

with 3,5-dimethoxy-4-hydroxybenzene and guaiacol, both of which carry hydrogen at position one (Melchers *et al.* 1989). The chalcone appears structurally dissimilar, however, it contains the active guaiacyl moiety as the chalcone B ring. The flavanoid glycosides apigenin-7-glucoside, myricetin-3-galactoside, narcissin, rutin were found to be active at approximately the same limited level as the native flavonol glycosides above (Zerback *et al.* 1989).

Genes and gene functions of the virulence region.

Using *vir:lacZ* reporter fusions, it was found that Ti plasmidless strains were unable to induce *beta*-galactosidase. By examining multiple plasmids containing different Ti plasmid segments, two loci *virA* and *virG*, were identified which allowed inducible expression of the reporter plasmid (Stachel and Zambryski 1986a, Winans *et al.* 1986). Mutations in *virA* or *virG* eliminated inducible expression, but mutation of *virB*, *virC*, *virD* or *virE* had no effect on induction (Winans *et al.* 1986).

VirA.

The efficacy of *virA* protein to serve as a regulator for *vir* gene induction was found to be strain dependent. Induction of a *virB:lacZ* construct from pTiA6 was barely detectable when introduced into the limited host range grapevine isolate Ag162. However, when the reporter plasmid contained in addition the *virA* sequence of pTiA6, efficient induction occurred (Leroux *et al.* 1987)

The *virA* protein has a transmembrane topology. Proteinase K treatment of bacterial spheroplasts converted the 91,000 dalton native *virA* protein to a 60,000 dalton fragment. Insertion of the structural gene for alkaline phosphatase into different regions of *virA* allowed the identification of bacterial colonies expressing different levels of alkaline

phosphatase activity. Sequence analysis of the clones showed that the highest activities were associated with insertions between the two hydrophobic regions of the *virA* protein, as would be expected if the hydrophobic regions delineated a periplasmic domain (Winans *et al.* 1989).

Mutations of the C-terminal domain of *virA* from strain B6 eliminated induction and tumorigenicity (Melchers *et al.* 1989). Substitution of 18 to 270 5'-nucleotides of the *virA* coding region by *E. coli tar* sequences resulted in an active *virA* protein. However, larger substitutions which encroached into the second N-terminal hydrophobic region resulted in loss of acetosyringone sensitivity (Melchers *et al.* 1989).

VirG.

The *virG* protein encoded by the A6 octopine Ti plasmid has recently been shown to bind to a twelve base pair sequence present in the 5'-untranslated region of open reading frames found in *virE* and *virB* (Jin *et al.* 1990b, Pazour and Das 1990). The conserved 12 base pair region (termed the "vir box") was found in promoter regions of genes in all *vir* loci (Das *et al.* 1986). Vir box sequences of strain A6 and nopaline strain C58 are similar but not identical (Steck 1988).

***VirA* and *virG* as two-component regulatory sensors.**

VirA and *virG* share amino acid homology with a broadly conserved set of two-component regulatory proteins which allow the bacterium to sense environmental stimuli. C-terminal domains of the sensor proteins virA, ntrB, envZ, phoR are conserved (Leroux *et al.* 1987). Regulator proteins virG, ntrC, ompR, phoB share N-terminal homology (Winans *et al.* 1986). With the exception of ntrC, the sensor proteins have two N-terminal hydrophobic transmembrane regions which define a periplasmic domain (Ronson *et al.*

1987). The mechanism of activation-deactivation appears to be one of phosphorylation-dephosphorylation. In the *ntrB-ntrC* system of *E. coli*, which responds to changes in nitrogen level, ntrB phosphorylates the ntrC protein thereby activating it (Ninfa and Magasanik 1986).

In keeping with homologous bacterial systems, recent work (Jin *et al.* 1990a, Huang *et al.* 1990) demonstrated that the C-terminal domain of the virA protein undergoes auto-phosphorylation. Mutation of the coding region to produce a glutamine in place of the histidine which undergoes phosphorylation resulted in a virA protein which failed to autophosphorylate, and failed to activate the *vir* gene induction cascade. Similar to the homologous systems, virA has been reported to phosphorylate virG, (in Jin *et al.* 1990b) .

VirB.

The *virB* locus of octopine strains A6 and 15955 encode 11 open reading frames (Ward *et al.* 1988, Thompson *et al.* 1988). Analysis of the deduced amino acid sequences identified signal peptide sequences which suggest a membrane location for many of the proteins. Three *virB* proteins were found associated with cell envelope fractions from *Agrobacterium* cells grown under inducing conditions (Engström *et al.* 1987). The abundance, location and regulation of expression of the virB protein has led to the hypothesis that the proteins function in export of T-DNA through the bacterial membrane (Engström *et al.* 1987).

VirC.

VirC1 is another binding protein encoded by the *vir* region. Recent work (Toro *et al.* 1989) showed that VirC1 of the A6 octopine Ti plasmid binds to a 23 bp region termed *overdrive*. *Overdrive* is located 15 to 16 base pairs to the right of the T-DNA right border

repeats (Peralta *et al.* 1986). *Overdrive* acts as a T-DNA transfer enhancer. Movement of *overdrive* up to 553 bp from the T-DNA right border either in the normal or inverted sequence still allowed enhanced transfer activity. Strains with *overdrive* accumulated markedly more T-strands than strains lacking *overdrive* (Ji *et al.* 1989). VirC1 binding to *overdrive* supports the position that these two elements act to distinguish the right T-DNA border from the left border, and thereby provide a mechanism for enhanced right border cleavage.

VirD.

VirD was found to function in T-DNA processing. Yamamoto *et al.* (1987) developed a highly selective system in which a plasmid carrying *beta*-lactamase (the enzyme responsible for kanamycin resistance) with an insertion mutation allowed the assay of T-DNA circularization. The insert into the *beta*-lactamase coding region contained left and right T-DNA border sequences, thus forming a miniature T-DNA. If T-DNA circularization occurred at the T-DNA borders, then the continuity of the *beta*-lactamase coding region was restored. Strains harboring the recircularized plasmid were therefore kanamycin resistant. Recombinant plasmids were found only in the presence of a *vir* gene inducer. Strains with mutations in *virA*, *virG* or *virD* failed to recircularize. The frequency of recircularization was low, wild-type strain A348 produced on average 2.5 recombinant bacteria per 10^7 cells.

VirD1 and virD2 act together as a specific T-DNA border endonuclease (Yanofski *et al.* 1986, Stachel *et al.* 1987, Jayaswal *et al.* 1987). VirD1 was found to encode a protein of molecular weight 16.2 kilodaltons (Yanofski *et al.* 1986) and was required for *virD* mediated nicking (Jayaswal *et al.* 1987). *E. coli* strains which harbored plasmids containing pTiA6 *virD1* were able to direct production of proteins which catalyzed the *in vitro*

conversion of supercoiled DNA to relaxed circular DNA, therefore acting as a topo-isomerase. A requirement for Mg^{2+} was identified, but not for ATP (Ghai and Das 1989).

The virD2 protein becomes tightly (covalently) associated to the T-strand. Ward and Barnes (1988) examined the attachment of *virD2* using a plasmid with many copies of tandem T-DNA sequences (multiple left border sequences of pTiT37). Additionally the *virD2* coding region was fused in frame to *lacZ*. In these strains, T-strands were bound to a larger protein than native strains, and the T-strands were immunoprecipitable by antibody to *beta*-galactosidase. In other studies (Herrera-Estrella *et al.* 1988, Young and Nester 1988, Dürrenburger *et al.* 1989) proteinase treatment of preparations of DNA from acetosyringone induced *Agrobacterium* cells allowed isolation of T-strands from the aqueous phase in aqueous/phenol extractions. In the absence of proteinase, T-strands were found in the interface region. Binding of T-strands to nitrocellulose membranes was competitively inhibited by denatured salmon sperm DNA if T-DNA preparations were proteinase treated, but only slightly inhibited if the proteinase treatment was omitted (Herrera-Estrella *et al.* 1988). Further, hybridization studies with DNA sequences spanning the T-DNA border sequence indicate that the protein is bound to the 5' side of the nick (Ward and Barnes 1988, Herrera-Estrella *et al.* 1988, Dürrenburger *et al.* 1989). Chaotropic agents such as 2 % SDS, 6 M urea, 6 M guanidine hydrochloride and piperidine were unable to dissociate the protein from the DNA (Herrera-Estrella *et al.* 1988). Attachment of virD2 reduced the rate of exonucleolytic degradation of the DNA (Dürrenburger *et al.* 1989).

Hybridization of T-strands to RNA probes indicated homology to the bottom strand of the T-DNA. (Albright *et al.* 1987, Yanofski *et al.* 1986) (Under standard conventions the Ti plasmid is diagrammed as a circle with the T-DNA at the top and vir region genes immediately to the left of the T-DNA). Multiple T-strands were generated from the

octopine Ti plasmid pTiA6. The six distinct strands were S1 nuclease sensitive and transferred to nitrocellulose membranes under nondenaturing conditions, indicating a single stranded character. The strand sizes were consistent with T-strand formation from each of the four pTiA6 border repeats (Stachel *et al.* 1987). T-strand production from the upper T-DNA strand was found if the T-DNA borders were reversed (Veluthambi *et al.* 1988). Investigators have found both single stranded nicking at T-DNA borders (Stachel *et al.* 1986, Albright *et al.* 1987) and double stranded cleavage (Veluthambi *et al.* 1987, Steck *et al.* 1989). There is a lack of agreement as to the relative significance of each type of cleavage for T-DNA transfer.

VirE

The *virE* locus of pTiC58 contains three open reading frames encoding proteins of 9, 7.1, and 63.5 kilodaltons. The deduced protein of the largest open reading frame is highly hydrophilic and shares 70 % homology with the largest open reading frame in the *virE* locus of pTiA6NC (Hirooka *et al.* 1987)

The function of pTiA6 *virE1* is unknown, however, in-frame insertions into the coding region resulted in strains with marked reduction of virulence on *Kalanchoe* leaves (McBride and Knauf 1988). The virE2 protein is the most abundant protein produced in *Agrobacterium* cells grown under conditions inducing *vir* gene expression (Engström *et al.* 1987). It is the longest open reading frame of the *virE* locus and acts as a single stranded DNA binding protein (Geitl *et al.* 1987, Citovsky *et al.* 1988a, Das 1988, Christie *et al.* 1988). Using band shift assays and immunoprecipitation studies, *virE2* was found to bind to single stranded copies of the T-DNA (Christie *et al.* 1988). Binding to DNA is cooperative, with a weight ratio of 6:1 protein to DNA and results in a more extended DNA protein complex than binding of *E. coli* single stranded binding protein to the same DNA. This

DNA-virE2 complex was resistant to cleavage by exonuclease VII and S1 nuclease (Citovsky *et al.* 1989). *VirE2* mutant strains can be complemented by coinoculation LBA4404, a strain harboring pTiAch5 carrying a deletion of its T-DNA (Christie *et al.* 1988). Although it is primarily found in cytoplasmic fractions, *virE2* is also exported, being found in membrane and periplasmic fractions. It has been postulated therefore, that *virE2* complementation occurs within the plant cell (Christie *et al.* 1988). Production of a single stranded DNA binding protein under the regulation of the *vir* cascade supports the hypothesis that the transferred copy of the T-DNA is single stranded (Stachel and Zambryski 1986b).

VirF

VirF was identified as a locus on pTiB6 which was required for a strongly tumorigenic response on tobacco and tomato but not on sunflower, pea or *Kalanchoe*. The locus was mapped on pTiB6 between virE and the T-DNA (Hooykaas *et al.* 1984).

VirH

Expression from *virH* (previously designated *pinF*) is subject to virA-virG mediated induction (Stachel and Nester 1986). *Agrobacterium* strain A348 carrying a mutation in the *virH* locus was attenuated in virulence on pea, especially with lower inoculum level. Sequence analysis of the 5.5 kb *virH* locus identified four open reading frames. Amino acid sequence comparisons of *virH1* and *virH2* indicated that they were similar to each other and to cytochrome P450 enzymes. Metabolism of bactericidal or bacteriostatic compounds produced by the plant has been suggested as possible role for the *virH* encoded enzymes (Kanemoto *et al.* 1989).

Tzs

Tzs, the second cytokinin synthetic prenyltransferase encoded on the C58 and T37 nopaline Ti plasmids is also under virA-virG regulation (Powell *et al.* 1988, John and Amasino 1988). Promoter sequences of *tzs* from strain C58 were found to share homology with *ompC*, an *E. coli* gene also under two-component regulation. Unlike *ipt* of the T-DNA, *tzs* is expressed in the bacterium and expression is inducible by acetosyringone (Powell *et al.* 1988).

A model for *Agrobacterium* transformation

A model of *Agrobacterium* mediated transformation has been proposed (Winans *et al.* 1986, Stachel and Zambryski 1986b, Citovsky *et al.* 1988b). The model is consistent with activities associated with the T-DNA and *vir* loci described above, and combines elements of bacterial conjugation and more viral-like transformation activities. The steps of the model are:

1. Attachment of the bacterium to the plant cell wall.
2. Induction of virulence gene expression.
3. Processing of the T-DNA and T-complex formation.
4. Export of the T-DNA complex to the plant cell.
5. Targeting the T-DNA to the plant nucleus.
6. T-DNA integration into the genome.
7. Expression of the transferred genes.
8. Response of the plant cell to the novel T-DNA encoded products.

Steps 1 to 4 are comparable to bacterial conjugation. Steps 5 to 8 are more reminiscent of eukaryotic viral infection.

In support of the conjugation aspects of the model, transfer of *E. coli* RSF1010 plasmid DNA from *A. tumefaciens* strain LBA4404 to tobacco occurred at frequencies approximately equivalent to T-DNA transfer. The pRSF1010 loci required for this transfer were found to be the origin of transfer (*ori*T), and the mobilization (*mob*) locus. Both loci are required for bacterium to bacterium conjugation (Buchanan-Wollaston *et al.* 1987).

The effects of *vir* gene mutations on conjugal transfer of the Ti plasmid offers additional support for the conjugal model of T-DNA transfer . Certain mutations in the *virA*, *virB*, *virC* and *virG* loci were found to reduced the rate of transfer by four orders of magnitude. However, the effect was dependent on the time of induction. The greatest effect of the mutations was seen after 18 hr of induction and almost no effect was seen after 48 hr of incubation. The basis for these effects is at present obscure (Gelvin and Habeck 1990).

Much of the fate of the T-DNA between T-strand processing and final integration is not presently understood. Examination of petunia protoplasts infected with *A. tumefaciens* showed that transfer of T-DNA took place in 2 to 4 hr, and that most of the DNA initially transferred was degraded (Virts and Gelvin 1985). The actual mechanism of T-DNA movement from the bacteria and its integration remains an open question. T-DNA sequences inserted into host genomic DNA exhibit a variety of structural and methylation patterns (Gheysen *et al.* 1987, Peerbolte *et al.* 1986). Further refinement of the transformation model awaits additional experimentation.

Conifer Transformation.

While research on the mechanisms of *Agrobacterium* transformation has focused on the bacterium and non-conifer hosts, during the course of the studies which make up this

volume, several research groups have evaluated specific conifers as hosts for infection by examining levels of tumor formation. In all studies transformation was confirmed, as a minimum, by identification of opine production in tumor tissue. Sederoff *et al.* (1986) working with *Pinus taeda* seedlings demonstrated tumor formation at low frequency (2.6 % for strain M2/73 and 17 % for strain U3). Clapham and Ekberg (1986) reported relatively high levels of tumor formation (65 %) by *A. tumefaciens* strain C58 on *Abies nordmanniana* and lower levels (20 %) on *Picea abies*. Dandekar *et al.* (1987) reported relatively low levels (average 13 %) of transformation for a pTiA6 based transformation vector inoculated on micropropagated *Pseudotsuga menziesii* shoots and *in vitro* germinated seedlings. Stomp *et al.* (1988) examined nine *Pinus* species, *Pseudotsuga menziesii*, and *Libocedrus decurrens* for tumor formation incited by five *Agrobacterium* strains. Tumorigenicity rates on *Pinus* were as high as 43 % (*P. ponderosa* inoculated with M2/73) but the average maximum level for the nine species was 25 %. *Libocedrus* supported the highest level of tumor formation (61 % when inoculated with M2/73). Ellis *et al.* (1989) examined 37 *Agrobacterium* strains for tumor formation on *Picea glauca*. High levels were found for certain strains (95 % of seedlings inoculated with K6/73, 94 % for strain B2/74).

AGROBACTERIUM INCITED GALL FORMATION ON CONIFERS.

INTRODUCTION

In order to develop *Agrobacterium* mediated transformation as an efficient DNA delivery system for pinaceous gymnosperms, strains must be identified which transform the hosts with high efficacy. Although *Agrobacterium* host range is broad (De Cleen and De Ley 1976), specific isolates vary in their host range (Anderson and Moore 1979). At the initiation of this project the only published reports of *A. tumefaciens* infection of conifer hosts were those of Smith (1935, 1942). The work of Hansen and Smith (1937) also established the susceptibility of *P. menziesii* to a bacterial gall forming pathogen. These gall forming strains are no longer available. If conifers indeed were hosts, it was unknown whether *A. tumefaciens* strains existed which were strongly tumorigenic and therefore likely to be highly efficient in transforming the hosts of interest.

This early phase of the project had two objectives. The first was verification of susceptibility of at least one conifer host to *Agrobacterium* infection. The second objective was identification of *Agrobacterium* strains which were strongly tumorigenic on a range of genera of the *Pinaceae*.

Strategy: The strategy was to inoculate several conifer hosts with a large set of *Agrobacterium* strains. In a preliminary study, 25 *A. tumefaciens* strains were inoculated

onto readily available *Pseudotsuga menziesii* seedlings from nursery stock. For a full study, four members of the *Pinaceae* were chosen. These were: *P. menziesii* (Douglas-fir), *Pinus ponderosa* (Ponderosa pine), *Tsuga heterophylla* (Western hemlock), and *Abies procera* (Nobel fir). The species represented a diverse group within the pine family, are widely distributed in the Pacific Northwest, and are commercially significant. A representative set of *A. tumefaciens* and *A. rhizogenes* strains was chosen for this study. It encompassed strains known to be capable of infecting a broad range of plant hosts, and included wild type agropine, nopaline, octopine, and succinamopine strains, and a number of transconjugants. Details of strain genotypes and the sources from which they were obtained are listed in the appendix. *Kalanchoe* plants were inoculated with the same strains in order to confirm the virulence of the isolates, and identify the opines produced. As described below, frequency of gall formation on the conifer hosts depended on the inciting *Agrobacterium* strain and was very high for selected strains and hosts.

METHODS

Conifer seed was obtained from commercial stocks (Brown Seed Co., Vancouver, WA; Simpson Timber Co., Sweethome, OR) which had been collected from the following Oregon and Washington seed zones (Schopmeyer 1974) *Pseudotsuga menziesii*, zone 251; *Pinus ponderosa*, zone 863; *Tsuga heterophylla*, zone 241; *Abies procera*, zone 051.

Agrobacterium strains are listed in Table 3, original plant hosts and references to the strains are listed in the appendix. Reagent grade chemicals were obtained from J. T. Baker

Chemical Co., Phillipsburg, NJ; Bethesda Research Laboratories, Gaithersburg, MD; or Sigma Chemical Co., St Louis, MO.

Bacteria, seedlings and inoculations for the preliminary screen.

Frozen bacterial stocks were streaked onto 523 agar plates[(Kado and Heskett 1970) modified by the addition of ammonium sulfate (2 gm/l)] and colonies were allowed to grow to approximately 3 mm prior to inoculation. *P. menziesii* seedlings (2-0 bare-root stock from the USDA Hood River nursery) were planted in sandy loam soil grown in environmental chambers (16 hr photoperiod, 25 °C). For inoculations, a sterile steel needle was used to pick up bacteria from a colony and then tangentially pierce the cambial layer of the main stem. Seedlings were inoculated three times at the base and additionally three time near the apex. One seedling was inoculated per strain. Ten weeks following inoculation the seedlings were examined for gall formation.

***Kalanchoe* virulence assay.**

Clonal *Kalanchoe daigremontiana* propagules were transplanted from large trays into plastic pots (100 ml) containing potting mix. Plants were inoculated once they attained a height of 5 cm, by excising the apical pair of leaves and applying to the cut surface a suspension of bacteria (5 μl, 10^8 cells) from colonies cultured on 523 medium. Two plants were inoculated for each strain. Tumor formation was scored at six weeks. Opines in the tumors were identified as described below.

Conifer seedling culture.

Seed was soaked for 24 hr to 36 hr in deionized water, surface sterilized in 3% hydrogen peroxide for 10 min, rinsed in sterile water, and stratified on moistened sterile

vermiculite at 4 °C for thirty days. After stratification, seeds were germinated on vermiculite at 30 °C under a 16 hr photoperiod for 7 to 10 days. Those seeds having emergent radicals were planted singly in seedling containers (2.3 x 15 cm, Conetainer Nursery, Aurora, OR) containing a 1:1 mixture of vermiculite and peat moss, subirrigated at 3 day intervals, and fertilized weekly using a mineral supplement developed for pine (Ingestad 1960).

Conifer inoculation and gall evaluation.

Seedlings were inoculated when the epicotyls were 1 to 3 cm in length (approximately six weeks after germination). A suspension of bacteria (5 μl, 10^8 cells) derived from colonies in rapid growth was applied to each seedling epicotyl and the epidermis was then punctured seven times through the suspension with a disposable 26 gauge needle to a depth approximately equal to the epicotyl radius. For each strain and host pair, two replications of ten seedlings were inoculated. The first replication was inoculated in March 1986, the second in June 1986. For each host, the seedlings were randomly assigned to positions in the culture trays. Temperatures were maintained at 28 °C for at least one week following inoculation. Gall diameter was measured twelve weeks after inoculation. Gall formation scores were calculated as the percentage of inoculated seedlings which produced galls, independent of gall size. The coefficient of variation between replications for the gall formation scores for each host was calculated as the mean of values for each bacterial strain. The correlation coefficient for the relationship between gall formation level and gall size was calculated by the method of least squares, and tested for significance from zero by the Student's *t* test (Snedecor and Cochran, 1967). Galls were excised and stored at -80 °C prior to opine analysis.

Opine determination.

Opines were identified by a modification of the procedure of Chilton *et al.* (1984). Frozen gall tissue (100 mg) was ground in liquid nitrogen and the powder was added to 200 μl Tris-acetate (20 mM, pH 8.0), EDTA (1 mM), allowed to thaw, and stirred vigorously. Following centrifugation (12000 x g, 1 min), an aliquot of the supernatant (5-8 μl) was applied to Whatman 3MM paper previously saturated with ammonium bicarbonate (50 mM, adjusted to pH 9.1 with ammonium hydroxide) or with a mixture of acetic acid (1.5 M) and formic acid (0.7 M). Electrophoresis was conducted at 22 V/cm for 1 hour. Electrophoretograms were examined for fluorescent compounds prior to application of visualization sprays. Nopaline and octopine were rendered visible by spraying with phenanthrenequinone in ethanolic NaOH (Yamada and Itano 1966). Photographs of the resulting fluorescent derivatives were made under 300 nm illumination using a transilluminator (Ultra Violet Products Inc., San Gabriel, CA) and a Polaroid MP4 camera system with type 55 positive/negative film. Exposures were for 10 min at f4.5. Agropine and mannopine were visualized by spraying sequentially with silver nitrate and ethanolic NaOH (Chilton *et al.* 1984) and photographed under visible illumination (1 sec, f4.5) using the same film.

RESULTS

Preliminary *P. menziesii* inoculations.

Scores for gall formation from the preliminary screen are shown in Table 1. Seven of the thirty strains produced tumor-like growths at one or more of the inoculation sites. Strains were scored as positive if there was tissue proliferation well in excess of the usually minimal wound callus production. A plus/minus score indicated a level of tissue proliferation which was greater than inoculations without bacteria, and less than the level

Table 1. Preliminary experiments on *Agrobacterium* tumorigenicity.

Strain	Gall formation	Strain	Gall formation	Strain	Gall formation
A203	-	C58	-	K6/73	-
A277	+	GV3160	+/-	M2/73	+
A281	+/-	I7/75	-	M3/73	+/-
A518	-	K26	+/-	NT1	-
A557	+	K32	-	RR5	-
A596	-	K41	-	S5/72	+/-
A723	+/-	K47	+	T28/73	-
B3/73	+	K49	-	T37	-
B4/74	+				

Agrobacterium strains were inoculated onto 2 yr old *P. menziesii* (2-0) seedlings. Following six weeks of growth, stems were examined for tissue hypertrophy.

typical of positive responses. Negative scores showed virtually no callus proliferation and where present merely lined the wound site.

***Kalanchoe* inoculations.**

In order to confirm the tumorigenicity of the *Agrobacterium* strains, and to identify which opine biosynthetic genes were encoded in the T-DNA, the strains were inoculated onto *Kalanchoe*. All strains formed galls, except seven, these were; 2657, 2659, A723, C58(pRi8196), S5/72, TP102 and TP2. Of the seven, the first two were narrow host range cucumopine strains, and lack of gall formation was not unexpected. Lack of gall formation by strain A723 was unexpected. However, pTi plasmid preparations of this isolate showed a reduced size, suggesting a deletion (data not shown). The nominal *A. pseudotsugae* strains, TP102 and TP2, appeared to lack Ti plasmids entirely (data not shown), and therefore would not be expected to incite galls. Strain S5/72 was received as *A. radiobacter*, and thus had been previously characterized as avirulent (L. Moore personal communication).

Tumor morphology differed between strains. Four classes were defined: unorganized, smooth callus; unorganized rough (often dry) callus; shooty with deformed leaves (appearing as a portion of a leaf blade); shooty with generally normally shaped leaves. Additionally rooty galls were formed by *A. rhizogenes* strains. Tumor character is listed with each strain in Table 2. Unorganized rough callus with a dry appearance was only found following inoculations of strain A281, an agropine strain. Octopine strains always incited unorganized smooth tumors with the exception of strain GV3245 which produced an unorganized rough callus. The two known succinamopine strains both incited shooty callus with irregular leaves. Nopaline strains incited all gall types except rooty.

Opines present in *Kalanchoe* galls are listed in Table 2. In most cases the nominal opine status of the strain was confirmed. Confirmation of octopine production was not common. In only one of the eight cases where octopine was expected in the tumor, was it identified (strain CG1C). In one case (strain K26) octopine was identified in tumor tissue incited by a strain with unknown opine status. Nopaline was commonly found in galls incited by strains uncharacterized for opine type. No unanticipated opines were detected in the tumors.

Gall morphology on conifers.

Contrary to expectations, many strains were tumorigenic at high frequency. Figure 2 illustrates typical tumors observed following infection of each of the four hosts. The hypertrophy characteristic of crown gall was clearly evident. Tumor morphology was almost always unorganized and galls exhibited either single (Fig. 1A) or multiple lobes (Fig. 1B, 1C, 1D). In most cases, swelling of the stem occurred below the gall. On *Abies* seedlings, galls commonly developed with two colors, either light green to brown, or white to cream. Browning of the epidermal layers increased with age. On seedlings inoculated with *A.*

Table 2. Tumor formation and opine identification in *Kalanchoe*.

Strain	Genotype	Gall Formation	Gall type	Opine Expected	Opine Identified
15955	15955(pTi15955)	+	US	octopine	nd
2655	2655(pRi2655)	+	rooty	cucumopine	nd
2657	2657(pRi2657)	-		cucumopine	nd
2659	2659(pRi2659)	-		cucumopine	nd
3667	3667(pTi3667)	+	US		nopaline
A175	C58(pTiC58)	+	US	nopaline	nopaline
A178	C58(pTiK27)	+	SD	nopaline	nopaline
A2	A2(pRiA2)	+	rooty	agropine	agropine
A203	C58(pTiNCPPB223)	+	US	nopaline	nopaline
A208	C58(pTiT37)	+	SD	nopaline	nopaline
A277	C58(pTiB6-806)	+	US	octopine	ni
A281	C58(pTiBo542)	+	UR	agropine	agropine
A4	A4(pRiA4)	+	rooty	agropine	nd
A518	C58(pTiEU6)	+	SD	succinamopine	ni
A519	C58(pTiAT181)	+	SD	succinamopine	ni
A557	A200(pTiIIBV7)	+	UR	nopaline	nopaline
A596	C58(pTiAch5)	+	US	octopine	ni
A6	A6(pTiA6)	+	US	octopine	ni
A723	C58(pTiB6-806)	-		octopine	nd
Ach5	Ach5(pTiAch5)	+	US	octopine	ni
B1/74	B1/74(pTiB1/74)	+	SN		nopaline
B234	B234(pTiB234)	+	SN	nopaline	nopaline
B3/73	B3/73(pTiB3/73)	+	US		nopaline
B4/74	B4/74(pTiB4/74)	+	US		nopaline
C2/74	C2/74(pTiC2/74)	+	US		nopaline
C58	C58(pTiC58)	+	SN	nopaline	nopaline
C58(pRi8196)	C58(pRi8196)	-		mannopine	nd
C58(pRiA4)	C58(pRiA4,pArA4a,pArA4c)	+	rooty	agropine	agropine
C58(pRiTR105)	C58(pRiTR105)	+	rooty	agropine	agropine
CG1C	CG1C(pTiCG1C)	+	US	octopine	octopine
G1/73	G1/73(pTiG1/73)	+	US		nopaline
GV3160	C58(pTiC58tra-c)	+	US	nopaline	nopaline
GV3245	LS1005(pTiB6S3)	+	UR	octopine	ni
I7/75	I7/75(pTiI7/75)	+	US		nopaline
IIBV7	IIBV7(pTiIIBV7)	+	SN	nopaline	nopaline
K108	K108(pTiK108)	+	US	nopaline	nopaline
K26	K26(pTiK26)	+	US		octopine
K27	K27(pTiK27)	+	SD	nopaline	nopaline
K32	K32(pTiK32)	+	US,SN		nopaline
K37	K37(pTiK37)	+	US		nopaline
K39	K39(pTiK39)	+	US		nopaline
K41	K41(pTiK41)	+	SD		nopaline
K47	K47(pRiK47)	+	rooty	agropine	agropine
K49	K49(pRiK49)	+	US		agropine
K6/73	K6/73(pTiK6/73)	+	SD		nopaline
M2/73	M2/73(pTiM2/73)	+	US		nopaline
M3/73	M3/73(pTiM3/73)	+	SD		nopaline
MFM83.4	MFM83.4(pTiMFM83.4)	+	US		nopaline
MFM84.1	MFM84.1(pTiMFM84.1)	+	SD		nopaline
MFM84.4	MFM84.4(pTiMFM84.4)	+	SN		nopaline
MFM84.5	MFM84.5(pTiMFM84.5)	+	SD		nopaline
MFM84.61	MFM84.61(pTiMFM84.61)	+	SD		nopaline
MFM84.63	MFM84.63(pTiMFM84.63)	+	SN		nopaline
MFM84.7	MFM84.7(pTiMFM84.7)	+	US		nopaline
MFM84.9	MFM84.9(pTiMFM84.9)	+	SD		nopaline
RR5	RR5(pTiRR5)	+	US		nopaline
S5/72	S5/72(pTiS5/72)	-			nd
T28/73	T28/73(pTiT28/73)	+	US		ni
T37	T37(pTiT37)	+	SD,SN	nopaline	nopaline
TP 102	no pTi	-			nd
TP 2	no pTi	-			nd
TR105	TR105(pRiTR105)	+	rooty	agropine	agropine
TR7	TR7(pRiTR7)	+	rooty	mannopine	nd

Abbreviations for gall type: SD, shooty with deformed leaves (generally appearing as partial leaf blades) SN, shooty with generally normally shaped leaves; US, unorganized smooth callus; UR, unorganized rough callus. Abbreviations for opine determinations: blank, unknown; nd not determined; ni, none identified (negative for agropine, manopine, nopaline and octopine). For references to strains see appendix.

