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# *Agrobacterium tumefaciens* and the susceptible plant cell

## The organization, expression, and regulation of the virulence genes of the A6 Ti plasmid

by

Scott E. STACHEL

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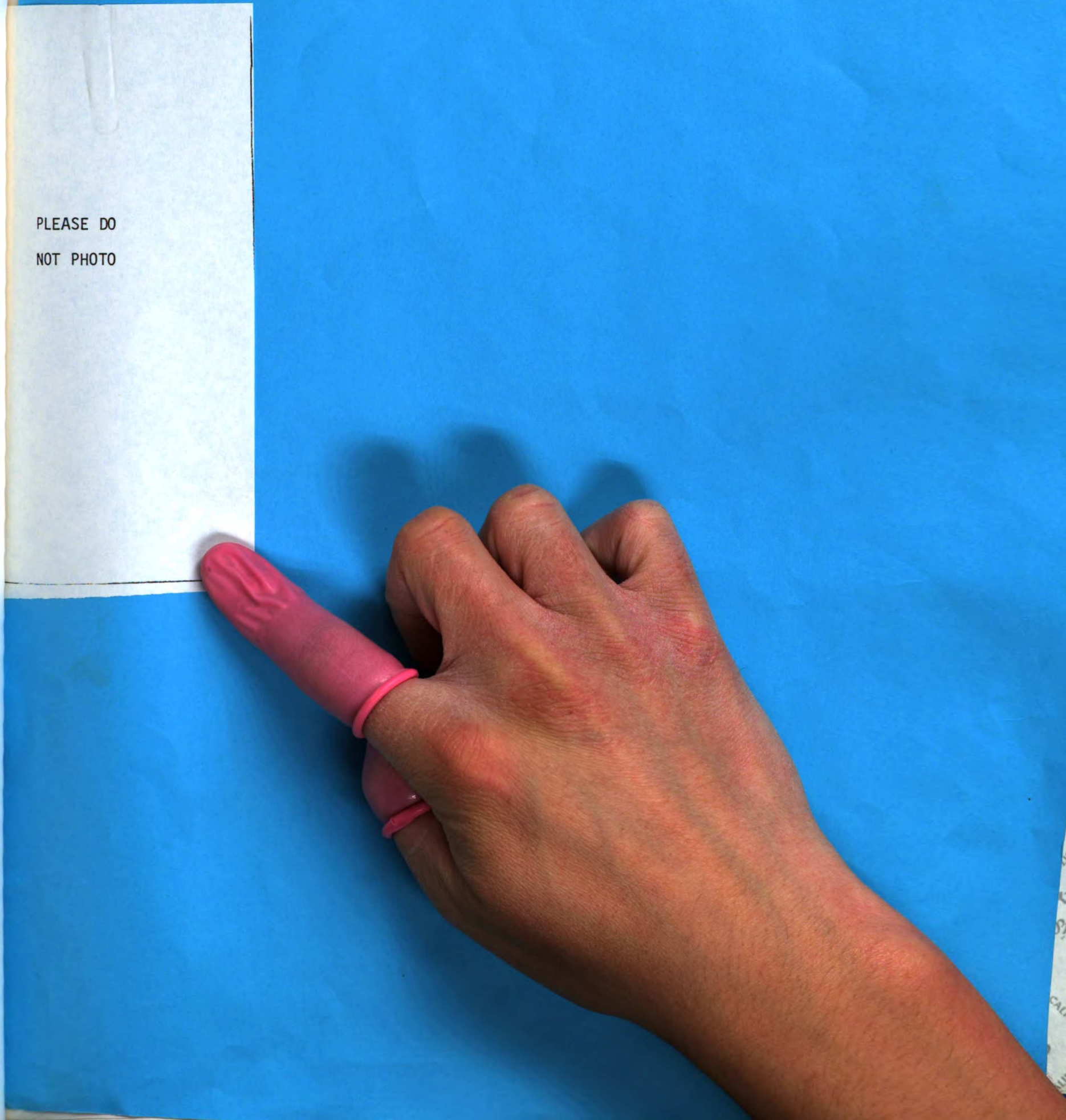

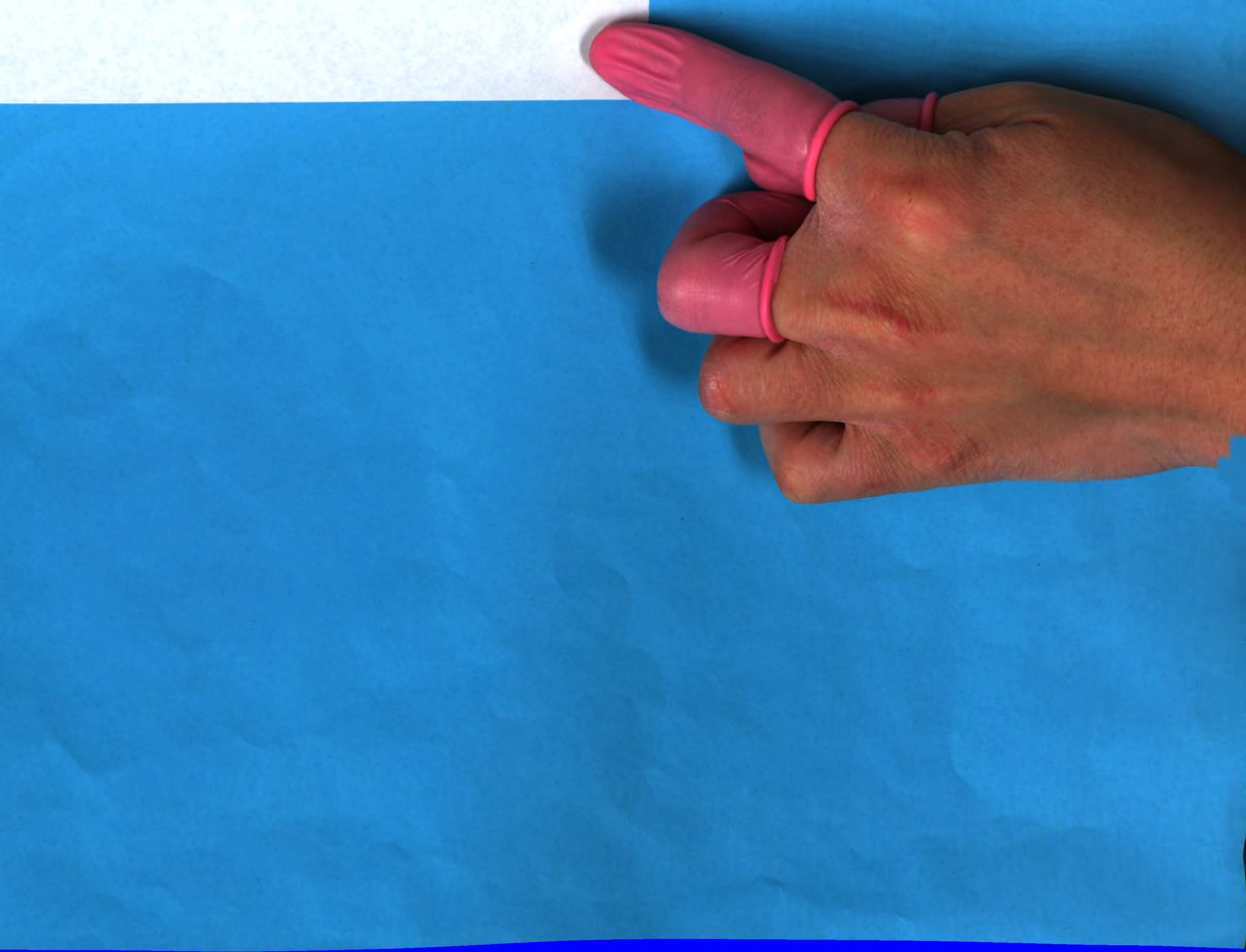

# AGROBACTERIUM TUMEFACIENS AND THE SUSCEPTIBLE PLANT CELL

## The organization, expression, and regulation of the virulence genes of the A6 Ti plasmid

Scott E. STACHEL

To Patti and Gynheung for their guidance, collaboration, and friendship; and to my dad.

PREFACE

The production of this thesis has perhaps been unusual – several years have passed and different laboratories have been passed through since it was begun. The research work (and researcher) was initiated in the laboratory of Howard Goodman at the University of California, San Fransisco, and has since travelled to the laboratory of Eugene Nester at the University of Washington (Seattle), and finally to the laboratory of Marc Van Montagu and Patricia Zambryski at the University of Gent (Belgium). I am greatly indebted to Howard, Gene, Marc and Patti for their encouragement and support during this sojourn, and also to the Department of Biochemistry at UCSF for allowing me the freedom to study in different laboratories. I would especially like to thank Harold Varmus for his support and willingness to serve as my thesis adviser, and to Sue Adams for her unshakable patience and help. I would also like to thank all of my colleagues for their advice, help, and refusal to allow me to quit the bench for the golden sands of a remote tropical island; and to Martine De Cock, Karel Spruyt, and Albert Verstraete for the preparation of this thesis and its figures.

The text of this thesis consists of seven chapters – introduction, five research chapters, and conclusion. The research chapters have been written for publication as research articles; chapters 2, 4, and 5, have been previously published while chapters 3 and 6 are presently under review (as of March 1986). A few sentences and figures not present in the original texts of these manuscripts have been added, and are set off by brackets. The text of chapter 2 is a

reprint of "A Tn*3* *lacZ* transposon for the random generation of β-galactosidase gene fusions : application to the analysis of gene expression in *Agrobacterium*", by S.E. Stachel, G. An, C. Flores, and E.W. Nester, as it appears in *EMBO J.* 4, 891-898 (1985); the text of chapter 4 is a reprint of "A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression" by S.E. Stachel, E.W. Nester, and P.C. Zambryski, as it appears in *Proc. Natl. Acad. Sci. USA* 83, 379-383 (1986); and the text of chapter 5 is a reprint of "Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*", by S.E. Stachel, E. Messens, M. Van Montagu, and P. Zambryski, as it appears in *Nature* (London) 318, 624-629 (1985). Chapters 3 and 6 have been submitted as articles by S.E. Stachel, and E.W. Nester, and by S.E. Stachel and P. Zambryski, respectively.

Because the research chapters were written as self-inclusive articles, there is some overlap between their contents, especially with regard to their introductions and references. Some overlap also exists between these introductions and chapter 1 (introduction). I apologize for this repetition and hope that it does not prove to be inconvenient or distracting to the reader.

During the time of my doctoral work I hope that I have begun to learn to be a scientist – to design, carry out, and analyze the results of experiments, and perhaps answer a few questions about how things work. If I have gained any of this knowledge it is because of the invaluable guidance of two people, Patricia Zambryski in Gent, and Gynheung An in Seattle, to whom this thesis (along with my dad), is dedicated.

# Agrobacterium tumefaciens and the susceptible plant cell
## The organization, expression, and regulation of the virulence genes of the A6 Ti plasmid

Scott E. Stachel

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ABSTRACT

The phytopathogen Agrobacterium tumefaciens genetically transforms wounded plant cells to cause crown gall disease. During the transformation process the bacterium transfers a specific segment of DNA, the T-DNA, from its large (> 200 kb) Ti plasmid to the plant cell where it becomes integrated into the nuclear genome. The Ti plasmid genes which direct these events are located outside the T-DNA within the large (> 40 kb) virulence (vir) region. This thesis examines the genetic and transcriptional organization of the pTiA6 vir region, and the relationship between the expression of its vir genes and plant cells susceptible to Agrobacterium transformation.

The Tn3-lac element Tn3-HoHo1, was constructed to characterize the pTiA6 vir region. This transposon mutagen is also a reporter for the expression of the sequences into which it has inserted since it generates lacZ gene fusions. Tn3-HoHo1 was inserted throughout the pTiA6 vir region to construct : (i) a set of vir::lac gene fusions used to establish that vir sequences become transcriptionally activated when Agrobacterium is cocultivated with plant cells; and (ii) a set of vir insertion mutations used to determine that the pTiA6 vir

region contains 6 vir complementation groups. Mutations in these loci eliminate (virA, virB, virD, virG) or restrict (virC, virE) Agrobacterium pathogenicity. Each vir locus corresponds to a single transcription unit : virA is constitutively expressed and non-inducible; virB, virC, virD, and virE are expressed only upon activation by plant cells; and virG is both constitutively expressed and plant-inducible.

Plant induction of vir requires two components : a signal produced by plant cells, and a bacterial regulatory system that allows the bacterium to recognize and respond to this signal. The vir::lac gene fusions and vir insertion mutants were used to characterize these components. (i) The vir-inducing molecules produced by Nicotiana tabacum root and leaf tissue were purified and identified to be the phenolics acetosyringone and α-hydroxy-acetosyringone. These compounds were seen to be specific to exudates of wounded and activity metabolizing plant cells, the natural targets of Agrobacterium. (ii) Mutations in virA and virG attenuate and eliminate vir induction, respectively. These loci encode trans-acting functions which together with plant signal molecules control vir expression in a complex fashion.

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| | |
|---|---|
| AS | acetosyringone |
| bp | base pair |
| Cb | carbenicillin |
| *chv* | chromosomal virulence genes |
| *cml* | conditioned medium-leaves |
| *cmr* | conditioned medium-roots |
| D | daltons |
| kb | $10^3$ bp |
| Km | kanamycin |
| Nal | nalidixic acid |
| *occ* | octopine catabolism genes |
| OH-AS | α-hydroxy-acetosyringone |
| ONPG | O-nitrophenyl-β-galactoside |
| SDS | sodium dodecyl sulfate |
| *vir* | Ti plasmid virulence genes |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

# CHAPTER 1

## Introduction

The soil phytopathogen Agrobacterium tumefaciens genetically transforms plant cells to cause crown gall, a neoplastic disease of dicotyledonous plants (reviewed in [1-3]). In nature, only plant cells that have been wounded are seen to be susceptible to crown gall. The interaction between Agrobacterium and these cells is remarkable since it is the only known example of the directed insertion of prokaryotic DNA into a eukaryotic genome. In the transformation process, the bacterium transfers a specific segment of DNA, the transfer-DNA, from its large (> 200 kb) tumor-inducing Ti plasmid to the suceptible plant cell. The T-DNA then becomes integrated into the plant nuclear genome, and expression of T-DNA genes within the transformed cell results in its neoplastic growth, and the synthesis of novel compounds, called opines. Opines are specific to crown gall cells, and Agrobacterium, unlike other soil microbes, has the ability to utilize these compounds as a sole source of carbon and nitrogen.

Besides the T-DNA, the Ti plasmid carries two other sets of genes important to the Agrobacterium/plant cell interaction : the opine utilization genes which allow the bacterium to catabolize opines, and the virulence (vir) genes which direct the events of the T-DNA transfer process. The analysis of the genetic and transcrip-

tional organization of the vir genes, and their regulated expression by plant cells susceptible to Agrobacterium infection, are the subjects of this thesis.

The interaction between Agrobacterium and plant cells presents a number of biologically interesting concepts and has been widely studied throughout the present century. For example, (i) because crown gall cells are neoplastic and display either an undifferentiated or teratomatic growth phenotype, they can provide potential insight into how growth and differentiation are controlled in plant cells, and perhaps also in animal cells; (ii) the T-DNA transfer process carries out a complex series of events that results in the unidirectional exchange of DNA between two widely different organisms, and understanding these events can provide insight into other DNA mobile-element phenomena, such as transposition and conjugation; (iii) the T-DNA transfer process provides a natural vector system for the transformation of plant cells with any DNA of interest; and 4) the Agrobacterium-plant cell interaction provides a model system for the study of how bacterial-plant cell interactions are initiated and maintained in the biologically and chemically complex soil environment. Each of these subjects is briefly discussed below.

## T-DNA-transforming genes

The neoplastic growth of crown gall tumor cells provided an early interest because of its possible relation to the neoplastic growth of virally-transformed animal tumor cells (reviewed in [4]). Both of these systems involve the integration of foreign DNA that

carries genes whose subsequent expression in the transformed cell leads to its neoplastic and often dedifferentiated phenotype. In both systems, the transforming genes encode functions involved in cellular growth and/or differentiation; however, the types of functions and their evolutionary origins are different.

The animal viruses, exemplified by the retroviruses, carry transforming genes that are typically derived from cellular prototypes which encode a variety of functions (for example, protein tyrosine kinases, adenylate cyclase regulators, growth factor analogues) that have a variety of cellular locations (for example, plasma membrane, golgi, cytoplasm, nucleus) [5-7, and references therein].

In contrast, the T-DNA transforming genes encode novel enzymatic pathways for the biosynthesis of the phytohormones auxin and cytokinin [8-14]. The elevated levels of these molecules in crown gall cells is responsible for their neoplastic and hormone-independent growth. Also in contrast to animal cell-transforming genes, the T-DNA-transforming genes appear to have an independent evolutionary origin than their host (plant) analogues; the plant-encoded enzymatic pathways for auxin and cytokinin biosynthesis carry out different chemical reactions than those specified by the T-DNA genes.

The elucidation of the T-DNA encoded phytohormone pathways has provided further insight into the metabolism of auxin and cytokinin in both normal and transformed plant tissues. For example, plant cells normally are able to control their auxin levels by converting auxin to conjugates that have only low growth factor activity. The

T-DNA-directed auxin pathway circumvents this control pathway by utilizing the auxin conjugates as substrate for auxin synthesis [15].

## T-DNA Transfer process

The T-DNA transfer process carries out a complex series of events : a specific segment of DNA is recognized in and mobilized from the Ti plasmid, transferred across the cell walls of the bacterium and plant cell, and integrated as a linear nonpermuted fragment into the plant nuclear genome. The molecular details of these events are largely unknown. For example, although only the T-DNA is seen to be integrated into the plant genome, it has not been established whether Agrobacterium transfers the T-DNA alone as a specific intermediate molecule, the entire Ti plasmid, or all of its DNA, to the plant cell. Research into the events of the T-DNA transfer process has focused on determining (i) what defines the T-DNA; (ii) what are the mechanisms of transfer; and (iii) what are the genetic requirements for transfer other than the T-DNA.

During transfer, the T-DNA functions solely as a structural element, since its internal portion can be fully deleted without affecting its transfer [16-18]. In the Ti plasmid the T-DNA is bounded by essentially identical 25-bp direct-repeats. These sequences fully define the T-DNA, since any DNA located between T-DNA borders will be efficiently transferred and integrated into the plant cell genome [19-23]. In fact, it is not required that the T-DNA be carried by the Ti plasmid : if a DNA segment containing

T-DNA borders is located on a separate plasmid or the Agrobacterium chromosome in a cell that also carries a complete complement of the genes which are essential for transfer, this DNA will be transferred to the plant cell during infection [24; A. Depicker, unpublished results; S. Stachel, unpublished results]. Because only DNA between the T-DNA borders is seen to be transferred to the plant genome, these border sequences likely are the structural substrates of the proteins that directly mediate the tranfer process.

To date there are only a few clues into the events related to the transfer and integration of the T-DNA. Genetic analyses of the 25 bp terminus sequences have demonstrated that these sequences are polar in function [25]. While deletion of the left border repeat has no significant effect on pathogenicity, deletion of the right repeat totally abolishes it. Furthermore, when the orientation of the right border is reversed with regard to its natural orientation, the efficient transfer and/or integration of the T-DNA sequences is greatly attenuated. These results suggest that the transfer of the T-DNA might occur in a polar fashion, determined by the orientation of the 25-bp border repeats.

With regard to T-DNA integration, structural analysis of the T-DNA/plant DNA junctions from independent integration events in different plant cell tumor lines has shown that the integration process is relatively precise with respect to the T-DNA border sequences [19-23]. For example, a number of studies have shown that the integration event, with respect to the T-DNA, can occur directly within, or in a few cases close to (from 7 to 92 bp inside of) the 25-bp sequence. These studies have also indicated that there is

little or no specificity for the plant sequences present at the integration site, and that deletions and/or rearrangements of plant sequences are often associated with T-DNA integration.

While the studies cited above have conclusively shown that the T-DNA borders are essential for T-DNA transfer and integration, they do not identify how they are recognized and utilized during the early stages of the infection process. Recent evidence has indirectly suggested that transfer might involve a circular T-DNA intermediate that is produced by a site-specific recombination event between the T-DNA borders [26]. Specifically, the T-DNA of the pGV3850 Ti-plasmid carries the E. coli cloning vehicle pBR322. When E. coli is transformed with total (undigested) DNA prepared from pGV3850 Agrobacteria, pBR322::T-DNA transformants are obtained if the bacteria have been cocultivated with plant cells susceptible to Agrobacterium infection. These transformants consist of the entire T-DNA region of pGV3850 that has been circularized and contains a single T-DNA border sequence. The circular junction site of this molecule occurs precisely within the 25 bp T-DNA border sequences of pGV3850. This circular T-DNA molecule is an attractive candidate for the molecule that Agrobacterium transfers to the plant cell. However, the detection of circular T-DNAs in E. coli only indirectly suggests that they are produced in Agrobacterium itself. Also, if T-DNA circles are formed in Agrobacterium they may serve either as the template for the synthesis of the actual T-DNA molecule to be transferred to the plant cell, or they may simply be a by-product of the molceular events which occur during the generation of the transferred molecule.

Several different systems that mediate the directed movement and/or rearrangement of specific DNA elements have been identified and studied. These mobile element systems include plasmid conjugation between bacteria [27], transposable elements in both prokaryotes (i.e., Tn3, Tn10) [28,29] and eukaryotes (i.e., Ac, Spm, copia, Ty1) [30-33], various types of viruses (i.e., Mu-type phages, lambdoid phages, retroviruses) [34-36], and a number of directed developmental gene rearrangements (i.e., mating-type switching in yeast, generation of immunoglobulin genes, phase-variation in Salmonella, antigenic variation in trypanosomes, antigenic variation in Borrelia) [38-41]. These systems can be classed according to a number of different properties, for example, whether genes are transferred between separate cells, whether the movement of the DNA element results in specific developmental changes, and whether as a part of its movement the mobile element integrates into genomic sequences or is episomally maintained.

While the Agrobacterium T-DNA transfer system shares certain features with several of these other systems, it is unique in being the only known example of the directed transfer and integration of DNA between widely different organisms. Whether the mechanisms of T-DNA transfer and integration are functionally similar to other mobile element systems cannot yet be determined. The structure of the T-DNA, with its direct repeats, is different than the structure of most transposon-like elements (such as the transposoble elements, retroviruses, immunoglobin gene segments) which are bounded by indirect repeats.

On the other hand, the T-DNA transfer system does share certain properties with other systems. For example, analogous to plasmid conjugation, T-DNA transfer appears to have a polar step; also, the circular T-DNA molecules which have been proposed to represent the transfer intermediate, has a similar structure to phage λ prior to its integration into the E. coli genome. Obviously much further work is required to define the T-DNA transfer process, and whether it shares mechanisms with other mobile element systems.

Three distinct sets of Agrobacterium genetic elements essential to the T-DNA transfer process have been defined, the T-DNA border repeats, the chromosomal virulence chv genes and the Ti plasmid virulence vir genes [42]. While the T-DNA border repeats are cis-acting elements, the chv and vir genes function in trans. The chv genes are organized within two separate genetic loci, chvA and chvB, located on a 12-kb segment of the Agrobacterium chromosome [43]. These genes appear to be involved in the specific binding of Agrobacterium to plant cells during the infection process, and their expression is constitutive.

The vir genes are organized within several distinct loci located on an approximatively 40-kb segment of the Ti plasmid (reviewed in chapter 3). The products encoded by these loci probably mediate the events of the T-DNA transfer process since their expression (as shown in the present work) is limited to when Agrobacterium is in the presence of specific phenolic compounds produced by plant cells susceptible to Agrobacterium transformation [44; chapter 5]. An understanding of the organization, expression, and regulation of the expression of the vir loci is essential to the understanding of the

T-DNA process. This thesis was initiated with the goal of providing this information.

## Use of *Agrobacterium* for the genetic engineering of plant cells

Even without knowing how the *vir* loci function to mediate T-DNA transfer, *Agrobacterium* has recently been developed as an important vector system for the genetic engineering of plant cells [3, 45-47]. Based on an understanding of the genetic organization of the T-DNA region (i.e., knowing which sequences specify the tumorous phenotype [48] and which sequences are required for transfer [25]), has led to the development of modified Ti plasmids that promote DNA transfer without causing crown gall in the transformed plant cell. Specifically, the T-DNA oncogenes were removed from the Ti plasmid, and T-DNA vectors were constructed such that any DNA of interest can be easily inserted between T-DNA border sequences. Several different vectors have been constructed in the past few years which allow the transfer and integration of foreign DNA into plant cells without interfering with the normal growth and differentiation of the transformant [16,18,49,50]. One of the unique characteristics of the *Agrobacterium* DNA transfer system is that there is no specific limit to the size of the transferred DNA, and DNA from 4.5 kb [51] to 37 kb [52] has been observed to be stably transferred.

To date the *Agrobacterium* system has been widely used as a tool in research aimed at understanding how the expression of specific plant genes is regulated. For example, the 5' regions of several genes involved in the photosynthetic pathway have been dissected *in*

vitro using recombinant DNA technology, and then reintroduced into plant cells using Agrobacterium to identify sequences which control the induction of gene expression in the presence of light [53-56]. Developmentally regulated sequences have also been investigated; for example, soybean-specific seed lectin genes, reintroduced into tobacco using Agrobacterium, have been observed to be correctly regulated in the heterologous tobacco cell (J. Okamuro, D. Jofuku, and R. Goldberg, unpublished results). This type of research is only in part limited to the availability of interesting genes to study; the other limitation is that not all plant species are amenable (i) to culture in the laboratory, or (ii) to infection by Agrobacterium. Presently, the vector potential of Agrobacterium has been applied to particular dicotyledonous plants which exhibit both these properties, and tobacco and petunia are the most frequently used source of plant material for Agrobacterium transformation. Presumably a better understanding of both the functions encoded by the vir loci and their transcriptional regulation might help lead to the application of the Agrobacterium transformation system to other plant species.

The genetic transformation of plant cells employing either the natural transfer system of Agrobacterium, or one of the other recently developed plant transformation techniques (i.e., direct DNA transfer [57-59]), has a significant experimental advantage over the genetic transformation of animal cells. A transformed plant cell, unlike an animal cell, can often be regenerated into a fully developed adult organism, to allow the analysis of the transformed gene at the multicellular level. For example, the differential expres-

sion of the gene can be assessed in all the cell types of the plant and the effect(s) of this expression on development can be determined. Furthermore, the "transgenic" plant is typically capable of setting seed so that the inheritance of the transformed gene in successive generations can be assessed.

While transgenic animals can also be generated (i.e., fruit flies, mice), this technology at present is laborious and large numbers of transformants can not be easily obtained. In contrast, the generation of transgenic plants for a number of different dicotyledonous species has proven to be relatively simple. Furthermore, large numbers of transformants that can be regenerated to whole plants (> $10^7$) can be readily obtained for study.

## Bacterial/plant cell interactions

Soil bacteria can often form specialized pathogenic or symbiotic interactions with plant cells, and typically a particular bacterium interacts with only one or a few species and/or types of plant cells [60]. For example, Pseudomonas syringae pathovar morsprunorum causes cankers of stone fruit trees, and particular isolates exhibit absolute specificity for either plum or cherry [61]; Pseudomonas savastanoi, which causes tumorous outgrowths, is restricted to olive and oleander [62]; and different species of Rhizobium, which invade the roots of certain plants (family Leguminosae) to effect the development of nitrogen-fixing root nodules (that the bacteria inhabit as endosymbionts termed bacteroids), exhibit great specificity for the particular plant species that is nodulated [63].

Bacterial/plant interactions are mediated, in part, by one or more bacterial genes, which are often carried within the bacterium on specialized plasmids [64]. These genes typically code for highly specialized functions which allow the bacterium to, for example, penetrate the external surface of the plant cell [65], synthesize phytohormones [66] or toxins [67] or direct the development of nitrogen-fixing nodules [68]. The constitutive expression of such functions would be disadvantageous to the bacterium, since it will not always be in the presence of plant cells susceptible to its interaction. Thus, it is important to the bacterium to be able to limit the expression of these genes to the appropriate environmental condition when their expression is beneficial to its increased survival. Such regulation requires at least two distinct components : an environmental signal that represents the susceptible plant cell, and a bacterial regulatory system that allows the bacterium to recognize and respond to this signal.

The rhizosphere is a complex biological and chemical environment, and the signals that mediate the initiation and development of an interaction between a bacterium and a specific plant cell have not been previously identified. Such signals could include soluble compounds produced in plant cell exudates, or might involve the direct physical contact of the bacterium with the plant cell. The mechanisms of bacterial recognition of and response to the plant signal also have not been identified. The Agrobacterium/plant cell interaction that results in crown gall represents a model system for both the identification of a plant signal, and the analysis of the components of a bacterial sensory/response system through which the

plant signal activates the expression of bacterial genes that mediate a bacterial/plant cell interaction.

In the case of Agrobacterium, these genes are the Ti plasmid vir genes, which direct the T-DNA transfer process. A major portion of my thesis work has been to identify and characterize both the plant signal which mediates the transcriptional activation of the vir loci, and the components of the Agrobacterium regulatory system that together with this signal direct the activation of vir expression and the T-DNA transfer process.

## Outline of thesis research

At the beginning of my thesis work, knowledge of the Ti plasmid virulence region was limited. This region had been identified as an approximately 40 to 50-kb segment of the Ti plasmid in which mutations resulted in either the loss or attenuation of bacterial pathogenicity. However, neither the detailed genetic organization of this region, nor its regulated expression were known. I set out to obtain this information, with the specific goal of understanding how the first steps of the T-DNA transfer process might be signalled by the plant cell.

The experiments described in chapters 2–6 are largely based on a single observation that the vir loci of the A6 Ti plasmid become transcriptionally activated when Agrobacterium is cocultivated with plant cells susceptible to its transformation. This observation has generated three lines of research, which include (i) the determination of the genetic and transcriptional organization of the vir loci

within the pTiA6 vir region; (ii) the purification, identification, and characterization of the plant molecules which signal vir induction and the activation of the T-DNA transfer process; and (iii) the identification and characterization of the vir genes whose products control the plant induction of the vir regulon.