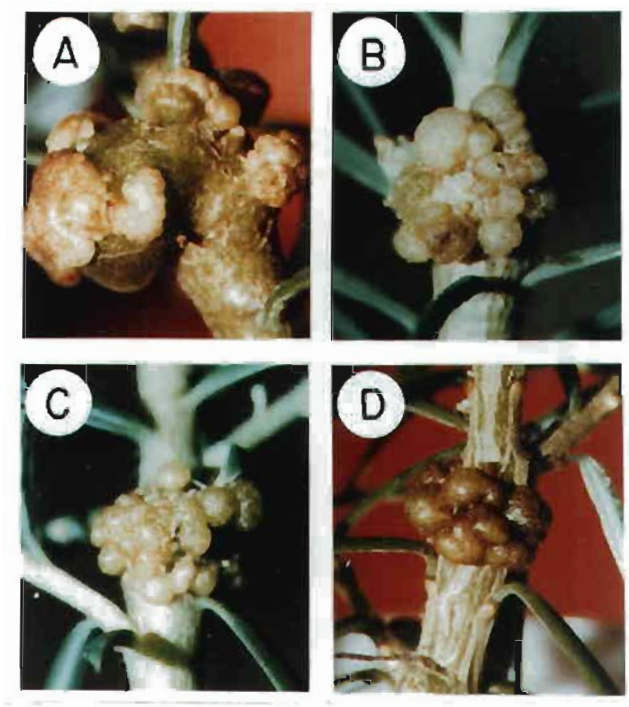

Figure 2. Typical galls incited by A. tumefaciens strains on pinaceous hosts. A. Strain A518 on *Abies procera*. **B.** Strain K27 on *Pinus ponderosa*. **C.** Strain B3/73 on *Pseudotsuga menziesii*. **D.** Strain K41 on *Tsuga heterophylla*. Photographs taken twelve weeks after inoculation.

rhizogenes, knob-like structures were commonly seen and, on *Tsuga* seedlings, rootlets occasionally developed which later withered. Galls were readily evident eight weeks after inoculation, and continued to proliferate during the final four weeks of the experimental period.

Galls were not produced in control experiments in which plants were wounded in the absence of bacteria or in which wound sites were infected with avirulent bacteria. There was no swelling at the wound site and wounds healed forming only a small scar on the stem.

Strain Efficacy.

Abies and *Pseudotsuga* were the most permissive of the four hosts whereas *Pinus* was the most restrictive, as judged by the overall frequency of gall formation (Table 3). Different *Agrobacterium* strains incited tumors at very different frequencies on each host. For example, the nopaline strain 3667 incited galls at high frequency (70 % and 100 %) on *Abies* and *Pseudotsuga* respectively, whereas the nopaline strain MFM84.61 was low (5 % or less) on all four hosts. Because both strains were capable of forming large tumors at high frequency on *Kalanchoe* (R. O. Morris personal communication, and above), it appeared likely that there was no defect in the T-DNA. The difference in tumorigenicity on gymnosperms may reflect an inherent difference in transformation ability.

For each host there was at least one, and often several strains that incited galls at high frequency. On *Abies* for example, seven strains (A4, A557, B1/74, K39, K47, K108, and M3/73) incited galls at frequencies greater than or equal to 85%. On *Pseudotsuga*, five strains (3667, B1/74, B3/73, K6/73, M2/73) incited galls at frequencies equal to 85 % or higher. On *Tsuga* two strains (A4 and K47) scored greater or equal to 80% infectivity. Whereas on *Pinus* only one strain was capable of efficient infection (TR105, 75 %). Usually, strains that incited galls at high frequency on one host also incited galls at high frequency on the other hosts, although this was not true for infection of *Pinus*. Gall formation frequency on this host was generally low. No consistent differences in tumorigenicity were noted between wild type and transconjugant strains although C58(pRiA4) and C58(pRiTR105) were certainly less tumorigenic than their parent strains. The coefficient of variation between replications for the frequency of gall formation scores was 0.52 for *A. procera*, 0.49 for *P. ponderosa*, 0.42 for *P. menziesii*, and 0.73 for *T. heterophylla*.

Table 3. Tumor frequency and tumor size on conifer hosts.

	Frequency of Gall Formation (%)				Average Gall Size (mm)			
Strain	Ap	Pm	Pp	Th	Ap	Pm	Pp	Th
15955	21	20	0	20	1.2	0.9	-	1.3
2655	5	0	5	0	0.9	-	0.8	-
2657	5	0	0	5	1.4	-	-	0.8
2659	0	0	0	5	-	-	-	1.2
3667	70	100	25	65	2.2	1.7	1.0	2.8
A175	40	35	10	10	1.5	1.4	0.9	1.1
A178	35	20	5	5	1.7	1.1	0.4	3.1
A2	70	55	25	30	1.2	0.9	1.1	1.2
A203	30	60	5	10	1.6	1.8	1.7	0.7
A208	33	35	8	8	0.7	1.3	0.9	0.8
A277	60	70	0	55	1.2	1.4	-	0.9
A281	55	58	5	17	2.0	1.8	1.6	1.7
A4	90	30	35	80	2.3	1.2	1.0	1.3
A518	55	50	20	20	2.4	1.8	2.0	1.2
A519	51	50	15	10	1.5	2.0	1.4	1.2
A557	85	75	20	55	2.0	2.1	1.7	1.7
A596	80	70	10	40	1.6	1.4	0.8	1.2
A6	32	10	0	25	1.5	0.9	-	0.9
A723	0	0	0	0	-	-	-	-
Ach5	55	40	0	25	1.4	1.6	-	0.6
Ach5C3	0	0	0	0	0.8	-	-	-
B1/74	85	85	30	75	2.0	1.7	0.8	1.5
B234	70	75	40	20	2.1	2.1	1.4	1.4
B3/73	90	100	30	70	2.4	2.1	2.1	2.1
B4/74	75	25	15	55	1.7	0.9	0.8	1.3
C2/74	70	80	60	50	1.7	1.3	1.4	1.2
C58	20	25	0	10	1.0	1.0	-	1.2
C58(pRi8196)	0	0	0	0	-	-	-	-
C58(pRiA4)	5	15	0	0	0.5	0.7	-	-
C58(pRiTR105)	15	5	0	5	1.1	0.7	-	0.9
CG1C	25	40	0	35	1.1	1.3	-	0.6
G1/73	65	55	20	10	2.1	1.4	0.9	1.6
GV3160	30	15	15	0	0.9	1.0	0.8	-
GV3245	70	65	5	40	1.2	1.3	0.6	1.0
I7/75	30	25	0	25	1.1	0.9	-	1.0
IIBV7	50	0	0	50	1.4	-	-	1.2
K108	85	35	30	65	1.9	1.1	1.1	1.9
K26	55	30	10	30	1.2	1.4	0.6	0.8
K27	80	65	55	55	2.8	1.6	2.1	2.1
K32	70	75	5	35	1.8	1.8	0.8	1.5
K37	64	70	15	30	3.0	1.4	1.0	1.0
K39	85	65	30	60	2.0	1.5	1.3	1.8
K41	70	75	25	70	1.9	1.1	0.9	2.2
K47	100	65	50	85	2.2	1.5	4.2	1.7
K49	10	5	0	5	1.0	0.7	-	0.7
K6/73	75	85	20	60	1.5	1.6	1.4	2.1
M2/73	75	80	45	60	2.4	1.9	1.0	1.8
M3/73	95	65	10	55	2.3	1.6	1.3	1.5
MFM84.1	10	15	5	10	1.4	0.9	1.0	0.8
MFM84.5	5	10	0	5	2.1	0.8	-	1.0
MFM84.61	5	0	0	5	0.7	-	-	1.5
MFM84.63	0	5	0	10	-	1.3	-	0.5
MFM84.7	20	10	0	5	1.1	1.2	-	1.7
MFM83.4	10	0	10	15	1.8	-	0.4	0.9
MFM84.4	15	20	5	5	1.3	1.0	1.6	1.3
MFM84.9	10	10	0	10	0.7	1.3	-	1.1
NT1	0	0	0	0	-	-	-	-
RR5	15	10	0	0	1.2	0.9	-	-
S5/72	20	5	5	0	1.1	0.3	1.1	-
T28/73	60	15	0	25	1.5	1.4	-	0.9
T37	10	0	0	10	0.8	-	-	1.1
TP 102	0	0	0	0	-	-	-	-
TP 2	0	0	0	0	-	-	-	-
TR105	60	50	75	20	1.4	1.9	1.7	0.8
TR7	5	0	0	0	1.6	-	-	-
control	0	0	0	0	-	-	-	-

Frequency determined as the percentage of seedlings with tumors twelve weeks following inoculation. Abbreviations: Ap, *A. procera*; Pm, *P. menziesii*; Pp, *P. ponderosa*; Th, *T. heterophylla*. For references to strains see appendix.

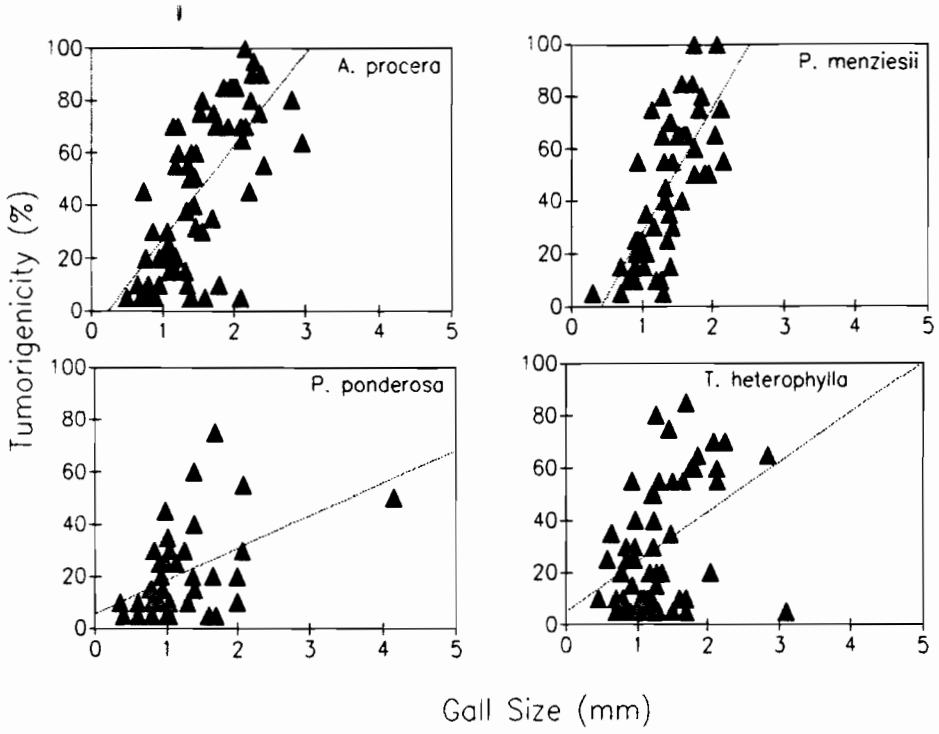

Figure 3. Relationship between tumorigenicity and tumor size. For each strain inciting tumors and each conifer host, the frequency of tumor formation was plotted against the average tumor size. With increasing tumorigenicity levels there were increasing tumor sizes for all four hosts. Correlation coefficients relating size to tumorigenicity were highest for the two most susceptible hosts, *A. procera* and *P. menziesii*.

Of the thirteen strains with the highest tumorigenicity, eleven were nopaline strains. However, the converse, that low scoring strains were not nopaline strains, was not true. Many nopaline strains had low gall formation scores. While a few agropine strains were included among the strains examined, many of these were associated with high frequency of gall formation. None of the octopine strains incited galls at high frequency.

Conifer Gall Size.

For each seedling, gall size was measured three months following inoculation. The average values for the two replications are given in Table 3. Strains which incited galls most frequently, generally produced the largest galls. This relationship is shown graphically in

Figure 3. For all four hosts, the correlation between the frequency of gall formation and gall size was significant at the 1 % level. The R^2 values were 0.46, 0.23, 0.54 and 0.18 for *A. procera*, *P. ponderosa*, *P. menziesii*, and *T. heterophylla* respectively.

Confirmation of conifer transformation.

Gall formation following inoculation with virulent Agrobacteria serves as a necessary indicator of transformation although it alone is not a sufficient criterion. Transformation was confirmed by assaying for the presence of opines in the tumors. Paper electrophoresis of gall extracts followed by phenathrenequinone or silver staining of the electrophoretograms identified nopaline and agropine in galls from among the most prolific of the host and strain combinations. For both nopaline and agropine, extracts were subjected to electrophoresis in both acidic and alkaline conditions. This was necessary because the conifer extracts contained interfering compounds which had the potential for confounding the opine identification. In addition, most conifer galls contained much lower concentrations of opines than the corresponding galls on *Kalanchoe* and extracts had to be concentrated ten-fold in order to detect them. Nevertheless, as shown in Figure 4, *Pseudotsuga* galls B1/74 and M2/73 contained nopaline whereas untransformed *Pseudotsuga* stem tissue did not. *Abies* 3667 and K108 galls also contained nopaline. Untransformed *Abies* stem tissue contained small amounts of a fluorescent contaminant with an electrophoretic mobility almost identical to that of authentic nopaline (Figure 4. , upper panel lane 6). However, the fluorescence of this compound was not enhanced by phenanthrenequinone nor was the color of its fluorescence identical to the blue-green fluorescence of authentic nopaline. *Tsuga* A281 and *Abies* A281 galls were found to contain agropine (Figure 4, lower panel). Untransformed *Tsuga* and *Abies* contained no visible amounts of agropine.

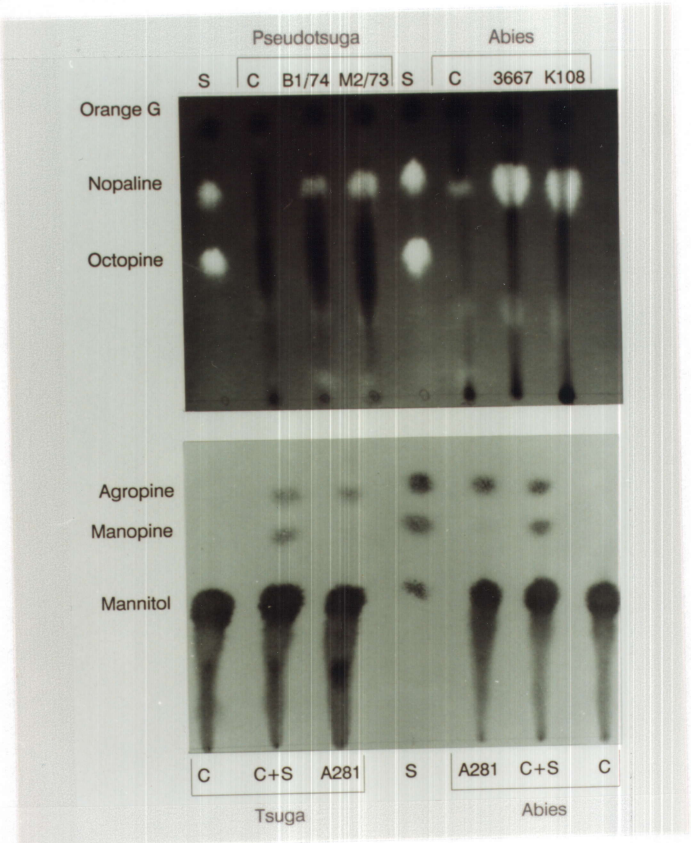

Figure 4. Opine identification in gall extracts following paper electrophoresis.
Upper panel: Identification of nopaline following electrophoresis at pH 9.1 and staining with phenanthrenequinone. Lanes 1) and 5), authentic nopaline and octopine (1 μg); lane 2), untransformed *Pseudotsuga* stem tissue; lanes 3) and 4), *Pseudotsuga* B1/74 and M2/73 galls (10 mg); lane 6), untransformed *Abies* tissue; lanes 7) and 8), *Abies* 3667 and K108 galls.
Lower panel: Identification of agropine following electrophoresis at pH 2.1 and silver staining. Lane 1), untransformed *Tsuga* stem tissue; lane 2), untransformed *Tsuga* tissue with added agropine and mannopine; lane 3), *Tsuga* A281 galls (100 mg); lane 4), agropine, mannopine, and mannitol; lane 5), *Abies* A281 galls; lane 6), untransformed *Abies* tissue with added agropine and mannopine; lane 7), untransformed *Abies* tissue. Abbreviations: S, standard; C, untransformed control tissue.

DISCUSSION

The results of the preliminary inoculation trial were encouraging, indicating that some strains were capable of transforming *P. menziesii*. It appeared likely, therefore, that a larger inoculation study would be successful in identifying one or more *Agrobacterium* strains which efficiently transformed conifer hosts.

This comprehensive study confirmed findings which appeared during the course of the study (Clapham and Ekberg 1986, Dandekar *et al.* 1987, Sederoff *et al.* 1986) that the host range of *Agrobacterium* extends to members of the *Pinaceae*. A wide range of tumorigenicity levels were associated with the strains examined. Certain strains were highly tumorigenic on these hosts. Most of the highly tumorigenic strains harbored nopaline Ti plasmids. However, the presence of a nopaline Ti plasmid was not a guarantee of high frequency of tumor formation. Some nopaline strains were inefficient, while one agropine strain was highly tumorigenic. These differences in tumorigenicity were not unexpected since host-range specificity among *Agrobacterium* strains has been repeatedly demonstrated for the angiosperms (Anderson & Moore, 1979, Yanofsky *et al.* 1985). What was a little surprising was the high frequency of infection observed here (average 33%, and as high as 100%) as compared to the frequency (2-17%) observed by Sederoff *et al.* (1986) for *Pinus taeda* (loblolly pine). Possible explanations of the higher frequencies may be the nature of the test plants, their difference in age, or in the strains of *A. tumefaciens* used for transformation. Plants used here were only six to eight weeks old and the epicotyls were still expanding. Those used by Sederoff *et al.* were six months old and may have been approaching dormancy. The positive correlation between tumor size and frequency of tumor formation was not unexpected and was the basis for calculating tumorigenicity values as the number of seedlings with galls and not simply the mass of tumor material. Why the correlation was highest in the most susceptible hosts, *P. menziesii* and *A. procera* is not

immediately apparent, but may be due to the reduced sample size for the *P. ponderosa* and *T. heterophylla* correlations.

From the practical point of view, much needs to be learned about these strains before they can be utilized for vector development. While certain strains (for example K47 and C2/74) incite tumors at high frequency, others (MFM83.4, RR5) do not. The molecular basis for the difference remains to be determined. Tumor formation is the end product of at least two independent events. An initial transformation event (in which the T-DNA is mobilized into the host cell) is followed by an expression event (in which the T-DNA genes responsible for phytohormone biosynthesis are expressed and the host cells respond). Transformation and tumor growth are thus independent, and the formation of a visible tumor by a given *Agrobacterium* strain must mean that both have occurred.

For the strains examined in this study, it is not clear whether tumorigenicity is limited by defects in the transformation event or in the expression event. Since all strains formed large tumors at high frequency on *Kalanchoe*, it is likely that the T-DNA genes were functional and capable of causing cell proliferation once integrated into any plant genome. If so, then the lack of tumorigenicity of the ineffective strains must be sought in the virulence region of the Ti plasmid (Nester *et al.* 1984) or in chromosomal loci such as *chvA* or *chvB* (Douglas *et al.* 1985, Miller *et al.* 1986). In order to determine the relative roles played by the T-DNA and virulence regions, it will be necessary to replace the T-DNA of ineffective strains with T-DNA derived from effective strains. Alternatively, the T-DNA could be replaced with a marker gene which could be expressed transiently in the plant cells and whose expression is not dependent upon the ability of the host cells to respond to T-DNA-encoded phytohormone biosynthesis. Transformation frequencies could then be

measured directly. The recently characterized beta-glucuronidase gene (Jefferson *et al.* 1987) has been used in a related application, and might prove useful here.

If indeed, the genes responsible for differences in tumorigenicity reside in the virulence region, at least two loci should be considered. In developing a model for T-DNA transfer, Stachel and Zambryski (1986a) postulate that the virA protein is the receptor for a plant phenolic inducer secreted at the wound site. VirA then initiates a cascade of vir gene expression which culminates in T-DNA transfer. A possible reason for the limited efficacy of some strains might then be that the virA protein does not recognize wound phenolics produced by these four hosts. Leroux *et al.* (1987) have proposed that an alteration in virA specificity explains the narrow host range of a grape-specific *A. tumefaciens* strain. Data presented in Chapters IV and V below are consistent with limited *vir* gene induction by the weakly tumorigenic strains.

A second element which may influence transformation efficiency is the *tzs* locus which is associated with the *vir* region (Morris and Powell 1987). *Tzs* expression, which is induced by plant phenolics, causes the bacteria to secrete high levels of cytokinins (G.K. Powell *et al.* 1988). While *tzs* was not mapped on the Ti plasmids of all the strains which formed galls with high frequency on gymnosperms, most of the strains were nopaline strains which usually carry *tzs* (Akiyoshi *et al.* 1987). Additional hybridization data verifying the presence of *tzs* on Ti plasmids of a set of the strongly tumorigenic strains is presented in Chapter III below. Cytokinin enhancement of Agrobacterium transformation has been postulated (Fillatti *et al.* 1987, Byrne *et al.* 1987). These authors report that vectors based on the nopaline strain C58 are more effective in transformation of *Populus* and soybean than vectors based on octopine strains which do not carry *tzs*.

In conclusion, it is clear that gymnosperms can be transformed at high frequency by selected *Agrobacterium* strains. Although these data from inoculation studies alone do not explain why some strains are more virulent than others, the data presented here provided a basis for investigation of the molecular details of differences in virulence presented below.

BACTERIAL ISOLATES FROM PSEUDOTSUGA MENZIESII GALLS.

INTRODUCTION

As described in Chapter I, it was unclear initially whether *Agrobacterium* strains would efficiently transform conifer hosts. Therefore, concurrent with the studies above on tumorigenicity of characterized *Agrobacterium* strains, efforts were directed toward identifying native *Agrobacterium* isolates of *P. menziesii* which were strongly tumorigenic. This was seen as an undertaking with limited prospects for success, as native isolates of *Agrobacterium* from *P. menziesii* were not currently recognized. Tumor formation incited by *(Agro)bacterium pseudotsugae* was demonstrated on *P. menziesii* (Hansen and Smith 1937). However, isolates with the characters of this strain were not extant (Bradbury 1986). DNA hybridization kinetics of an *Agrobacterium pseudotsugae* isolate to DNA of the *Agrobacterium* reference strain indicated that this isolate was only distantly related to *A. tumefaciens* and probably not *Agrobacterium* (Heberlein *et al.* 1967). Studies of metabolic characters of *A. pseudotsugae* showed it to cluster with most of the examined *Arthrobacter* species and apart from the *Agrobacterium* cluster (Skyring *et al.* 1971).

If found, a native *Agrobacterium* isolate might be virulent on a broad range of conifer hosts, and more efficient in T-DNA transfer than isolates from angiosperm hosts. Although rare, galls were known to occur on *P. menziesii* which were potentially of bacterial origin (E. Hansen, Botany Department, Oregon State University, personal communication). The objective of the studies described in this chapter was to identify *native Agrobacterium* strains highly tumorigenic on conifers.

Strategy: The strategy was to isolate bacteria from *P. menziesii* galls found in the wild, and to identify those isolates which were virulent *Agrobacterium* strains. In order to isolate bacteria, several collections of galls were made from different *P. menziesii* stands and bacteria cultured from them. Several *Agrobacterium* characteristics were examined. Colony lysates were hybridized to the T-DNA oncogene *ipt*. Plasmid content was characterized. Virulence on *Kalanchoe*, *P. menziesii* and *P. ponderosa* was examined.

The successes achieved with the characterized *Agrobacterium* strains described in Chapter I allowed the project to move forward, to focus on questions of why certain *Agrobacterium* strains were more tumorigenic than others. Therefore efforts on native strain characterization were set aside, and the results described below are incomplete. Although preliminary, the data described in this chapter may serve as a basis for investigations of very rapid tissue hypertrophy incited by native bacterial isolates of *P. menziesii* galls.

METHODS

Gall collections.

Four groups of galls were collected from *P. menziesii* trees. These were the MP series from Mary's Peak, in the Oregon Cascades, and three separate collections from trees located near Hoodsport, Washington. The latter three collections were generously provided by Mike McWilliams, USDA Forest Sciences Laboratory, Corvallis, Oregon. Except for one of the galls of the MP series which occurred on the main stem, all the galls were located on branches. Galls varied in size from less than 1 cm to greater than 3 cm in diameter.

Isolation of Bacteria from Galls.

Small pieces of the galls (approximately 5 mm in diameter) were surface sterilized in ethanol (95 %, 2 min.) followed by sodium hypochlorite (0.5 %, 7 min.) and washed with sterile water. The pieces were added to 50 ml of sterile water and partially disrupted using a tissue homogenizer (Virtus). Fragments were allowed to settle and 1 ml of the suspension was spread on selective media for each of the three *Agrobacterium* biotypes (Brisbane and Kerr 1983). Plates were incubated at 28 °C. Once colonies were visible, they were restreaked on corresponding selective biotype plates. Single colonies from this second set of selective plates were restreaked on to plates of 523 media. Single colony isolates were restreaked on to new plates until colonies appeared homogeneous in size and color (3 to 4 colony isolations). Strains were stored frozen (-70 °C) in 15 % glycerol stocks.

***Ipt* probe construction and colony hybridization.**

Plasmid pAR13 (N. Hommes and R.O. Morris personal communication) contains a 4300 bp insert from *A. tumefaciens* strain Ach5 in pUC 18 which includes the 723 bp of *ipt*. Plasmid DNA (9 μg) was digested with restriction enzymes *Hin*dIII and *Nde*I following the recommendations of the supplier (BRL) to yield a 1.5 kb fragment containing *ipt*, 430 bp 5' and 390 bp 3' of the gene, and additional vector fragments. The digestion products were separated by gel electrophoresis (0.7 % agarose, 120 V, 2 hr, in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM ethylenediaminetetraacetic acid, pH 8.3). The 1.5 kb *ipt* band was cut from the gel and the slice extruded through a 23 gauge needle to fracture the gel. A volume of phenol (preequilibrated with 0.1 M Tris pH 8.0) equal to the volume of the gel slice was added, the sample mixed, frozen (-70 °C, 10 min), centrifuged (12,000 x *g*, 3 min), the supernatant removed and extracted twice with phenol and twice with chloroform. DNA was precipitated from solution with two volumes of ethanol. DNA (100 ng) was radioactively labelled using [alpha ^{32}P] dCTP and a hexamer primer labelling

protocol (Feinberg and Vogelstein 1984, see appendix for a detailed protocol). Labelled product was separated from unincorporated nucleotides using a gel filtration spin column (Sephadex G50, 1 ml bed volume, centrifugation 200 x *g*, 3 min). Specific activity of the DNA probe was 3.1×10^8 dpm/μg.

Bacterial DNA was transferred to nitrocellulose filters from individual colonies using the alkaline lysis protocol of Maniatis *et al.* (1982). The protocol for filter hybridization was adapted from Denhardt (1966) and Grunstein and Hogness (1975). Filters were prehybridized 3 hr at 42 °C in a solution of 2.5 ml 20X SSC (1X SSC is 150 mM sodium chloride, 15 mM sodium citrate), 4.5 ml formamide, 1.0 ml 50X Denhardt's [50X Denhardt's contains 10 gm each of ficoll (average molecular weight 400,000), polyvinylpyrrolidone (average molecular weight 360,000), and bovine serum albumin (fraction V) per liter.], 1.0 ml sodium phosphate (0.5 M, pH 6.5), 1.0 ml denatured salmon sperm DNA (10 mg/ml). Filters were hybridized 16 hr at 42 °C in a solution of 2.5 ml 20X SSC, 4.5 ml formamide, 0.2 ml 50X Denhardt's, 0.4 ml sodium phosphate (0.5 M, pH 6.5), 2 ml sodium dextran sulfate (50 % w/v), 40 μl *ipt* probe stock (5 μCi). The low stringency washing protocol was as follows: once in 2X SSC, 1X Denhardt's for 15 min at 22 °C; three times in 2X SSC, 0.1 % (w/v) sodium dodecyl sulfate (SDS) for 20 min at 22 °C; three times in 0.1X SSC, 0.1 % SDS for 20 min at 42 °C. Filters were wrapped in plastic wrap and placed in film cassettes. Film (Kodak XOMAT) was exposed 24 hr at -70 °C to the filters prior to development.

Plasmid Isolation:

Plasmid complement of the gall isolates was characterized by a protocol adapted from Kado and Lui (1981). Cells were grown in 523 medium 16 hr to 18 hr. An aliquot (1 ml) was centrifuged (12000 g, 2 min), the cell pellet resuspended in 10 μl TE buffer (20 mM

Tris-acetate, 1 mM EDTA, pH 8.0). Lysis buffer (50 mM Tris base, 3 % SDS, pH 12.6, 200 μl) was added and gently mixed. Following incubation (40 min, 65 °C), 20 μl sodium acetate (3 M , pH 4.8) and 10 μl potassium chloride (2.5 mM) was added, and the resulting precipitate pelleted by centrifugation (12000 g, 3 min, 4 °C). The supernatant was extracted with phenol and chloroform, and the DNA precipitated as described above. *A. tumefaciens* pTiC58 was prepared with each native strain series as a positive control for the procedure. The plasmids were separated using gel electrophoresis (0.7 % agarose, 120 V, 2 hr, in TBE buffer) and visualized using UV fluorescence (300 nm illumination from an Ultra Violet Products Transilluminator) after staining with ethidium bromide (0.4 μg/ml in TBE buffer).

Plant inoculations and opine examination:

Bacterial inoculations onto *Kalanchoe*, *P. menziesii*, and *P. ponderosa* were as described in Chapter I for *A. tumefaciens* inoculations. Examination of gall extracts for opines followed the protocol from Chapter I.

RESULTS

Bacterial Isolation.

The four collections of galls collected from *P. menziesii* trees growing in the wild served as rich sources of bacteria. Examples of the galls are depicted in Figure 5. A list of the bacterial isolates and their source materials are provided in Table 4. Names of the isolates reflect location of gall origin and the biotype selective medium use in isolation. The MP series strains were isolated from galls collected on Mary Peak in the Oregon Cascades. The letters V, G, and R refer to the color of the indicators in the biotype I, II, and III selective media respectively. The number in the isolate name relates to the gall of origin

Figure 5. Native galls of P. menziesii. The center gall served as source material for bacterial isolates of the Washington-3 collection. Similar, but smaller galls, collected from different trees served as source material for Washington-2 and Washington-1 collections.

(gall A, numbers 1-15; gall B, numbers 16-30; gall C, numbers 31-45). The Washington-1 series of strains are named beginning with the letter W. The second letter designates the gall of origin, the third the biotype. Washington-2 and Washington-3 strain names begin with a digit corresponding to the selective biotype medium upon which the strain was isolated.

Growth rates varied substantially among the isolates. Washington-1 strains grew more slowly on 523 medium than the other native isolates which generally grew at rates comparable to *A. tumefaciens* strain C58. In general the native isolates were cream-colored. A subset of the isolates were further examined for other traits characteristic of *Agrobacterium* strains in addition to growth on the selective media.

Table 4. Native isolates and their sources.

Isolate	Collection/Source	Biotype	Colony Character
1MBA1	Wash. 2, Gall A	1	cream
1MBA2	Wash. 2, Gall A	1	cream
1MBC1	Wash. 2, Gall C	1	cream
1MBC2	Wash. 2, Gall C	1	cream
1MBC3	Wash. 2, Gall C	1	cream
1MCA1	Wash. 3, Gall A	1	cream
1MCA2	Wash. 3, Gall A	1	cream, spreading, slow growth
1MCA3	Wash. 3, Gall A	1	cream
1MCA4	Wash. 3, Gall A	1	pale yellow
1MCA5	Wash. 3, Gall A	1	cream
1MCA6	Wash. 3, Gall A	1	yellow
2MBA1	Wash. 2, Gall A	2	cream, slow growth
2MBA2	Wash. 2, Gall A	2	cream, slow growth
2MBA3	Wash. 2, Gall A	2	cream
2MBB1	Wash. 2, Gall B	2	cream
2MBC1	Wash. 2, Gall C	2	cream, slow growth
3MBA1	Wash. 2, Gall A	3	cream
3MBA2	Wash. 2, Gall A	3	cream
3MBA3	Wash. 2, Gall A	3	cream
3MBB1	Wash. 2, Gall B	3	cream
3MBB2	Wash. 2, Gall B	3	cream
3MBB3	Wash. 2, Gall B	3	cream
3MBC1	Wash. 2, Gall C	3	cream, very slow growth
3MBC2	Wash. 2, Gall C	3	cream
3MBC3	Wash. 2, Gall C	3	cream
3MCA1	Wash. 3, Gall A	3	cream
3MCA2	Wash. 3, Gall A	3	cream
3MCA3	Wash. 3, Gall A	3	cream
3MCA4	Wash. 3, Gall A	3	cream
3MCA5	Wash. 3, Gall A	3	cream
3MCA6	Wash. 3, Gall A	3	cream
3MCA7	Wash. 3, Gall A	3	cream
3MCA8	Wash. 3, Gall A	3	cream
LBWAG	Wash. 1, Gall A	2	Grows on LB medium
LBWBV	Wash. 1, Gall B	1	Grows on LB medium
MPG 1	MarysPeak, Gall A	2	pink
MPG 2	MarysPeak, Gall A	2	pink
MPG 3	MarysPeak, Gall A	2	pink
MPG 4	MarysPeak, Gall A	2	pink
MPG 5	MarysPeak, Gall A	2	pink
MPG 6	MarysPeak, Gall A	2	slow growth pink
MPG 7	MarysPeak, Gall A	2	slow growth pink
MPG 8	MarysPeak, Gall A	2	slow growth pink
MPG 9	MarysPeak, Gall A	2	slow growth pink
MPG10	MarysPeak, Gall A	2	slow growth pink
MPG11	MarysPeak, Gall A	2	slow growth pink
MPG12	MarysPeak, Gall A	2	cream
MPG13	MarysPeak, Gall A	2	slow growth cream
MPG14	MarysPeak, Gall A	2	slow growth cream
MPG15	MarysPeak, Gall A	2	slow growth cream
MPG16	MarysPeak, Gall B	2	cream
MPG17	MarysPeak, Gall B	2	cream
MPG17	MarysPeak, Gall B	2	cream
MPG18	MarysPeak, Gall B	2	cream
MPG19	MarysPeak, Gall B	2	cream
MPG20	MarysPeak, Gall B	2	cream
MPG21	MarysPeak, Gall B	2	cream
MPG22	MarysPeak, Gall B	2	cream
MPG22	MarysPeak, Gall B	2	cream
MPG23	MarysPeak, Gall B	2	cream
MPG24	MarysPeak, Gall B	2	cream
MPG25	MarysPeak, Gall B	2	cream
MPG26	MarysPeak, Gall B	2	cream
MPG27	MarysPeak, Gall B	2	cream
MPG28	MarysPeak, Gall B	2	cream
MPG29	MarysPeak, Gall B	2	cream
MPG30	MarysPeak, Gall B	2	cream
MPG31	MarysPeak, Gall C	2	cream
MPG32	MarysPeak, Gall C	2	cream
MPG33	MarysPeak, Gall C	2	cream
MPG34	MarysPeak, Gall C	2	cream
MPG35	MarysPeak, Gall C	2	cream
MPR 1	MarysPeak, Gall A	3	cream
MPR 2	MarysPeak, Gall A	3	cream
MPR 3	MarysPeak, Gall A	3	cream
MPR 4	MarysPeak, Gall A	3	cream
MPR 5	MarysPeak, Gall A	3	cream

continues.

Table 4. contined.

Isolate	Collection/Source	Biotype	Colony Character
MPR 6	MarysPeak, Gall A	3	cream
MPR 7	MarysPeak, Gall A	3	cream
MPR 8	MarysPeak, Gall A	3	cream
MPR 9	MarysPeak, Gall A	3	cream
MPR10	MarysPeak, Gall A	3	cream
MPR11	MarysPeak, Gall A	3	cream
MPR12	MarysPeak, Gall A	3	cream
MPR13	MarysPeak, Gall A	3	cream
MPR14	MarysPeak, Gall A	3	cream
MPR15	MarysPeak, Gall A	3	cream
MPR16	MarysPeak, Gall B	3	cream
MPR17	MarysPeak, Gall B	3	cream
MPR18	MarysPeak, Gall B	3	cream
MPR19	MarysPeak, Gall B	3	cream
MPR20	MarysPeak, Gall B	3	cream
MPR21	MarysPeak, Gall B	3	cream
MPR22	MarysPeak, Gall B	3	cream
MPR23	MarysPeak, Gall B	3	cream
MPR24	MarysPeak, Gall B	3	cream
MPR25	MarysPeak, Gall B	3	cream
MPR26	MarysPeak, Gall B	3	cream
MPR27	MarysPeak, Gall B	3	cream
MPR28	MarysPeak, Gall B	3	cream
MPR29	MarysPeak, Gall B	3	cream
MPR30	MarysPeak, Gall B	3	cream
MPR31	MarysPeak, Gall C	3	cream
MPR32	MarysPeak, Gall C	3	cream
MPR34	MarysPeak, Gall C	3	cream
MPR35	MarysPeak, Gall C	3	cream
MPR36	MarysPeak, Gall C	3	cream
MPR37	MarysPeak, Gall C	3	cream
MPR38	MarysPeak, Gall C	3	cream
MPR39	MarysPeak, Gall C	3	cream
MPR40	MarysPeak, Gall C	3	cream
MPR41	MarysPeak, Gall C	3	cream
MPR42	MarysPeak, Gall C	3	cream
MPR43	MarysPeak, Gall C	3	cream
MPR44	MarysPeak, Gall C	3	cream
MPR45	MarysPeak, Gall C	3	cream
MPV 1	MarysPeak, Gall A	1	cream
MPV 2	MarysPeak, Gall A	1	cream
MPV 3	MarysPeak, Gall A	1	cream
MPV 4	MarysPeak, Gall A	1	cream
MPV 5	MarysPeak, Gall A	1	cream
MPV 6	MarysPeak, Gall A	1	cream
MPV 7	MarysPeak, Gall A	1	cream
MPV 8	MarysPeak, Gall A	1	cream
MPV 9	MarysPeak, Gall A	1	cream
MPV10	MarysPeak, Gall A	1	cream
MPV11	MarysPeak, Gall A	1	cream
MPV12	MarysPeak, Gall A	1	cream
MPV13	MarysPeak, Gall A	1	cream
MPV14	MarysPeak, Gall A	1	cream
MPV15	MarysPeak, Gall A	1	cream
MPV16	MarysPeak, Gall B	1	cream
MPV17	MarysPeak, Gall B	1	cream
MPV18	MarysPeak, Gall B	1	cream
MPV19	MarysPeak, Gall B	1	cream
MPV20	MarysPeak, Gall B	1	cream
MPV21	MarysPeak, Gall B	1	cream
MPV22	MarysPeak, Gall B	1	cream
MPV23	MarysPeak, Gall B	1	cream
MPV24	MarysPeak, Gall B	1	cream
MPV25	MarysPeak, Gall B	1	cream
MPV26	MarysPeak, Gall B	1	cream
MPV27	MarysPeak, Gall B	1	cream
MPV28	MarysPeak, Gall B	1	cream
MPV29	MarysPeak, Gall B	1	cream
MPV30	MarysPeak, Gall B	1	cream
MPV31	MarysPeak, Gall C	1	yellow, slow growth
MPV32	MarysPeak, Gall C	1	yellow, slow growth
MPV34	MarysPeak, Gall C	1	yellow, slow growth
MPV35	MarysPeak, Gall C	1	yellow, slow growth
MPV36	MarysPeak, Gall C	1	yellow, slow growth
MPV37	MarysPeak, Gall C	1	yellow, slow growth
MPV38	MarysPeak, Gall C	1	yellow, slow growth
MPV39	MarysPeak, Gall C	1	yellow, slow growth
MPV40	MarysPeak, Gall C	1	yellow, slow growth

continues

Table 4. continued

Isolate	Collection/Source	Biotype	Colony Character
MPV41	MarysPeak, Gall C	1	yellow, slow growth
MPV42	MarysPeak, Gall C	1	yellow, slow growth
MPV43	MarysPeak, Gall C	1	yellow, slow growth
MPV44	MarysPeak, Gall C	1	yellow, slow growth
MPV45	MarysPeak, Gall C	1	cream
WAG1	Wash. 1, Gall A	2	cream, slow growth
WAG2	Wash. 1, Gall A	2	cream
WAG3	Wash. 1, Gall A	2	cream
WAG4	Wash. 1, Gall A	2	yellow, slow growth
WAG5	Wash. 1, Gall A	2	yellow, slow growth
WAG6	Wash. 1, Gall A	2	yellow, slow growth
WAG7	Wash. 1, Gall A	2	cream
WAR1	Wash. 1, Gall A	3	cream
WAR2	Wash. 1, Gall A	3	cream
WAR4	Wash. 1, Gall A	3	cream
WAR5	Wash. 1, Gall A	3	cream
WAR6	Wash. 1, Gall A	3	cream
WAV1	Wash. 1, Gall A	1	cream, spreading
WAV2	Wash. 1, Gall A	1	cream, spreading slow growth
WAV3	Wash. 1, Gall A	1	yellow, slow growth
WAV4	Wash. 1, Gall A	1	cream, spreading
WAV5	Wash. 1, Gall A	1	yellow, slow growth
WAV6	Wash. 1, Gall A	1	cream, spreading very slow growth
WBG1	Wash. 1, Gall B	2	cream
WBG2	Wash. 1, Gall B	2	cream
WBG3	Wash. 1, Gall B	2	cream
WBG4	Wash. 1, Gall B	2	cream
WBG5	Wash. 1, Gall B	2	slow growth, cream
WBG6	Wash. 1, Gall B	2	cream
WBR1	Wash. 1, Gall B	3	yellow
WBR2	Wash. 1, Gall B	3	yellow
WBR3	Wash. 1, Gall B	3	yellow
WBR4	Wash. 1, Gall B	3	cream
WBR5	Wash. 1, Gall B	3	cream
WBR6	Wash. 1, Gall B	3	cream
WBV1	Wash. 1, Gall B	1	cream, very slow growth
WBV2	Wash. 1, Gall B	1	yellow, slow growth
WBV3	Wash. 1, Gall B	1	cream, very slow growth
WBV4	Wash. 1, Gall B	1	cream, highly convex
WBV5	Wash. 1, Gall B	1	cream, highly convex
WBV6	Wash. 1, Gall B	1	cream, slow growth
WCG1	Wash. 1, Gall C	2	cream
WCG2	Wash. 1, Gall C	2	cream
WCG3	Wash. 1, Gall C	2	cream
WCG4	Wash. 1, Gall C	2	cream
WCG5	Wash. 1, Gall C	2	pink,slow growth
WCG6	Wash. 1, Gall C	2	pink,slow growth
WCR1	Wash. 1, Gall C	3	cream
WCR2	Wash. 1, Gall C	3	cream, with yellow, ring
WCR3	Wash. 1, Gall C	3	cream, with yellow, ring
WCR4	Wash. 1, Gall C	3	cream
WCR5	Wash. 1, Gall C	3	cream
WCR6	Wash. 1, Gall C	3	cream
WCV1	Wash. 1, Gall C	1	cream
WCV2	Wash. 1, Gall C	1	pink, very slow growth
WCV3	Wash. 1, Gall C	1	orange, slow growth
WCV4	Wash. 1, Gall C	1	pink,slow growth
WCV5	Wash. 1, Gall C	1	cream
WCV6	Wash. 1, Gall C	1	cream

Homology to *ipt*

DNA from many of the MP isolates, especially those isolated on biotype III media (MPR strains), hybridized strongly to the *ipt* probe. Figure 6 shows the autoradiogram from the colony lysates of the biotype three strains, which showed the largest number of positive colonies of the three biotypes. Only one biotype I strain (MPV15) produced a positive signal while eight biotype II strains (MPG isolates) and eighteen biotype III strains (MPR isolates) were positive (Table 5). Most isolates which showed hybridization were isolated from gall B of the MP collection. None of the bacteria isolated from gall C produced positive hybridization signals.

Characterization of the MP strains.

Plasmid complement and plasmid electrophoretic mobility relative to pTiC58 is indicated in Table 5. Of the 47 strains examined, 22 had large Ti-sized plasmids. Four isolates harbored two plasmids, two of which (harbored by MPR23 and MPG29) were relatively small (Figure 7). There was no consistent relation between plasmid content and *ipt* hybridization. Thirteen of the strains with strong hybridization signals harbored large plasmids, while an equal number did not.

A subset of the MP strains (MPG28, MPG31, MPG35, MPR12, MPR2, MPR25, MPR29, MPR35, MPR41, MPR5, MPV45) were inoculated onto *P. menziesii*. These generally formed a small swollen knot at the inoculation site, without the callus or lobed appearance typical of *A. tumefaciens* infections (Figure 8).

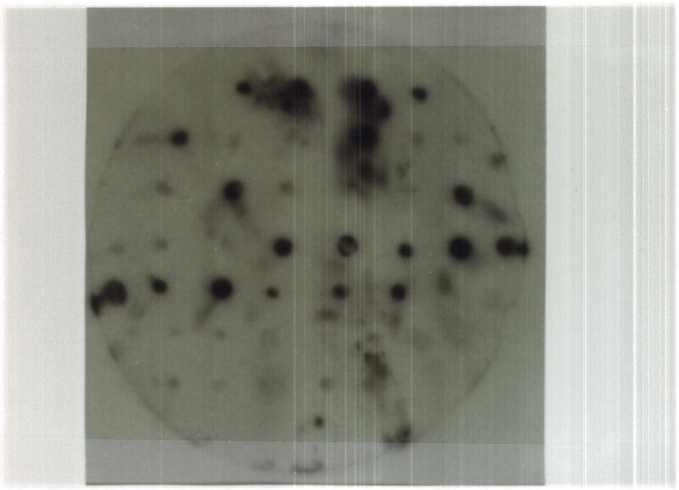

Figure 6. Colony hybridization of native bacterial isolates. Colonies of MP strains were lysed, the DNA transferred to nitrocellulose, and hybridized to [^{32}P]-labelled *ipt* of pTiAch5. This filter hybridization pattern developed from the biotype III colonies following autoradiography.

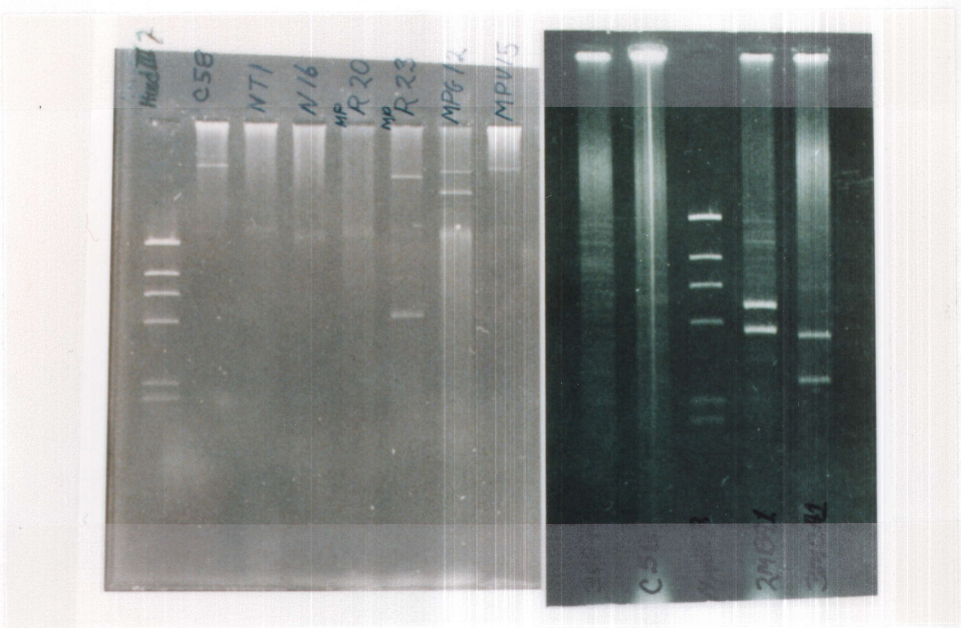

Figure 7. Large and small plasmids of native bacterial isolates. Left. Plasmid preparations of *Agrobacterium* and native gall isolates. Ti-sized plasmids were found in native isolates MPR23, MPG12, and MPV15 among others. Lanes; 1. *Hin*dIII digested lambda, 2. *A. tumefaciens* C58, 3. *A. tumefaciens* A136 (Ti plasmid cured), 4. strain NAB16, 5. gall isolate MPR20, 6. gall isolate MPR23, 7. gall isolate MPG12, 8. gall isolate MPV15. **Right.** Plasmid preparations of isolates 3MCA1 and 2MBA1 were digested with *Eco*RI. Electrophoresis of the products of showed a background ladder typical of large plasmids and two intense bands consistent with the presence of small multicopy plasmids. Lanes are: 1, *A. tumefaciens* 3667; 2, *A. tumefaciens* C58; 3, HindIII digested lambda; 4, gall isolate 2MBA1; 5, gall isolate 3MCA1.

Table 5. Hybridization and plasmid status of native isolates.

Strain	ipt hybridization	pA	pB	Strain	*ipt* hybridization	pA	pB
MPR1	+	1.19		MPG2	-	ni	
MPR2	+	1.21		MPG4	-	ni	
MPR3	+	1.07		MPG6	-	0.33	1.33
MPR4	+	nd		MPG8	-	1.33	
MPR5	+	1.19		MPG12	+	1.04	1.52
MPR8	++	1.07		MPG16	-	ni	
MPR10	-	ni		MPG17	++	ni	
MPR12	++	1.04		MPG20	++	ni	
MPR16	+	ni		MPG22	++	ni	
MPR20	+	ni		MPG23	+	ni	
MPR21	+	ni		MPG24	++	ni	
MPR22	-	ni		MPG28	+	1.19	
MPR23	++	1.17	3.2	MPG29	+	1.18	4.71
MPR24	+	ni		MPG31	-	1.00	
MPR25	+	1.23		MPG33	-	1.00	
MPR26	+	ni		MPG35	-	1.00	
MPR27	+	ni					
MPR28	+	ni					
MPR29	+	1.21		MPV6	-	ni	
MPR30	+	ni		MPV8	-	ni	
MPR31	-	1.02		MPV9	-	ni	
MPR35	-	1.19		MPV15	+	0.98	
MPR38	-	ni		MPV31	-	ni	
MPR41	-	1.15		MPV34	-	ni	
MPR44	-	ni		MPV45	-	1.02	

Plasmid mobilities are given relative to pTiC58 which is approximately 210 kb in size, larger values indicate smaller plasmids . Abbreviations; pA, largest plasmid; pB, second largest plasmid; ni, none identified; nd, not determined;

Plasmid content and virulence of the Washington-1 strains.

Selected strains of the Washington-1 collection were also examined for large plasmids. As shown in Table 6, twelve of the 32 strains harbored large plasmids. A subset of these strains were examined for virulence on *P. menziesii*. Three seedlings were used for each strain. Gall formation was incited by all the examined strains (Table 6). As seen in Figure 8, these isolates incited large galls at the inoculation site, frequently with enlarged stems below the gall. The rate of tissue proliferation surpassed the rates seen following inoculations of known *A. tumefaciens* strains. Two of the Washington-1 strains, WBR5

Table 6. Plasmid content and tumorigenicity of native isolates.

Gall A			Gall B			Gall C		
Isolate	Plasmid	Gall	Isolate	Plasmid	Gall	Isolate	Plasmid	Gall
LBWAG	+	nd	WBR1	-	nd	WCG2	-	nd
WAG1	+	nd	WBR2	-	nd	WCG5	+	+
WAG2	-	nd	WBR3	-	nd	WCG6	+	+
WAG3	-	nd	WBR4	+	+			
WAG4	+	nd	WBR5	+	+	WCR1	+	+
			WBR6	+	+	WCR3	+	+
WAR2	+	+				WCR4	+	+
WAR4	+	+				WCR5	+	+
WAR5	+	+				WCR6	+	+
WAR6	+	+						
						WCV2	-	nd
WAV1	-	nd				WCV3	-	nd
WAV2	-	nd				WCV4	-	nd
WAV4	+	nd				WCV5	+	+
WAV6	-	nd				WCV6	+	+

and WCR3 were inoculated onto *P. ponderosa* stems, resulting in large galls. Figure 8 shows a gall on *P. ponderosa* incited by WCR3.

Washington-2 and Washington-3 characterizations.

The isolates from the Washington-2 and Washington-3 collections were obtained after efforts were focused on the characterized *Agrobacterium* strains, and little characterization was undertaken. However, as shown in Figure 7, plasmid preparations of two strains, 2MBA1 and 3MCA1, when digested with *Eco*RI, yielded two intense bands in each of the digests and a ladder of fainter bands. This banding pattern suggested the presence of a large low copy plasmid (or plasmids) and different multicopy small plasmids. The sizes of the small plasmids were estimated as 7.2 kb and 4.4 kb for isolates 2MBA1 and 3MCA1 respectively.

Figure 8. Galls incited on conifers by native bacterial isolates. Left. Gall incited by strain MPV15 on *P. menziesii.* **Center.** Gall incited by strain WCR3 on *P. menziesii.* **Right.** Gall incited by strain WCR3 on *P. ponderosa.*

Only isolates 1MCA4, 2MBA1 and 3MCA1 of the Washington-2 and Washington-3 collections were inoculated onto stems of *P. menziesii* seedlings. No hypertrophy was noted two months following inoculation (data not shown)

Gall induction on Angiosperm hosts.

Kalanchoe leaves were inoculated with the Mary's Peak isolates; MPR1, MPG12, MPV15, MPR23, MPG29, MPR31 and MPG33. Decapitated *Kalanchoe* stems were inoculated at the wound site with Washington-1 isolates WAG1, WAG4, WAR2, WAR4, WAR5, WAR6, WAV4, WBR4, WBR5, WBR6, WCR1, WCR3, WCR4, WCR5, WCR6, WCV5 and WCV6. Decapitated tobacco plants were inoculated at the wound site with Washington-1 isolates WAR6, WCG5, and WCR3.

None of the inoculations incited any obvious tissue proliferation within two months (data not shown).

Opine Characterizations:

Extracts from galls incited by Washington-1 isolates and parental Washington-2 and Washington-3 galls failed to indicate the presence of octopine, nopaline, agropine or manopine. Silver staining of gall extracts from the parental Washington-2 gall separated by electrophoresis at pH 9.2 did yield a faint positively staining compound with a mobility between that of agropine and manopine which was not found in untransformed control tissue (data not shown). The nature of the compound remains unknown.

DISCUSSION

Isolates of *P. menziesii* galls have many attributes of *Agrobacterium* strains. However, confirmation of *A. tumefaciens* identity is still lacking. Growth on *Agrobacterium* selective plates, positive *ipt* hybridization signals, presence of large Ti-sized plasmids, and the ability to incite galls on the host of origin argue in favor of the isolates being *Agrobacteria*. The most convincing evidence for this hypothesis was their ability to incite gall formation following stem inoculation. Other than *A. tumefaciens*, there are no reports of bacterial gall-forming pathogens of *P. menziesii* (Bradbury 1986).

The very rapid gall development on *P. menziesii* stems following inoculation with isolates of the Washington-1 collection was unexpected. *Agrobacterium* incited galls were approximately 1.0 mm in size three months after inoculation (Chapter II). Galls of this dimension took only two to three weeks to develop when incited by Washington-1 strains.

Assuming this gall development was the result of transformation, it is impossible to separate rapid growth rate due to more cells being transformed, from increased growth rate due to greater proliferation of the same number of transformed cells. The high consistency of gall formation (100 % for the 3 to 6 seedlings inoculated per isolate) lends support to the idea of high transformation efficiency and therefore large numbers of cells transformed. If these strains are indeed more efficient in transforming tissue at the cellular level, then they may provide a means of examining genetic determinants required for highly efficient transformation of conifer tissue.

The failure to form galls on *Kalanchoe* is difficult to reconcile with the ability to form galls on conifers. However, this is not with out precedence. Strain S5/72 which was isolated from the conifer *Libocedrus decurrens* failed to form galls on *Kalanchoe*, yet did form galls on three of the four conifer hosts inoculated (Chapter II, Table 3). The basis for this host range specificity is unknown.

While the failure to identify opines in extracts of the native galls is evidence against the isolates belonging to the genus *Agrobacterium*, extracts were not examined for all known opines. Also, novel opine production from "null tumors" has been reported (Guyon *et al.* 1980), and new opines continue to be identified (Szegedi *et al.* 1988, Isogai *et al.* 1988). Further, the absence of opine production by tissue transformed by T-DNA of octopine strains was commonly found in the *Kalanchoe* transformations described in Chapter II. Thus, a lack of detectable opine may indicate simply the lack of an appropriate assay, or that there was a failure in the transfer and expression of the T-DNA genes encoding opine biosynthesis, perhaps due to a divided T-DNA.

The presence of multiple plasmids in some of the native strains indicated compatible replication origins were extant. Although the genes located on the plasmids are uncharacterized, and may be of interest in the biology of the bacteria, the simple presence of the plasmids is of interest in terms of vector development. The small plasmids of 2MBA1 (7.2 kb) and 3MCA1 (4.2 kb) if converted to cosmids, would allow the insertion of larger DNA segments compared to the cosmid pVK102 (23 kb Knauf and Nester 1982). Inserts the size of C58 T-DNA (22.7 kb Lemmers *et al.* 1980) would yield plasmids of a size easily manageable in bacterial electroporation or direct transformation protocols.

A wide variety of opportunities exist in further characterization the native isolates. Answers to several questions should be sought. Are the isolates indeed *A. tumefaciens* strains, and is transformation occurring? What is the basis for the lack of tumorigenicity on *Kalanchoe* and tobacco? Is the relation between slow bacterial growth and rapid gall proliferation related to a host hypersensitive response? What is the efficiency of transformation at the cellular level? Judging from sequence homology, what known genes (T-DNA, virulence, opine metabolism, or other) are encoded on the plasmids? How may the small plasmids best be used in development of cloning or transformation vector development? The native isolates while only characterized to a limited extent as yet, hold potential for development of highly efficient transformation vectors for conifers.

INDUCTION OF THE *AGROBACTERIUM VIR* CASCADE.

INTRODUCTION

While the tumorigenicity screen for gall production described in Chapter II identified a select group of *A. tumefaciens* strains that were highly tumorigenic on conifers, the basis for the differences among strains remained obscure. As elaborated in Chapter I, tumor formation is the culmination of a series of steps, attachment, signal transduction, T-DNA processing, T-complex export, nuclear T-DNA targeting and chromosome integration, and expression of T-DNA encoded oncogenes. Although attachment deficiencies may reduce the virulence of a strain, attachment was not likely to be a limitation during the inoculation studies described above. The bacteria were applied directly to the wound site and no activities, such as watering from above, were conducted which would remove the inoculum. From the model, the next stage of tumor formation is signal transduction and virulence gene induction. It was possible that the level of induction of expression of virulence region genes was different between the strongly and weakly tumorigenic strains, and that this difference in expression was the basis for the difference in tumorigenicity. Two independent measures of activation of *vir* region genes were considered. The first was the induction of cytokinin biosynthesis. The second was the induction of a reporter fusion introduced into the *Agrobacterium* strains.

Cytokinin biosynthesis by *Agrobacterium* results from expression of *tzs*, the locus responsible for secretion of zeatin (Regier and Morris 1982). It has been found on all

nopaline Ti plasmids examined to date (Akiyoshi *et al.* 1987, Beaty *et al.* 1986, John and Amasino 1988). This gene encodes a prenyl transferase responsible for the formation of iso-pentenyladenosine-5'-monophosphate (IPM) from iso-pentenyladenosine and dimethylallyl pyrophosphate (Akiyoshi *et al.* 1984). Hydroxylation of the prenyl moiety and dephoshporylation yields zeatin riboside. Induction of *tzs* expression is dependent on the same *virA-virG* regulatory cascade as induction of the other *vir* genes (John and Amasino 1988, Powell *et al.* 1988). For cells which carry *tzs*, increased cytokinin levels in the culture medium in the presence of a *vir* gene inducer indicates *vir* gene induction.

The second, more rapid, assay of *vir* gene induction was originally developed by Stachel and Nester (1986). They described reporter constructs which contained translational fusions between *vir* promoters and the structural region of the *E. coli lacZ* gene, which encodes *beta*-galactosidase. *Beta*-galactosidase may be measured colorimetrically (Miller 1972). Among many *lacZ:vir* fusions, one of the most highly inducible fusions was the *virE2:lacZ* fusion contained on plasmid pSM358. Plasmid pSM358 has been used to identify the efficacy of various phenolics as *A. tumefaciens vir* gene inducers (Spencer and Towers 1988) and also to identify flavanoid inducers in pollen (Zerback *et al.* 1989).

The goal of the studies in this chapter was to test the hypothesis that the level of *vir* gene induction was directly correlated with strain tumorigenicity. Specific objectives were as follows:

1. To identify culture conditions suitable for measurement of *vir* induction.
2. To measure acetosyringone induced *tzs* expression in a set of *Agrobacterium* strains.

3. To measure acetosyringone induced expression of *virE:lacZ* in *Agrobacterium* strains harboring pSM358.
4. To correlate the above measures with strain tumorigenicity on conifers.

METHODS

Development of *Vir* Induction Media.

To evaluate the relationship between different carbon sources and the degree of bacterial cell agglutination, strain A348(pTiA6,pSM358), generously provided by P. Zambryski, was grown in liquid VIM1 with the compounds listed in Table 7 substituted gram for gram for sucrose. In the case of glycerol, the substitution was 1 ml per 1 gm. Following incubation on a rotary shaker for 18 hr at 22 °C the relative growth and level of agglutination were visually assessed.

A study was undertaken to evaluate the effectiveness of several media components in increasing the growth rate of B3/73. Components were added to a minimal media (DEF1, appendix) modified from the nopaline media for *Agrobacterium* selection (Hooykaas *et al.* 1979). The components examined, and their final concentrations were: biotin, 100 μg/l; glycerol, 5 ml/l; mannitol, 5 gm/l; yeast extract 0.5 gm/l and 1.0 gm/l (Difco); vitamin mix (appendix), 0.1X normal concentration; vitamin mix, 0.5X normal concentration; casaminoacids, 0.5 gm/l and 1.0 gm/l (Difco); Murashige and Skoog salts, 0.5 gm/l (Gibco); myo-inositol, 0.1 gm/l. The first seven of these components and unsupplemented control medium were applied in a two dimensional array across all of the components to identify two-component interactions. Cultures were inoculated at low

density (A_{650} = 0.03) in a micro-well assay plate and incubated 34 hr at 22 °C. Final cell densities were measured at 650 nm

Assay for *tzs* induction.

Strains were streaked onto 523 plates from stocks stored at -70 °C. Following three days of incubation at room temperature, a loop of bacteria was inoculated into 1.5 ml VIM1 medium containing 200 μM acetosyringone. Cultures were incubated 18 hr on a rotary shaker at 25 °C. Cells were removed by centrifugation (12000 g, 2 min), and the zeatin/zeatin riboside content of an aliquot of the supernatant (10 μl) was determined by ELISA.

ELISA assay for zeatin/zeatin riboside.