To begin these studies, the Tn3-lac transposon mutagen, Tn3-HoHo1, was constructed, and a method for using this element to study Agrobacterium gene expression was developed. This work is described in chapter 2 [69]. Briefly, Tn3-HoHo1 is both an insertion mutagen and a reporter for the expression of the sequences into which it has inserted. This element carries a promoterless lacZ gene that is in-frame through its left-end inverted-repeat. Transposition of Tn3-HoHo1 into target DNA sequences results in the random generation of insertional mutations in, and lac fusions to, genes within the target sequences, and both transcriptional and translational lacZ fusions can be generated with this element. The transcriptional fusions express a β-galactosidase protein (the lacZ gene product) completely encoded within Tn3-HoHo1 that is dependent on external transcription; the translation fusions express chimeric β-galactosidase fusion proteins that depend both on external transcription and translation. This element was initially applied to the analysis of the expression of the octopine-inducible octopine catabolism operon (opine utilization genes), and of the T-DNA genes, in Agrobacterium.

As presented in chapter 3 [70], Tn3-HoHo1 was then applied to the analysis of the vir region of the A6 Ti plasmid. In the course of these experiments two sets of experimental tools were generated – a set of vir mutant strains that allowed both the determination of

the vir region sequences required for virulence, and the genetic organization of these sequences; and a set of gene fusions between each of the vir loci and lacZ, that allowed the determination of the vir region sequences which are transcribed, the transcriptional orientation of these sequences, and the conditions under which their expression occurs. Also, these gene fusions were essential to the discovery that plant cells signal the transcriptional activation of particular vir region sequences.

The results of the experiments presented in chapter 3 demonstrated that the pTiA6 vir region is composed of 6 distinct vir complementation groups, virA, virB, virC, virD, virE, and virG. Mutations in these loci eliminate (virA, virB, virD, virG) or restrict (virC, virE) the ability of Agrobacterium to transform plant cells. Each of the vir loci corresponds to a single vir transcription unit : virA is constitutively expressed and non-inducible; virB, virC, virD, and virE are expressed only upon activation by plant cells; and virG is both constitutively expressed and plant-inducible. The pTiA6 vir region also contains plant-inducible pin loci which are non-essential for virulence.

The two sets of experimental tools generated in the studies of chapter 3 also provided the foundation for the experiments presented in chapters 4, 5, and 6. In these chapters the two components of the bacterial/plant interaction – the plant cell factor and the vir regulatory system – that together mediate the plant-induced activation of Agrobacterium vir gene expression are characterized.

In chapter 4 [71], the initial analysis of the plant cell factor is described. In these experiments induction of vir gene

expression was assayed by monitoring β-galactosidase activity in a set of Agrobacterium strains carrying gene fusions between each of the vir loci and lacZ. The results of these experiments demonstrated that vir gene expression is specifically induced by a small molecular weight (< 1000 Da) diffusible plant cell metabolite present in limiting quantities in the exudates of a variety of plant cell cultures. Active plant cell metabolism is required for the synthesis of the vir-inducing factor, and the presence of bacteria has no effect on this production. Initial characterization of the factor indicated that it is composed of one or more chemically stable organic molecules. The inducing factor present in the exudates of N. tabacum root culture was partially purified on silica C-18, and this material was observed to induce both the expression in Agrobacterium of each of the plant-inducible vir region loci, and also the production of T-DNA circular molecules, possible intermediates of the T-DNA transfer process.

Chapter 5 [44] describes the chemical purification and identification of the plant-synthesized vir-inducing factor. Compounds having vir-inducing activity (as measured by increased β-galactosidase activity expressed from vir::lac gene fusions) were purified from the exudates of both root and leaf disc cultures by reverse-phase chromatography on a fast-performance liquid chromatography system. Employing a variety of analytical techniques these compounds were identified to be acetosyringone (AS) and α-hydroxyacetosyringone (OH-AS) [4-acetyl-2,6-dimethoxyphenol, and 4-(2-hydroxyacetyl)-2,6-dimethoxyphenol, respectively]. Both these compounds were seen to efficently stimulate the induction of the entire vir

regulon and also the production of T-DNA circles.

It was then interesting to try to understand why Agrobacterium has evolved to specifically recognize and respond to these plant molecules to activate vir expression and the T-DNA transfer machinery. AS and OH-AS had not been previously described as natural products of plant cells, so information regarding their synthesis and role in plant cells was not known. Further experiments presented in chapter 5 were carried out to obtain this information. The results demonstrated that AS and OH-AS are specifically produced in the exudates of wounded and actively metabolizing plant cells. Since such cells are the natural targets of Agrobacterium infection it appears that Agrobacterium has evolved to couple the activation of its T-DNA transfer process to molecules which represent plant cells susceptible to Agrobacterium transformation.

For plant molecules to induce vir expression, Agrobacterium must carry genes whose products enable it to recognize and respond to these molecules. The experiments presented in chapter 6 [72] were initiated to identify and characterize these genes. Briefly, different vir::lac gene fusions were placed into a complete set of vir and chv (chromosomal virulence) mutant strains to identify loci which encode functions that control the regulation of vir expression. The results of these experiments demonstrated that the vir loci are organized as a single regulon whose plant-inducible expression is positively controlled by the gene products of two loci, virA and virG.

Further experiments were carried out to assess (i) the respective vir expression phenotype of virA and virG mutant cells, and

(ii) the vegetative and plant-induced transcription of the virA and virG loci. The results of these experiments indicated that the virA and virG gene products direct different steps of the plant-activated induction of the vir regulon; and that this induction is a mechanistically complex process.

In summary, the bacterial/plant interaction that results in the genetic transformation of plant cells by Agrobacterium tumefaciens is diagrammed in Figure 1.1. The development of this interaction is, in part, directed by the gene products of the Ti plasmid vir loci, which mediate the transfer and integration of the T-DNA into the plant cell genome. As presented in this thesis, the activation of the T-DNA transfer process is directed by the susceptible plant cell itself, since vir expression becomes activated when Agrobacterium is in the presence of such cells. This plant induction of vir expression is the initial event of the T-DNA transfer process.

Vir induction has both a bacterial and plant component, and these components are the primary subjects of this thesis. The results presented in chapters 2–6 provide a detailed understanding of the first step of the Agrobacterium/plant cell interaction, and, as discussed in chapter 7 (conclusion), provide a foundation for the future dissection of the vir-directed events of the T-DNA transfer process.

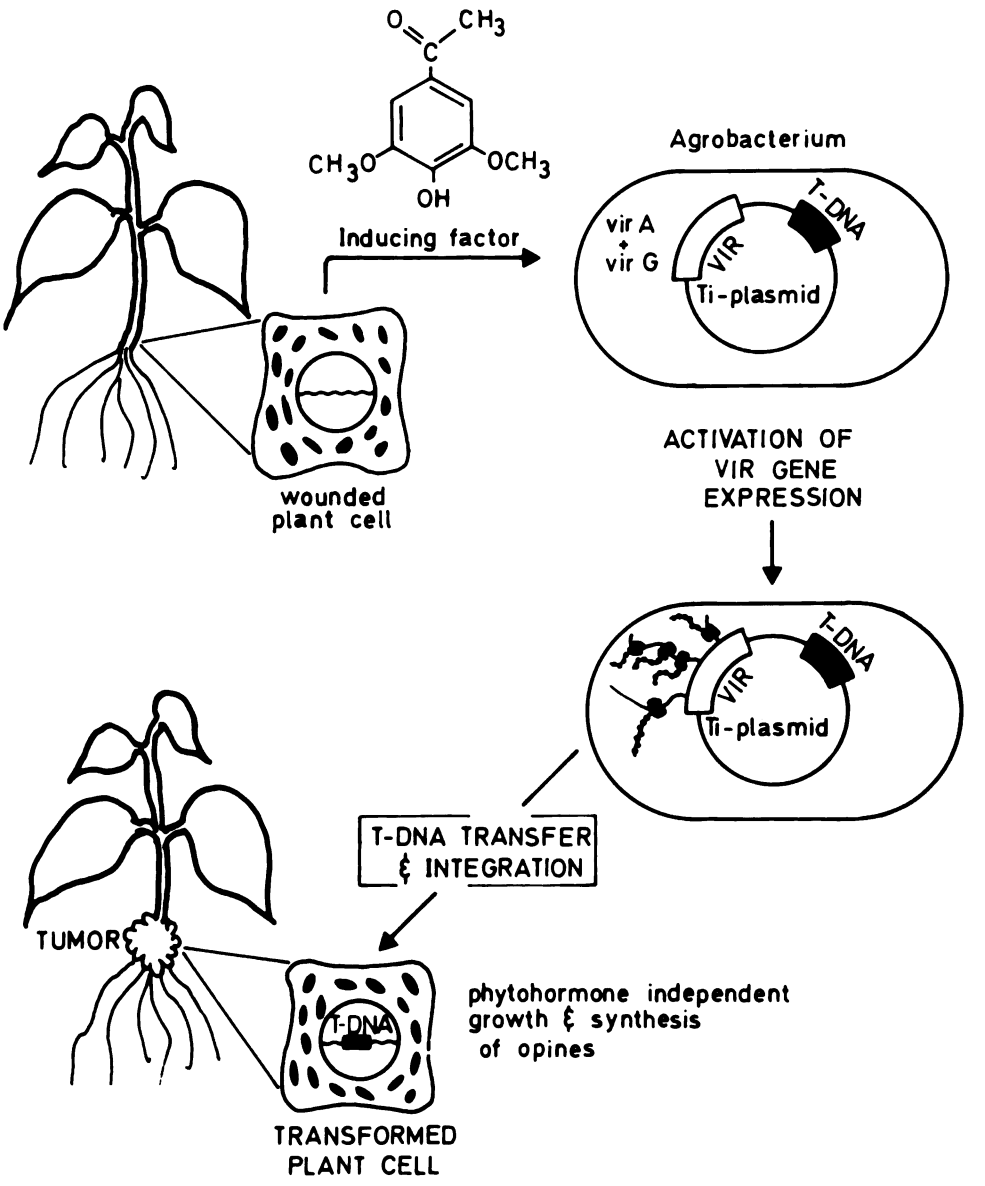


Figure 1.1. The Agrobacterium/plant cell interaction.

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# CHAPTER 2

## A Tn3 lacZ Transposon for the Random Generation of β-galactosidase Gene Fusions : Application to the Analysis of Gene Expression in Agrobacterium


### Abstract

The construction and use of a Tn3-lac transposon, Tn3-HoHo1, is described. Tn3-HoHo1 can serve as a transposon mutagen and provides a new and useful system for the random generation of both transcriptional and translational lacZ gene fusions. In these fusions the production of β-galactosidase, the lacZ gene product, is placed under the control of the gene into which Tn3-HoHo1 has inserted. The expression of the gene can thus be analyzed by monitoring β-galactosidase activity. Tn3-HoHo1 carries a non-functional transposase gene; consequently, it can transpose only if transposase activity is supplied in trans, and is stable in the absence of this activity. A system for the insertion of Tn3-HoHo1 into sequences specifically contained within plasmids is described. The applicability of Tn3-HoHo1 was demonstrated studying three functional regions of the Agrobacterium tumefaciens A6 Ti plasmid. These regions code for octopine catabolism, virulence, and plant tumor phenotype. The regulated expression of genes contained within each of these regions was analyzed in Agrobacterium employing Tn3-HoHo1 generated lac fusions.

## Introduction

Transposon mutagenesis and the generation and analysis of gene fusions have been widely used in the study of gene organization and expression of a variety of organisms. Transposon mutagenesis allows the mutation, identification, and isolation of genes contained within large genetic targets, including prokaryotic (Ruvkun and Ausubel, 1981) and eukaryotic genomes (Bingham *et al.*, 1981; Copeland *et al.*, 1983), while gene fusions allow the analysis and manipulation of the expression and regulation of genes whose functions are unknown or difficult to assay (Bassford *et al.*, 1978; Franklin, 1978).

In gene fusions, the control sequences of a gene of interest are placed in front of the coding sequences of a "reporter" gene whose product can be readily assayed. Thus, expression of the gene can be monitored by measuring the reporter gene product, and genetic and environmental factors that affect the expression can be determined and manipulated. The *E. coli* β-galactosidase structural gene, *lacZ*, has often been employed as the reporter gene in the construction of gene fusions. β-galactosidase is easily and quantitatively assayed, is amenable to genetic manipulations, and is active in a variety of organisms and when contained within hybrid proteins (Bassford *et al.*, 1978). Employing *lacZ*, two types of gene fusions can be made. Transcriptional fusions are generated using sequences that contain the *lacZ* gene including its translational initiation signal; expression of a transcription fusion results in the production of wild-type β-galactosidase. In a translational fusion, the

translational initiation signal and the amino terminal coding sequences of a gene are linked 5' directly to the lacZ coding sequences; expression of a translational fusion results in the production of a chimeric protein with β-galactosidase activity. Both transcriptional and translational fusions are useful for studying prokaryotic gene expression. Translational fusions also have a number of additional applications. They can be used to study gene expression in eukaryotic systems, because translation of the prokaryotic reporter gene is placed under the control of eukaryotic translational control sequences (Lis et al., 1983). They also can be used to determine the site of translational initiation of a gene and to analyze its control (Lee et al., 1982; Casadaban et al., 1982). Furthermore, a hybrid protein produced from a translational fusion can be useful for studying properties of the original protein encoded by the target gene (Schuman et al., 1980; Hall and Silhavy, 1981).

Transposable elements that contain lacZ coding sequences combine the techniques of transposon mutagenesis and the generation of lac gene fusions; such elements allow the random insertion of lacZ into a wide variety of DNA sequences. LacZ transposons that generate transcriptional gene fusions (Casadaban and Cohen, 1979; Kroos and Kaiser, 1984), or translational gene fusions (Casadaban and Chou, 1984) have been previously constructed, and used to determine the location (Kenyon and Walker, 1980) and the transcriptional orientation of genes (Dixon et al., 1980; Wanner et al., 1981), and to analyze their expression (Kenyon et al., 1982; Stern et al., 1984). In this paper we describe the construction and use of a new

Tn3-lacZ element, Tn3-HoHo1. This transposon can be used in the generation of both transcriptional and translational lac fusions with plasmid DNA sequences, and these fusions are stable, as Tn3-HoHo1 cannot self-transpose.

To test our Tn3-lac system and to provide novel information about an organism in which questions of gene organization and gene expression were relatively unstudied, we chose to study different genetic regions of the Agrobacterium tumefaciens Ti plasmid. A. tumefaciens is a phytopathogen that is able to transfer a specific DNA segment, the T-DNA, from its large (200 kb) Ti plasmid to plant cells (Caplan et al., 1983; Nester et al., 1984). The genes contained within the T-DNA do not have a known function within the bacterial cell, but their expression within the transformed plant cell results in altered growth of the plant cell and synthesis of novel compounds, called opines. The Ti-plasmid genes involved in T-DNA transfer and integration are not contained within T-DNA sequences but are located on another region of the plasmid designated the virulence (vir) region. The pTiA6 plasmid also contains a genetic region (occ) that codes for the catabolism of octopine, a specific opine. Tn3-HoHo1 was used to study the expression of these three genetic regions (T-DNA, vir, occ) of the Ti plasmid within Agrobacterium.