An ELISA protocol (adapted from Maldiney *et al.* 1986) was used for measuring cytokinin levels in aliquots of the culture medium. Briefly, micro-well assay plates were coated with a bovine serum albumin/zeatin riboside conjugate. Aliquots of culture supernatants (5 μl or 10 μl) and zeatin/zeatinriboside-specific mouse monoclonal antibody (R.O. Morris, clone #16) were added to the wells and incubated at 37 °C. The wells were washed, and an alkaline phosphatase/second antibody conjugate (anti mouse antibody) was added. After incubation and washing, phosphatase substrate (p-nitrophenyl phosphate, Sigma) was added. Phosphatase activity (measured as an absorbance increase, A_{405}) reflected the amount of the second antibody bound, and was inversely proportional to the concentration of cytokinin present in the sample. The antibody binds both zeatin and zeatin riboside, thus the presence of zeatin, zeatin riboside or a combination of the two compounds can not be distinguished. Results are expressed as zeatin riboside equivalents. Full details of the protocol and solutions needed are given in the appendix.

***Tzs* hybridization probe preparation.**

The *tzs* gene of *A. tumefaciens* C58 was obtained from the plasmid pTZ120 (Beaty *et al.* 1986). Digestion with *Bam*HI and *Hin*dIII as instructed by the supplier (BRL) yielded a 1.4 kb fragment containing the entire 729 bp *tzs* open reading frame. The fragment was electrophoretically separated (110 V, 2 hr, in TBE buffer) from the vector on an agarose gel (0.7 %), stained with ethidium bromide (0.4 μg/ml), excised, placed in a dialysis tube and electroeluted (110 V, 30 min, in TBE buffer). Following phenol-chloroform extraction and ethanol precipitation as described in Chapter III, the DNA (120 μg) was used as template in a random hexamer primed DNA polymerase reaction using [alpha^{32}P] dCTP (Feinberg and Vogelstein 1984). Full details of the labelling protocol are in the appendix. Specific activity of the probe was approximately 5 x 10^8 dpm/μg.

***Tzs* hybridization to Ti plasmid digests.**

The highly tumorigenic strains examined were; 3667, B1/74, B3/73, C2/74 and M2/73. C58 and T28/73 were also examined as positive *tzs* controls. *A. tumefaciens* Ti plasmids were isolated using a protocol modified from that of Koekman et al.(1980). DNA content of the preparations was estimated using bis-benzimidazole (Hoechst 33258) in a protocol described by the fluorimeter manufacturer (Hoefer Science Instruments, San Francisco). Equal amounts of Ti plasmid DNA (0.3 μg) were digested using the restriction enzyme *Hin*dIII as instructed by the supplier (BRL). DNA samples were mixed (4:1, v/v) with a loading dye (bromophenol blue, 0.5 mg/ml in glycerol:TBE, 9:1, v/v), loaded into an agarose gel (0.7 %) in TBE buffer, and electrophoretically separated by application of 45 V for 14 hr. Following electrophoresis, the DNA was stained with ethidium bromide (0.4 μg/ml in TBE buffer), visualized under 300 nm light (Ultra Violet Products Transilluminator) and photographed using a Polaroid MP4 camera system and type 667 film. The DNA was transferred to nylon hybridization membrane (Biorad Zetaprobe)

following an initial depurination step. (0.25 M HCl, 0.6 M NaCl for 15 min.). Capillary transfer using a solution of 0.4 M NaOH, 0.6 M NaCl, was complete after 8 hr. DNA was linked to the membrane by incubating it at 37 °C for 16 hr. A low stringency hybridization protocol (adapted from Chapter III) was followed. The membrane was prehybridized 10 hr at 42 °C in 10 ml prehybridization solution; 50 % formamide (v/v), 1 % SDS (w/v). Hybridization proceeded for 16 hr at 42 °C following the addition of the boiled *tzs* probe solution; 5 μl probe stock (1.4 μC), 100 μl salmon sperm DNA (10 mg/ml), 400 μl $H_2$0. The membrane was washed twice in 2X SSC at 23 °C (5 min), and four times in 2X SSC, 1 % SDS at 65 °C (10 min). Following washing, the membrane was exposed to X-ray film (Kodak Xomat) at -70 C for 18 hr prior to development.

Transformation of A. tumefaciens strains with pSM358.

Plasmid pSM358 was isolated from A348(pSM358) using the alkaline lysis protocol described in Chapter III scaled up to a volume of 500 ml. The plasmid was used to transform competent *E. coli* DH5alpha cells (BRL) following instructions for freeze-thaw transformation provided by the supplier. The resulting transformed strain, CRV982 [*E. coli* DH5alpha (pSM358)], was used as a donor in triparental matings (Ditta *et al.* 1980) using *E. coli* HB101(pRK2013) as helper, and *Agrobacterium* wild-type strains as recipients. The liquid-plate cocultivation protocol was conducted according to a modification developed in the laboratory (appendix). Where putative transformants were nopaline strains, they were confirmed as *Agrobacterium* by growth on minimal media containing nopaline as the sole nitrogen source (Hooykaas *et al.* 1979) and kanamycin (50 μg/ml) and carbenicillin (50 μg/ml) to select for pSM358.

Virulence Gene Induction Assay.

The assay for *beta*-galactosidase was adapted from Miller (1972) and is described in detail in the appendix. Briefly, log-phase liquid cultures were diluted into VIM4 medium containing a test compound or standard phenolic inducer dissolved in dimethyl sulfoxide (DMSO). After 12 hr or at specific time points, cells were centrifuged, resuspended in the original volume of VIM4, the culture density was determined (A_{650}) and Z buffer was added to permeabilize the cell membranes. Following incubation (10 min 22 °C), enzyme activity was assayed by addition of *o*-nitrophenyl-*beta*-D-galactopyranoside and measurement of color development (A_{405}, 2 min, VMAX Microplate Reader, Molecular Devices). *Beta*-galactosidase activity (nmol *o*-nitrophenol released /min) was calculated per unit density of the cell culture to compensate for differences in growth rate. Most assays were performed on bacteria cultured in 100 μl of medium. For the time course assays, however, bacteria were cultured in 10 ml or 20 ml of media and aliquots (100 μl) were removed to micro-well plates for assay.

Effect of acetosyringone concentration on *beta*-galactosidase induction.

Concentration dependency of acetosyringone induction of *beta*-galactosidase were developed by conducting the micro-well plate assay protocol with serial (50 %) dilutions of acetosyringone into VIM4 (50 μl) containing 2 % DMSO. Final concentrations were 3 μM to 500 μM. An equal volume of bacterial culture (50 μl, A_{650} = 0.03 was added to the wells of the plate to initiate the assay. The level of *beta*-galactosidase activity was determined after 16 hr or 18 hr.

Effect of initial cell culture density on *beta*-galactosidase induction.

Initial cell culture densities were adjusted by dilution from VIM4 liquid shake cultures. Bacteria were grown at 22 °C for 16 hr in VIM4 containing 200 μM

acetosyringone and 1 % DMSO or 1 % DMSO without inducer. Micro-well cultures (100 μl) were assayed as described above.

Statistical Correlations.

The correlation coefficient was calculated by the method of least squares for the relationships between *virE:lacZ* induction and *tzs* induction, between *virE:lacZ* induction and strain tumorigenicity, and between *tzs* induction and strain tumorigenicity. The correlation coefficient was tested for significance from zero by the Student's *t* test (Snedecor and Cochran, 1967).

RESULTS

Results in this chapter fall into three sections; media development, *tzs* expression and *virE:lacZ* expression.

Media development.

In order to accurately assess *vir* induction, it was necessary to accurately measure cell growth. The sucrose based medium originally described (Stachel *et al.* 1985b) supported substantial agglutination by some of the test strains, especially MFM83.4, leading to inaccurate measures of cell density. Substitution of sucrose by other carbon sources reduced cell agglutination (Table 7). Substantial agglutination was seen with malic acid. Little agglutination was seen with maleic acid, but there was less overall growth than with glycerol as a carbon source. Glycerol provided the best growth with little or no agglutination. *Vir* gene induction medium containing glycerol (VIM2) was used in the *tzs* induction, and time course induction assays described below.

Table 7. Effect of carbon source on growth of *A. tumefaciens* strain B3/73.

Carbon source	Growth	Agglutination
Citric Acid	2	+
Glycerol	3	-
Maleic Acid	1	-
Malic Acid	1	++
Mannitol	2	+
Succinic Acid	2	+
Tartaric Acid	2	+

Sucrose was substituted as a carbon source in VIM1 medium, and relative growth and agglutination assessed after 18 hr incubation. Growth was scored as: 1. low; 2. intermediate; 3. high. Plus characters indicate cultures which showed agglutination.

Table 8. Growth optimization of *A. tumefaciens* strain B3/73.

		Second Media Component							
		DEF1	Biotin	Glycerol	Mannitol	YE 0.5X	YE 1X	Vit 0.1	Vit 0.5
First Media component									
1	DEF1	2.0							
2	Biotin	2.3	2.7						
3	Glycerol	2.3	2.1	3.0					
4	Mannitol	3.3	4.2	4.1	4.2				
5	YE 0.5X	6.7	6.4	4.6	5.2	7.6			
6	YE 1X	5.6	5.8	8.6	11.2	7.0	8.4		
7	Vitamin 0.1X	3.0	2.9	3.2	4.3	6.2	7.9	3.6	
8	Vitamin 0.5X	2.4	2.3	2.9	4.8	5.9	8.0	3.2	2.9
9	CA 0.5X	4.5	2.6	3.8	7.5	5.1	9.4	4.7	4.6
10	CA 1X	4.5	3.3	3.5	7.3	6.0	6.0	4.2	3.9
11	M&S	2.5	2.9	3.4	5.0	8.5	13.0	3.6	3.1
12	Inositol	1.4	2.1	2.4	2.7	4.4	7.1	3.1	1.6

Values represent the average fold increase in culture density (A_{650}) following a 34 hr incubation period. Component concentrations are defined in the text. DEF1 composition is in the appendix. Abbreviations: YE, yeast extract; CA, casaminoacids; Vit, vitamin mix; M&S, Murashige and Skoog salts.

In order to optimize the growth of the test strain B3/73, its growth was measured on minimal medium and minimal medium containing several supplements. An initial inoculum (A_{650}=0.015) required 34 hr of incubation to double in minimal medium (Table 8). When yeast extract (1 gm/l) was added, culture densities increased 5.6 fold in the same period. When yeast extract and Murashige and Skoog (M&S) salts were added together, culture densities increased 13.0 fold. A medium containing mannitol together with yeast extract yielded a large increase in culture density (11.2 fold). However, this combination and others with mannitol exhibited high levels of agglutination. Based on these data VIM4 was developed. It contains per liter:

M&S salts	0.5 gm	$(NH_4)_2SO_4$	1.0 gm
K_2HPO_4	1.0 gm	$CaCl_2 2H_2O$	0.07 gm
$MgSO_4$	0.1 gm	Yeast extract	1.0 gm
NaCl	0.2 gm	Glycerol	10.0 ml

The medium is adjusted to pH 5.6. This medium provided enhanced growth relative to VIM1, and minimal agglutination. All assays for the comparison of induction of *beta*-galactosidase and zeatin/zeatin riboside equivalents, and the data developed in Chapter V used VIM4.

Expression of *tzs*.

Acetosyringone-induced expression of *tzs* (a measure of *vir* induction) was found in few of the examined *Agrobacterium* strains (Table 9). Strains are scored simply as -, +, or ++, corresponding to zeatin riboside equivalent production in the ranges of < 0.8 μM, 0.8 to 2.0 μM, and > 2.0 μM . Among those not showing appreciable levels of induction were all of the strains with the highest overall levels of gall formation. For example strains B3/73, B1/74, M2/73, C2/74, and 3667 which had average tumorigenicity levels of 73 %, 69 %, 65 %, 65 % and 65 % respectively, had no detectable induction of secretion of zeatin/zeatin riboside by acetosyringone even though they were all nopaline strains. In contrast, T28/73, C58, MFM84.9 and MFM84.5 which had average tumorigenicity levels of

Table 9. Acetosyringone induced cytokinin production in *Agrobacterium* strains .

Strain	Genotype	Opine Class	Cytokinin Class
0362	0362(pTi0362)	nd	+
1001	1001(pTi1001)	(octopine)	-
13333	13333(pTi13333)	nd	-
15834	15834(pRi15834)	nd	-
15955	15955(pTi15955)	(octopine)	-
25818(TR7)	25818(pRi25818)	(mannopine)	-
2655	2655(pTi2655)	(cucumopine)	-
2657	2657(pTi2657)	(cucumopine)	-
2659	2659(pTi2659)	(cucumopine)	-
8196	8196(pRi8196)	nd	-
A136	C58C1	avirulent	-
A175	C58C1(pTiC58)	nopaline	+++
A178	C58C1(pTiK27)	nopaline	++
A2	A2(pRiA2)	agropine	-
A203	C58C1(pTi223)	nopaline	++
A208	C58C1(pTiT37)	nopaline	++
A21/75	A21/75(pTiA21/75)	nd	+
A25/75	A25/75(pTiA21/75)	nd	-
A277	C58C1(pTiB6-806)	(octopine)	-
A281	C58C1(pTiBo542)	agropine	-
A348	C58C1(pTiA6NC)	(octopine)	-
A350	C58C1(pTiB2A)	(octopine)	-
A4	A4(pRiA4)	agropine	-
A518	C58C1(pTiEU6)	(succinamopine)	++
A519	C58C1(pTiAT181)	(succinamopine)	+++
A527	C58C1(pTiCG1C)	(octopine)	-
A543	C58C1(pTiAT4)	(agropine)	-
A557	A200(pTiIIBV7)	nopaline	-
A596	C58C1(pTiAch5)	(octopine)	-
A6	A6(pTiA6)	(octopine)	-
A723	C58C1(pTiB6-806)	(octopine)	-
Ach5	Ach5(pTiAch5)	(octopine)	-
AT1	AT1(pTiAT1)	nd	-
B1/74	B1/74(pTiB174)	nopaline	+
B2/74	B2/74(pTiB2/74)	nopaline	-
B234	B234(pTiB234)	nopaline	-
B2A	B2A(pTiB2A)	(octopine)	-
B3/73	B3/73(pTiB3/73)	nopaline	-
B4/73	B4/73(pTiB4/73)	nopaline	-
B6	B6(pTiB6)	(octopine)	-
B6-806	B6-806(pTiB6-806)	(octopine)	-
Bo542	Bo542(pTiBo542)	(agropine)	-
C2/74	C2/74(pTiC2/74)	nopaline	-
C3/74	C3/74(pTiC3/74)	nd	-
C58	C58(pTiC58)	nopaline	+++
C58C1	C58C1	avirulent	-
C58C1(pRi8196)	C58C1(pRi8196)	(mannopine)	-
C58C1(pRiA4)	C58C1(pArA4a,pRiA4,pArA4c)	agropine	-
C58C1(pRiTR105)	C58C1(pRiTR105)	agropine	-
CG1C	CG1C(pTiCG1C)	octopine	-
E8/73	E8/73(pTiE8/73)	nd	-
EHA101	C58C1(pEHA101)	avirulent	-
G1/73	G1/73(pTiG1/73)	nopaline	++
GMI9023	C58C1	avirulent	-
GV3140	C58C1(pTiC58)	(nopaline)	+++
GV3160	C58C1(pTiC58tra-c)	nopaline	+
GV3245	LS1005(pTiB6S3)	(octopine)	-
GV3560	S1005(pTiK14)	(octopine)	-
H100	H100(pTiH100)	(nopaline)	+++
I1/75	I1/75(pTiI1/75)	nopaline	-
I10/75	I10/75(pTiI1/75)	nd	-
IIBV7	IIBV7(pTiIIBV7)	nopaline	-
K108	K108(pTiK108)	nopaline	-
K15/73	K15/73(pTiK15/73)	nd	++
K21	K21(pTiK21)	nd	-
K26	K26(pTiK26)	octopine	-
K27	K27(pTiK27)	nopaline	-
K305	K305(pTiK305)	nd	-
K308	K308(pTiK308)	nd	-
K32	K32(pTiK32)	nopaline	-
K34	K34(pTiK34)	nd	-
K35	K35(pTiK35)	nd	-
K36	K37(pTiK36)	nd	-
K37	K37(pTiK37)	nopaline	-
K39	K39(pTiK39)	nopaline	-

continues

Table 9 continued

Strain	Genotype	Opine Class	Cytokinin Class
K40	K40(pTiK40)	nd	+
K41	K41(pTiK41)	nopaline	-
K46	K46(pRiK46)	nd	-
K47	K47(pRiK47)	agropine	-
K49	K49(pRiK49)	agropine	-
K6/73	K6/73(pTiK6/73)	nopaline	-
K9/73	K9/73(pTiK9/73)	nd	-
LBA4404	Ach5(pTiAch5::Tn904)	avirulent	-
M2/73	M2/73(pTiM2/73)	nopaline	-
M3/73	M3/73(pTiM3/73)	nopaline	-
MFM1	MFM84.1(pTiMFM84.1)	nopaline	+
MFM5	MFM84.5(pTiMFM84.5)	nopaline	+++
MFM61	MFM84.61(pTiMFM84.61)	nopaline	+
MFM63	MFM84.63(pTiMFM84.63)	nopaline	-
MFM7	MFM84.7(pTiMFM84.7)	nopaline	++
MFM83.4	MFM83.4(pTiMFM83.4)	nopaline	-
MFM84.4	MFM84.4(pTiMFM84.4)	nopaline	+
MFM84.9	MFM84.9(pTiMFM84.9)	nopaline	++
N4/73	N4/73(pTi/73)	nd	-
R3	R3(pTiR3)	nd	-
RR5	RR5(pTiRR5)	nopaline	+
S1/73	S1/73(pTiS1/73)	nd	-
S2/73	S2/73(pTiS2/73)	nd	-
S5/72	S5/73(pTiS5/72)	nd	-
S7/73	S7/73(pTiS7/73)	nd	-
T10/73	T10/73(pTiT10/73)	nd	+
T28/73	T28/73(pTiT28/73)	nd	+++
T3/73	T3/73(pTiT3/73)	nd	-
T37	T37(pTiT37)	nopaline	+++
TR105	TR105(pRiTR105)	agropine	-
TT133	TT133(pTiT133)	nd	++

Strains were inoculated into liquid VIM1 with 200 μM acetosyringone and grown 18 hr. Relative zeatin/zeatin riboside level were measured by ELISA. Opines in parentheses were not confirmed. Cytokinin class defined in text. Abbreviation: nd, not determined.

25 %, 18 %, 8 %, and 5 % secreted relatively high levels of zeatin riboside-like material. However, later assay of B3/73 with a controlled level of inoculum (Table 10) did show acetosyringone mediated induction of *tzs*. Strain K47, with the highest average tumorigenicity level (75 %) showed no consistent induction of zeatin/zeatin riboside secretion (Table 10).

That *tzs* was present in the strongly tumorigenic nopaline strains was determined by DNA hybridization. DNA from both strongly tumorigenic strains 3667, B1/74, B3/73, C2/74, and less tumorigenic strains T28/73 and C58 produced hybridization signals at the same relative position in the gel (Figure 9). *Tzs* is located on *Hin*dIII fragment 9 of pTiC58

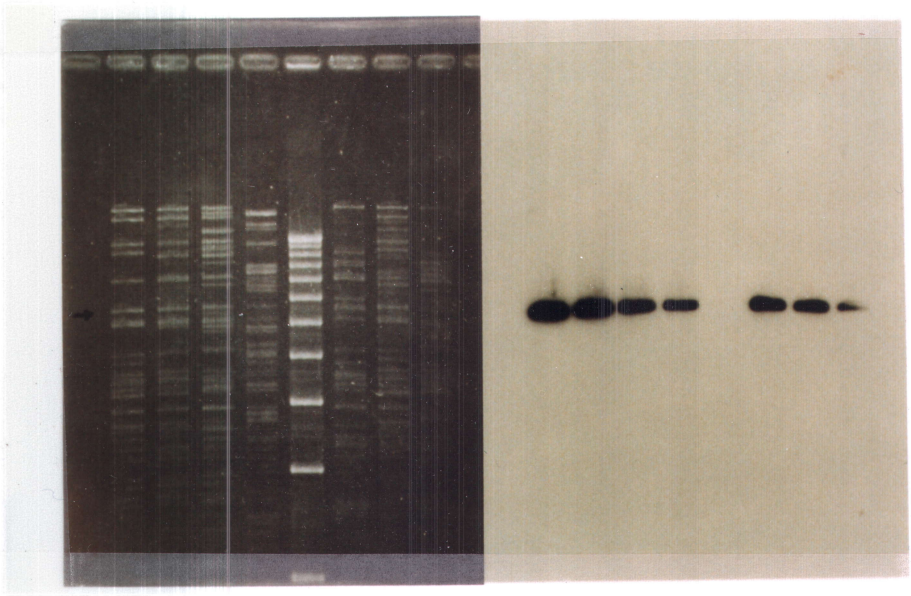

Figure 9. Ti plasmid digests and tzs hybridization. Left. Ti plasmid restriction fragments from *Hin*dIII digestion separated in a 0.7 % agarose gel. **Right.** Hybridization of [^{32}P]-labelled *tzs* from *A. tumefaciens* strain C58 to DNA following capillary transfer to nylon membrane The single band in each lane corresponds to a DNA fragment 6.5 kb in size. Lanes are (from left to right): 1, B1/74; 2, M2/73; 3, 3667; 4, T28/73; 5, 1 kb ladder (BRL); 6, C58; 7, B3/73; 8, C2/74.

(Beaty *et al.* 1986) which is 6.5 kb in size (Depicker *et al.* 1980). No hybridization was seen to *ipt*, present on *Hin*dIII fragment 22 of pTiC58 (3.3 kb) (Beaty *et al.* 1986). Therefore absence of *tzs* was not the basis for the lack of zeatin/zeatin riboside secretion. In these strains its expression was simply not induced, as measured by zeatin/zeatin riboside secretion.

Expression of *virE:lacZ*

In order to use *virE:lacZ* expression as a tool to measure *vir* induction, the plasmid pSM358 was first transferred to *E. coli* and then by triparental mating procedures to *Agrobacterium*. Transformation efficiency of pSM358 (60 kb) into *E. coli* DH5alpha was

low as expected. Subsequent plasmid isolations and restriction digests confirmed plasmid transfer (data not shown). This *E. coli* strain, CRV982, served as donor in triparental matings (Ditta *et al.* 1980) to recipient *Agrobactera*; 3667, A518, B3/73, C58, K26, K41, K47, K108, MFM83.4, M2/73, RR5, T28/73. Plasmid isolation and electrophoresis of restriction enzyme digests confirmed pSM358 transfer. Selection of transconjugant *Agrobacteria* from among the *E. coli* colonies proved to be difficult for several of the more slow growing strains [3667(pSM358), B3/73(pSM358), K41(pSM358), K108(pSM358), M2/73(pSM358)]. To alleviate the problem, strains were confirmed as *Agrobacterium* by growth on an indicator medium (BTB, appendix) containing nopaline as the sole nitrogen source. On this medium, colonies grew relatively rapidly and were bright yellow orange in color, confirming nopaline catabolism and medium acidification.

Effect of acetosyringone concentration on *virE:lacZ* induction.

In order to determine the sensitivity of the induction assay to different acetosyringone concentrations, a concentration versus response curve was developed for the strongly tumorigenic strain B3/73(pSM358). Increasing the acetosyringone level from 3 μM to approximately 500 μM yielded increases in *beta*-galactosidase induction (Figure 10). Activity increased linearly within the acetosyringone concentration range from 5 μM to 100 μM, and then plateaued.

Effect of initial cell density on *virE:lacZ* induction.

In order to determine the effect of initial cell density on the induction of *virE:lacZ*, an initial density versus response curve was determined (Figure 11). The growth rate (factor increase in cell density) and the level of *beta*-galactosidase induction was inversely proportional to the initial cell density (Figure 11). *Beta*-galactosidase induction was highly dependent on the initial culture density. The lower initial densities exhibited the greatest

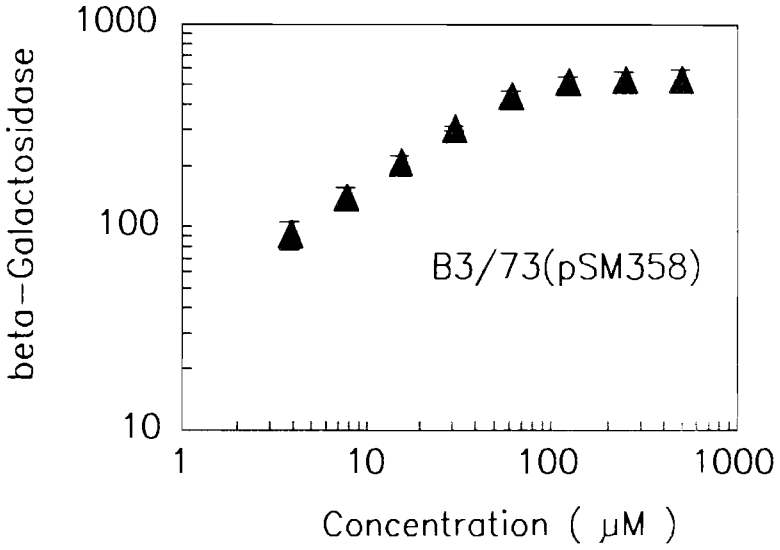

Figure 10. Effect of acetosyringone concentration on *vir* gene induction. The ability of different concentrations of acetosyringone to induce the expression of *beta*-galactosidase in strain B3/73(pSM358) was measured as described in the text.

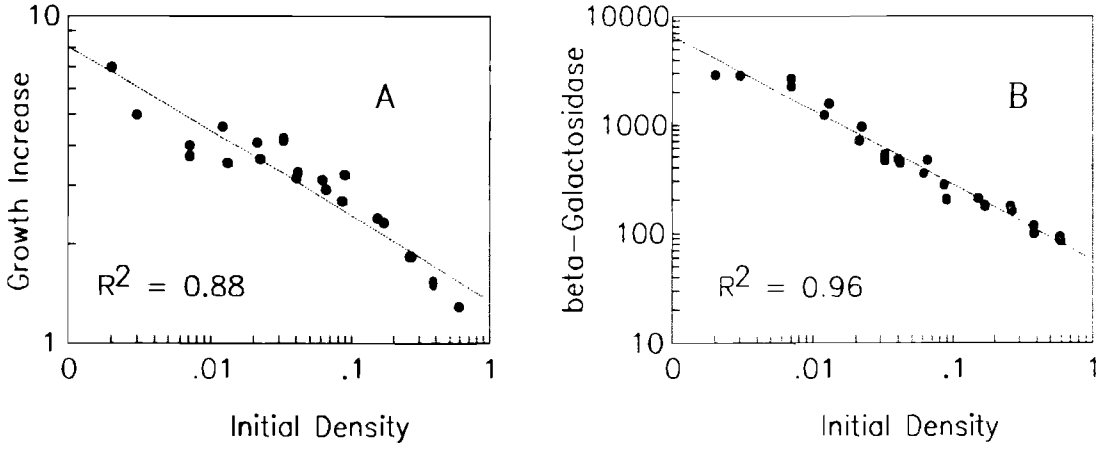

Figure 11. Effect of initial culture density on cell growth and virulence gene induction. **A.** Growth of B3/73(pSM358) in liquid VIM4 containing 200 μM acetosyringone in microwell assay plates at different initial densities. Growth increase was determined as the factor increase in cell density (A_{650}) during the 14 hr 28 °C incubation period. **B.** *virE:lacZ* directed galactosidase activity (nmol ONPG/min/unit of culture density) associated with the cultures.

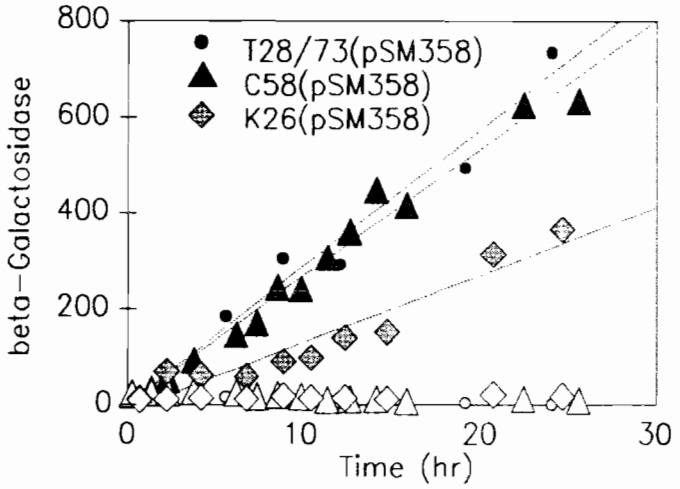

Figure 12. Effect of incubation time on virulence gene induction. Induction of *beta*-galactosidase (nmol/min/unit of culture density) was measured in strains harboring pSM358 as described in the text. Induction was approximately linear for each of the strains examined up to 24 hr. However, the rate of increase was dependent on the *Agrobacterium* strain. Filled symbols indicate activity in the presence of 200 μM acetosyringone. Open symbols indicate activity in the absence of acetosyringone.

specific *vir* induction in the presence of acetosyringone. A strong linear relation existed between the initial cell density and the specific induction on a log-log scale ($R^2 = 0.96$).

Time course of *virE:lacZ* induction.

The time course of *beta*-galactosidase expression in the presence of acetosyringone is presented in Figure 12 for three strains. Activities of strains T28/73(pSM358), C58(pSM358) and K26(pSM358) increased linearly over the 24 hr time period. R^2 values were 0.98, 0.98, and 0.92 for T28/73(pSM358), C58(pSM358), and K26(pSM358) respectively. Rates of increase varied from strain to strain with T28/73(pSM358) having the highest rate and K26(pSM358) the lowest. From a practical stand point, *virE:lacZ* gene induction was best assayed following 12 hr incubation in VIM4 medium containing 200 μM acetosyringone, with a low initial inoculum ($A_{650} = 0.015$).

Correlation of *virE:lacZ* induction, and *tzs* expression.

The two measures of *vir* induction were poorly correlated when comparing different strains (Table 10). Strains which secreted high levels of zeatin/zeatin riboside into the medium (C58, T28/73, B3/73, MFM83.4) did not necessarily express high levels of *beta*-galactosidase. Strain K47 at 36 hr had a very high level of *beta*-galactosidase activity, but no detectable zeatin riboside equivalents. Strains M2/73(pSM358) and K108(pSM358) failed to show inducible *beta*-galactosidase expression, for reasons which are not clear.

The pattern of strain responses at 12 hr and 36 hr is shown graphically in Figure 13. Strains not examined for cytokinin secretion (M2/73, K108, non-inducing; K26 an octopine strain) were omitted. The major difference between the two time points was an increase in the zeatin/zeatin riboside concentration for some strains, particularly C58(pSM358), T28/73(pSM358), B3/73(pSM358) and K41(pSM358). The R^2 values for both the 12 hr and 36 hr time points were not statistically significant at the 5 % level. Thus *virE:lacZ* induction can not substitute as a gauge for zeatin/zeatin riboside secretion and vice versa. The values which were plotted in Figure 13 are given in Table 11 together with the average tumorigenicity scores for the strains calculated from Table 3. Tumorigenicity correlated better with *beta*-galactosidase induction than with zeatin/zeatin riboside biosynthesis. For the 12 hr time point, the correlation of *beta*-galactosidase with tumorigenicity was significant at the 5 % level (R^2= 0.74, degrees of freedom = 7). This correlation deteriorated for the 36 hr time point (R^2= 0.40). Correlation of tumorigenicity with the level of zeatin riboside equivalents was not significant at the 5 % level for either the 12 hr or 36 hr time point.

Table 10. Acetosyringone induced *beta*-galactosidase activity and zeatin/zeatin riboside secretion.

		beta-galactosidase (nmol/min/cell A_{650})		zeatin riboside (nM equivalent)	
Strain	Hr	+AS	-AS	+AS	-AS
3667	8.5	123	7	<20	<20
	12	345	15	<20	<20
	18	218	25	<20	<20
	24	88	16	<20	<20
	36	35	10	<20	<20
A518	8.5	73	<2	36	34
	12	41	2	126	25
	18	24	5	126	22
	24	61	<2	133	36
	36	54	3	168	29
B3/73	8.5	629	6	72	84
	12	411	9	113	<20
	18	133	11	110	24
	24	399	28	135	53
	36	347	47	387	33
C58	8.5	109	2	87	50
	12	128	5	246	38
	18	137	5	824	43
	24	118	5	864	63
	36	131	6	920	33
K26	8.5	151	<2	37	53
	12	94	2	77	24
	18	106	5	34	40
	24	124	5	<20	56
	36	114	5	<20	<20
K41	8.5	522	2	61	52
	12	305	4	157	<20
	18	103	9	79	35
	24	128	18	46	40
	36	240	26	233	<20
K47	8.5	484	17	41	51
	12	764	31	82	<20
	18	932	55	41	28
	24	1058	68	26	37
	36	966	140	<20	22
K108	8.5	<2	<2	nd	nd
	12	<2	<2	nd	nd
	18	<2	<2	nd	nd
	24	<2	<2	nd	nd
	36	<2	<2	nd	nd
M2/73	8.5	<2	<2	nd	nd
	12	<2	<2	nd	nd
	18	<2	6	nd	nd
	24	<2	<2	nd	nd
	36	<2	<2	nd	nd
MFM83.4	8.5	69	2	42	59
	12	60	2	152	<20
	18	95	2	369	35
	24	69	<2	414	39
	36	65	4	343	24
RR5	8.5	25	2	46	54
	12	18	5	87	<20
	18	18	7	86	22
	24	16	7	65	34
	36	18	5	50	60
SM358	8.5	46	2	nd	nd
	12	46	4	nd	nd
	18	47	4	nd	nd
	24	38	4	nd	nd
	36	42	5	nd	nd
T28/73	8.5	42	<2	49	26
	12	66	3	288	<20
	18	63	4	687	<20
	24	69	4	721	25
	36	54	<2	566	<20

Strains transformed with pSM358 were grown in VIM4 containing acetosyringone (200 μM) and aliquots were harvested at five time points following subculture (initial density, 0.015 A_{650}). *Beta*-galactosidase assay and zeatin riboside ELISA as described in the text.

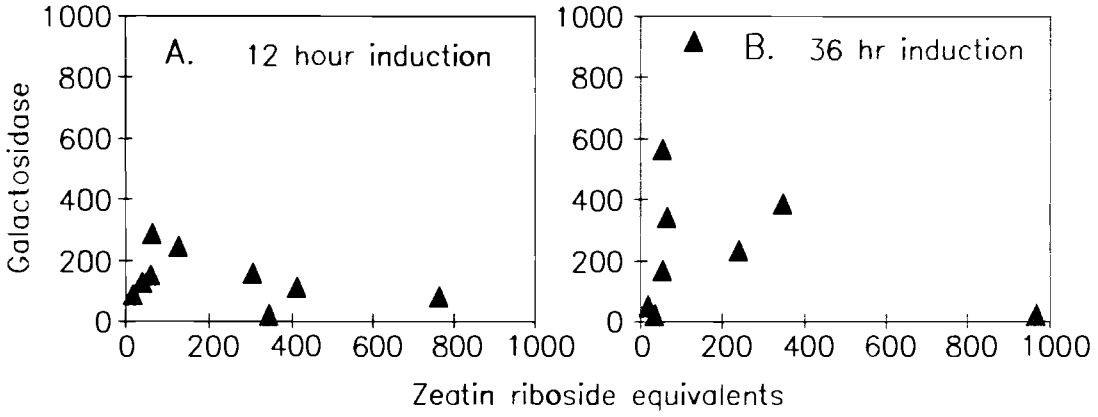

Figure 13. Correlation of acetosyringone induced beta-galactosidase activity and cytokinin production. *Agrobacterium* strains harboring pSM358 were cultured in VIM4 medium containing 200 μM acetosyringone for 12 hr or 36 hr. *Beta*-galactosidase activity (nmol/min/unit density of cell culture) and zeatin riboside equivalents (nM) measured as described in the text.

Table 11. Tumorigenicity and virulence gene induction of *Agrobacterium* strains.

Strain	Tumorigenicity (%)	Induction 12 hr Galactosidase	Induction 12 hr ZR equivalent	Induction 36 hr Galactosidase	Induction 36 hr ZR equivalent
K47	75	764	82	966	<20
B3/73	72.5	411	113	347	387
3667	65	345	<20	35	<20
K41	60	305	157	240	233
A518	32.5	41	126	54	168
T28/73	25	66	288	54	566
C58	13.75	128	246	131	920
RR5	6.25	18	86	18	50
MFM83.4	3.75	60	152	65	343

Tumorigenicity values from Table 3, *beta*-galactosidase and zeatin riboside equivalents from Table 10.

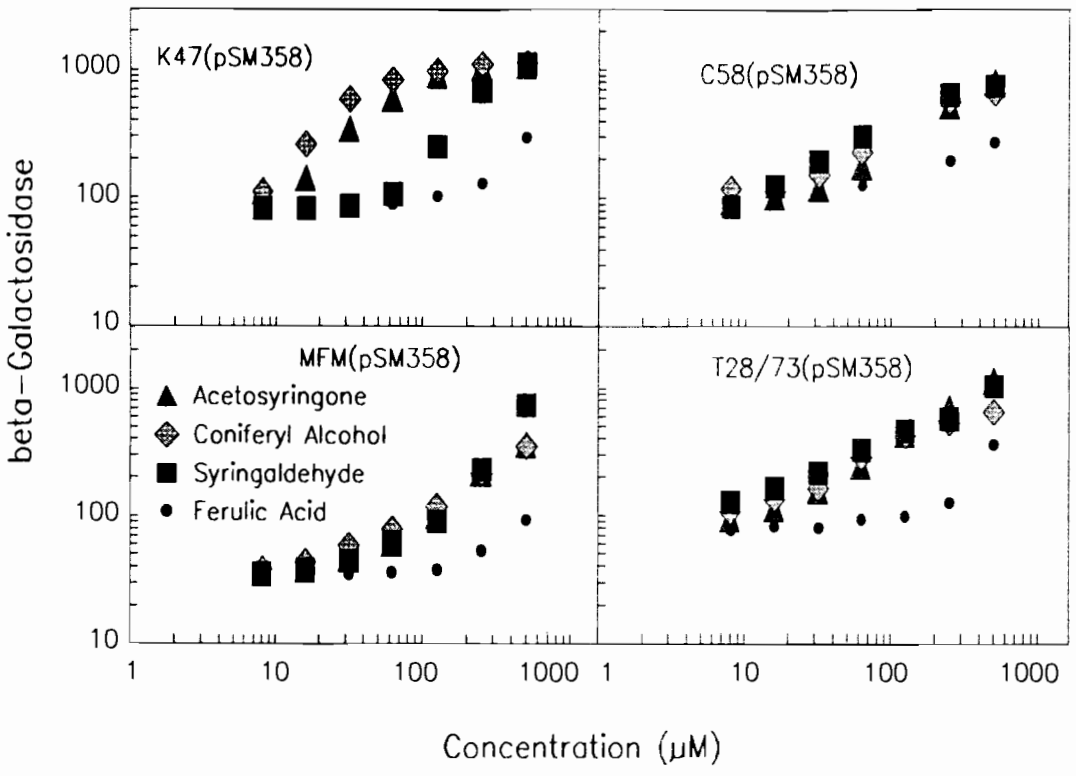

Figure 14. ***Agrobacterium*** **virulence gene induction by four phenolic compounds.** Acetosyringone, coniferyl alcohol, sinapic acid and ferulic acid were used to induce *virE:lacZ* expression in *A. rhizogenes* strain K47(pSM358) and *A. tumefaciens* strains C58(pSM358), K26(pSM358), and MFM83.4(pSM358). For a given strain, acetosyringone and coniferyl alcohol were highly active while ferulic acid was least effective of the four compounds. Sinapic acid was generally highly active except for strain K47(pSM358), where it was intermediate in activity.

Induction of *virE:lacZ* by different phenolic compounds.

In order to evaluate the effectiveness of different phenolic compounds in inducing *vir* gene expression in different strains, several characterized inducers (Spencer and Towers 1988) were used in addition to acetosyringone. The efficacy of acetosyringone, coniferyl alcohol, ferulic acid, and syringaldehyde to induce *beta*-galactosidase activity in strains B3/73(pSM358) C58(pSM358), K47(pSM358) and MFM83.4(pSM358) at concentrations from 5 to 500 μM is shown in Figure 14. acetosyringone and coniferyl alcohol were most effective. Ferulic acid was least effective, especially at the lower concentrations. Of the

four strains shown, K47 was the most sensitive . Further, K47 discriminated against syringaldehyde relative to acetosyringone and coniferyl alcohol (approximately a five fold difference in response at 50 μM) which the other strains did not.

DISCUSSION

The results in this chapter allow several conclusions to be drawn regarding *vir* gene induction.

1. Acetosyringone-induced *tzs* expression was highly variable from strain to strain. Secretion of zeatin/zeatin riboside in nopaline strains varied from nearly 1 μM [C58(pSM358)] to undetectable [3667(pSM358)], at the same time point even though both Ti plasmids encode *tzs*.
2. Acetosyringone induced expression of *virE:lacZ* was also highly variable from strain to strain. *Beta*-galactosidase activities varied widely from 40 nmol/min for A348(pSM358) to approximately 1 μmol/min for K47(pSM358).
3. A significant correlation between the two measures of *vir* gene induction was not found.
4. Acetosyringone induced *vir* gene expression is not well correlated with strain tumorigenicity on conifers. Zeatin/zeatin riboside secretion was not significantly correlated to strain tumorigenicity. Significant correlation of *VirE:lacZ* induction with strain tumorigenicity was found, but was dependent on the incubation time.
5. Different strains may have virA proteins which vary in sensitivity to phenolic compounds. Strain K47 was more sensitive to acetosyringone and coniferyl alcohol, and less sensitive to syringaldehyde than C58 or T28/73.

The disparity between the two measures of *vir* induction may have several explanations First, the strains may not contain *tzs*. This is likely true for octopine strains such as K26, However this was not true for K47 and 3667. *Tzs* hybridization studies (K47, Akiyoshi *et al.* 1987; 3667, this chapter) indicated *tzs* presence.

Second, the *tzs* gene may be deficient or other genes required for secretion of zeatin/zeatin riboside may be absent. A deficient *ipt* gene (the prenyl transferase of the T-DNA) has been found in the narrow host grape strain Ag162 (Yanofsky *et al.* 1985a), a similar defect in *tzs* is plausible. Strains which lacked the prenyl hydroxylase might secrete iso-pentenyl adenine rather than zeatin which would not be detected in the ELISA. Strains K47 and 3667 appear to fit into this zeatin/zeatin riboside secretion-deficient category as both *tzs* containing strains exhibited inducible virE:lacZ expression, but little or no induction of zeatin/zeatin riboside secretion.

Third, a heterologous interaction, which may be limiting, is required for *virE:lacZ* induction, but not for induction of zeatin/zeatin riboside secretion. There is a heterologous interaction at one of two places. The native virA protein may interact with the native virG protein which activates the heterologous *virE:lacZ* gene. Otherwise, the native virA protein may interact with the heterologous virG protein encoded on pSM358 which then activates the *virE:lacZ* gene. Strains M2/73 and K108 may fit this category since neither showed *virE:lacZ* induction.

The basis for the lack of a strong relationship between both *vir* gene induction measures and strain tumorigenicity is not readily determined, but may in part be due to the inducer. Acetosyringone may not serve as an appropriate surrogate for the native inducer. Strain specific virA sensitivity is an appealing hypothesis as it would help to explain why

strains such as MFM83.4 with highly inducible *tzs* genes and relatively high levels of *virE:lacZ* induction are only weakly tumorigenic on conifers. As determined in subsequent experiments (Chapter V) the major inducer of *P. menziesii* does have a novel character.

Despite the limitations of the assay, the *virE:lacZ* induction assay developed here allowed determination of strain to strain differences in virulence induction and virA sensitivity to phenolic inducers. At the wound site, these differences may translate into differences in strain tumorigenicity. Strain characters in combination with the phenolics present at the wound site will determine the level of *vir* gene induction. As described in Chapter V, below, the *virE:lacZ* induction assay provided a means to characterize the phenolic complement of the conifer host, *P. menziesii*, providing additional insight into the conifer specific induction of *Agrobacterium* virulence genes.

CHARACTERIZATION OF INDUCING COMPOUNDS IN *PSEUDOTSUGA MENZIESII* EXTRACTS.

INTRODUCTION

Inducible T-strand mobilization from the Ti plasmid of *A. tumefaciens* to the genome of a plant host is mediated by virulence proteins regulated in an expression cascade. The cascade is initiated by the interaction of a plant phenolic and the *A. tumefaciens* virA gene product. Several plant phenolics have been shown to initiate this first step of the virulence gene expression cascade (Bolton *et al.* 1986, Melchers *et al.* 1989, Spencer and Towers 1988). Nevertheless, the only native inducers active at micromolar concentration which have been identified to date are acetosyringone and hydroxyacetosyringone, which were isolated from wounded tobacco leaves and tobacco root cultures (Stachel *et al.* 1985b).

Experiments described in Chapter II indicated that certain *A. tumefaciens* strains could transform conifers at high frequency. Other strains, which were equally tumorigenic on *Kalanchoe*, were much less tumorigenic on conifers. It was not known whether this specificity arose because conifers possessed a set of phenolic inducers which differed chemically from those of herbaceous dicotyledonous species or was due to other causes. Two objectives were initially developed for this part of the project.

1. Identify the major *vir* gene inducer present in *P. menziesii* extracts.
2. Measure the ability of this compound to induce *vir* genes of strongly and weakly tumorigenic *A. tumefaciens* strains.

From the identification of the inducer, a third objective was developed.

3. Measure the level of bacterial glucosidase expression in strongly and weakly tumorigenic *A. tumefaciens* strains.

Strategy:

The strategy was to employ the *A. tumefaciens* strains which harbored the *virE:lacZ* reporter plasmid (described in Chapter IV) in the *beta*-galactosidase induction assay to identify active compounds from conifer extracts. In order to isolate inducing compounds, a purification protocol would be developed in which activity of the crude extract was retained. Solvent partitioning and HPLC fractionation would provide a highly purified sample to allow identification of the compound by mass spectrum- and proton magnetic resonance-analyses.

METHODS

Plant Material and Bacterial Strains.

Lateral shoots (10 cm) of the current season's growth were collected from young (20 yr) *P. menziesii* trees located in Corvallis, Oregon and stored at 4 °C for 4 days prior to extraction of phenolics. Shoots were collected in July 87, December 1988 and May 1989. References to wild-type *Agrobacterium* strains are given in the appendix. Bacteria harboring the *virE:lacZ* marker plasmid pSM358 are described in Chapter IV.

Tissue Extraction and Initial Characterization.

A methanolic extract of *P. menziesii* shoots was prepared by bruising the tissue (300 gm) with the aid of a metal rod and incubating in 80 % methanol (2 liters). Following 18 hr of incubation on a rotary shaker, the solution was filtered (Whatman number 1) and

Table 12. Chromatography columns and gradients.

	COLUMNS	
	Dimensions	Packing
1	180 x 24 mm	40 μm BondElute, Analytichem
2	150 x 4.6 mm	5 μm Ultrasphere ODS, Beckman
3	250 x 4.6 mm	5 μm Ultrasphere ODS, Beckman

	GRADIENTS					
	Mobile Phases		Initial	Final	Gradient	Hold
	A	B	Conditions (%B)	Conditions (%B)	Duration (min)	(min)
1	TEA*	Acetonitrile	5	90	40	0
2	TEA	Acetonitrile	1	100	40	0
3	TEA	Methanol	15	90	120	40
4	TEA	Methanol	15	50	40	0
5	TEA	Methanol	15	30	40	0
6	TEA	Methanol	20	20	40	0

TEA* = 40 mM acetic acid raised to pH 3.35 with triethylamine.

stored at -70 °C. The extract was evaporated to 1 % of the original volume (Büche Rotovapor) and the concentrated extract was stored at -70 °C. An aliquot (10 μl) was fractionated by reversed phase HPLC (Table 12; column 3, gradient 1), and the fractions assayed for *beta*-galactosidase induction activity in strains B3/73(SM358) and MFM83.4(SM358)

HPLC.

Samples were fractionated using a Beckman 322 liquid chromatograph. Solvents, gradients and columns used are listed in Table 12. Solvent flow was 1 ml/min. Eluted materials were detected with a Waters 440 absorbance detector operating at 254 nm. Analogue to digital signal processing was performed by a Radiomatic A200 radioactive flow monitor (2 sec update time). Fractions were collected with a Gilson Model 203 fraction collector.

***Beta*-galactosidase assay of chromatographic fractions.**

Fractions of the HPLC column eluates (0.3 ml to 2.0 ml) were evaporated to dryness, redissolved in DMSO, and aliquots (1 μl) placed in microwell plates. Cultures of B3/73(SM358) or MFM83.4(SM358) (100 μl, A_{650} = 0.020) growing in VIM4 medium were added, mixed, and incubated 12 hr in an orbital shaker (Lab-line Model 3525) operating at 225 rpm and 28 °C. Following incubation, *beta*-galactosidase activity was assayed as described in Chapter IV.

Polyvinylpolypyrrolidone chromatography.

Polyvinylpolypyrrolidone (PVPP, Sigma) was prepared as described (Loomis 1974). PVPP was boiled in 10 % HCl for 10 min, filtered, and washed once with water. Additional water was added to produce a slurry and the pH raised with potassium hydroxide to pH 5.0. Fines were decanted following a 10 min settling period. The slurry was washed with an additional 10 volumes of water. The PVPP was dried at 60 °C prior to storage at room temperature.

Dry PVPP was added to 5 volumes of methanol and allowed to swell. The slurry was poured to form a packed volume (2 ml) in a polypropylene syringe (3 ml) and preequilibrated with methanol (5 ml, containing 1mM ascorbate). An aliquot (100 μl) of the concentrated *P. menziesii* extract was loaded on the column, eluted with methanol (6 ml containing 1mM ascorbate), the eluate evaporated to dryness under vacuum (Savant SpeedVac), and redissolved in methanol to the original volume (100 μl).

Aliquots (40 μl) of the PVPP eluate and unpurified extract were fractionated using a reverse phase HPLC (Table 12, column 2 eluted by gradient 2). Fractions were collected

(0.35 min) and evaporated to dryness under vacuum (Savant SpeedVac). *Beta*-galactosidase induction was assayed as described above.

Preliminary partitioning.

An aliquot (100 μl) of the concentrated extract was diluted with 2 ml ascorbate (10 mM, pH 9.5). The aqueous solution was mixed vigorously with 1 ml hexane for 30 sec and the phases separated by centrifugation (1 min, 1000 x *g*). The hexane phase was removed to a separate container, and partitioning was repeated two additional times. An ether partition followed the protocol for hexane. After acidification of the aqueous phase (pH 2), a second ether partition was carried out. Water was removed from the organic phases by addition of anhydrous sodium sulfate (1 gm), and volumes reduced by evaporation.

Following organic solvent extraction, the aqueous phase was passed through an ODS column (10 mm x 8 mm, 40 μm BondElute, Analytichem) which had been preequilibarated sequentially with methanol, triethylamineacetate (0.1 M, pH 6.5) and ascorbate (10 mM, pH 6.5). The column was washed with 6 ml of ascorbate (10 mM, pH 6.5), and retained materials were eluted with 6 ml methanol. Methanolic and aqueous fraction were evaporated under vacuum (Savant SpeedVac).

Each partition fraction was further separated by gradient HPLC (Table 12, column 2 eluted by gradient 2), and fractions were assayed for the presence of virulence-inducing compounds as described above.

Large scale purification of the active inducer.

Concentrated methanolic extract (10 ml, from approximately 150 gm *P. menziesii* tissue) was diluted with an equal volume of ascorbic acid (10 mM, pH 6.5), and partitioned

against hexane, ethyl ether, and ethyl acetate using five 3 ml volumes for each solvent. Prior to ethyl acetate partitioning, saturating amounts of sodium chloride were added to the aqueous phase. Following organic solvent partitioning, the aqueous phase was passed through an ODS column (80 x 10 mm BondElute 40 μm, Analytichem International) and the column was washed with ascorbic acid (10 mM, pH 6.5). Retained compounds were eluted with three column volumes of methanol and evaporated to dryness. The dried eluates were fractionated by preparative chromatography on ODS (Table 12, column 1, gradient 3). Fractions active in the *beta*-galactosidase induction assay were combined and further purified using a sequence of HPLC steps: from Table 12; column 2 with gradient 4, column 3 with gradient 4, column 3 with gradient 5, and column 3 with gradient 6.

Beta-glucosidase hydrolysis of the major inducer.

After purification, two aliquots of the active material were evaporated to dryness, one not treated further while the second was redissolved in 100 μl sodium acetate (20 mM pH 5.0) containing 1.0 unit *beta*-glucosidase (Sigma). Following incubation (1 hr 37 °C), the enzyme was precipitated with 3 volumes of methanol, the sample centrifuged, and the supernatant evaporated to dryness. Both dried samples were rechromatographed (Table 12, column 3, gradient 4) and HPLC fractions were assayed for induction activity.

Mass spectra.

Trimethylsilyl (TMS) derivatives of authentic coniferin and the active compound were prepared using trimethylsilylimidazole (20 μl, Pierce) in anhydrous pyridine (200 μl) incubated for 20 min at 37 °C . Electron impact spectra were acquired on a Kratos MS 50 S mass spectrometer (Kratos, Urmston, Manchester, U.K.) interfaced with a Carlo Erba Model 4160 gas chromatograph. Spectra were recorded at 70 eV with an ionization current

of 50 μA, a source temperature of 250 °C, and a transfer temperature of 290 °C. The gas chromatograph was fitted with an OV-7 fused silica capillary column (60 m x 0.25 mm) operating under isothermal conditions at 290 °C with injector and detector temperatures at 310 °C.

Nuclear magnetic resonance spectra.

Nuclear magnetic resonance spectra of underivatized authentic coniferin and the active compound were acquired using a Nicolet NT 300-WB spectrometer operating at 300.06 mHz and equipped with a 5 mm proton probe. Transients were accumulated using a 9 μsec pulse and 1 sec relaxation time. Field shifts were referenced to tetramethylsilane and the solvent ([$^2H_4$]-methanol).

Bacterial *beta*-glucosidase assay.

The glucosidase assay was adapted from the *beta*-galactosidase assay (above). Bacterial cultures grown in VIM4 to log phase (A_{650} = 0.3) were mixed with 0.2 volumes of 5X modified Z buffer. After incubation (10 min, 22 °C), p-nitrophenyl-*beta*-D-glucopyranoside (Sigma) was added (5.8 mM). For values in Table 13, enzyme activity proceeded for 10 min and the reaction was stopped by the addition of Na_2CO_3 (1 M, 50 μl). Color development was measured as the difference between A_{405} and A_{650}. For values in Table 15, color development was measured kinetically at 405 nm for 10 min. *Beta*-glucosidase activity was expressed as nmol/min per unit cell culture density. The correlation coefficient for the relationship between *beta*-glucosidase activity and mean strain tumorigenicity was calculated by the method of least squares, and tested for significance from zero by the Student's *t* test (Snedecor and Cochran, 1967).

RESULTS

Extracts contain an active inducer.

When the strongly tumorigenic strain B3/73(pSM358) was used to assay HPLC fractions of the unpurified phenolic extract from *P. menziesii*, a response was seen to a compound (PM1, Figure 15a) eluting early in the gradient (12 min). In contrast, when aliquots of the same fractions were assayed with the weakly tumorigenic strain MFM83.4(pSM358) no significant response was seen (Figure 15b). The differential response was not due to a failure of the *virE:lacZ* reporter in MFM83.4(pSM358) as both strains gave approximately equivalent responses to acetosyringone.

Preliminary purification of active inducers

Two approaches were examined for preliminary purification prior to HPLC. First was adsorption of UV absorbing materials to PVPP. The effectiveness of PVPP as an adsorbent was verified (Figure 16a and 16c). Unfortunately, despite the polarity of PM1, it too was adsorbed by PVPP as shown by the drop in *virE:lacZ* induction following treatment (Figure 16b and 16d).

The second approach for preliminary purification was solvent partitioning. Assay of HPLC fractions from the five partition fractions for compounds active in the *virE:lacZ* induction assay (Figure 17) indicated no activity in hexane or ether fractions. An active compound was found in the methanol eluate of the ODS column. Activity in the aqueous flow through of the ODS column may be attributed to overloading of the column.

Isolation and characterization of PM1.

In order to identify PM1, a large scale preparation of *P. menziesii* phenolics was undertaken. Fresh shoots were extracted with methanol and the extracts were subjected to

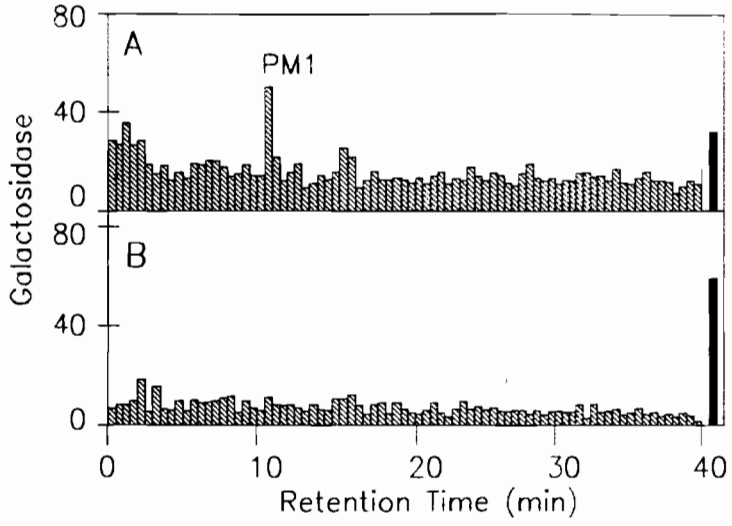

Figure 15. Induction of the vir gene cascade in B3/73(pSM358) and MFM83.4(pSM358) by compounds present in P. menziesii extracts. An aliquot of a methanolic extract from *P. menziesii* shoots was fractionated using reverse phase HPLC and assayed for induction of *beta*-galactosidase as described in the text. A relatively polar compound (PM1) was effective in inducing B3/73(pSM358) but was ineffective in inducing MFM83.4(pSM358). Solid bar, induction by 200 μM acetosyringone.

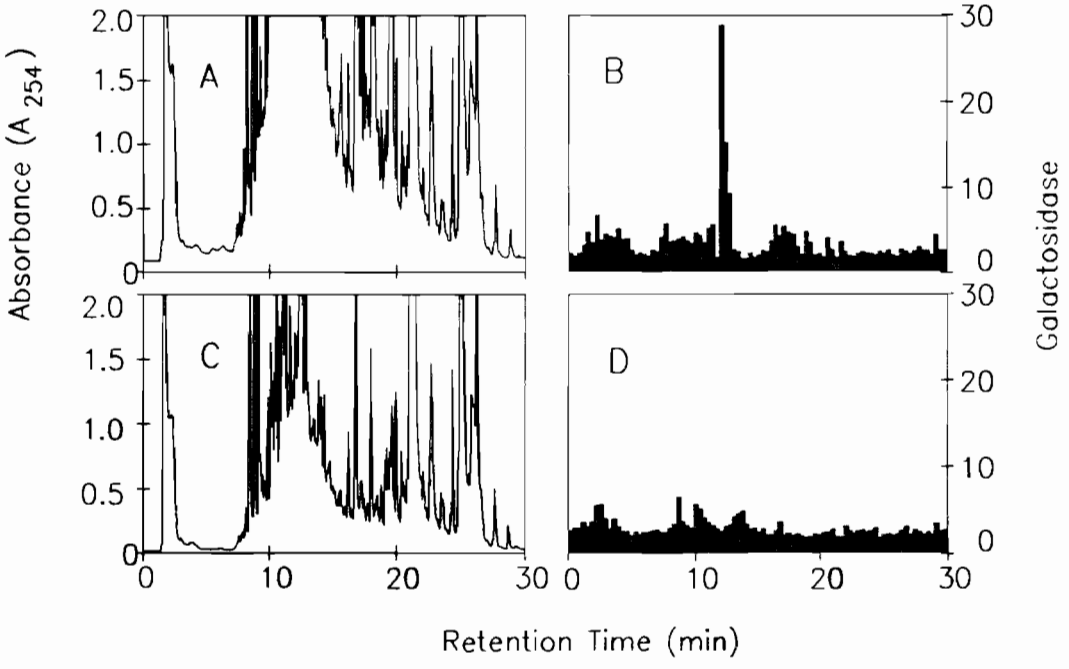

Figure 16. Efficiency of polyvinylpolypyrrolidone (PVPP) in enrichment of inducing compounds. An aliquot of concentrated methanolic extract was diluted in methanol and passed through PVPP column. The material eluting from the column and a second equivalent aliquot of the methanolic extract were both fractionated by HPLC and assayed for *beta*-galactosidase induction as described in the text. PVPP was effective in reducing the amount of UV absorbing material in the sample, but also effectively removed the inducing compound.

solvent partitioning and HPLC. No active compounds were partitioned into hexane or ether, some activity (PM3) was found in the ethyl acetate phase, and the bulk of the active material was retained in the aqueous phase and was purified by adsorption to ODS followed by elution with methanol (Figure 18). HPLC of this fraction (Figure 18) showed that two compounds (PM1 and PM2) were present.

Serial HPLC fractionation of PM1 over ODS provided homogeneous material possessing biological activity. A typical HPLC profile of purified PM1 is illustrated in Figure 19a. The compound had a retention time of 17.2 min, considerably shorter than that of syringic acid (23.0 min), coniferyl alcohol (28.0 min), acetosyringone (31.0 min) or sinapic acid (33.1 min). The early retention time together with its insolubility in hexane, ether, and ethyl acetate indicated that it was quite polar. In light of this polarity it seemed possible that it might be a glucoside. Treatment with *beta*-glucosidase confirmed this supposition (Figure 19b). After glucosidase treatment, full (or perhaps enhanced) activity was retained but the retention time of the active species increased to 28 min. The retention time of the hydrolysis product coincided with that of authentic coniferyl alcohol.

The structure of PM1 was determined from mass spectra and proton nuclear magnetic resonance spectra. The TMS derivative gave a homogeneous peak on gas chromatography with a retention time identical to that of authentic TMS-coniferin. The electron impact mass spectra of TMS-PM1 and TMS-coniferin were identical (Figure 20). TMS-PM1 had a base peak at m/z = 73 and major fragment ions at m/z = 103, 147, 217, 324, and 361. The spectrum of TMS-coniferin also contained the same peaks with the same relative intensities.