## Results and discussion

### a) Tn3::lac constructions

We set out to construct a molecule that can be used to generate fusions between cloned DNA sequences of interest and the β-galactosidase lacZ gene. Such a molecule should be able to transpose at high frequency into plasmid DNA, generate both transcriptional and translational hybrid lacZ gene fusions, and be stable after transposition. Stability could be achieved by requiring that transposition can occur only if transposase activity is provided in trans. The bacterial transposon Tn3 is well suited for such a construct. Tn3 preferentially transposes into plasmid versus chromosomal DNA sequences (F. Heffron, unpublished results) at a frequency approximately $10^{-3}$/cell (Heffron et al., 1977), and its DNA sequence and genetic organization have been determined (Heffron et al., 1979).

The Tn3-lac transposon, Tn3-HoHo1, which is carried by the plasmid pHoHoI, was constructed as shown in Figure 2.1A. pFH33 served as the starting point in this construction. pFH33 is a pMB8::Tn3 derivative that contains an EcoRI linker inserted 84 bp inside of the left inverted repeat ($IR_L$) of Tn3 (Heffron et al., 1979). This linker mutation results in the abolition of transposase activity and provided a convenient restriction site closely adjacent to $IR_L$ for the insertion of lac operon sequences. The lac sequences within Tn3-Hoho1 are oriented such that gene expression which initiates in sequences into which the element has transposed and proceeds through $IR_L$, will result in β-galactosidase production.

Transcriptional control sequences do not precede the lac sequences within Tn3-HoHo1; therefore, β-galactosidase production is dependent on expression that initiates outside of and reads into this element.

Tn3-HoHoI was constructed to serve in the generation of both transcriptional and translational lac fusions. The lacZ portion of the pGA300-derived lac fragment in Tn3-HoHo1 encodes a hybrid β-galactosidase protein of approximately $M_r$ 146,000 D, whose amino terminus is composed of sequences derived from tufB and rpoB fused in-frame to the eighth amino acid of lacZ (Lee et al., 1981; An et al., 1982). This fragment has been inserted into Tn3 such that translation into and through $IR_L$ is open and in-frame; therefore, such translation can result in the production of a hybrid β-galactosidase protein whose amino terminus is encoded by the gene into which Tn3-HoHo1 has inserted. Generation of active gene::lac fusions with this element, however, does not depend on in-frame translation. At least six methionine codons occur in-frame 5' of the lacZ structural gene in the construct, and translation can apparently initiate at one or more of these codons if transcription occurs across this region. Because the pGA300-derived lac fragment used in the construction of Tn3-HoHo1 contains sequences encoding the SV40 early transcript acceptor splice site and polyadenylation signal (An et al., 1982; Figure 2.1A), these sequences occur within Tn3-HoHo1 downstream of and in the same orientation as the lacZYA sequences (Figure 2.1C). Thus, lac fusions functional in both prokaryotic and eukaryotic systems can potentially be generated. Tn3-HoHo1 is 14.25 kb in size.

Tn3-HoHoI is defective for transposase activity. To effect Tn3-HoHo1 transposition, pSShe was constructed (Figure 2.1B) to supply tnpA activity in trans. pSShe contains Tn3 deleted for sequences rightward of the Tn3 BamHI site; thus, it encodes transposase activity, but cannot be transposed due to the absence of $IR_R$. The structures of Tn3-HoHo1 and pSShe, and the nucleotide sequence of the left terminus of Tn3-HoHo1 are shown in Figure 2.1.

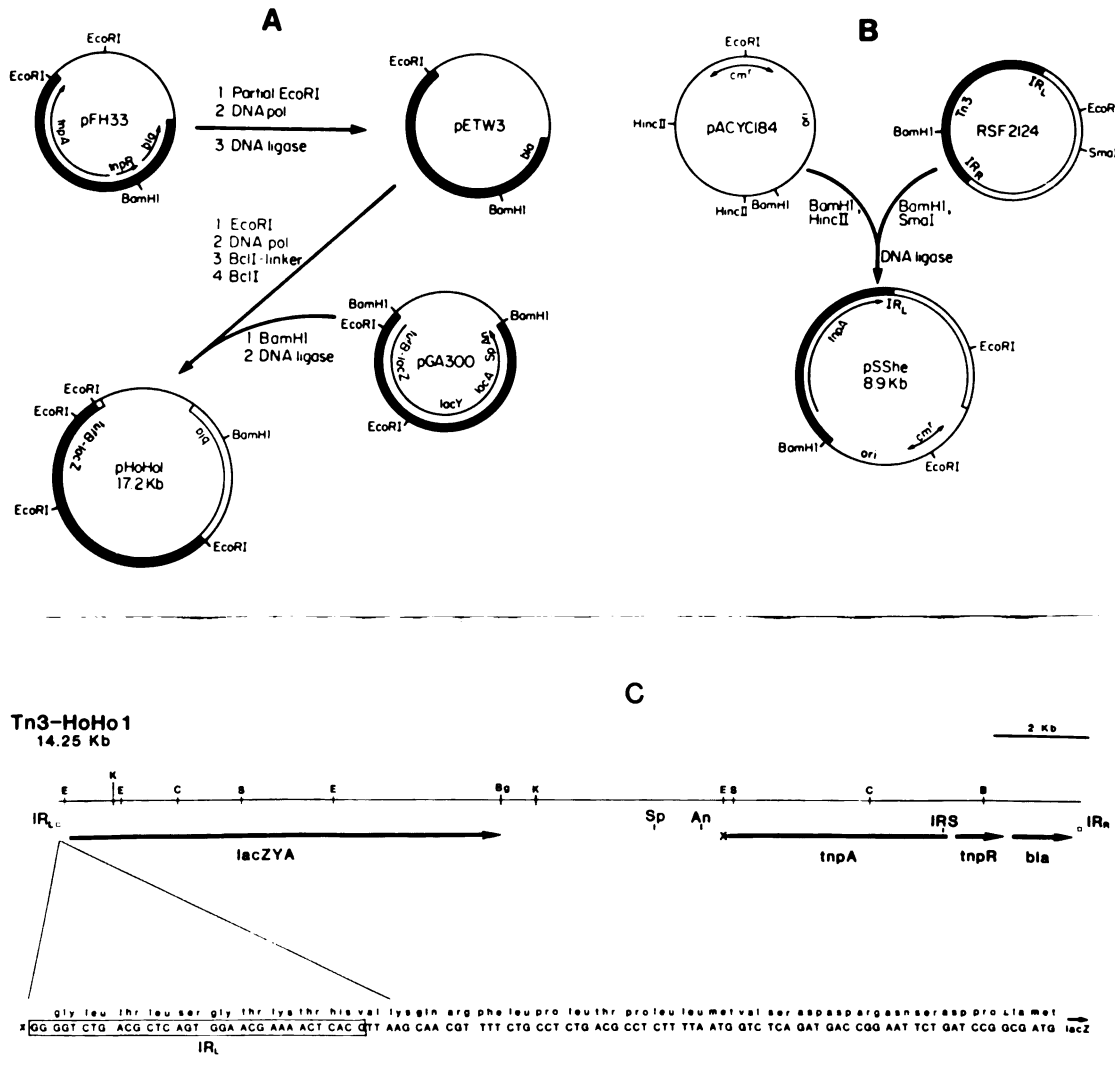



Figure 2.1. Tn3-lac constructs.

Legend to Figure 2.1.

A. Construction of pHoHo1.

The pMB8 EcoRI site of pFH33 (Heffron et al., 1978) was removed to yield pETW3 by partial digestion with EcoRI, incubation with DNA polymerase (Klenow fragment) in the presence of dATP and dTTP, and circularization with T4 DNA ligase. The EcoRI site of pETW3 was converted to a BclI site by digestion with EcoRI, filling this site by Klenow treatment, followed by the addition of 8-mer BclI linkers and T4 DNA ligase. This molecule was subsequently digested with BclI and ligated to the 9.3-kb BamHI fragment of pGA300 (An et al., 1982) to give pHoHo1. The above treatments generated new EcoRI sites that flank the lac sequences inserted into Tn3 on pHoHo1. pHoHo1 is 17.2 kb in size. In the plasmid representations, single lines designate pMB8 sequences. Black areas represent Tn3 sequences in pFH33 and pETW3, and lac sequences in pGA300. In the pHoHo1 diagram, the Tn3-lac transposon, Tn3-HoHo1, is represented by the open and black areas together. The transcriptional orientations of the Tn3 tnpA, tnpR and bla genes, and the tufB-lacZ gene, are indicated by arrows.

B. Construction of pSShe.

The BamHI-SmaI digest of the Tn3::ColE1 plasmid RSF2124 (So et al., 1975) was ligated to the BamHI-HindIII digest of pACYC184 (Chang et al., 1978) to yield pSShe. pSShe is 8.9 kb in size, and carries the tnpA coding region of Tn3. In the plasmid representations single lines designate pACYC184 sequences; open areas, ColE1 sequences; black areas, Tn3 sequences; ori, the pACYC184 origin of replication; and $Cm^R$, the chloramphenicol resistance gene.

Legend to Figure 2.1. (cont'd)

C. Structure and organization of Tn*3*-HoHo1.

The vertical lines indicate the positions of various restriction sites in Tn*3*-HoHo1. *Hin*dIII and *Sal*I sites do not occur within the element. The genetic organization of Tn*3*-HoHo1 is shown below the restriction map. The respective coding region of each gene within the element is indicated by a bold line, and the transcriptional orientation of each gene is indicated by an arrow. *tnp*R and *bla* are wild-type, while *tnp*A is non-functional owing to the *lac* sequences inserted into its 3' end. The *lac* operon sequences are intact but lack a functional promoter. The DNA sequences of the left end of Tn*3*-HoHo1 was determined by the method of Maxam and Gilbert (1980). The DNA sequence, along with the predicted amino acid sequence, up to the native initiation codon of the *tuf*B-*lac*Z-encoded protein, are shown in the bottom of the figure; the Shine-Dalgarno sequence of *tuf*B has been eliminated in Tn*3*-HoHo1 and translation can potentially initiate at any ATG that occurs upstream of the *lac*Z structural gene. The $IR_L$ sequence is boxed, and X represents sequences leftward of $IR_L$ into which Tn*3*-HoHo1 has inserted. Symbols : B, *Bam*HI, Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; S, *Sst*I; $IR_L$, left-inverted repeat; $IR_R$, right-inverted repeat; IRS, internal resolution site; Sp, eukaryotic splice site; An, eukaryotic polyadenylation signal; *lac*ZYA, *E. coli lac* operon; *tnp*A, transposase; *tnp*R, resolvase; *bla*, β-lactamase.

b) Tn3-lac transposition

Tn3-HoHo1 insertions into target sequences that were cloned into the mobilizable wide-host range plasmid, pVCK102 were selected in E. coli using the transconjugant procedure described in detail in Materials and Methods [and diagrammed in Figure 2.2]. In brief, the transposon donor strain which contained pHoHo1, pSShe and the target plasmid was mated with the recipient strain, SF800, and transconjugants containing Tn3-HoHo1::target plasmids were obtained at a frequency of approximately $10^{-4}$. The mating efficiency of the target plasmid into SF800 is approximately $10^{-1}$, so Tn3-HoHo1 transposes at a frequency of approximately $10^{-3}$. Transconjugants were obtained at a frequency of approximately $10^{-7}$ when pSShe was omitted from the donor transposon strain. These frequencies are similar to those obtained by Heffron et al. (1977) for complementation of Tn3 tnpA mutations in trans. Also, more than 99% of the transconjugants contained Tn3-HoHo1 inserted into the target plasmid, and deletions were associated with approximately 1% of the Tn3-HoHo1::target plasmids.

An alternative method that we have employed to select for transposition of Tn3-HoHo1 into target cosmid plasmids is the in vivo packaging procedure of White et al. (1983). Using this method, Tn3-HoHo1 transductants were obtained at a frequency of approximately $5 \times 10^{-4}$. When the initial target cosmid was greater than 40 kb in size, a majority of the resultant Tn3-HoHo1::target transductants contained deletions. Thus, this method can be used to generate random deletions in a target plasmid.

We tested Tn3-HoHo1 by using the element to study the organization and regulated expression of three separate genetic regions of the A6 Ti plasmid within Agrobacterium tumefaciens.

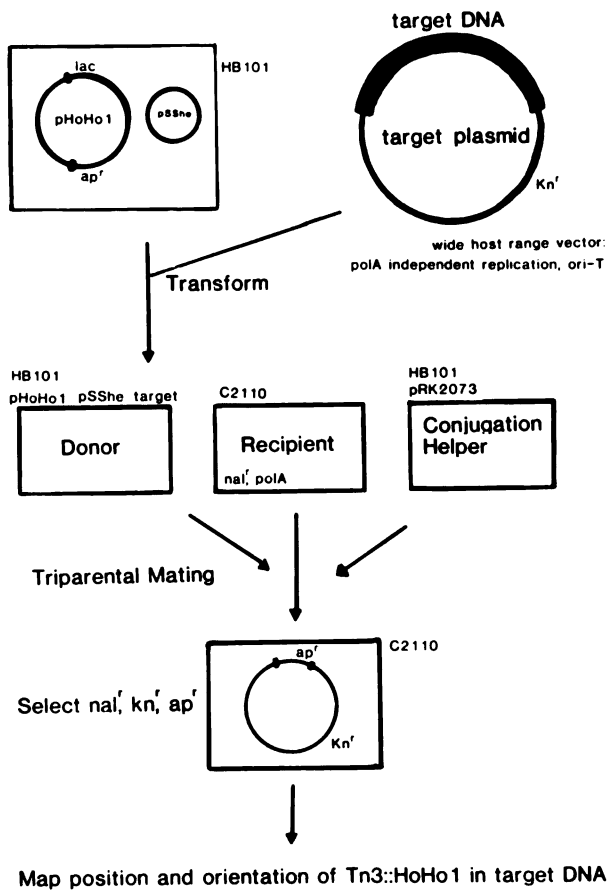



Figure 2.2. Procedure for isolation of Tn3 insertions into plasmid sequences.

c) Analysis of occ::lac gene fusion

The occ genes confer upon Agrobacterium the ability to utilize both octopine and arginine as sole carbon and nitrogen sources, and expression of these genes is induced by octopine (Montoya et al., 1977; Klapwijk and Schilperoort, 1979); this induction appears to be mediated through a negative regulatory system (Klapwijk and Schilperoort, 1979). Knauf and Nester (1982) previously constructed pVCK261, which contains the complete occ region of pTiA6 cloned into the wide-host range cosmid pVCK102. Their studies suggested that occ is encoded at minimum by the 6.2-kb EcoRI fragment 8 (De Vos et al., 1981), and is probably expressed from the rightward to leftward direction with respect to the map of pVCK261 shown in Figure 2.3.

pVCK261 was mutagenized with Tn3-HoHoI and seven pVCK261::Tn3-HoHo1 derivatives, pSM100 to pSM106, were introduced by conjugation into Agrobacterium strains A348 and A136, that contain or lack pTiA6, respectively. pSM100 carries Tn3-HoHo1 within the vector portion of the plasmid, while pSM101 to pSM106 carry Tn3-HoHo1 within the cloned occ region (Figure 2.3). These Agrobacterium strains were grown to logarithmic phase in liquid minimal media and in liquid minimal media supplemented with 100 μg/ml octopine for 6 hours. Bacteria were then harvested and their β-galactosidase activities were determined (Figure 2.4). The parental A136 and A348 strains have negligible levels of β-galactosidase activity (less than 1 unit), and all pSM strains exhibitied similar low activities when grown in minimal media. The pSM102, pSM105, and pSM106 strains displayed increased β-galactosidase activities when grown in the

presence of octopine (Table 2.1), demonstrating that expression of octopine catabolism genes occurs in a rightward to leftward orientation (Figure 2.3). The β-galactosidase activity measured for each of the occ::lac strains grown in octopine medium was independent of the presence or absence of wild-type occ carried in trans on the Ti plasmid (data not shown), suggesting that none of the Tn3-HoHoI insertions affected genes involved in the regulation of occ expression. The time course of octopine induction of β-galactosidase activity for the strain A348(pSM102) was determined and is shown in Figure 2.4. These kinetics closely follow those seen for octopine-induced octopine uptake by A348 (Montoya et al., 1977; Klapwijk et al., 1977). Interestingly, although pVCK261 does not confer octopine utilization upon E. coli, pSM102, pSM105 and pSM106 produced low levels of octopine inducible β-galactosidase activity in the E. coli lac deletion strain MC1061. Octopine induction of occ in Agrobacterium is repressed in the presence of 0.5% casamino acids (A. Montoya, personal communication; S. Stachel, unpublished results); occ induction in E. coli was similarly seen to be amino acid repressible, suggesting that some mechanisms for gene control might be analogous between these two organisms.

The A136(pSM) strains were grown on octopine minimal media agar (Hooykaas et al., 1979) and on arginine minimal media agar (Knauf and Nester, 1982) containing 10 μg/ml octopine to determine the effect of Tn3-HoHo1 insertions on octopine and arginine utilization, respectively (Table 2.1). The pSM100, pSM101, and pSM103 strains grew on both these media, while the pSM102, pSM104, pSM105, and pSM106 strains did not. This suggested that a regulated occ promoter

occurs within the 1-kb region bounded by the pSM103 and pSM106 insertions. The regulation of this promoter was not affected by the pSM102, pSM105, and pSM106 insertions. These experiments with occ indicated that Tn3-HoHo1 efficiently generates lac fusions that can be used to analyze gene organization and expression in Agrobacterium.

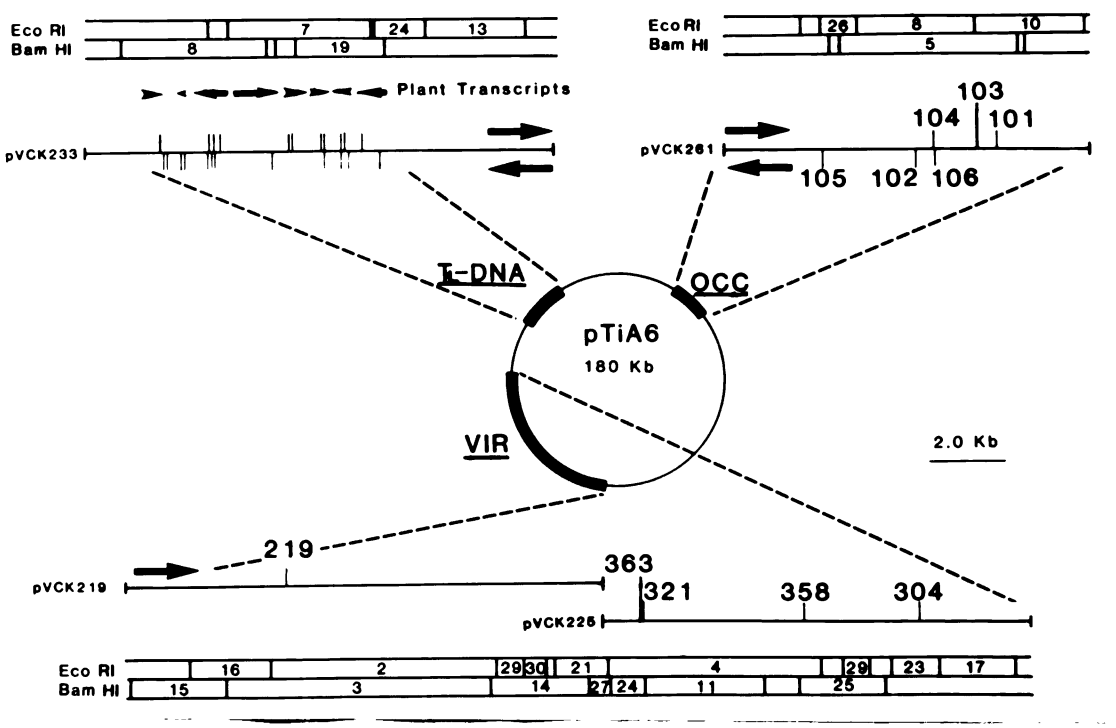



Figure 2.3. Tn3-HoHo1 insertions within TL-DNA, occ and vir sequence of pTiA6.

Legend to Figure 2.3.

The BamHI and EcoRI restriction maps (De Vos et al., 1981) of TL-DNA, occ and vir, and the respective location of each of these regions within pTiA6 (Garfinkel and Nester, 1980; De Greve et al., 1981) are shown. The genetic location, size, and transcriptional orientation of the TL-DNA genes known to be expressed in transformed plants (Garfinkel et al., 1981; Willmitzer et al., 1982) are indicated as "plant transcripts" under the restriction map of the TL-DNA region. TL-DNA, occ, and vir sequences have been separately cloned into the cosmid vector pVCK102 (Knauf and Nester, 1982), and the complete TL-DNA and occ regions are carried by pVCK233 and pVCK261, respectively, and vir sequences are carried by pVCK219 and pVCK225. These four cosmid clones are represented in the figure by horizontal lines. Vertical lines above and below the horizontal lines represent separate Tn3-HoHo1 insertions into the cloned sequences. Tn3-HoHo1 can insert into sequences in either of two orientations in regards to its lacZ coding sequences. In the figure, the lacZ orientation of an insertion represented by an upper line is leftward to rightward while that by a lower line is rightward to leftward as indicated by the arrows.

Table 2.1. Characteristics of A136(*occ*::*lac*) strains

| Strain | β-galactosidase units | | Utilization | |
|---|---|---|---|---|
| | - octopine | + octopine | octopine | arginine |
| A136/ 100 | 2.54 | 2.47 | + | + |
| 101 | 1.35 | 2.37 | + | + |
| 102 | 0.992 | 102.6 | - | - |
| 103 | 3.24 | 1.56 | + | + |
| 104 | 0.943 | 18.93 | - | - |
| 106 | 1.34 | 82.4 | - | - |

Seven A136(pVCK261::Tn*3*-HoHo1) strains were characterized for octopine-inducible β-galactosidase activity and carbon source utilization. The β-galactosidase activity of cell grown six hours in AB/glucose liquid medium and AB/glucose liquid medium supplemented with 100 μg/ml octopine was determined. Carbon source utilization was assessed by scoring growth on AB agar containing 100 μg/ml octopine and AB agar containing 600 μg/ml arginine and 10 μg/ml octopine.

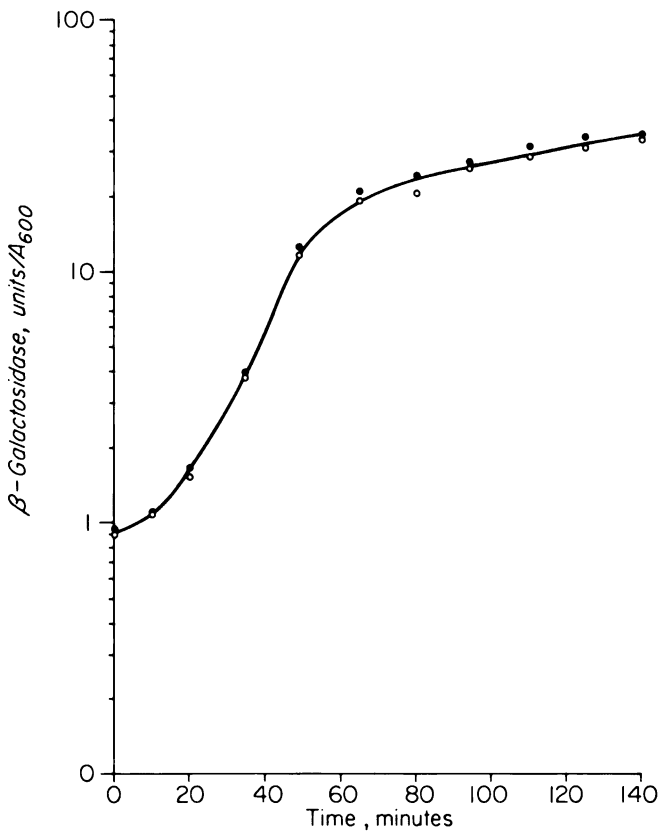



Figure 2.4. Kinetics of octopine induction of β-galactosidase in A348(pSM102).

Cells were grown in AB/glucose liquid medium. 100 μg/ml octopine was added to two exponential phase cultures, represented by o and o. Aliquots were periodically removed and the β-galactosidase activity in each culture as a function of time following octopine addition was determined.

d) Analysis of translational vir::lacZ gene fusions

The ability of Tn3-HoHo1 to specifically generate translational lac fusions was demonstrated using the vir genes of the Agrobacterium pTiA6 plasmid as a model system. The vir region of pTiA6 spans approximately 50 kb of the plasmid and contains at least 7 separate transcriptional units (chapter 3). Transposon insertions in these loci result in loss or attenuation of virulence (Garfinkel et al., 1980; Ooms et al., 1980; Hooykaas et al., 1984; Klee et al., 1983; Hille et al., 1984; chapter 3).

Adjacent segments of the pTiA6 vir region, plasmid clones pVCK219 and pVCK225 (Knauf and Nester, 1982; Figure 2.3), were mutagenized with Tn3-HoHo1 and introduced into A348. Five A348(vir::lac) transconjugants, pSM219, pSM304, pSM321, pSM358 and pSM363 (Figure 2.3) are analyzed here in detail. These strains were grown both in minimal medium and minimal medium supplemented with octopine, and the units of β-galactosidase activity were determined. Three of these strains, pSM321, pSM363 and pSM358, displayed significantly higher units of β-galactosidase activity for growth in minimal medium than other vir:: lac strains or than the occ::lac strains (Figure 2.5, Table 2.1). These results suggest that the regions of Tn3-HoHo1 insertion in these strains are constitutively expressed in Agrobacterium. Indeed, Gelvin et al. (1981) have reported that steady-state transcription occurs from the same region of vir as that of the Tn3-HoHo1 insertions in pSM321 and pSM363. None of the vir::lac strains exhibited octopine-induced β-galactosidase activity.

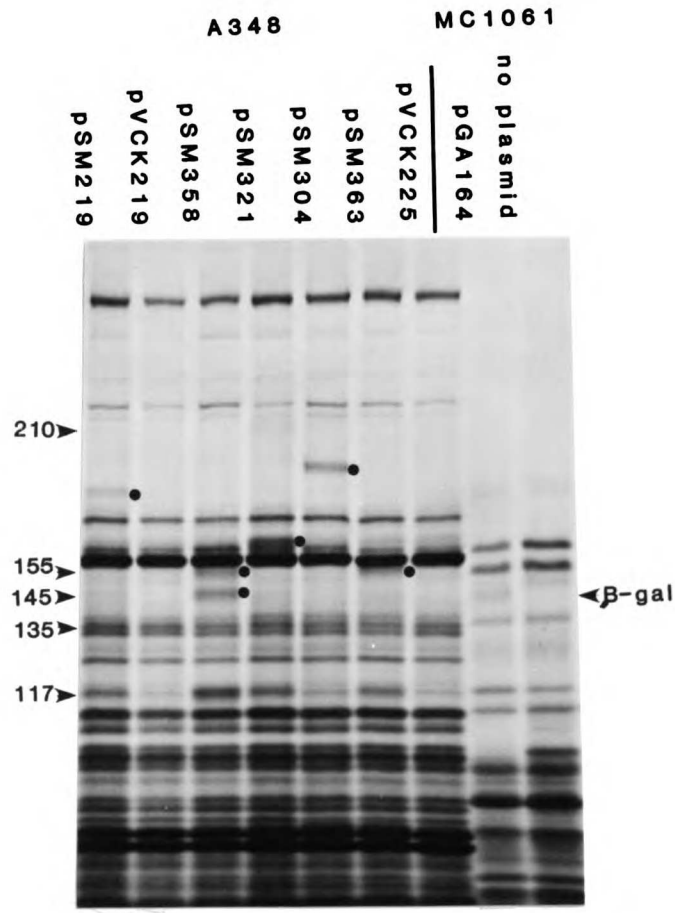
Recently it has been seen that plant cells produce a small molecular weight factor(s) that specifically induces the expression of Agrobacterium vir genes (Stachel et al., 1984; chapter 3). Regenerating mesophyll protoplasts of Nicotiana tabacum Xanthi, which are susceptible to high efficiency transformation by Agrobacterium (An et al., 1985) produce this factor. Vir induction has been seen by direct assay of vir RNA and has also been assessed using vir::lac fusions. In the latter experiments, the levels of β-galactosidase activity in A348(vir::lac) strains are seen to markedly increase, in respect to other growth conditions, as a result of cocultivation with Xanthi protoplasts.

The lacZ gene used in the construction of Tn3-HoHo1 encodes a β-galactosidase protein of approximately $M_r$ 146,000. To demonstrate that translational fusions can be generated with Tn3-HoHo1; it is necessary to visualize hybrid proteins greater than this size synthesized from Tn3-HoHo1-generated lac fusions. Because the five vir::lac strains discussed above exhibited comparatively high levels (7–30-fold over the levels of octopine-induced β-galactosidase activity exhibited by the occ::lac strains) of plant-induced β-galactosidase activity (Figure 2.5; Table 2.1), they were examined for the presence of hybrid β-galactosidase proteins. These strains, along with the parent plasmid strains A348(pVCK219) and A348(pVCK225), were radiolabelled for 16 hours with $^{35}$S-methionine during cocultivation with plant cells, and total bacterial protein was subsequently analyzed by SDS/polyacrylamide gel electrophoresis (Figure 4). Each of the vir:: lac strains used in this experiment, but not the control strains, yielded a novel protein band that varied in size from

approximately $M_r$ 147,000 to $M_r$ 193,000, and the production of these presumptive *vir*::β-galactosidase hybrid proteins depended on cocultivation (data not shown).

A348(pSM358) produced two fusion proteins of $M_r$ 156,000 and $M_r$ 147,000. When an A348(pSM358) cocultivation was pulse-labelled for 5 minutes and bacterial protein was immediately isolated, the majority of the radiolabelled fusion protein occurred in the 156,000 D band (data not shown). This suggests that the 147,000 D protein might be derived from the 156,000 D protein.