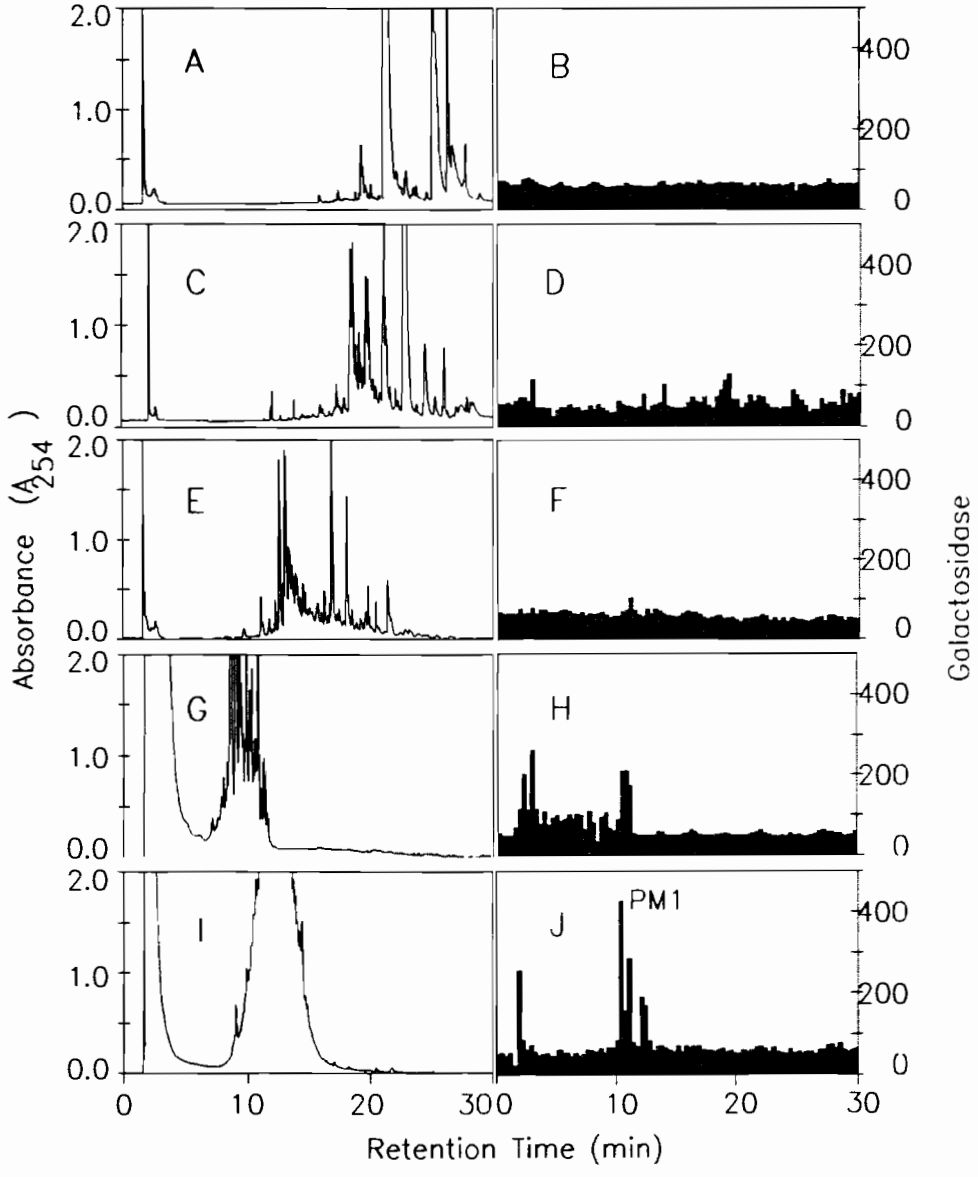

Figure 17. Presence of inducing compounds in solvent partition fractions. A. and B. UV trace and fraction activities of hexane partition. **C. and D.** UV trace and fraction activities of neutral ether partition. **E. and F.** UV trace and fraction activities of acidic ether partition. **G. and H.** UV trace and fraction activities of ODS column aqueous eluate. **I. and J.** UV trace and fraction activities of ODS column methanolic eluate. The major inducing activity was found in the methanolic eluate of the ODS column.

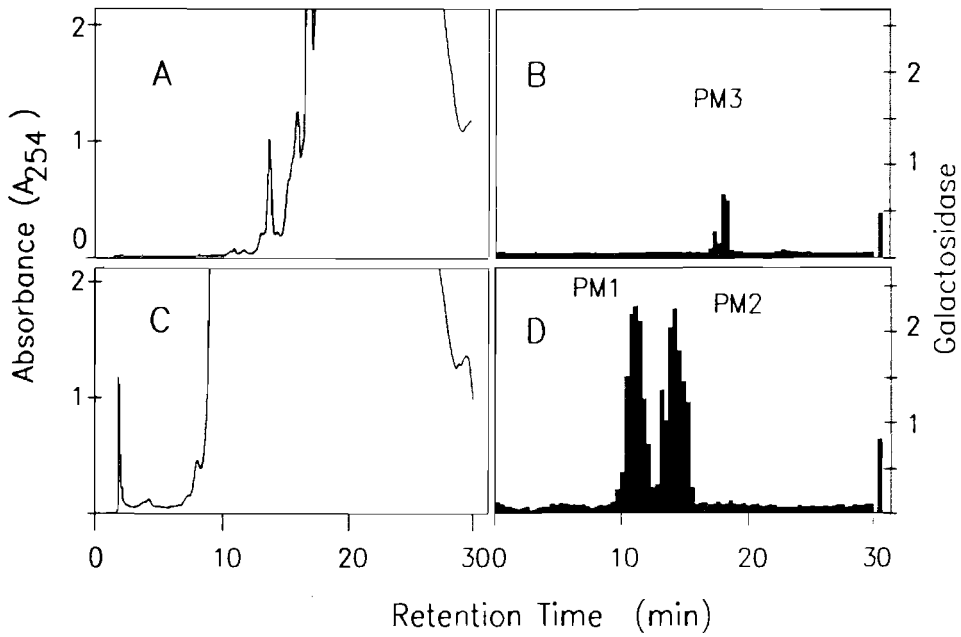

Figure 18. Active compounds in *P. menziesii* shoot extract. Large scale preparations of shoots collected in December 1989. Three peaks of activity were identified. Two compounds PM1 and PM2 were identified in the material adsorbed by the initial ODS preparative column. PM3 was found in the ethyl acetate partition fraction. **A and B.** UV absorbance trace and activity profile of ethyl acetate partition fraction. C. and D. UV absorbance trace and activity profile of ODS column methanolic eluate.

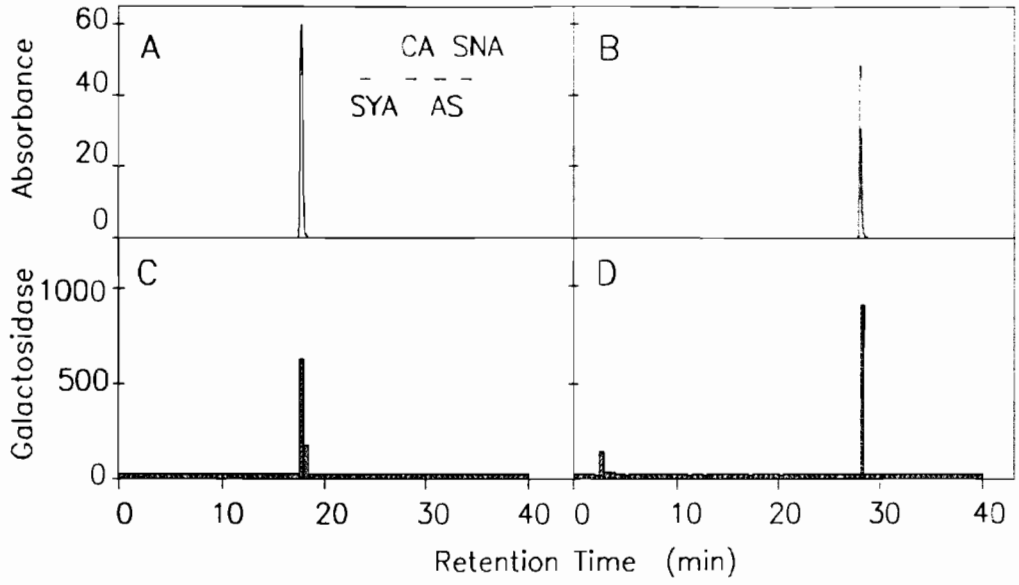

Figure 19. Effect of *beta*-glucosidase on purified PM1. The retention times and inducing activity of two equivalent aliquots of PM1 were measured. **A.** UV trace of untreated PM1. **B.** UV trace of *beta*-glucosidase treated PM1. **C.** Induction profile of untreated PM1. **D.** Induction profile of *beta*-glucosidase treated PM1. Induction activity assayed using strain B3/73(pSM358). Marked retention times are; acetosyringone AS, coniferyl alcohol CA, sinapic acid SNA, syringic acid SYA.

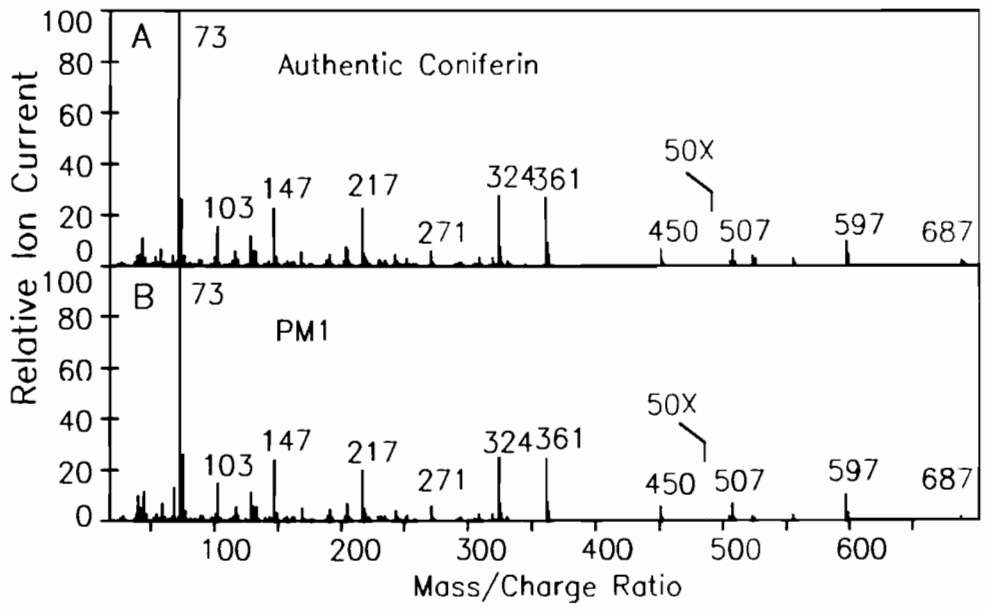

Figure 20. Mass spectra of PM1 and coniferin. Spectra of trimethylsilyl derivatives of PM1 and authentic coniferin were obtained as described in the text. The spectra were essentially identical.

The proton magnetic resonance spectrum of PM1 confirmed the structural assignment. The NMR spectrum was identical to that of coniferin (Figure 21). Resonances were present at chemical shifts (ppm) -6.9 to -7.15 (ring protons), -6.3 to -6.6 (exocyclic protons) and -3.9 (ring methoxy protons). Solvent resonances were present at -3.2 and -4.9 ppm.

In order to demonstrate that the biological activity of PM1 matched that of authentic coniferin and was not merely coeluting with it, the concentration-dependencies of virulence induction by PM1 and by coniferin were compared (Figure 22). Activity profiles were not different within experimental error.

On the basis of biological activity, HPLC and gas chromatographic retention times and, mass and proton magnetic resonance spectra the major *A. tumefaciens* virulence-inducing substance found in young *P. menziesii* shoots was therefore coniferin.

Strain specific response to coniferin.

To confirm the original differential response to coniferin by B3/73(pSM358) and MFM83.4(pSM358) and to confirm the response to coniferyl alcohol, both strains were examined for *beta*-galactosidase induction by coniferin, coniferyl alcohol and acetosyringone. While the aglycones were effective on both strains, coniferin was effective only on B3/73(pSM358) (Figure 23).

Strongly tumorigenic strains express *beta*-glucosidase.

As a glucoside, coniferin has a unique position among *A. tumefaciens* virulence gene inducers. All previously characterized phenolic inducers have been aglycones and possess free phenolic hydroxyl groups (Melchers *et al.* 1989, Spencer and Towers 1988, Stachel *et*

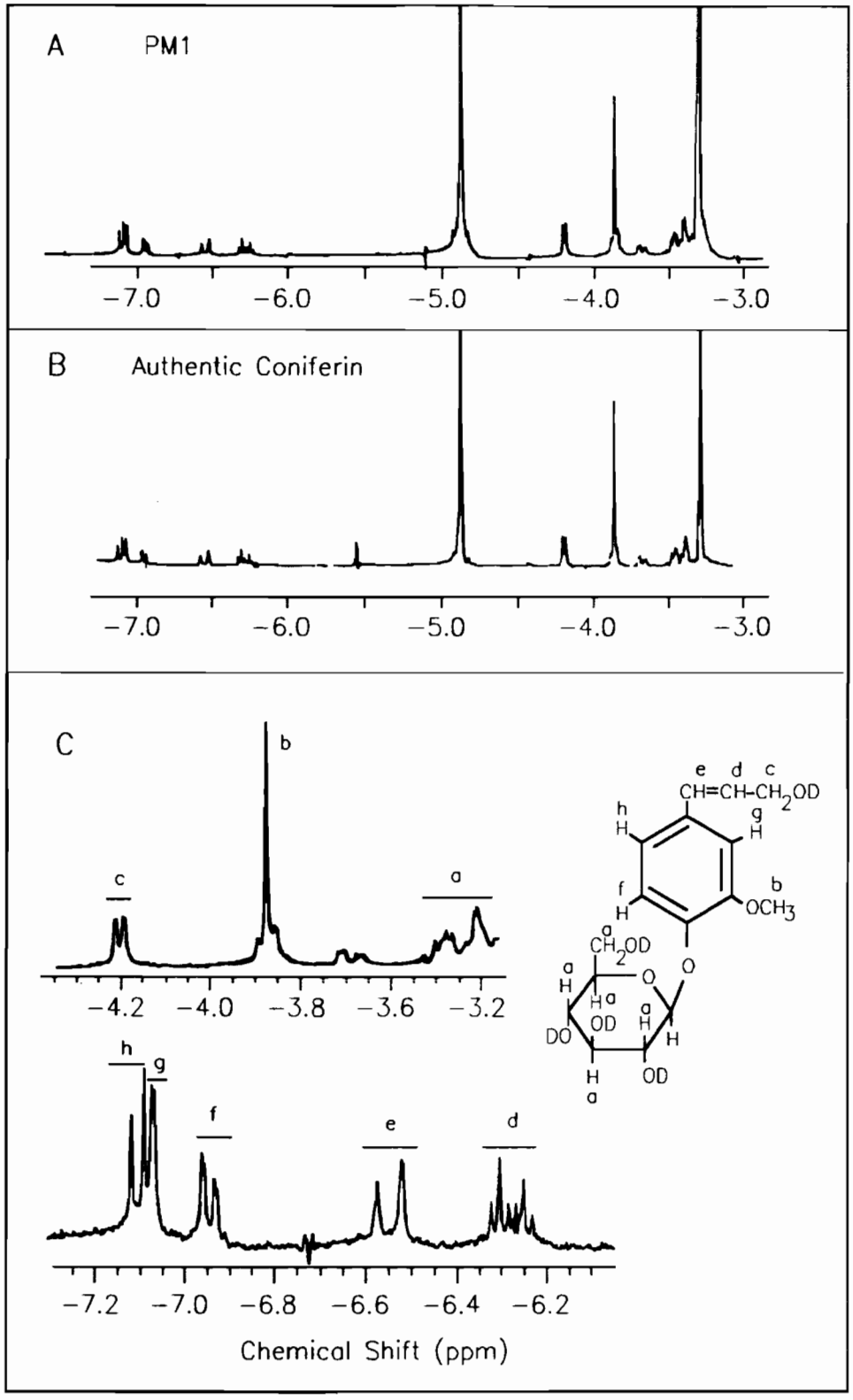

Figure 21. Nuclear magnetic resonance spectra of PM1 and coniferin. Spectra were obtained as described in the text and were essentially identical. **A.** Coniferin. **B.** PM1. **C.** Proton assignments for absorbance peaks. Peak assignments of PM1 were made by comparison to published spectra of similar compounds (Sadtler 1980).

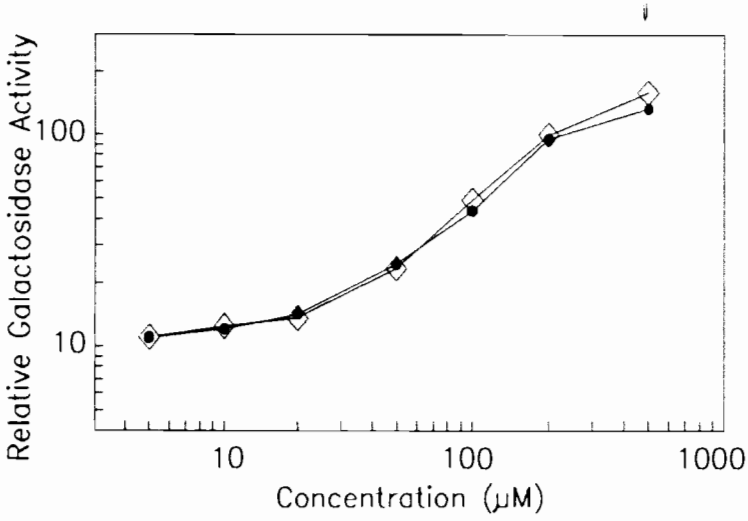

Figure 22. Induction of virulence gene expression by PM1 and coniferin. Equal amounts (based on A_{254} absorbance) of PM1 and coniferin were assayed for activity over a range of concentrations using the protocol described in the text. Average scaled values from three independent trials are plotted. Coefficient of variation of triplicate samples was 0.46.

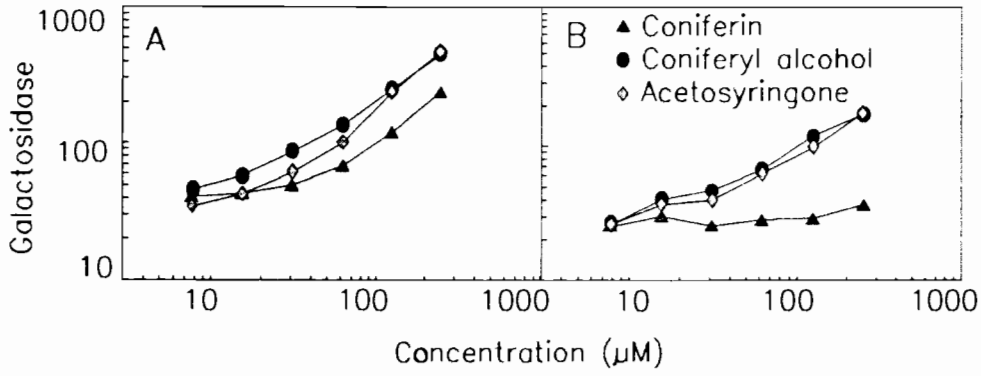

Figure 23. Strain specific induction by coniferin. The strongly tumorigenic strain B3/73(pSM358) and the weakly tumorigenic strain MFM83.4(pSM358) were cultured in VIM4 containing a range of inducer concentrations; coniferin (triangles), coniferyl alcohol (circles), and acetosyringone (diamonds). Although coniferin and acetosyringone were effective inducers for both strains. Only B3/73(pSM358) was highly induced by coniferin.

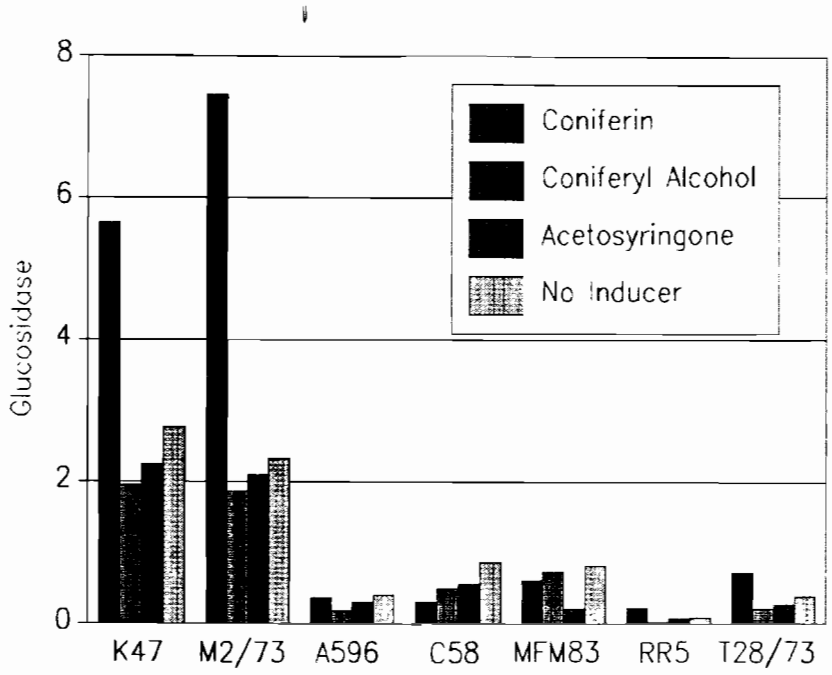

Figure 24. Induction of *Agrobacterium* beta-glucosidase expression. Wild-type *Agrobacterium* strains were grown in VIM4 containing coniferin, coniferyl alcohol, acetosyringone (all 200 μM) or no inducer. Glucosidase activity was measured after 12 hr incubation as described in the text.

al. 1985b) Therefore, the question arose as to whether coniferin was the immediate inducer or whether it was first converted by the bacteria to coniferyl alcohol, which had previously been shown to induce the *A. tumefaciens* virulence region genes (Spencer and Towers 1988).

In order to investigate the potential role of *A. tumefaciens* glucosidases in the virulence induction process, the glucosidase activity of several *A. tumefaciens* strains was examined. Initially, glucosidase was measured in a set of 8 wild-type strains with differing tumorigenicities on conifers. Strongly tumorigenic strains were found to have higher levels of *beta*-glucosidase activity than the weakly tumorigenic strains (Figure 24). Also shown in the figure was the approximately two fold increase in *beta*-glucosidase activity found in the

presence of coniferin relative to levels in the absence of inducer. Basal glucosidase expression in a larger set of strains is listed in Table 13. A plot of these *beta*-glucosidase activities versus the corresponding strain tumorigenicity is shown in Figure 25. The correlation is significant at the 1 % level. As indicated by the value of R^2 (0.36) glucosidase expression alone accounts for a third of the variation in tumorigenicity level.

A correlation was sought between the ability of coniferin to act as a *vir* gene inducer, and glucosidase activity of the individual bacterial strain. The *A. tumefaciens* strains which harbored pSM358, were cultured in the presence of coniferin or coniferyl alcohol. Cultures were examined for *beta*-glucosidase, and both measures of *vir* gene induction, *virE:lacZ* directed *beta*-galactosidase activity and secretion of zeatin riboside-like compounds. The levels of *virE:lacZ* induction were generally less in incubations with coniferin than with coniferyl alcohol, this was especially evident for the earlier time points (Table 14). In the presence of coniferin, levels of induction greater than 100 nmol/min/unit cell culture density were found only for the highly tumorigenic strains B3/73(pSM358), K41(pSM358) and K47(pSM358) which also expressed high levels of *beta*-glucosidase (Table 15). By 18 hr, the difference between induction levels induced by coniferyl alcohol and those induced by coniferin were largely eliminated. Only strains 3667(pSM358), C58(pSM358), RR5(pSM358), and T28/73(pSM358) had *beta*-galactosidase levels in the presence of coniferin that were not within 20 % of the levels found in the presence of coniferyl alcohol. The latter three strains expressed no detectable level of *beta*-glucosidase (Table 15).

In agreement with the *virE:lacZ* expression as a measure of *vir* gene induction, biosynthesis of zeatin/zeatin riboside was generally lower in incubations with coniferin than with coniferyl alcohol (Table 14). In the presence of coniferyl alcohol, high levels (greater

Table 13. *Beta*-glucosidase activity of *Agrobacterium* strains.

Strain	Glucosidase	Strain	Glucosidase	Strain	Glucosidase
15955	0.7	B3/73	8.4	K6/73	2.3
3667	5.0	B4/74	1.8	M2/73	4.7
A178	1.0	C2/74	11.2	M3/73	6.1
A2	6.7	C58	1.0	MFM83.4	0.7
A203	1.4	C58C1(pRiA4)	0.4	MFM84.1	0.5
A208	1.0	C58C1(pRiTR105)	0.7	MFM84.4	0.5
A277	1.0	CG1C	1.1	MFM84.5	0.8
A281	1.1	G1/73	2.4	MFM84.61	0.5
A4	3.0	I7/75	10.1	MFM84.63	0.4
A518	0.6	IIBV7	0.3	MFM84.7	1.3
A519	1.4	K108	7.9	MFM84.9	0.8
A557	1.5	K26	0.5	NT1	1.0
A596	0.6	K27	2.6	RR5	0.8
A6	0.2	K32	1.8	S5/72	2.3
A723	0.6	K37	2.1	T28/73	0.4
Ach5	0.4	K39	2.4	T37	0.6
Ach5C3	1.1	K41	8.0	TR105	3.4
B1/74	6.4	K47	6.0	TR7	1.8
B234	7.8	K49	3.4		

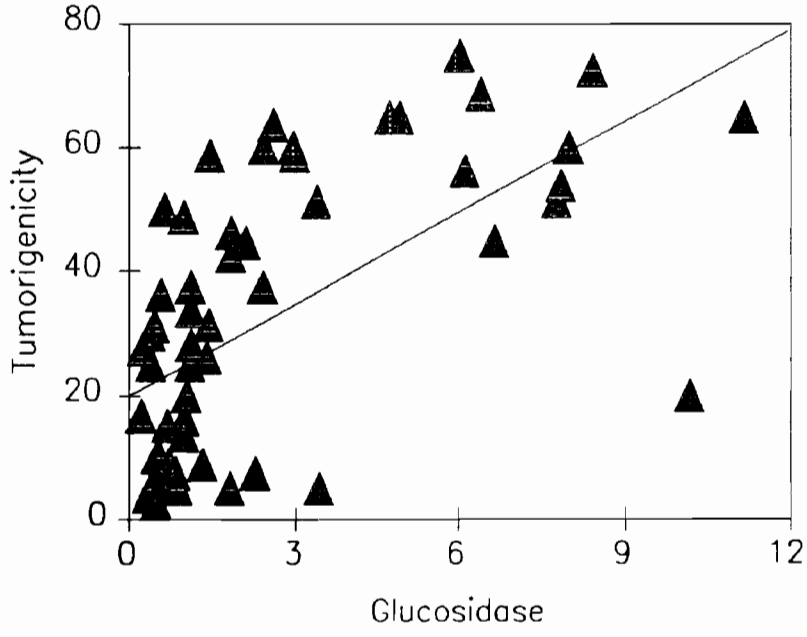

Figure 25. Relationship between tumorigenicity and glucosidase expression. For each strain, the glucosidase activity expressed by strains included in Table 13 was plotted against the average frequency of tumor formation on all four hosts. Generally, the strongly tumorigenic strains expressed higher glucosidase activity than the weakly tumorigenic strains.

Table 14. Virulence gene induction by coniferyl alcohol and coniferin.

		beta-galactosidase (nmol/min/cell A_{650})			zeatin riboside (nM equivalent)		
Strain	Hr	+CA	+CN	Control	+CA	+CN	Control
3667(pSM358)	8.5	160	38	36	60	69	26
	12	140	33	34	35	18	2
	18	66	26	24	69	41	28
	24	40	21	15	15	55	29
	36	36	50	27	24	44	20
A518(pSM358)	8.5	61	5	5	93	65	47
	12	29	7	3	170	70	25
	18	21	25	5	153	77	40
	24	13	10	0	72	80	38
	36	20	21	4	226	95	65
B3/73(pSM358)	8.5	625	161	16	136	95	68
	12	356	153	13	157	79	14
	18	473	490	13	271	241	41
	24	697	670	29	443	547	49
	36	643	444	42	915	642	61
C58(pSM358)	8.5	138	7	4	123	82	56
	12	153	34	8	222	85	38
	18	110	46	4	674	185	70
	24	112	48	4	547	180	65
	36	123	55	5	1160	225	74
K26(pSM358)	8.5	193	2	2	79	73	58
	12	93	10	2	147	90	24
	18	98	82	3	72	93	55
	24	68	59	4	40	52	61
	36	72	63	4	127	73	45
K41(pSM358)	8.5	401	45	7	150	135	59
	12	157	67	7	190	92	11
	18	202	195	7	88	192	54
	24	424	512	18	297	439	50
	36	414	491	24	784	570	24
K47(pSM358)	8.5	678	186	55	58	58	53
	12	792	449	56	64	45	11
	18	847	708	63	127	44	48
	24	1073	964	67	38	33	38
	36	1381	1461	133	50	51	36
MFM83.4(pSM358)	8.5	138	6	5	99	49	47
	12	73	23	4	87	40	7
	18	41	69	1	306	242	43
	24	36	52	0	330	250	46
	36	40	66	4	477	425	58
RR5(pSM358)	8.5	24	9	7	39	47	49
	12	20	9	6	65	40	7
	18	13	9	6	72	56	37
	24	11	7	6	72	56	40
	36	16	11	6	110	66	74
T28/73(pSM358)	8.5	96	3	2	86	24	35
	12	81	34	2	303	29	3
	18	59	33	2	794	173	25
	24	66	40	2	691	169	32
	36	84	50	2	962	299	17

Strains harboring pSM358 were grown in VIM4 medium containing coniferyl alcohol (+CA, 200 μM) or coniferin (+CN, 200 μM) or no inducer (Control). *Beta*-galactosidase activity (nmol o-nitrophenol/min/unit culture density) was measured as described in the text. Zeatin riboside equivalents (nanomolar) determined by ELISA as described in the text. Coefficient of variation for ELISA samples greater than 300 nM was 0.19. For samples between 10 nM and 60 nM it was 0.54.

Table 15. *Beta*-glucosidase activity in the presence of coniferyl alcohol and coniferin.

Strain	Incubation Time (hr)	Coniferyl Alcohol	Coniferin	Control
3667	18	2.1	2.1	1.2
	38	1.5	1.9	1.7
A518	18	<0.3	<0.3	<0.3
	38	<0.3	<0.3	<0.3
B3/73	18	1.9	3.0	2.6
	38	2.9	3.7	3.3
C58	18	<0.3	<0.3	<0.3
	38	<0.3	<0.3	<0.3
K26	18	<0.3	<0.3	<0.3
	38	<0.3	<0.3	<0.3
K41	18	1.3	1.9	1.6
	38	2.6	3.1	2.9
K47	18	2.4	4.9	3.0
	38	2.2	2.9	2.7
MFM83.4	18	<0.3	<0.3	<0.3
	38	<0.3	<0.3	<0.3
RR5	18	<0.3	<0.3	<0.3
	38	<0.3	<0.3	<0.3
SM358	18	<0.3	<0.3	<0.3
	38	<0.3	<0.3	<0.3
T28/73	18	<0.3	<0.3	<0.3
	38	<0.3	<0.3	<0.3

A. tumefaciens transconjugants which harbored pSM358 were grown in VIM4 media containing inducers (200 μM). Glucosidase activity (nmol p-nitrophenyl phosphate/min/unit culture density. The coefficient of variation for triplicate samples was 0.06.

than 400 nM) of zeatin/zeatin riboside were found in strains B3/73(pSM358), C58(pSM358), K41(pSM358), MFM83.4(pSM358), and T28/73(pSM358). In only two cases where levels were above 100 nM, did the zeatin/zeatin riboside levels in the presence of coniferin match (within 20 %) the levels found in the presence of coniferyl alcohol; B3/73 at 24 hr and unexpectedly MFM83.4(pSM358) at 36 hr.

Glucosidase levels were only measurable for 3667(pSM358), B3/73(pSM358), K41(pSM358) and K47(pSM358), the four most tumorigenic strains of the eleven examined (Table 15). At both time points the glucosidase levels were found to be highest in cultures incubated in the presence of coniferin compared to corresponding cultures incubated in the presence of coniferyl alcohol or in the absence of inducer. The exception was strain 3667(pSM358) at 18 hr where levels were the same. With one exception, 3667(pSM358) at 18 hr, *beta*-glucosidase levels were higher in the absence of inducer than in the presence of coniferyl alcohol.

DISCUSSION

Several conclusions may be drawn from the results of this chapter.

1. The major inducer of the *Agrobacterium vir* gene induction cascade in *P. menziesii* extracts is the glucoside coniferin.
2. The ability of coniferin to act as an inducer is strain dependent.
3. Strains expressing high levels of *beta*-glucosidase activity are effectively induced by coniferin.
4. Strains expressing low levels of *beta*-glucosidase activity are not effectively induced by coniferin.

5. *Vir* gene induction by coniferin during log phase growth of the bacterium is lower than induction by equimolar coniferyl alcohol.
6. A statistically significant correlation exists between *beta*-glucosidase activity and bacterial tumorigenicity on conifers.

These conclusions indicate, but do not establish, a basis for differential strain tumorigenicity on conifers. It appears that hydrolysis of the glucoside inducers which are present in *P. menziesii* (and by analogy in other conifers) is required before interaction with the virA gene product can occur. Strains which express low levels of *beta*-glucosidase activity, therefore do not initiate their *vir* gene induction cascade, or do so at low level due to the low concentration of active compound.

In order to establish a role for *beta*-glucosidase in defining tumorigenicity, suitable mutagenisis and complementation studies must be performed. These would include transferring the *beta*-glucosidase gene (or genes) into weakly tumorigenic strains, deleting the *beta*-glucosidase gene from strongly tumorigenic strains, and complementing the mutation by reintroduction of the gene.

PROJECT SUMMARY

This project was initiated during a period when plant gene regulation and genetic regulation of plant physiology were rapidly being advanced by improvements in DNA manipulation and transfer. Transformation of model plant systems allowed greater insight into gene function in the plant, and allowed the possibility of altering phenotypic traits. However, conifers had in large part been left out of gene transfer studies. The goal of the project was to begin to build a pathway to include conifers in the promises of genetic transfer.

As explained in the introduction, this project focused on two themes. First, was the identification of *Agrobacterium* strains strongly tumorigenic on conifers. Inoculation studies described in Chapter II identified many tumorigenic strains, more than expected. Additionally, the intriguing possibility exists that native gall isolates may be even more efficient in conifer transformation as described in Chapter III.

The second theme was understanding the basis for the differential responses seen among strains. Why strains differ in transformation ability is most certainly as multifaceted as the transformation process itself. As described in Chapter IV, the level of vir gene induction differs from strain to strain as does the sensitivity to different inducing compounds.

Identification of coniferin as the major native inducer found in *P. menziesii* extracts helped unravel the relationship between bacterial *beta*-glucosidase expression and induction by the native conifer inducer. Conclusive demonstration of the importance of

beta-glucosidase expression in affecting strain tumorigenicity awaits additional experimental evidence.

Taken in its entirety, this project succeeded in demonstrating that conifers are suitable study systems for *Agrobacterium* mediated transformation. Future work will extend the findings of this project. At the tissue level (the inoculated stem) this project identified strains of *Agrobacterium* which are efficient in transformation. It may be that transformation rates on a cell by cell basis can be further enhanced. A high percentage of transformed cells is important because the major limitation in gene transfer technology for conifers is efficient regeneration of transformed cells. Presently, regeneration of plantlets from somatic conifer tissues is labor intensive at best, and efficient foreign DNA integration into cells capable of regeneration has not been achieved. If conifer culture is to realize the potential benefits from gene transfer (potential increases in pest resistance, herbicide tolerance, wood quality, and other modifications of growth and development) the hurdle of efficiently regenerating transformed cells must be surmounted.

APPENDICES

BIBLIOGRAPHY

Akiyoshi, D.E., Morris, R.O., Hinz, R., Mischke, B.S., Kosuge, T, Garfinkel, D., 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.

Akiyoshi, D.E., Regier, D.A. & Gordon, M.P. (1987). Cytokinin production by *Agrobacterium* and *Pseudomonas spp.* Journal of Bacteriology 169: 4242-4248.

Albright, L.M., Yanofsky, M.F., Leroux, B., Ma, D. and Nester, E.W. (1987) Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. J. Bacteriol. 169: 1046-1055.

Amasino, R.M. and Miller, C.O. (1982) Hormonal control of tobacco crown gall tumor morphology. Plant Physiol. 69: 389-392.

Anderson, A.R. (1978). Taxonomy and host specificity of the genus Agrobacterium. Doctoral thesis, Oregon State University, Corvallis, Oregon. 61 p.

Anderson, A.R. and Moore, L.W. (1979). Host specificity in the genus Agrobacterium. Phytopathology 69, 320-323.

Barton, K.A., Binns, A.N., Matzke, A.J.M. and Chilton, M.-D. (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32: 1033-1043.

Beaty, J.S., Powell, G.K., Lica, L., Regier, D.A., MacDonald, E.M.S., Hommes, N.G. and Morris, R.O. (1986) *Tzs*, a nopaline Ti plasmid gene from *Agrobacterium tumefaciens* associated with *trans*-zeatin biosynthesis. Mol. Gen. Genet. 203: 274-280.

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

Bomhoff, G., Klapwijk, P.M., Kester, H.C.M. and Schilperoort, R.A. (1976) Octopine and nopaline systhesis and breakdown genetically controlled by plasmid of *Agrobacterium tumefaciens*. Mol. Gen. Genet. 145: 177-181.

Bradbury, J.F. (1986) Guide to plant pathogenic bacteria. CAB International Mycological Institue, Farnham Royal U.K. 332p.

Braun, A. (1958) A physiological basis for autonomous growth of the crown-gall tumor cell. Proc. Natl. Acad. Sci. USA 44: 344-349.

Braun, A.C. and White, P.R. (1942) Bacteriological sterility of tissues derived from secondary crown-gall tumors. Photopath. 33: 85-100.

Brisbane, P.G. and Kerr, A.K. (1983) Selective media for three biovars of Agrobacterium. J. Appl. Bact. 54: 425-431.

Brown N.A. and Gardner, F.E. (1936) Galls produced by plant hormones, including a hormone extract from *Bacterium tumefaciens*. Phytopath. 26: 708-713.

Buchholz, W.G. and Thomashow, M.F. (1984a) Comparison of T-DNA oncogene complements of *Agrobacterium tumefaciens* tumor-inducing plasmids with limited and wide host ranges. J. Bacteriol. 160: 319-326.

Buchholz, W.G. and Thomashow, M.F. (1984b) Host range encoded by the *Agrobacterium tumefaciens* tumor-inducing plasmid pTiAg63 can be expanded by modification of its T-DNA oncogene complement. J. Bacteriol. 160: 327-332.

Byrne, M.C. McDonnel, R.E., Wright, M.S. and Carnes, M.G. (1987) Strain and cultivar specificity in the *Agrobacterium*-soybean interaction. Plant Cell, Tissue and Organ Culture 8: 3-15.

Cangelosi, G.A., Hung, L., Puvanesarajah, V., Stacey, G., Ozga, D.A., Leigh, J.A., Nester E.W. (1987) Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccaride synthesis and their roles in plant interaction. J. Bact. 169: 2086-2891.

Cangelosi, G.A., Martinetti, G., Leigh, J.A., Lee, C.C., Theines, C. and Nester, E.W. (1989) Role of *Agrobacterium tumefaciens* ChvA protein in export of beta-1,2-glucan. J. Bacteriol. 171: 1609-1615.

Chen, C.-M., Eckert, R.L. and McChesney, J.D. (1976) Isopentenyl adenosine from crown gall tumor tissue of *Vinca rosea*. Phytochem. 15: 1565.

Chilton, M-D., Drummond, M., Merlo, D., Sciaky, D., Montoya, A., Gordon, M.P. and Nester, E.W. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis for crown gall tumorigenesis. Cell 11: 263-271.

Chilton, M.-D., Drummond,M.H., Merlo, D.J. and Sciaky, D. (1978) Highly conserved DNA of Ti plasmids overlaps T-DNA maintained in plant tumours. Nature 275: 147-149.

Chilton, S.W., Tempé, J., Matzke, M. & Chilton, M-D. (1984). Succinamopine: a new crown gall opine. Journal of Bacteriology. 157, 357-362.

Christie, P.J., Ward, J.E., Winans, S.C., Nester, E.W. (1988) The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-DNA. J. Bacteriol. 170: 2659-67.

Citovski, V., Howard, E., Winsor, B. and Zambryski, P. (1989a) Proteins that mediate DNA transfer by *Agrobacterium tumefaciens* to plant cells: summary and perspectives. *In* Molecular Biology of Plant-Pathogen Interactions. B. Stakawicz, P. Ahlquist, O. Yoder, eds. Alan R. Liss, Inc. New York.

Citovski, V., Wong, M.L., Zambryski, P. (1989b) Cooperative interaction of *Agrobacterium* virE2 protein with single-stranded DNA: implications for the T-DNA transfer process. Proc. Natl. Acad. Sci. USA 86: 1193-1197.

Clapham. D.H. & Ekberg, I. (1986). Induction of tumours by various strains of *Agrobacterium tumefaciens* on *Abies nordmanniana* and *Picea abies*. Scandinavian Journal of Forest Research 1, 435-437.

Dahl, G.A., Guyon, P.A., Petit, A. and Tempe J. (1983) Silver nitrate-positive opines in crown gall tumors. Plant Sci. Lett. 32: 193-203.

Dandekar, A.M., Gupta, P.K., Durzan, D.J. & Knauf, V. (1987). Transformation and foreign gene expression in micropropagated Douglas-fir (*Pseudotsuga menziesii*). Bio/technology 5, 587-590.

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

Das, A., Stachel, S., Ebert, P., Allenza, P., Montoya, A. and Nester, E. (1986) Promoters of *Agrobacterium tumefaciens* Ti-plasmid virulence genes. Nucleic Acids Res. 14: 1355-1364.

Davioud, E., Quirion, J.-C., Tate, M.E., Tempe, J. and Husson, H.-P. (1988) Structure and synthesis of cucmopine, a new crown gall and hairy-root opine. Heterocycles 27: 2423-2429.

De Ropp, R.S. (1947) The response of normal plant tissues and of crown-gall tumor tissues to synthetic growth hormones. Amer. J. Bot. 34: 53-62.

DeCleene, M. & DeLey, J. (1976). The host range of crown gall. Botanical Review 42, 389-466.

Denhardt, D.T. (1966) A membrane-filter technique for the detection of complementary DNA. Biochem Biophys. Res. Comm. 23: 641-646.

Depicker, A., DeWilde, M., DeVos, G., DeVos, R., VanMontagu, M. and Schell, J. (1980) Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. Plasmid 3: 193-211.

Depicker, A., Van Montagu and Schell, J. (1978) Homologous DNA sequences in different Ti-plasmids are essential for oncogenicity.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980) Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. 77, 7347-7351.

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

Drummond, M.H. and Chilton, M.-D. (1978) Tumor-inducing (Ti) plasmids of *Agrobacterium* share extensive regions of DNA homology. J. Bacteriol. 136: 1178-1183.

Dürrenburger F., Crameri, A., Hohn, B., Koukolikova-Nicola (1989) Covalently bound virD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proc. Natl. Acad. Sci. USA 86: 9154-9158.

Ellis, D., Roberts, D., Sutton, B., Lazaroff, W., Webb, D. and Flinn, B. (1989) Transformation of white spruce and other conifer species by *Agrobacterium tumefaciens*. Plant Cell Rep. 8: 16-20.

Ellis, J.G., Kerr, A., Petit, A. and Tempe, J. (1982) Conjugal transfer of nopaline and agropine Ti-plasmids - the role of agrocinopines. Mol. Gen. Genet. 186: 269-274.

Ellis, J.G. and Murphy, P.J. (1981) Four new opines from crown gall tumours - their detection and properties. Mol. Gen. Genet. 181: 36-43.

Engler, G., Depicker, A., Maenhaut, R., Villarroel, R., Van Montagu, M. and Schell, J. (1981) Physical mapping of DNA base sequence homologies between an octopine and a nopaline Ti plasmid of *Agrobacterium tumefaciens*. J. Mol. Biol. 152: 183-208.

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

Fillatti, J.J., Sellmer, J., Mccown, B., Hassig, B. & Comai, L. (1987). *Agrobacterium* mediated transformation and regeneration of *Populus*. Molecular and General Genetics 206, 192-199.

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

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

Garfinkel, D.J., Simpson, R.B., Ream, L.W., White, F.F., Gordon, M.P. and Nester, E.W. (1981) Genetic analysis of crown gall: fine structure map of the T-DNA by site directed mutagenesis. Cell 27: 143-153.

Gasser, C.S. and Fraley, R. T. (1989) Genetically engineering plants for crop improvement. Science 244: 1293-1299.

Gelvin, S.B. and Habeck, L.L. (1990) *Vir* genes influence conjugal transfer of the Ti plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. 172: 1600-1608.

Ghai J. and Das, A. (1989) The *virD* operon of *Agrobacterium tumefaciens* Ti plasmid encodes a DNA-relaxing enzyme. Proc. Natl. Acad. Sci. USA 86: 3109-3113.

Gheysen, G., Van Montagu, M. and Zambryski, P. (1987) Integration of *Agrobacterium tumefaciens* transfer DNA (T-DNA) involves rearrangements of target plant DNA sequences. Proc. Natl. Acad. Sci. USA 84: 6169-6173.

Gietl, C., Koukolikova-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: 9006-90010.

Grimsley, N.H., Hohn, T., Davies, J.W. & Hohn, B. (1987) *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. Nature 325, 177-179.

Grunstein, M. and Hogness D.S. (1975) Colony hybridization: a method for the isolation of coloed DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA 72: 3961-3965.

Guyon, P., Chilton, M.-D., Petit, A. and Tempe, J. (1980) Agropine in "null-type" crown gall tumors: evidence for generality of the opine concept. Proc. Natl. Acad. Sci. USA 77: 2693-2697.

Hansen, H.N. and Smith, R.E. (1937) A bacterial gall disease of Douglas-fir, *Pseudotsuga taxifolia*. Hilgardia 10: 569-577.

Heberlein, G.T., De Ley, J. and Tijtgat, R. (1967) Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. J. Bacteriol. 94: 116-124.

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

Herrera-Estrella, A., Chen,Z. Van Montagu, M. and Wang, K. (1988) VirD proteins of *Agrobacterium tumefaciens* are required for the formation of a covalent DNA-protein complex at the 5' terminus of T-strand molecules. EMBO J. 7: 4055-4062.

Hirooka, T., Rogowsky, P.M. and Kado, C.I. (1987) Characterization of the *virE* locus of *Agrobacterium tumefaciens* plasmid pTiC58. J. Bacteriol. 169: 1529-1536.

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.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) a binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179-180.

Holmes, B. and Roberts, P. (1981) The classification, identification and nomenclature of agrobacteria. J. Appl. Bacteriol. 50: 443-467.

Holsters, M., Hernalsteen, J.P., Van Montagu, M. and Schell, J. (1982) Ti plasmids of *Agrobacterium tumefaciens*: the nature of the TIP. *In* Molecular Biology of Plant Tumors, G. Kahl and J. Schell eds. Academic Press, New York, NY pp 269-298.

Hood, E.E., Helmer, G.L., Fraley, R.T. and Chilton, M-.D. (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside the T-DNA. J. Bacteriol. 168: 1291-1301.

Hooykaas, P.J.J. (1989) Transformation of plant cells via *Agrobacterium*. Plant Molec. Biol. 13: 327-336.

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

Hooykaas, P.J.J. Roobol, C. and Schilperoort, R.A. (1979) Regulation of the transfer of Ti plasmids of *Agrobacterium tumefaciens*. J. Gen. Microbiology 110: 99-109.

Huang, Y., Morel, P., Powell, B. and Kado, C. (1990) VirA, a coregulator of Ti-specified virulence genes, is phosphorylated in vitro. J. Bacteriol. 172: 1142-1144.

Huffman, G.A., White, F.F., Gordon, M.P. and Nester, E.W. (1984) Hairy-root-inducing plasmid: plasmid map and homology to tumor-inducing plasmids. J. Bacteriol. 157: 269-276.

Ingestad, T. (1960). Studies on the Nutrition of Forest Tree Seedlings. III Mineral Nutrition of Pine. Physiologia Plantarum 13, 513-533.

Isogai, A. Fukuchi, N., Hayashi, M., Kamada, H., Harada, H. and Suzuki, A. (1988) Structure of a new opine, mikiopine, in hairy root induced by *Agrobacterium rhizogenes*. Agric. Biol. Chem. 52: 3235-3237

Jayaswal, R.K., Veluthambi, K., Gelvin, S.B. and Slightom, J.L. (1987) Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in *Escherichia coli* by a virD-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. J. Bacteriol. 169: 5035-5045.

Jefferson, R., Kavanagh, T.A. and Bevan, M.W. (1987). GUS Fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal 6: 3901-3907.

Ji, J.M., Martinez, A., Dabrowski, M., Veluthambi, K., Gelvin, S.B., Ream, W. (1989) The overdrive enhancer sequence stimulates production of T-strands from the *Agrobacterium tumefaciens* tumor-inducing plasmid. *In* Molecular Biology of Plant-Pathogen Interactions. B. Stakawicz, P. Ahlquist, O. Yoder, eds. Alan R. Liss, Inc. New York.

Jin, S., Roitsch, T., Ankenbauer, R.G., Gordon, M.P. and Nester, E.W. (1990) 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. (1990) 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.C. 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.

Joos, H., Inze, D., Caplan, A., Sormann, M., Van Montagu, M. and Schell, J. (1983) Genetic Analysis of T-DNA transcripts in nopaline crown galls. Cell 32: 1056-1067.

Kado, C.I. & Heskett, M.G. (1970). Selective media for isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas and Xanthomonas. Phytopathology 60, 969-976.

Keane, P.J., Kerr, A. and New, P.B. (1970) Crown gall of stone fruit II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23: 585-595.

Kerr, A., Manigault, P. and Tempe, J. (1977) Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium*. Nature 265: 560-561.

Kersters, K. and De Ley, J. (1984) *Agrobacterium*. *In* Bergey's manual of systematic bacteriology. N.R. Kreig ed., Williams and Wilkins, Baltimore.

Klapwijk, P.M., Scheulderman, 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. Journal of Bacteriology 136: 775-785.

Klee, H.J., Gordon, M.P. and Nester, E.W. (1982) Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid mutations affecting oncogenicity. J. Bacteriol. 150: 327-331.

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

Knauf, V.C. and Nester, E.W. (1982) Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid 8: 45-54.

Knauf, V.C., Panagopoulos, C.G. and Nester, E.W. (1982) Genetic factors controlling the host range *Agrobacterium tumefaciens*. Phytopath. 72: 1545-1549.

Knauf, V.C., Yanofsky, M., Montoya, A. and Nester, E.W. (1984) Physical and functional map of an *Agrobacterium tumefaciens* tumor-inducing plasmid that confers a narrow host range. J. Bacteriol. 160: 564-568.

Koekman, B.P., Hooykaas, P.J.J. & Schilperoort, R.A. (1980). Localization of the replication control region on physical map of the octopine Ti plasmid. Plasmid 4: 184-195.

Kosslak, R.M., Bookland, R., Barkei, J., Paaren, H.E. and Appelbaum,E.R. (1987) Proc. Natl. Acad. Sci. 84: 7428-7432.

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.

Lemmers, M., De Beukeleer, M., Holsters, M., Zambryski, P., Depicker, A., Hernalsteens, J.P., Van Montagu, M. and Schell, J. (1980) Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline crown gall tumours. J. Mol. Biol. 144: 353-376.

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

Lin, B-C. and Kado, C.I. (1977). Studies on Agrobacterium tumefaciens. VII Avirulence induced by temperature and ethidium bromide. Canadian Journal of Microbiology 23: 1554-1561.

Liu, S.-T., Perry, K.L., Schardl, C.L., and Kado, C.I. (1982) *Agrobacterium* Ti plasmid indoleacetic acid gene is required for crown gall oncogenesis. Proc. Natl. Acad. Sci. 79: 2812-2816.

Loomis, D. (1974) Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods in Enzymology 31: 528-544.

Loper, J.E. and Kado, C.I. (1979) Host range conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. 139: 591-596.

Maldiney, R., Leroux, B., Sabbagh, I., Sotta, B., Sossountzov, L. and Miginiac, E. (1986) A biotin-avidin-based enzyme immunoassay to quantify three phytohormones: auxin, abscisic acid and zeatin-riboside. J. Immunol. Meth. 90: 151-158.

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

Matthysse, A.G. (1987) Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. J. Bacteriol. 169: 313-323.

McBride, K.E. and Knauf, V.C. (1988) Genetic analysis of the *vir*E operon of the *Agrobacterium* Ti plasmid pTiA6. J. Bact. 170: 1430-1437.

Melchers, L.S., Regensburg-Tuïnk, T.J.G., Bourret, R.B., Sedee, N.J.A., Schilperoort, R.A. and Hooykaas, P.J.J. (1989) Membrane topology and functional analysis of the sensory portein virA of *Agrobacterium tumefaciens*. EMBO J. 8: 1919-1925.

Melchers, L.S., Regensburg-Tuïnk, T.J.G., Schilperoort, R.A. and Hooykaas, P.J.J. (1989) Specificity of signal molecules in the activation of *Agrobacterium* virulence gene expression. Mol. Microbiol. 3: 969-977.

Melchers, L.S., Thompson, D.V., Idler, K.B., Neuteboom, S.T.C., de Maagd, R.A., Schilperoort, R.A. and Hooykaas, P.J.J. (1987) Molecular characterization of the virulence gene *vir*A of the *Agrobacterium tumefaciens* octopine Ti plasmid. Plant Molecular Biology 9: 635-645.

Miller, I.S., Fox, D., Saeed, N., Borland, P.A., Miles, C.A. and Sastry, G.R.K. (1986). Enlarged map of *Agrobacterium tumefaciens* C58 and the location of chromosomal regions which affect tumorigenicity. Molecular and General Genetics 205: 153-159.

Miller, J.H. (1972) Experiments in Molecular Genetics. Cold SpringHarbor Laboratory, Cold Spring Harbor, NY. 466p.

Montoya,A.L., Chilton, M.-D., Gordon, M.P., Sciaky, D. and Nester, E.W. (1977) Octopine and nopaline metabolism in *Agrobacterium tumefaciens* and crown gall tumor cells: role of plasmid genes. J. Bacteriol. 129: 101-107.

Moore, L., Warren, G. and Strobel, G. (1979). Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterum rhizogenes*. Plasmid 2, 617-626.

Morris, R.O. & Powell, G.K. (1987). Genes specifying cytokinin biosynthesis in prokaryotes. Bioessays 6, 23-28.

Morris, R.O. (1977) Mass spectroscopic identification of cytokinins. Plant Physiol. 59: 1029-1033

Nakajima, H., Yokota, T., Takahashi, N., Matsumoto, T. and Noguchi, M. (1981) Changes in endogenous ribosyl-*trans*-zeatin and IAA levels in relation to the proliferation of tobacco crown gall cells. Plant Cell Physiol. 22: 1405-1410.

Nester, E.W., Gordon, M.P., Amasino, R.N. and Yanofsky, M.F. (1984). Crown Gall: a molecular and physiological analysis. Annual Review of Plant Physiology 35: 387-413.

Ninfa, A. and Magasanik, B. (1986) Covalent modification of the glnG product, NR1, by the glnL product, NRII, regulates transcription of the glnALG operon in *E. coli*. Proc. Natl. Acad. Sci USA 83: 5909-5913.

Ooms, G., Hooykaas, P.J.J., Moolenaar, G. and Schilperoort, R.A. (1981) Crown gall tumors of abnormal morphology, induced by *Agrobacterium tumefaciens* carrying mutated octopine Ti plasmids; analysis of T-DNA functions. Gene 14: 33-50.

Ooms, G., Hooykaas, P.J.J., Van Veen, R.J.M., Van Beelen, P., Regensburg-Tuïnk, T.J.G., Schilperoort, R.A. (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of T-region. Plasmid 7: 15-29.

Otten, L., De Greve, H., Leemans, J., Hain, R., Hooykaas, P.J.J. and Schell, J. (1984) Restoration of virulence of *vir* region mutants of *Agrobacterium tumefaciens* strain B6S3 by coinfection with normal and mutant *Agrobacterium* strains.

Otten, L., Piotrowiak, G., Hooykaas, P., Dubois, M., Szegedi, E. and Schell, J. (1985) Identification of an *Agrobacterium tumefaciens* pTiB6S3 vir region fragment that enhances the virulence of pTiC58. Mol. Gen. Genet. 199: 189-193.

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.

Peerbolte, R., Leenhouts, K., Hooykaas-van Slogteren, G.M.S., Wullems, G.J., and Schilperoort, R.A. (1986) Clones form a shooty tobacco crown gall tumor II: irregular T-DNA structures and organization, T-DNA methylation and conditional expression of opine genes. Plant Mol. Biol. 7: 285-299.

Peralta, E.G., Hellmiss, R. and Ream, W. (1986) *Overdrive*, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid. EMBO J. 5: 1137-1142.

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

Peterson, J.B. and Miller, C.O. (1977) Glucosyl zeatin and glucosyl ribosylzeatin from *Vinca rosea* L. crown gall tumor tissue. Plant Physiol. 59: 1026-1028.

Petit, A., David, C., Dahl, G.A., Ellis, J.G. and Guyon, P. (1983). Further extension of the opine concept: plasmids in Agrobacterium rhizogenes cooperate for opine degradation. Molecular and General Genetics 190: 204-214.

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

Porter, S.G., Yanofsky, M.F. and Nester E.W. (1987) Molecular characterization of the *virD* operon from *Agrobacterium tumefaciens*. Nucl. Acid. Res. 15: 7503-7517.

Powell, G.K., Hommes, N.G., Kuo, J., Castle, L.A. and Morris, R. O. (1988) Inducible expression of cytokinin biosynthesis in *Agrobacterium tumefaciens* by plant phenolics. Molec. Plant-Microbe Interact. 1: 235-242.

Regier, D.A. and Morris, R.O. (1982) Secretion of trans-zeation by *Agrobacterium tumefaciens*: a function determined by the nopaline Ti plasmid. Biochem. Biophys. Res. Comm. 104: 1560-1566.

Ronson, C.W., Nixon, B.T. Ausubel, F.M. (1987) Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell 49: 579-581.

Rosenberg, C. and Huguet, T. (1984) The pAtC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumour induction. Mol. Gen. Genet. 196: 533-536.

Ryder, M.H., Tate, M.E. and Jones, G.P. (1984) Agrocinopine A, a tumor-inducing plasmid-coded enzyme product, is a phosphodiester of sucrose and L-arabanose. J. Biol. Chem. 259: 9704-9710.

Sadtler (1980) Standard Spectra Collection, Sadtler Research Laboratories, Inc. Division of BioRad Inc.

Schell, J. and Van Montagu, M. (1977) Transfer, Maintenance, and expression of bacterial Ti-plasmid DNA in plant cells transformed with *A. tumefaciens*. Genetic Interaction and Gene Transfer, Brookhaven Symposia in Biology 29: 36-49.

Schopmeyer, C.S. (1974) Seeds of woody plants of the United States, Agriculture Handbook No. 450, US Government Printing Office, Washington, D.C. 883p.

Schröder, J., Schröder, G., Huisman, H., Schilperoort, R.A. and Schell, J. (1981) The mRNA for lysopine dehydrogenase in plant tumor cells is complementary to a Ti-plasmind fragment. FEBS Lett. 129: 166-168.

Schroth, M.N., Thompson, J.P., and Hildebrand, D.C. (1965) Isolation of *Agrobacterium tumefaciens - A. radiobacter* group from soil. Phytopath. 55: 645-647.

Sciaky, D., Montoya, A.L. and Chilton, M-D. (1978). Fingerprints of Agrobacterium Ti plasmids. Plasmid 1: 238-253.

Sederoff, R., Stomp, A-M., Chilton, W.S. and Moore, L.W. (1986). Gene transfer into loblolly pine by *Agrobacterium tumefaciens*. Bio/technology 4: 647-649.

Sinkar, V.P., Pythoud, F., White, F.F., Nester, E.W., Gordon, M.P. (1988) rolA locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants. Genes & Develop. 2: 688-697.

Skyring, G.W., Quadling, C. and Rouatt, J.W. (1971) Soil bacteria: principal component analysis of physiological descriptions of some named cultures of *Agrobacterium*, *Arthorbacter*, and *Rhizobium*. Can. J. Microbiol. 17: 1299-1311.

Smith, C.O. (1935). Crown gall on conifers. Phytopathology 25: 894.

Smith, C.O. (1942). Crown gall on species of *Taxaceae*, *Taxodiaceae*, and *Pinaceae*, as determined by artificial inoculations. Phytopathology 32: 1005-1009.

Snedecor, G.W. and Cochran W.G. (1967) Statistical Methods. Iowa State University Press, Ames, Iowa 593 p.

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

Stachel, S.E., An, G., Flores, C., and Nester, E.W. (1985a) A Tn*3* *lac*Z transposon for the random generation of *beta*-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. and Nester, E.W. (1986) The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. 5: 1445-1454.

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' *virD* gene products. EMBO J. 6: 857-863

Stachel, S.E., Timmerman, B. and Zambryski, P.C. (1986) Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature 322: 706-712.

Stachel, S.E. and Zambryski, P.C. (1986a). virA and virG 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.

Steck, T.R., Close, T.J. and Kado, C.I. (1989) High levels of double-stranded transferred DNA (T-DNA) processing from an intact nopaline Ti plasmid. Proc. Natl. Acad. Sci. USA 86: 2133-2137.

Steck, T.R., Morel, P. and Kado, C.I. (1988) Vir box sequences in *Agrobacterium tumefaciens* pTiC58 and A6. Nucl. Acids Res. 16: 8736.

Stomp, A.-M. Loopstra, C., Sederoff, R., Chilton, S., Fillatti, J., Dupper, G., Tedeschi, P. and Kinlaw, C. (1988) Development of a DNA transfer system for pines. *In*: The genetic manipulation of woody plants. J.W. Hanover and D. E. Keathley eds. Plenum Press, N.Y. pp231-241.

Szegedi, E., Czako, M., Otten, L. and Koncz, C. (1988) Opines in crown gall tumore induced by biotype 3 isolates of *Agrobacterium tumefaciens*. Physiol. Mol. Plant Physiol. 32: 237-247.

Thomashow, M.F., Karlinsey, J., Marks, J.R., Hurlbert, R.E. (1987) Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide 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., Nutter, R., Montoya, A., Gordon, M.P. and Nester, E.W. (1980a) Integration and organization of Ti plasmid sequences in crown gall tumors. Cell 19: 729-739.

Thomashow, M.F., Panagopoulos, C.G., Gordon, M.P. and Nester, E.W. (1980b) Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid. Nature (London) 283: 794-796.

Toro, N., Datta, A., Carmi, O.A., Young, C., Prusti, R.K. and Nester, E.W. (1989) The *Agrobacterium tumefaciens virC1* gene product binds to overdrive, a T-DNA transfer enhancer. J. Bacteriol. 171: 6845-6849.

Unger, L., Ziegler, S.F., Huffman, G.A., Knauf, V.C., Peet, R., Moore, L.W. Gordon, M.P. and Nester, E.W. (1985) New class of limited-host-range *Agrobacterium* mega-tumor-inducing plasmids lacking homology to the transferred DNA of a wide-host-range, tumor inducing plasmid. J. Bacteriol. 164: 723-730.

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

Veluthambi, K., Ream, W. and Gelvin, S.B. (1988) Virulence genes, boarders, and overdrive generate single-stranded T-DNA molecules from the A6 Ti plasmid of *Agrobacterium tumefaciens*. J. Bact. 170: 1523-1532.

Virts, E.L. and Gelvin, S.B. (1985) Analysis of transfer of tumor-inducing plasmids from *Agrobacterium tumefaciens* to *Petunia* portoplasts. J. Bacteriol. 162: 1030-1038.

Ward, E.R. and Barnes, W.M. (1988) VirD2 protein of *Agrobacterium tumefaciens* very tightly bound to the 5' end of the T-strand DNA. Science 242: 927-930.

Ward, J.E., Akiyoshi, D.A., Regier, D.A., Datta, A., Gordon, M.P. and Nester, E.W. (1988) Characterazation of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. 263: 5804-5814.

Watson, B., Currier, T.C., Gordon, M.P. Chilton, M.-D. and Nester, E.W. (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. 123: 255-264.

Watson, D.R.W. (1981) *In* Plant disease division culture collection catalogue, New Zealand Department of Scientific & Industrial Research, Auckland, New Zealand.

Weiler, E.W., and Spanier, K. (1981) Phytohormones in the formation of crown gall tumors. Planta 153: 326-337.

White, F.F. and Nester, E.W. (1980a) Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. J. Bacteriol. 141: 1134-1141.