The above data demonstrate that translational gene fusions to *lacZ* can be randomly generated with Tn*3*-HoHo1. If no post-translational processing has occurred, one can determine from the size of a hybrid β-galactosidase fusion protein where translation has initiated within the sequences into which Tn*3*-HoHo1 has inserted. Such determinations for the five *vir*::*lac* fusions examined above have been borne out by DNA sequence analysis. Tn*3*-HoHo1 also generates transcriptional gene fusions that initiate β-galactosidase translation from one of the six ATG codons internal to the Tn*3*-HoHo1 *lacZ* gene. This conclusion is based on the analysis of more than one hundred independent *vir*::*lac* gene fusions (chapter 3); each fusion that carries *lac* orientated in the direction of transcription of the *vir* gene to which it is fused displays *vir*-inducible *lacZ* expression. If Tn*3*-HoHo1 only generates translational fusions, then statistically only 1/3 of the fusions would be in frame and thus inducible. Since all fusions are active, transcriptional as well as translational fusions are present.




| Strain | | β-Galactosidase Units − plant cells | + plant cells | β-Galactosidase Fusion M.W. ($\times 10^{-3}$) |
|---|---|---|---|---|
| A348/ | pVCK219 | 2.2 | 1.9 | - |
| | pVCK225 | 1.8 | 2.0 | - |
| | pSM219 | 10.3 | 726 | 182 |
| | pSM304 | 8.2 | 859 | 193 |
| | pSM321 | 81 | 1050 | 164 |
| | pSM358 | 46 | 3120 | 156,147 |
| | pSM363 | 72 | 1063 | 156 |
| MC1000/no plasmid | | 1.2 | - | - |
| | pGA164 | 1320 | - | 146 |

**Figure 2.5. Analysis of hybrid β-galactosidase proteins encoded by translational *vir*::*lac* fusions.**

Legend to Figure 2.5.

Radiolabelled total cell extracts were analyzed by one-dimensional 7% SDS polyacrylamide gel electrophoresis. Agrobacterium strains are A348 containing different plasmids. pSM219 is derived from pVCK219, and pSM304, pSM321, pSM358, and pSM363 are derived from pVCK225. The Tn3-HoHo1 insertions in pSM304, pSM321, pSM358, and pSM363 are located within vir genes and cause an avirulent phenotype when recombined into the Ti plasmid. The pSM219 insertion occurs within a plant-inducible sequence that is not required for virulence (chapter 3). The tufB-lacZ coding sequences contained within Tn3-HoHo1 encode a protein of 146,000 D that has 226 amino acids of tufB and approximately 50 amino acids of rpoB fused to the eighth amino acid of β-galactosidase. This protein was expressed in the E. coli strain MC1061 from the plasmid pGA164 (Lee et al., 1981), and is indicated by the arrow on the right. This plasmid in not expressed in Agrobacterium. The units of β-galactosidase activity expressed by the Agrobacterium strains in AB/glucose liquid medium (minus plant cells) and in cocultivation (plus plant cells), along with the estimated size of the induced hybrid β-galactosidase proteins, are shown in the lower section of the figure.

e) Expression of TL-DNA sequences within Agrobacterium

The expression of T-DNA sequences within Agrobacterium has been previously reported. Gelvin et al. (1981) observed that the pTiB6-806 T-DNA region is transcribed at a low steady-state level in Agrobacterium, and that the pattern of this transcription appears to be less specific than that seen for the same sequences in crown gall cells. Similar results were obtained by Janssens et al. (1984) for the T-DNA region of pTiC58. Also, Schröder et al. (1984) demonstrated that the transcript 2 gene of the pTiAch5 T-DNA is functionally expressed in Agrobacterium. At present, however, no data exists which indicates that an Agrobacterium cell which harbors T-DNA genes has a selective advantage over a cell which does not. If T-DNA genes do serve a functional role in Agrobacterium their expression might be expected to be specifically regulated within the bacterial cell. We have sought to find evidence for such regulation. T-DNA:: lacZ fusions generated with Tn3-HoHo1 were used to examine the expression of T-DNA sequences within Agrobacterium under a variety of conditions.

The complete pTiA6 TL-DNA (Thomashow et al., 1980) has been cloned into pVCK102 to yield pVCK233 (Knauf and Nester, 1982; Figure 2.3). This plasmid was mutagenized with Tn3-HoHo1, and the twenty-three pVCK233:: Tn3-HoHo1 derivatives shown in Figure 2.3 were introduced into A136 and A348. Twelve of these derivatives carry Tn3-HoHo1 within seven of the eight pTiA6 T-DNA genes with the lacZ coding sequences oriented in the direction of transcription of these genes (Willmitzer et al., 1982; Barker et al., 1983; Gielen et

al., 1984). Each of the transconjugant strains was grown under a variety of different conditions and the β-galactosidase activity units were determined. In minimal media, activities varied between 3 and 15 units for the different strains. These low values suggest that the expression in Agrobacterium of the fused T-DNA sequences is negligible or low. Also, the differences in β-galactosidase activity seen between the different strains appeared to be unrelated to the position or orientation of the Tn3-HoHo1 insertions in regard to the T-DNA genes. A136(pVCK233::Tn3-HoHo1) strains and their respective A348 strains exhibited identical activities, indicating that pTiA6 likely does not carry a repressor of T-DNA transcription.

No growth conditions were found that caused a consistent and marked (more than 2-fold) increase or decrease in the levels of β-galactosidase activity of each A348 (pVCK233::Tn3-HoHo1) strain. Growth conditions included Mg/L medium and minimal medium, and these media supplemented separately with octopine, arginine, indole-acetic-acid, trans-zeatin or tobacco cell wall extract. Bacteria from mesophyll protoplast cocultivations also failed to exhibit increased expression. To date, we have been unable to find any evidence of regulated expression of T-DNA sequences within Agrobacterium using T-DNA::lac fusions generated with Tn3-HoHo1.

## Conclusion

We describe the construction of a Tn3-lac transposon, Tn3-HoHo1, and its application in the random generation of lac fusions useful in the study of gene organization and expression. In such fusions,

the production of β-galactosidase is placed under the control of sequences into which Tn*3*-HoHo1 has transposed. This element, like other transposon-*lac* constructs, is useful in the determination of the location and transcriptional orientation of genes, and in the analysis of factors affecting gene expression. Tn*3*-HoHo1 has a number of properties which make it novel. It is derived from Tn*3*, and thus transposes with high specificity into plasmid DNA sequences. It does not carry a functional transposase and cannot self-transpose. Transposition only occurs if transposase is supplied *in trans* by a plasmid that encodes functional transposase; Tn*3*-HoHo1 insertions are stable in the absence of this plasmid.

The *lacZ* coding sequences within Tn*3*-HoHo1 are open and in-frame to the end of the Tn*3* left terminal repeat, and both transcriptional and translational gene fusions can be generated with this element. When Tn*3*-HoHo1 inserts into a gene so that the coding sequences of the gene are in frame to the *lacZ* coding sequences, expression of the gene will result in the production of a hybrid β-galactosidase protein. If insertion occurs so that the coding sequences are not in-frame, gene expression will result in the production of a non-hybrid β-galactosidase protein that initiates translation within the transposon *lacZ* sequences. We have also constructed a second Tn*3*-*lacZ*, Tn*3*-HoHo2, that can be used in the generation of transcriptional, but not translational fusions, because several stop codons occur between $IR_L$ and the initial ATG of the *lacZ* gene (S. Stachel, unpublished results).

Tn*3*-HoHo1 was used to study the organization and expression of the octopine catabolism, virulence, and T-DNA genetic regions of the

A6 Ti plasmid in Agrobacterium tumefaciens. This element can also be applied to studies of gene expression in a wide variety of other prokaryotic organisms, and has recently been used in Rhizobium sesbania (M. Holsters, personal communication). Furthermore, because Tn3-HoHo1 contains a eukaryotic polyadenylation sequence, this element might also be applicable to expression studies in eukaryotic organisms.

## Materials and methods

### a) Reagents

Restriction and DNA modification enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. X-gal was from Bethesda Research Laboratories. ONPG, indole-3-acetic acid, and trans-zeatin were from Sigma. $^{35}$S-methionine (1000 Ci/mMol) was purchased from New England Nuclear. L-octopine was the generous gift of Robert L. Jensen.

### b) Bacterial strains and media

E. coli strains are HB101 : recA$^-$ hsr$^-$ hsm$^-$ (Boyer and Roulland-Dussoix, 1969), MC1061 : (lacIPOZY) (ara, leu) galU$^-$ galK$^-$ hsr$^-$ hsm$^+$ (Casadaban and Cohen, 1980), and SF800 : Nal$^R$ polA$^-$ (Heffron et al., 1977). E. coli were grown in LB liquid medium and on LB agar (Miller, 1972), at 37°C, unless otherwise specified. Carbenicillin, kanamycin, chloramphenicol, and nalidixic acid were used at 200,

100, 100, and 60 μg/ml, respectively. Agrobacterium strains are A136 : C58C1 $Nal^R$ $Rif^R$, and A348 : A136 carrying pTiA6 (Garfinkel et al., 1981). Agrobacteria were grown on AB minimal agar, and in AB/glucose and in Mg/L liquid media (Chilton et al., 1974), at 28°C, unless otherwise specified. Carbenicillin and kanamycin were each used at 100 μg/ml.

c) Procedures

Plasmid DNA transformation was by the method of Cohen et al. (1972), and triparental matings were performed by the method of Ditta et al. (1980). Transposon insertions into target DNA sequences were selected essentially by the transconjugant procedure of Heffron et al. (1977). In this scheme, sequences to be mutagenized were first cloned into pVCK102 (Knauf and Nester, 1982), to yield the target plasmid. pVCK102 is a wide-host range cosmid vector that carries kanamycin and tetracycline resistance genes, can be mobilized for conjugal transfer by the helper plasmid, pRK2013 (Figurski and Helinski, 1979), and carries a polA-independent origin of replication. The target plasmid was transformed into the transposon donor strain, HB101(pHoHo1, pSShe) and the resultant strain was mated with HB101(pRK2013) and the ($Nal^R$)polA recipient strain, SF800. The mating mixture was plated on LB agar containing Nal, Cb, and Km to select for SF800 (target plasmid::transposon) transconjugants. Replication of pHoHo1, pSShe, and pRK2013 is strictly dependent on DNA polymerase I, the polA gene product. Plasmid DNA was isolated from single transconjugants by the method of Birnboim and

Doly (1979), and the position and orientation of the transposon within the target plasmid was mapped. Single target plasmid::transposon isolates were transferred by conjugation into Agrobacterium tumefaciens, and Agrobacterium transconjugants were selected on AB agar containing Cb and Km, and 0.1% X-gal. X-gal was included to identify insertions into actively transcribed regions.

Standard recombinant DNA (Maniatis et al., 1982) and Maxam and Gilbert (1980) sequencing procedures have been described. β-galactosidase activity was determined essentially as described by Miller (1972). Bacteria were collected by centrifugation, and suspended in 600 µl of Z-buffer. Cell density was determined by measuring the absorbance of 100 µl of cells at 600 nm (1 cm), and the remaining bacteria were vortexed with 20 µl 0.05% SDS and 20 µl $CHCl_3$ for 10 seconds and then incubated for 10 minutes at 28°C. 100 µl of ONPG (4 mg/ml) was added to start the assay reaction. Reactions were then incubated at 28°C and terminated by adding 250 µl of 1 M $Na_2CO_3$. Bacteria were removed by centrifugation and the absorbance of the solution at 420 nm (1 cm) was determined. Specific units of β-galactosidase activity were calculated using the formula :

$$\frac{A_{420} \times 10^3}{t(\text{minutes}) \times A_{600}}$$

Mesophyll protoplasts used for cocultivations were prepared from Nicotiana tabacum Xanthi by the method of Wullems et al. (1981). Two ml of cells at a density of $10^5$/ml were regenerated in 60 mm dishes for 72 hours and exponentially growing bacteria were then added to a density of $5 \times 10^7$/ml. The cocultivation mixture was

incubated for 16 hours at 28°C. During this period, initial protoplast cell division occurred. Bacteria were subsequently separated from the plant cells by passing the mixture through Miracloth® (Calbiochem), or by differential centrifugation, and the units of β-galactosidase activity were determined as described above.

Radiolabelled agrobacteria were prepared from cocultivations grown in the presence of 14 μCi/ml $^{35}$S-methionine. Following cocultivation the bacteria were washed in 10 mM Tris, 1 mM EDTA, pH 7.4, and extracts were made by sonication. E. coli were radiolabelled overnight in sulfate-free medium containing 10 μCi/ml $^{35}$S-methionine. SDS/polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Fixed gels were dried onto Whatman 3MM paper before exposure to XRP-1 X-ray film.

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# CHAPTER 3

## The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of Agrobacterium tumefaciens


### Abstract

Agrobacterium tumefaciens genetically transforms plant cells and this process is mediated by the genes of the Ti plasmid vir region. To determine the genetic and transcriptional organization of the vir region of pTiA6, vir plasmid clones were saturated with insertion mutations of a Tn3-lacZ transposon. This element is both an insertion mutagen and a reporter for the expression of the sequences into which it has inserted. One-hundred and twenty-four vir::Tn3-lac insertions were analyzed for their mutagenic effect on Agrobacterium virulence, and for their expression of β-galactosidase activity, the lacZ gene product, in vegetative bacteria and in bacteria cocultivated with plant cells. These data in conjunction with genetic complementation results show that the pTiA6 vir region is composed of 6 distinct vir complementation groups, virA, virB, virC, virD, virE, and virG. Mutations in these loci eliminate (virA, virB, viD, virG) or restrict (virC, virE) the ability of Agrobacterium to transform plant cells. Each of the vir loci corresponds to a single vir transcription unit : virA is constitutively expressed and non-inducible; virB, virC, virD, and virE are expressed only upon activation by plant cells; and virG is both constitu-

tively expressed and plant-inducible. The two largest vir operons, virB and virD, are probably polycistronic. The pTiA6 vir region also contains plant-inducible loci (pin) which are non-essential for virulence.

## Introduction

The soil phytopathogen, Agrobacterium tumefaciens, genetically transforms plant cells to cause crown gall, a neoplastic disease of dicotyledonous plants (Caplan et al., 1983; Nester et al., 1984; Gheysen et al., 1985). During transformation the bacterium transfers a specific segment of DNA, the T-DNA, from its large (> 200 kb) Ti plasmid to the plant cell nucleus. Expression of T-DNA genes within the transformed cell results in its neoplastic growth, and the synthesis of novel compounds, called opines. The Agrobacterium system is the only known example of the programmed insertion of prokaryotic DNA into a eukaryotic genome.

The T-DNA transfer process is remarkable in that it carries out a complex series of events : a specific segment of DNA is recognized in and mobilized from the Ti plasmid, transferred across the cell walls of the bacterium and plant cell, and integrated as a linear nonpermuted fragment into the plant nuclear genome. The molecular details of these events are presently unknown, and research has focused on the three sets of genetic elements identified to be essential for transfer – the T-DNA border sequences, the chromosomal virulence chv loci, and the Ti plasmid virulence vir loci. The vir loci likely encode functions that directly mediate the events of the

transfer process. As a first step in understanding how these genes function in this process, the genetic and transcriptional organization of the vir loci of the octopine-type Ti plasmid, pTiA6, are characterized here in detail.