White, F.F. and Nester, E.W. (1980b) Relationship of plasmids responsible for hairy root and crown gall tumorigencity. J. Bacteriol. 144: 710-720.

White, F.F., Taylor, B.H., Huffman, G.A., Gordon, M.P. and Nester, E.W. (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J. Bacteriol. 164: 33-44.

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. 83: 8278-8282

Winans, S.C., Kerstetter, R.A., Ward, J.E. and Nester, E.W. (1989) A protein required for transcriptional regulation of *Agrobacterium* virulence genes spans the cytoplasmic membrane. J. Bacteriol. 171: 1616-1622.

Yamada, S. and Itano, H.A. (1966). Phenanthrenequinone as an analytical reagent for arginine and other monosubstituted guanidines. Biochimica Biophysica Acta 130: 538-540.

Yamada, T., Palm, C.J., Brooks, B. and Kosuge, T. (1985) Nucleotide sequences of the *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. Proc. Natl. Acad. Sci. USA 82: 6522-6526.

Yamamoto, A., Iwahashi, M. Yanofsky, M.F. and Nester, E.W. (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.

Yang, F.-M., Montoya, A.L., Nester, E.W. and Gordon, M.P. (1980) Plant tumor reversal associated with the loss of foreign DNA. In Vitro 16: 87-92.

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

Yanofsky, M., Montoya, A., Knauf, V., Lowe, B., Gordon, M.P. and Nester, E.W. (1985b) Limited-host-range plasmid of *Agrobacterium tumefaciens*: molecular and genetic analyses of transferred DNA. J. Bacteriol. 163: 341-348.

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

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

Zambryski, P. (1988) Basic Processis underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu. Rev. Genet. 22: 1-30.

Zambryski, P., Tempe, J. and Schell J. (1989) Transfer and function of T-DNA genes from Agrobactium Ti and Ri plasmds in plants. Cell 56: 193-201.

Zerback, R., Dressler, K. and Hess, D. (1989) Flavonoid compounds from pollen and stigma of *Petunia hybrida*: inducers of the *vir* region of the *Agrobacterium tumefaciens* Ti plasmid. Plant Science 62: 83-91.

Zorreguieta, A., Geremia, R.A., Cavaignac, S., Cangelosi, G.A., Nester, E.W. and Ugalde, R.A. (1988) Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. Molecular Plant-Microbe Interactions 1: 121-127.

AGROBACTERIUM STRAINS.

Strain	Genotype	Reference
0362	0362(pTi0362)	Moore personal communication
1001	1001(pTi1001)	Sciaky *et al.* 1978
13333	13333(pRi13333)	ATCC
15834	15834(pRi15834)	White and Nester 1980a
15955	15955(pTi15955)	Sciaky *et al.* 1978
25818(TR7)	25818(pRi25818)	White and Nester 1980b
3667	3667(pTi3667)	PDDCC
8196	8196(pRi8196)	Tepfer personal communication
A136	C58C1	Chilton personal communication
A175	C58C1(pTiC58)	Nester personal communication
A178	C58C1(pTiK27)	Sciaky *et al.* 1978
A2	A2(pRiA2)	Moore personal communication
A203	C58C1(pTiNCPPB223)	Sciaky *et al.* 1978
A208	C58(pTiT37)	Sciaky *et al.* 1978
A21/75	A21/75(pTiA21/75)	Moore personal communication
A25/75	A25/75(pTiA25/75)	Anderson and Moore 1979
A277	C58C1(pTiB6-806)	Sciaky *et al.* 1978
A281	C58C1(pTiBo542)	Sciaky *et al.* 1978
A348	C58C1(pTiA6NC)	Garfinkel *et al.* 1980
A350	C58C1(pTiB2A)	Nester personal communication
A4	A4(pRiA4)	Moore *et al.* 1979
A518	C58C1(pTiEU6)	Sciaky *et al.* 1978
A519	C58C1(pTiAT181)	Sciaky *et al.* 1978
A527	C58C1(pTiCG1C)	Nester personal communication
A543	C58C1(pTiAT4)	Sciaky *et al.* 1978
A557	A200(pTiIIBV7)	Sciaky *et al.* 1978
A596	C58C1(pTiAch5)	Nester personal communication
A6	A6(pTiA6)	Sciaky *et al.* 1978
A723	C58C1(pTiB6806)	Sciaky *et al.* 1978
Ach5	Ach5(pTiAch5)	Lin and Kado 1977
Ach5C3	Ach5	Nester personal communication
AT1	AT1(pTiAT1)	Moore personal communication
B1/74	B1/74(pTiB1/74)	Anderson
B2/74	B2/74(pTiB2/74)	Moore personal communication
B234	B234(pTiB234)	Moore personal communication
B2A	B2A(pTiB2A)	Sciaky *et al.* 1978
B3/73	B3/73(pTiB3/73)	Anderson and Moore 1979
B4/74	B4/74(pTiB4/74)	Anderson 1978
B6	B6(pTiB6T)	Sciaky *et al.* 1978
B6-806	B6-806(pTiB6-806)	Sciaky *et al.* 1978
Bo542	Bo542(pTiBo542)	Sciaky *et al.* 1978
C2/74	C2/74(pTiC2/74)	Anderson 1978
C3/74	C3/74(pTiC3/74)	Moore personal communication
C58	C58(pTiC58)	Sciaky *et al.* 1978
C58(pRiA4)	C58C1(pArA4a,pRiA4,pArA4c)	Ryder personal communication
C58(pRi8196)	C58C1(pRi8196)	Ryder personal communication
C58(pRiTR105)	C58C1(pRiTR105)	Ryder personal communication
C58C1	C58C1	Tepfer personal communication
CG1C	CG1C(pTiCG1C)	Dahl *et al.* 1983

E8/73	E8/73(pTiE8/73)	Moore personal communication
EHA101	C58C1(pEHA101)	Hood *et al.* 1986
G1/73	G1/73(pTiG1/73)	Anderson 1978
GMI9023	C58C1	Rosenberg and Huguet 1984
GV3140	C58C1(pTiC58)	Schell personal communication
GV3160	C58C1(pTiC58tra-c)	Schell personal communication
GV3245	LS1005(pTiB6S3)	Schell personal communication
GV3560	S1005(pTiK14)	Schell personal communication
H100	H100(pTiH100)	Sciaky *et al.* 1978
I1/75	I1/75(pTiI1/75)	Anderson 1978
I10/75	I10/75(pTiI10/75)	Anderson 1978
I7/75	I7/75(pTiI7/75)	Anderson 1978
IIBV7	IIBV7(pTiIIBV7)	Sciaky *et al.* 1978
K108	K108(pTiK108)	Ryder personal communication
K15/73	K15/73(pTiK15/73)	Anderson 1978
K21	K21(pTiK21)	Moore personal communication
K26	K26(pTiK26)	Moore personal communication
K27	K27(pTiK27)	Anderson 1978
K308	K308(pTiK308)	Ryder personal communication
K32	K32(pTiK32)	Ryder personal communication
K34	K34(pTiK34)	Moore personal communication
K35	K35(pTiK35)	Moore personal communication
K36	K36(pTiK36)	Moore personal communication
K37	K37(pTiK37)	Moore personal communication
K39	K39(pTiK39)	Moore personal communication
K40	K40(pTiK40)	Moore personal communication
K41	K41(pTiK41)	Moore personal communication
K46	K46(pRiK46)	Moore personal communication
K47	K47(pRiK47)	Moore personal communication
K49	K49(pRiK49)	Moore personal communication
K6/73	K6/73(pTiK6/73)	Anderson and Moore 1979
LBA4404	Ach5(pTiAch5::Tn904)	Hoekema *et al.* 1983
M2/73	M2/73(pTiM2/73)	Anderson and Moore 1979
M3/73	M3/73(pTiM3/73)	Anderson 1978
MFM83.4	MFM83.4(pTiMFM83.4)	Michel personal communication
MFM84.1	MFM84.1(pTiMFM84.1)	Michel personal communication
MFM84.4	MFM84.4(pTiMFM84.4)	Michel personal communication
MFM84.5	MFM84.5(pTiMFM84.5)	Michel personal communication
MFM84.61	MFM84.61(pTiMFM84.61)	Michel personal communication
MFM84.63	MFM84.63(pTiMFM84.63)	Michel personal communication
MFM84.7	MFM84.7(pTiMFM84.7)	Michel personal communication
MFM84.9	MFM84.9(pTiMFM84.9)	Michel personal communication
N4/73	N4/73(pTiN4/73)	Moore personal communication
NCPPB2655	NCPPB2655(pTiNCPPB2655)	Ryder personal communication
NCPPB2657	NCPPB2657(pTiNCPPB2657)	Ryder personal communication
NCPPB2659	NCPPB2659(pTiNCPPB2659)	Ryder personal communication
NT1	C58C1	Nester personal communication
R3	R3(pTiR3)	Moore personal communication
RR5	RR5(pTiRR5)	Anderson and Moore 1979
S1/73	S1/73(pTiS1/73)	Anderson and Moore 1979
S2/73	S2/73(pTiS2/73)	Anderson and Moore 1979
S5/72	S5/72(pTiS5/72)	Anderson 1978
S7/73	S7/73(pTiS7/73)	Anderson and Moore 1979

T10/73	T10/73(pTiT10/73)	Moore personal communication
T28/73	T28/73(pTiT28/73)	Anderson 1978
T3/73	T3/73(pTiT3/73)	Anderson and Moore 1979
T37	T37(pTiT37)	Sciaky *et al.* 1978
TP102	TP102	ATCC
TP2	TP2	ATCC
TR105	TR105(pRiTR105)	White and Nester 1980a
TR7	TR7(pRiTR7)	White and Nester 1980a
TT133	TT133(pTiT133)	Kerr personal communication

HOST PLANTS OF ORIGIN AND *AGROBACTERIUM* STRAIN SUPPLIERS.

Strain	Host of Origin	Supplier
0362	Soil isolate	Moore
1001		Nester
13333		Nester
15834		Nester
15955	Lycopersicon	Nester
25818(TR7)	Malus	Lippincott
3667	Rosa	Moore
8196		Tepfer
A136		Chilton
A175		Nester
A178		Nester
A2		Moore
A203		Nester
A208		Nester
A21/75	Prunus	Moore
A25/75	Prunus	Moore
A277		Chilton
A281		Tempé
A348		Nester
A350		Nester
A4	Rosa	Moore
A518		Nester
A519		Nester
A527		Nester
A543		Nester
A557		Moore
A596		Nester
A6	Rubus	Kerr
A723		Schell
Ach5	Prunus	Nester
Ach5C3		Nester
AT1		Moore
B1/74	Prunus	Moore
B2/74	Prunus	Moore
B234		Moore
B2A		Nester
B3/73	Acer	Moore
B4/74	Acer	Moore
B6	Malus	Nester
B6-806		Nester
Bo542		Nester
C2/74	Prunus	Moore
C3/74	Prunus	Moore
C58	Prunus	Schell
C58(pRi8196)		Ryder
C58(pRiA4)		Ryder
C58(pRiA4)		Ryder
C58(pRiTR105)		Ryder
C58C1		Tepfer
CG1C	Prunus	Moore
E8/73	Dahlia	Moore
EHA101		Hood
G1/73	Prunus	Moore
GMI9023		Nester
GV3140		Schell
GV3160		Schell

GV3245		Schell
GV3560		Schell
H100	Humulus	Nester
I1/75	Rosa	Moore
I10/75	Rosa	Moore
I7/75	Rosa	Moore
IIBV7	Chrysanthemum	Nester
K108		Ryder
K15/73	Salix	Moore
K21	Prunus	Moore
K26	Malus	Moore
K27	Prunus	Moore
K308		Ryder
K32	Prunus	Moore
K34	Prunus	Moore
K35	Prunus	Moore
K36	Prunus	Moore
K37	Prunus	Moore
K39	Prunus	Moore
K40	Prunus	Moore
K41	Prunus	Moore
K46		Moore
K47	Prunus	Moore
K49	Prunus	Moore
K6/73	Salix	Moore
LBA4404		Schilperoort
M2/73	Betula	Moore
M3/73	Betula	Moore
MFM83.4	Prunus	Michel
MFM84.1	Populus	Michel
MFM84.4	Populus	Michel
MFM84.5	Populus	Michel
MFM84.61	Populus	Michel
MFM84.63	Populus	Michel
MFM84.7	Populus	Michel
MFM84.9	Populus	Michel
N4/73	Rubus	Moore
NCPPB2655	Cucumis	Ryder
NCPPB2657	Cucumis	Ryder
NCPPB2659	Cucumis	Ryder
NT1		Nester
R3	Prunus	Moore
RR5	Rubus	Moore
S1/73	Lippia	Moore
S2/73	Lippia	Moore
S5/72	Libocedrus	Moore
S7/73	Lippia	Moore
T10/73	Rosa	Moore
T28/73	Rosa	Moore
T3/73	Rosa	Moore
T37	Juglans	Moore
TP102		ATCC
TP2		ATCC
TR105		Ryder
TR7		Ryder
TT133		Kerr

SUPPLIERS OF BACTERIAL STRAINS.

M.-D. Chilton, Ciba-Geigy Biotechnoloy Unit, Research Triangle Park, North Carolina

A. Kerr, Department of Plant Pathology, Waite Agricultural Research Institiute, Glen Osmond, South Australia

M.-F. Michel, Station d'Amelioration des Arbres Forestiers, INRA, Orleans, France

L. Moore, USDA Ornamentals Laboratory, Corvallis, OR

E.W. Nester, University of Washington, Department of Microbiology, Seattle, Washington

M. Ryder, CSIRO, Division of Soils, Glen Osmond, South Australia

J. Tempé, Institut des Sciences Végétales, C.N.R.S., Gif sur Yvette, France

D. Tepfer, Station de Genetique d'Amelioration des Plantes, I.N.R.A., Route de Saint-Cyr, Versailles, France

J. Schell, Max-Planck Institut für Zuchtungsforschung, Köln, West Germany

P. Zambryski, Molecular Plant Biology Department, University of California, Berkeley, California

American Type Culture Collection (ATCC), Rockville, Maryland

HEXAMER PRIMER PROCEDURE FOR PREPARATION OF RADIOLABELLED DNA PROBES.

Based on A.P. Feinberg and B. Vogelstein (1984) A technique for radiolabelling DNA restriction fragments to high specific activity. Anal Biochem 132: 6-13.

Solutions:

1. TM: 250 mM Tris-HCl pH 8.0

   25 mM MgCl2

   50 mM 2-mercaptoethanol

   For 10 ml; 2.5 ml 1 M Tris-Cl, pH 8.0, 0.25 ml 1 M $MgCl_2$, and 39 μl 2-mercaptoethanol, was mixed and diluted to volume with water.

2. OL: 90 units/ml hexadeoxynucleotides (Pharmacia-PL) in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Stored at -20°C.

3. DTM: 100 μM dATP, dTTP, dGTP in TM.

   For 0.2 ml; 40 μl 0.5 mM dATP, 40 μl 0.5 mM dTTP, 40 μl 0.5 mM dGTP, 80 μl TM was mixed and stored at -20°C..

4. Hepes 6.6: 1 M Hepes buffer was titrated to pH 6.6 with NaOH and stored at 4°C.

The solutions above were used to make up:

5. LS: 50 μl Hepes; 50 μl DTM; 14 μl OL. The solution was aliquotted (25 μl) and stored at -70°C.

6. Stop buffer: 25 mM EDTA; 100 mM NaCl; 10 mM Tris-Cl (pH 8.0)

7. STE: 10 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA.

Reaction:

1. Linearized DNA was denatured by boiling in water for 5 min.

2) Tubes containing DNA were cooled on ice for 5 min to 15 min.

3. The reaction mixture was set up as:

    11.4 μl LS

    1.0 μl BSA (225 μg/ml)

    5.0 μl [^{32}P]dCTP (50 μCi)

    62.5 ng DNA in 7 μl volume

    0.5 μl Klenow fragment (BRL)

    25.0 μl total volume

4. The mixture was incubated at room temperature 3 hr.
5. The reaction was stopped by addition of 75 μl stop buffer.
6. This solution was applied to the top of a 1 ml column of Sephadex G50 (Pharmacia, packed in a 1 ml syringe plugged with glass wool). The column was prepared by hydrating the Sephadex in STE, transfering the column material to the syringe, spinning 3 min at 1100 rpm (room temperature), washing twice with 100 μl STE.
7. The DNA was passed through the column by centrifuging 3 min at 1100 rpm and collected in the eluate (100 μl) in a 1.5 ml eppendorf tube.
8. Specific activity was estimated by determining TCA precipitable counts in 5 μl of the eluate. (Aliquots were spotted on glass fiber filters (Whatman) and allowed to dry. One filter was washed with 50 ml of 5% trichloroacetic acid/sodium pyrophosphate (20 mM). Radioactivity remaining on both filters was measured in NEN 963 scintillation fluid with a Packard Tricarb scintilation counter.
9. The probe was stored at -20°C.

CYTOKININ ELISA PROTOCOL.

Adapted from the protocol of:
R. Maldiney, B. Leroux, I. Sabbagh, B. Sotta, L. Sossountzov and E. Miginiac (1986) A biotin-avidin-based enzyme immunoassay to quantify three phytohormones: auxin, abscisic acid and zeatin-riboside. J. Immunol. Meth. 90: 151-158.

Solutions:

1. Coating buffer (1X): per 1 liter.

$NaHCO_3$	4.2 gm (0.05 M)
Na_2CO_3	5.3 gm (0.05 M)

Adjusted to pH 9.6 with sodium bicarbonate or HCl

2. PBS-tween (10X): per 1 liter.

NaCl	80 gm
KCl	2 gm
KH_2PO_4	2 gm
Na_2HPO_4	11.5 gm
Tween20	5 ml
NaN_3	2 gm

Adjusted to 7.2 and filtered. Stored at room temperature. Diluted to 1X for use.

3. Diethanolamine buffer (1X): per 1 liter.

Diethanolamine	97 ml
NaN_3	0.2 g
$MgCl_2.6H_2O$	100 mg

Adjusted to pH 9.8 with HCl and stored at 4°C in the dark.

4. Blocking Reagent:

PBS-tween	100 ml
Ovalbumin	1 gm

Made fresh. Centrifuged (12000 x g, 5 min) to remove particulates.

5. PBS-BSA: (used to dilute cytokinins and antibody solutions)

PBS (no tween)	100 ml
BSA	5 mg

Made fresh from 10x PBS stock.

6. Zeatin-monoclonal antibody:

Clone 16, prepared 1:100 dilution in PBS-BSA as a stock solution. Stored at -20 °C.

Diluted 1:200 with PBS-BSA before use. (Final dilution = 1:20,000).

7. PNPP:

p-nitrophenylphosphate (Sigma)	1 pellet (5 mg).
diethanolamine buffer	5 ml.

Made fresh.

8. 5N NaOH

Micro-well plate coating:

1. Coat:

   Aliquots (200 μl) of ZR-ovalbumin (0.25 μg/ml in coating buffer) were placed in each well and the plates incubated 16 hr at 4 °C.

2. Wash:

   Plates were washed four times with PBS-Tween.

3. Block:

   Each well was filled with PBS-1% ovalbumin solution and incubated 2 hr at 37 °C. Plates were covered to prevent evaporation.

4. Wash:

   Plates were washed twice with PBS-tween and drained. Covered plates were stored at -20 °C.

Assay protocol.

(Assay range for tZR = 10 fmoles to 10 pmoles)

1. To the wells were added sequentially:

   A. The samples (5 μl or 10 μl diluted to 100 μl PBS-tween before addition),

   B. Zeatin-monoclonal antibody(50 μl).

   Plate was mixed and incubated 20 min at 37 °C.

2. The sample/monoclonal antibody mixture was removed and the plate washed four times with PBS-tween.

3. An aliquot (200 μl) of rabbit anti-mouse antibody/alkaline phosphatase (Sigma, diluted 1:1000 in PBS-tween) was added to each well, and the plate incubated 25 min at 37 °C.

4. The phosphatase solution was removed and the plate washed four times with PBS-tween.

5. PNPP (200 μl) was added to each well and the plate incubated at 37 °C for 25 min.

6. Color development was stopped with addition of NaOH (5N, 20 μl) and read at A_{405}.

Concentration curve for zeatin riboside

A solution of zeatin riboside (0.067 nmol/ml in PBS-tween) was used to make a response curve for comparison to samples of unknown concentration.

ZR/well (femto mole)	ZR Solution (μl)	PBS-tween/BSA (μl)
10,000	1,000	0
5,000	500	500
1,000	200	800
500	500	500
100	200	800
50	500	500
10	200	800
5	500	500
0	0	1,000

TRI-PARENTAL MATING PROTOCOL.

Adapted from L. Brown (Microbiology Dept, Oregon State University, Corvallis Oregon. personal communication) and,

G. Ditta, S. Stanfield, D. Corbin and D.R. Helinski (1980) Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. 77, 7347-7351.

1. Cultures of helper strain HB101(pRK2013) kanamycin^r, donor strain DH5alpha(pSM358) kanamycin^r, ampicillin^r and recipient *Agrobacterium* strains were grown in 2 ml 523 media plus respective antibiotics to mid-log phase.
2. Cultures were centrifuged (3 min, 12000 g), and the pellet washed twice in minimal media (MM) broth.
3. Cells were resuspended in 1 ml MM broth to concentrate cells.
4. An aliquot (0.1 ml) of helper, donor, and recipient strains were placed in the center of a 523 plate and mixed, but not spread out across the plate.
5. Cells were incubated at 37°C for 4 hours.
6. Cells were washed from the plate with 1 ml MM broth.
7. An aliquot (100 μl) of the cell suspension was placed on a MM plate with antibiotics (kanamycin 50 μg/ml, ampicillin 50 μg/ml) and spread evenly.
8. Plates were incubated at 28 °C for 3 days to 6 days.
9. Colonies that grew up were transferred to 523 plates with antibiotics.

VIRE:LACZ INDUCTION ASSAY.

Protocol modified from:
J.H. Miller (1972) Assay of beta-galactosidase *In* Experiments in molecular genetics. Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. pp 352-355.

S.E. Stachel, G. An, C. Flores and E.W. Nester (1985) A Tn*3* *lacZ* transposon for the random generation of *beta*-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. 4: 891-898.

Solutions:

1. Inducing compound stocks: Stocks (20 mM) were dissolved in dimethylsulfoxide. Add 10 μl of stock per ml media after autoclaving. (Add an equal amount of dimethyl-sulfoxide to media for control cultures.)

2. Z buffer salts (5X concentrate):

   0.25 M sodium phosphate pH 7.0
   0.05 M KCl
   0.005 M $MgSO_4$

3. SDS: sodiumlauralsulfate 10% (w/v) in water

4. Z5 buffer (make fresh):

Z buffer salts	20 ml
2-mercaptoethanol	270 μl
SDS	1.0 ml

5. ONPG: o-nitrophenyl-beta-D-galactoside 4 mg/ml in 1X Z buffer

6. Stop Buffer: Na_2CO_3 (1.0 M)

Methods:

Cell Culture

1. A starter culture was prepared by inoculating 10 to 50 ml VIM-4 containing kanamycin (50 μg/ml) and ampicillin [50 μg/ml or 10 μg/ml for strains B3/73(pSM358), M2/73(pSM358), K108(pSM358), 3667(pSM358), K47(pSM358), K41(pSM358)] Shake cultures were and grown 16 to 36 hours.

2. An aliquot (5 μl to 50 μl) of samples to be assayed for induction activity were placed in micro-well plates and the solvent evaporated (Savant SpeedVac). Dried plates were stored at -20 °C.
3. Cells of the starter culture were centrifuged (3 min 12000 g) and resuspended in a volume of VIM4 to produce a density of 0.005 (per 100 μl) as read by the micro-well plate reader (Molecular Devices VMAX, A_{650}).
4. The cell culture (100 μl) was added to sample wells, and the plates incubated 12 hr at 28 °C on a rotary shaker (Labline).

Note: For time course assays, cultures were inoculated into flasks (125 ml or 50 ml) containing VIM4 media (25 ml or 10 ml) with the inducing compound (200 μM). After incubation, aliquots (100 μl) were removed to micro-well plates and assayed as described below.

Beta-galactosidase assay:

1. Cells were pelleted by centrifugation of the micro-well plate (10 min, Savant Speed-vac) and the supernatant aspirated off.
2. Cells were washed by adding 100 μl VIM4, mixing, and repeating centrifugation and aspiration steps. (This wash step was necessary for removal of compound present in *P. menziesii* partition fractions which turned color with the addition of base.)
3. VIM4 (100 μl) was added, the cells resuspended with a vortex mixer, and culture density read with the micro-well plate reader (A_{650}).
4. Z5 buffer (20 μl) was added, mixed and the plate incubated 10 min at room temperature.
5. ONPG (50 μl) was added and color development was immediately measured (VMAX kinetic reading A_{405}) for 2 min.
6. For end point assays the reaction was stopped after 3 min by adding sodium carbonate (50 μl), and color development measured as A_{405} minus A_{650}.

7. The specific culture activity was calculated as:

(Activity per well)/(Culture density per well)

Color development was converted to nanomoles of product using the factors below. As measured on the VMAX reader in 100 μl volumes of Z-buffer adjusted to the appropriate pH, an absorbance reading of 1.0 measured at 405 nm is produced by:

130.6 nmol o-nitrophenol at pH 7

94.5 nmol o-nitrophenol at pH 10

30.3 nmol p-nitrophenol at pH 7

17.9 nmol p-nitrophenol at pH 10

BACTERIAL CULTURE MEDIA

523 Medium

For maintenance of *Agrobacteria* and *E. coli*.

Reference:
Kado, C.I. and Heskett, M.G. (1970) Selective media for isolation of *Agrobacterium* Corynebacterium, Erwinia, Pseudomonas, and Xanthomonas. Phytopathology 60: 969-976.

For 1.0 liter:

Casamino acids	8.0 gm
Yeast extract	4.0 gm
K2HPO4	2.0 gm
MgSO4.7H2O	0.3 gm
(NH4)2SO4	2.0 gm
sucrose	10.0 gm
agar	15.0 gm

Note: ammonium sulfate is an addition to the original formulation.

BTB Medium: Bromothymolblue Indicator Medium

Reference:
Hooykaas, P.J.J. Roobol, C. and Schilperoort, R.A. (1979) Regulation of the transfer of Ti plasmids of *Agrobacterium tumefaciens*. J. Gen. Microbiology 110: 99-109.

For 1.0 liter:

K2HPO4	0.09 gm
NaCl	0.15 gm
MgSO4.7H2O	0.50 gm
CaCl2.2H2O	0.07 gm
Glucose	2.0 gm
Nopaline	0.1 gm
Agar	18.0 gm

Use NaOH to adjust the pH to 7.1

Agrobacterium colonies which are able to catabolize the nopaline appear yellow-orange due to a local drop in the pH. The original formulation uses octopine rather than nopaline, and is therefore suitable for octopine strains.

DEF1:

Medium for *A. tumefaciens* culture based on BTB medium (above).

For 1.0 liter:

K_2HPO_4	0.09 gm
NaCl	0.15 gm
$MgSO_4.7H_2O$	0.1 gm
$CaCl_2.2H_2O$	0.07 gm
$NaNO_3$	0.15 gm
Sucrose	2.0 gm

Adjust the pH to 7.0

Selective Media for *Agrobacterium* isolation.

Reference:

Brisbane and Kerr (1983) Selective media for three biovars of *Agrobacterium*. J. Applied Bacteriol. 54: 425-431.

Kerr Biovar I For 1.0 liter

L-arabitol	3.04 gm
NH_4NO_3	0.16 gm
KH_2PO_4	0.54 gm
K_2HPO_4	1.04 gm
$MgSO_4.7H_2O$	0.25 gm
Sodium taurocholate	0.29 gm
Crystal violet (0.1% aqueous)	2.0 ml
Agar	15.0 gm

Autoclave 20 min. Cool to 50 °C
Before pouring plates, add:

cyclohexamide (actidione, 2% aqueous)	10.0 ml
$NaSeO_3.5H_20$ (1% aqueous)	10.0 ml

Kerr Biovar II For 1.0 liter

erythritol	3.05 gm
NH_4NO_3	0.16 gm
KH_2PO_4	0.54 gm
K_2HPO_4	1.04 gm
$MgSO_4.7H_2O$	0.25 gm
Sodium taurocholate	0.29 gm
yeast extract (1% aqueous)	1.0 ml
Malachite green (0.1% aqueous)	5.0 ml
agar	15.0 gm

Autoclave 20 min. Cool to 50 °C
Before pouring plates, add:

cyclohexamide (actidione, 2% aqueous)	10.0 ml
$NaSeO_3.5H_20$ (1% aqueous)	10.0 ml

Kerr Biovar III (to prepare 1 liter)

Solution A:

water	500 ml
sodium tartrate.2H2O	5.75 mg
D glutamic acid (4% aqueous, pH 7.0)	15.0 ml
$NaH_2PO_4.2H_2O$	6.24 gm
Na_2HPO_4	4.26 gm
NaCl	5.84 gm
$MgSO_4.7H_2O$	0.25 gm
Sodium taurocholate	0.29 gm
yeast extract (1% aqueous)	1.0 ml
Congo red (1% aqueous)	2.5 ml

Solution B:

water	500 ml
$MnSO_4.4H_2O$	1.12 gm
agar	15 gm

Autoclave separately, 20 min. Cool to 50 °C.

Before pouring plates, add to solution B:

1. cyclohexamide (actidione, 2% aqueous) 10.0 ml
2. $NaSeO_3.5H_20$ (1% aqueous) 5.0 ml
3. solution A

Mix well to evenly distribute the precipatate which forms)

MM media for triparental mating:
From L. Brown (Microbiology Dept, Oregon State University, Corvallis Oregon)

A) 100X salts: per liter

1.0 g NaCl
2.0 g MgSO4-7H2O
5.0 g NH4Cl
2.3 g KH2PO4
2.3 g Na2HPO4

Mix and autoclave

B) 500X trace minerals: per 100 ml

2.5 g CaCl2-2H2O
72.5 mg H3BO3
62.5 mg FeSO4-7H2O
30.0 mg CoCl2-6H2O
2.5 mg CuSO4-5H2O
2.5 mg MnCl2-4H2O
55.0 mg ZnSO4-7H2O
70.0 mg Na2MoO4-2H2O
120.0 mg FeEDTA

Note: not all solids dissolve completely, filter sterilize as is.

C) 1000X vitamin mixture: per 50 ml

10mg each of riboflavin, para-aminobenzoic acid, nicotinic acid, biotin, thiamine-HCl, Pyridoxine-HCl, Ca-pantothenate and *myo*-inositol.

Filter sterilize.

For 500 ml media:

1. Add 5 ml 100X salts to 245 ml ddH_2O
2. Combine 7.5 g bactoagar, 5.0 g mannitol and 250 ml ddH2O
3. Autoclave both of these solutions, cool to 60°C, and then combine.
   -add 0.5 ml 1000X vitamins
   -add 1.0 ml 500X trace elements
   -add antibiotics
   -pour plates

Agrobacterium vir gene induction media.
Modified from: Stachel, S.E. and Nester, E.W. (1986) The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. 5: 1445-1454

VIM1	For 1.0 liter
M&S salts	0.5 gm
K2HPO4	0.23 gm
KH2PO4	1.8 gm
myo-inositol	1.0 gm
Bacto-tryptone	1.0 gm
Yeast extract	0.5 gm
Sucrose	10.0 gm

Variations on VIM1:

VIM-G

For sucrose substitute

Glycerol	10.0 ml

VIM-GC

For tryptone, yeast extract and sucrose substitute:

Casamino acids	1.0 gm
Glycerol	10.0 ml

VIM4	For 1.0 liter
M&S salts	0.5 gm
K_2HPO_4	1.0 gm
$MgSO_4$	0.1 gm
NaCl	0.2 gm
$(NH4)_2SO_4$	1.0 gm
$CaCl_2.2H_2O$	0.07 gm
Yeast extract	1.0 gm
Glycerol	10.0 ml

For all VIM formulations pH adjusted to 5.6 with HCl or NaOH as required.