During transfer the T-DNA acts solely as a structural element, since its internal portion can be fully deleted without affecting its transfer (Zambryski et al., 1983; de Framond et al., 1983; An et al., 1985). In the Ti plasmid the T-DNA is defined and bounded by identical 25-bp direct-repeats (Wang et al., 1984, and the references therein). Because only DNA between these T-DNA borders is seen to be transferred to the plant genome, these sequences likely are the structural substrates of the proteins that directly mediate the transfer process. While the T-DNA borders are cis-acting sequences, the chv and vir loci function in trans. Chv mutant bacteria are avirulent on plants (Garfinkel and Nester, 1980), and the chvA and chvB loci have been shown to specify binding of Agrobacterium to plant cells. These loci are constitutively expressed in the bacterium (Douglas et al., 1985); in contrast, the expression of the vir loci appears to be highly regulated. The vir loci are located within the Ti plasmid vir region, and it has recently been shown that the expression of vir region sequences becomes induced to high levels when Agrobacteria are cocultivated with actively growing plant cells (chapters 2 and 4). This induction is specifically mediated by low molecular weight and wound-specific metabolites produced by these cells (chapter 5). Specific T-DNA-associated molecular events are also observed to occur during this induction (Koukolíková-Nicola et al., 1985; chapters 4 and 5). These results

are evidence that the T-DNA transfer process is active only when Agrobacterium is in the presence of plant cells susceptible to infection; and that the products of genes contained within the vir region direct the events of this process.

While the importance of the vir region as the mediator of T-DNA transfer is clear, an understanding of the genetic and transcriptional organization of this region is incomplete. To date, mutational analyses have indicated that a continuous region of roughly 40 kb is essential for virulence (Garfinkel and Nester, 1980; Holsters et al., 1980; Ooms et al., 1980). While several studies on the genetic organization of this region in a number of different Ti plasmids have been reported (Klee et al., 1982, 1983; Iyer et al., 1982; Hille et al., 1984; Knauf et al., 1984; Lundquist et al., 1984), a complete and accurate picture of this organization is not presently available; the vir region has not been fully saturated by mutations, and the organization of the existing vir mutations into distinct vir complementation groups has not been firmly established. Preliminary vir expression studies have suggested that certain vir region sequences are constitutively transcribed (Gelvin et al., 1981), while other sequences are plant-activated (chapter 4; however, these studies do not provide a comprehensive view of the transcription of the vir region during either vegetative growth or cocultivation with plant cells.

Since the vir loci are essential for T-DNA transfer, a detailed understanding of the genetic and transcriptional organization of the vir loci within the vir region is essential to an understanding of the T-DNA transfer process itself. The present work was initiated

with the aim of producing this information for the pTiA6 vir region. The results of this study provide a foundation for future studies into the functions specified by the vir loci and their role in the transformation process, and also into the regulation of vir expression (chapter 6).

## Results

### a) Experimental approach

To study the genetic and transcriptional organization of the pTiA6 vir region, the experimental strategy shown in Figure 3.1 was followed. This strategy is based on a set of vir::lac insertion molecules, generated by inserting a promoterless lacZ gene throughout plasmid clones of pTiA6 vir regions sequences. Each vir::lacZ insertion is both a potential vir mutation and vir::lacZ fusion gene, and as outlined in Figure 3.1 and described below, the insertion set allows the determination of (i) the vir region sequences required for virulence, and the genetic organization of these sequences; and (ii) the vir region sequences which are transcribed, the transcriptional orientation of these sequences, and the conditions under which their expression occurs.

To assess the mutagenic effect of a particular insertion it is introduced onto pTiA6 by homologous recombination in Agrobacterium and the resultant recombinant is tested for its ability to cause tumors on plants; if a recombinant displays altered virulence the insertion that it carries is presumed to be within sequences that

encode a vir function. Vir insertion mutations are subsequently organized into separate vir loci in genetic complementation experiments using vir merodiploid strains. Each merodiploid contains a pTiA6 vir mutant, and a complementing plasmid that carries a vir::lac insertion or a vir deletion; if wild-type virulence is restored in a merodiploid, the two vir mutations that it carries are presumed to occur in separate vir loci. The structural limits of each vir locus are determined from the positions of flanking insertions that are either virulent or within adjacent vir loci.

The promoterless lacZ element used as the insertion mutagen is also a reportor for the transcription of the sequences into which it has inserted. Expression at the insertion site can be monitored by assaying for β-galactosidase activity, the lacZ gene product. Because Agrobacterium does not have its own β-galactosidase activity (Plessis et al., 1985; chapter 2), a significant level of activity present in a cell that carries a vir::lac insertion indicates that the lacZ sequences of the insertion element are located downstream of, and in the same transcriptional orientation, as a vir region promoter undergoing active transcription. Such expressed insertions are designated vir::lac fusion genes. By identifying which of the insertions are vir::lac fusions and which are not, the transcribed sequences, and thus the transcription units, of the vir region are determined.

It has been shown that the expression of vir region sequences becomes activated when Agrobacterium is cocultivated with actively growing plant cells (chapters 2 and 4). It is also likely that other vir region sequences are constitutively expressed in both

vegetatively grown and cocultivated bacteria. Thus, it is necessary to analyze vir expression under two different conditions, vegetative growth and cocultivation with plant cells. In the experimental strategy, the level of β-galactosidase activity expressed by each insertion of the insertion set is determined for these two conditions. The results of these experiments define two classes of expressed lac insertions, and thus two classes of expressed vir loci. Insertions effecting significant levels of vegetative β-galactosidase activity are designated constitutive insertions and identify constitutively expressed sequences. Insertions that express β-galactosidase activity altered by cocultivation with plant cells are designated plant-inducible insertions and identify plant-inducible sequences. Each expressed insertion has three distinct properties, its location, orientation, and the condition(s) under which it is expressed.

In total, each vir::lac insertion mutation is tested for its functional (i.e., affect on tumor-inducing phenotype) and its transcriptional (i.e., β-galactosidase expression) activity. The results of these complementary tests for the entire insertion set provide a detailed analysis of both the genetic organization, and the vegetative and plant-activated transcriptional organization, of the pTiA6 vir region.

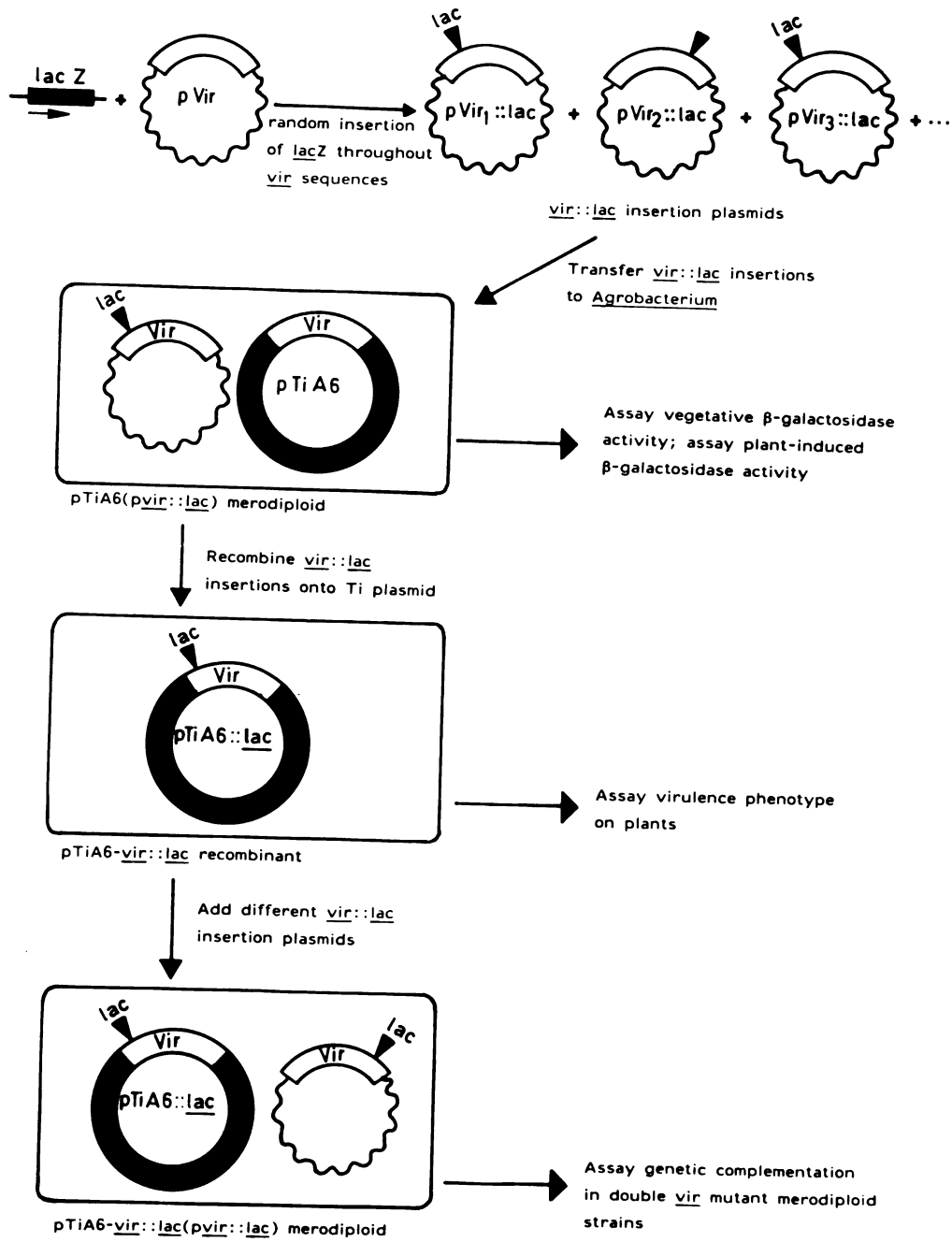


**Figure 3.1. Experimental strategy for the analysis of the genetic and transcriptional organization of the vir region of the A6 Ti plasmid of Agrobacterium.**

b) Construction of vir::lac insertions

The vir::lac insertions were generated in E. coli by Tn3-HoHo1 transposon mutagenesis (chapter 2) of three vir cosmid clones, pVCK219, pVCK221, and pVCK225 (Knauf and Nester, 1982; Figure 3.2), which contain overlapping portions of the pTiA6 vir region, replicate in both E. coli and Agrobacterium, and are readily transferred by conjugation between these two organisms. Tn3-HoHo1 is a Tn3-lac transposon that carries a promoterless lacZ gene that is in-frame through the left-end of the element. This element transposes only when transposase is supplied in trans, and Tn3-HoHo1 insertions are stable in the absence of transposase. Transposition of Tn3-HoHo1 into target DNA sequences results in the random generation of insertional mutations in, and lac fusions to, genes within the target sequences; both transcriptional and translational lacZ fusions can be generated with this element. The transcriptional fusions express a β-galactosidase protein completely encoded within Tn3-HoHo1 that is dependent on external transcription; the translation fusions express chimeric β-galactosidase fusion proteins that depend both on external transcription and translation. Statistically, one out of three gene fusions generated by Tn3-HoHo1 will be translational.

Following isolation of vir::Tn3-HoHo1 insertion plasmids, the position and orientation of each insertion was determined by restriction analysis. One hundred and twenty-four independent insertion plasmids were chosen as a representative set of vir::Tn3-HoHo1 insertions. An attempt was made to have insertions of both orientations of Tn3-HoHo1 every 0.5 kb across the pTiA6 vir region, from

SalI fragment 4 through SalI fragment 21. This criterion was not absolutely fulfilled as some sequences proved refractory to Tn3-HoHo1 mutagenesis. The set of insertion plasmids contains 37 pVCK219, 31 pVCK221, and 56 pVCK225 derivatives (Figure 3.2). An identifying number has been assigned to each insertion, and in Figure 2 the position of an insertion is indicated by this number and a vertical line drawn either above or below the horizontal line that represents the vir plasmid clone in which the insertion was isolated. The transcriptional orientation of the Tn3-HoHo1 lacZ gene of insertions represented by lines drawn above the plasmid clones is left to right, and below these clones is right to left. These two classes of insertions are designated LR and RL insertions, respectively. In the following presentation, insertions are used in two different molecular environments : within their original insertion plasmids, and within their pTiA6 recombinants. These molecules are respectively indicated by the identifying number of the insertion preceded by the prefixes pSM and pTi.

c) Insertion mutagenesis of the pTiA6 vir region

The 124 insertion plasmids were initially transferred to Agrobacterium strain A348 which contains wild-type pTiA6 to give a set of 124 independent A348(pSMvir::lacZ) merodiploid strains. Throughout the rest of the present study these strains are referred to as pTiA6(pSMvir ::lac) strains, to indicate that they carry the wild-type A6 Ti plasmid, and a vir::lac insertion plasmid. In 112 of the pTiA6(pSMvir:: lac) strains the vir:: Tn3-HoHo1 insertion was intro-

duced onto pTiA6 by double homologous recombination, and two independent pTiA6::Tn*3*-HoHo1 recombinants per insertion were isolated. The recombinant strains were inoculated onto leaf wounds of *Kalanchoe daigremontiana* to assess their virulence, and each duplicate strain was independently tested on two plants.

The results of the insertional mutation analysis of the pTiA6 *vir* region are presented in Figure 3.2. In this analysis, the *vir*::*lac* recombinants displayed three general phenotypes for tumor formation, wild-type virulence, avirulence, and attenuated virulence. These phenotypes are represented in Figure 3.2 by open circles, closed circles, and closed triangles, respectively. Forty-eight recombinants were fully virulent and 60 were avirulent, and both isolates of each recombinant displayed a single and identical phenotype. As shown in Figure 3.2 (closed circles), the avirulent insertions fall into four discrete regions that are separated by several virulent insertions. Two of the regions are bisected by single virulent insertions : insertion 41 in *Sal*I fragment 13b and insertion 324 in *Sal*I fragment 9, and each of these insertions is closely flanked by avirulent insertions. The virulent phenotype of two segments of approximately 1.0 kb within the pTiA6 *vir* region were not probed by insertional mutagenesis. These segments are between insertions 231 and 219 in *Sal*I fragment 4, and between insertion 368 and 363 in *Sal*I fragment 13b.

Four insertions produced attenuated virulence; pTi364, pTi365, pTi379, and pTi361 induce tumors that begin to appear only 30 days post-inoculation. After 60 days, tumors of wild-type size are seen; however, the tumor tissue does not occur throughout the wound site,

but is limited to one or two sites that from several test inoculations appear to be randomly distributed. The 364, 365 and 379 insertions are located within a 1.2-kb region of SalI fragment 7. The attenuated 361 insertion is located within SalI fragment 21, while more rightward SalI fragment 21 mutations are avirulent when tested on Kalanchoe.

Several of the avirulent and attenuated virulent recombinants were also inoculated on Nicotiana tabacum leaf discs and Nicotiana glauca stem wounds. The tumor-inducing phenotype of each tested recombinant on these two assay systems is identical to that observed on Kalanchoe with the exception that the pTi364, pTi365, and pTi379 strains are fully virulent on N. glauca stem wounds, and all the SalI fragment 21 mutations, scored as avirulent on Kalanchoe, give attenuated virulence on N. tabacum leaf discs. These results suggest that these two groups of insertion mutations are within vir loci that encode host range determinants of Agrobacterium pathogenicity, or accessory functions of the T-DNA transfer process.

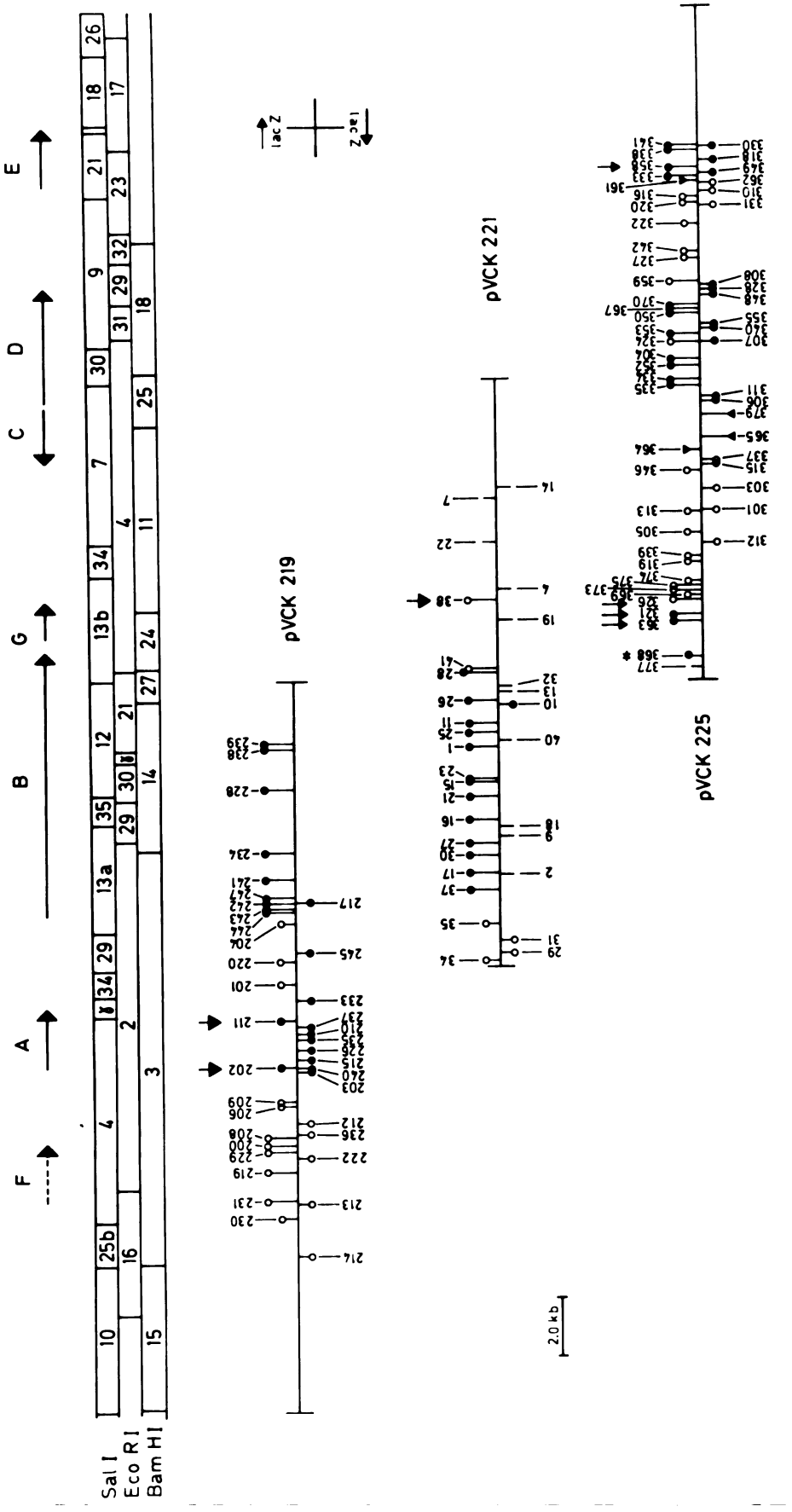


Figure 3.2. Genetic and transcriptional organization of the vir region of pTiA6.

Legend to Figure 3.2.

The respective map positions, defined genetic boundaries, and transcriptional orientation of the vir region loci pinF, virA, virB, virG, virC, virD, and virE are indicated by arrows above the SalI, EcoRI, and BamHI restriction enzyme maps of the pTiA6 vir region. pVCK219, pVCK221, and pVCK225, three cosmid clones that contain pTiA6 vir region sequences are shown below as horizontal lines. These clones were mutagenized with the Tn3-lacZ transposon, Tn3-HoHo1, to generate 124 independent pSM(vir:: lacZ) insertion plasmids. The figure is drawn to show 4 distinct properties for each Tn3-HoHo1 insertion – its map position, the orientation of its lac sequences, the β-galactosidase expression phenotype of its corresponding pTiA6(pSMvir::lac) merodiploid strain, and the virulence phenotype of its corresponding pTiA6 recombinant strain. Each insertion has been assigned an identifying number, and its map position is indicated by the vertical line adjacent to its number. Those insertions whose Tn3-HoHo1 lac sequences are oriented, with respect to the pTiA6 vir map, leftward to rightward (LR), are drawn above, and those oriented rightward to leftward (RL) are drawn below, the horizontal lines of the cosmid clones, respectively. The length of the vertical line that represents an insertion indicates whether its pTiA6(pSMvir::lac) strain exhibits plant inducible β-galactosidase activity (> 4-fold change from its vegetative activity) : insertions represented by short lines are non-inducible, and by long lines are plant-inducible. The LR insertions located within pinF, virB, virG, virD, and virE are plant-inducible, and thus these

Legend to Figure 3.2 (cont'd)

loci are transcribed left to right. The virC RL insertions are plant-inducible, and thus virC is transcribed right to left. The vertical arrows indicate insertions that give high-levels (> 25 U) of vegetative β-galactosidase activity. The left to right transcriptional orientation of the noninducible virA locus was determined from the orientation of its insertions displaying this phenotype. The asterisk (*) above insertion 368 indicates that this insertion is noninducible when located on its insertion plasmid (pSM368), and becomes inducible when recombined onto pTiA6 (pTi368). The vir::lac insertions were recombined onto pTiA6 to assess their effect on virulence. Insertions marked with open circles are fully virulent, with closed circles are avirulent, and with closed triangles are partially virulent. Unmarked insertions were not tested for virulent phenotype. The separate vir complementation groups are defined by the complementation analysis presented in Table 3.1 and Figure 3.3. Note that insertions 34, 35, 37, 38, and 41, are carried by pVCK242; pVCK242 is a pTiA6 vir clone that is identical to pVCK221 except that, it also has SalI fragment 34a at its leftward end and SalI fragment 30 at its rightward end (Knauf and Nester, 1982).

d) Genetic organization of the pTiA6 *vir* region

In the above analysis the areas of the pTiA6 *vir* region that encode virulence functions are identified. In the results of the complementation analysis presented in Figure 3.3 and Table 3.1, these areas are determined to be organized as six separate *vir* complementation groups, *vir*A, B, C, D, E, and G. An important aspect of this analysis is that Tn*3*-HoHo1 is both a direct and polar mutagen; insertion of Tn*3*-HoHo1 into a locus directly disrupts coding sequences, and, when within a polycistronic unit, disrupts the functional regulated expression of downstream cistrons. Thus, the mutant phenotype of each Tn*3*-HoHo1-generated pTiA6 *vir* mutant should only be complemented by molecules that carry a complete and intact copy of the particular *vir* locus that the insertion is within. While this rule is generally valid, exceptions can occur, as presented below.

(i) *vir*A

Complementation results obtained with the avirulent mutants pTi202, pTi226, and pTi211, which carry insertions within a 1.8-kb region of *Sal*I fragment 4, define the *vir*A locus. These mutants are complemented to wild-type virulence by pSM219 and pSM233, pVCK219 derivatives which carry Tn*3*-HoHo1 insertions outside of the 1.8-kb *vir*A region, but not by pSM226, which carries an insertion within this region. The positions of the wild-type insertions 203 and 201 (Figure 3.2) indicate that the maximum size of *vir*A is 2.5 kb.

(ii) virB

The virB locus is defined by complementation results obtained for 10 avirulent mutations (Figure 3A) that span an 8.5-kb region from SalI fragment 13a through the left end of SalI fragment 13b. Only plasmids that carry a complete and intact copy of this region (with the exception of pSM41, see below) complement these mutants.

The virB region has been reported to contain six separate complementation groups (Iyer et al., 1982), one of which cannot be trans-complemented (Klee et al., 1982; Iyer et al, 1982). In the present analysis this region behaves as a single locus, and results presented below (Table 3.3) on the expression of the virB locus further support this conclusion. Also, all virB mutants were observed to be trans-complementable. The maximum leftward extent of virB is defined by the position of the wild-type insertion 35 (Figure 3.2). The maximum rightward extent of virB is given by the position of insertion 363 which is within virG, and from the position of the leftward terminus of virG, defined by DNA sequence analysis (Ebert et al., 1986). The maximum size of virB is 9.5 kb.

Results obtained with insertion 41, which is located within SalI fragment 13b (0.1 kb to the right of the virB insertion mutation 28), are at odds with those obtained with other virB insertions. pTi41 is fully virulent and pSM41 complements virB mutants, suggesting that insertion 41 is located outside and to the right of virB; however, three pTiA6 mutants, A1010, pTi368, and A1061 (Figure 3.3), which carry transposon mutations located approximately 0.1, 0.6, and 0.7 kb, respectively, to the right of insertion 41, complement as virB mutants. Thus, insertion 41 must be within virB

sequences that do not encode a vir function, such as an untranslated intercistronic region. Also, since this insertion is closely flanked by avirulent mutations, it must be functionally nonpolar for virB transcription.

A possible explanation for this nonpolarity is that in certain instances Tn3-HoHo1 might be able to promote the functional expression of sequences downstream of its site of insertion. If this occurs for an insertion located within an intercistronic region of an operon, the insertion will be nonpolar for downstream cistrons and display a wild-type phenotype. It is noted that since the bla gene of Tn3-HoHo1 is located adjacent to the rightward inverted-repeat of Tn3-HoHo1 (chapter 2), the inefficient termination of bla transcription of an insertion could result in the transcription of sequences downstream of its right end.

(iii) virG

Three avirulent mutants, pTi363, pTi19, and pTi321, carry insertion mutations within a 0.2-kb region of SalI fragment 13b. These mutations, which do not cross-complement, define the virG locus. This locus is closely adjacent to the right end of virB and results of several complementations demonstrate that virG and virB are genetically distinct : pVCK222, pVCK223, and Tn3-HoHo1 derivatives of pVCK225, which lacks the left 8 kb of virB, restore virulence to virG, but not virB mutants; also, virG and virB mutants cross-complement. Furthermore, virG and virB mutants can be distinguished phenotypically as plant-induced vir expression occurs in virB but not virG mutant bacteria (chapter 6). The virG locus has

maximum size 1.2 kb. Its leftward end has been defined by DNA sequence analysis (Ebert et al., 1986), and the maximum extent of its rightward end by the position of insertion 38 (Figure 3.2).

(iv) virC

The insertions 364, 365, and 379 define the virC locus. As previously discussed, pTi364, pTi365, and pTi379 display an attenuated virulent phenotype. pTi365 is complemented by pVCK225 derivatives other than pSM364, pSM365, or pSM379. VirC is proximal to virD, and these loci were determined to be separate on criteria of phenotype, complementation, and transcriptional orientation : unlike virC mutants, virD mutants are avirulent; the virC mutant pTi365 is complemented by several virD insertion cosmids, and several virD mutants are complemented by pSM365; and, virC and virD are divergently transcribed (Table 3.1, and Figure 3.2). The positions of virulent insertion 337 and of virD insertion 306, respectively, mark the maximum leftward and rightward boundaries of virC (Figure 3.2). VirC has maximum size 2.0 kb.

(v) virD

The virD locus is defined by the complementation results obtained for insertion mutations located within a 4.5-kb region that extends rightward from the right end of SalI fragment 7, through SalI fragment 30, and into the left end of SalI fragment 9. With the exceptions of insertions 304 and 324 (see below), virD mutations do not cross-complement. Furthermore, complementation of several insertions within the 4.5-kb virD regions is not obtained if the

complementing vir cosmid carries only a portion of this region. The position of the virulent pTi359 insertion marks the maximum rightward extent of virD. The maximal leftward extent of virD is defined by the position of the virC insertion 379.

Results obtained with insertion 324 are contrary to those obtained with other virD Tn3-HoHo1 insertions. While insertion 324 is in the middle of virD, pTi324 is fully virulent and virD mutants are complemented by pSM324. Thus, this insertion is analogous to the virulent virB insertion 41, and potentially identifies the position of a virD intercistronic sequence. Further anomalous results are obtained with the avirulent insertion 304. Insertion 304 is located within SalI fragment 30, and is bounded to its left by avirulent insertion 352, and to its right by virulent insertion 324 and avirulent insertion 307. pTi304 does not complement like other virD mutants, since it is complemented by plasmids which do not carry an intact virD locus but only the leftward portion of virD (SalI fragments 7 and 30). Furthermore, pSM304, is able to restore virulence to virD mutants that map rightward, but not leftward, of the site of the 304 insertion. These results, in total, can be interpreted to indicate that the virD locus is two distinct complementation loci that respectively fall to the left and right of the site of insertion 324; however, this interpretation conflicts with the extensive complementation results that were obtained for the other virD mutations (Table 3.1). Another possible explanation for the 304 complementation results is that insertion 304 disrupts the same virD encoded function that is disrupted by mutations that fall leftward of insertion 324, but, unlike these mutations, inser-

tion 304 allows the functional expression of the virD sequences that fall rightward of insertion 324. This expression may be promoted by the Tn3-HoHo1 element, as discussed above for the wild-type virB insertion 41.

(vi) virE

Complementation results obtained with mutants pTi361, pTi358, and pTi341 define the virE locus. Insertions 361, 358, and 341 are located within a 1.2-kb region of SalI fragment 21 and do not cross-complement. The leftward boundary of virE is marked by virulent insertion 362 (Figure 3.2), and is located approximately 300 bp rightward of the left end of SalI fragment 21. pVCK223 (Figure 3.3), which carries vir sequences inclusive and leftward of SalI fragment 21, does not complement pTi358; thus the right end of virE extends beyond this fragment. Since Tn3-HoHo1 insertions located just within the right end of SalI fragment 21 are avirulent on Kalanchoe and complement as virE mutations, and insertions located just within the left end of SalI fragment 18 are fully virulent (P. Totten and S.E. Stachel, unpublished data); the right terminus of virE probably occurs within the approximately 150-bp SalI fragment located between SalI fragments 21 and 18 (Figure 3.2). VirE has maximum size 2.0 kb. As described in the previous section (c) above, virE mutants are avirulent on Kalanchoe but give attenuated virulence on N. tabacum leaf discs. Thus, virE, like virC, is not absolutely essential for Agrobacterium transformation of all plant species; however, the respective functions encoded by these loci are probably different since the attenuation of virulence caused by virE mutations is much greater than by virC mutations.

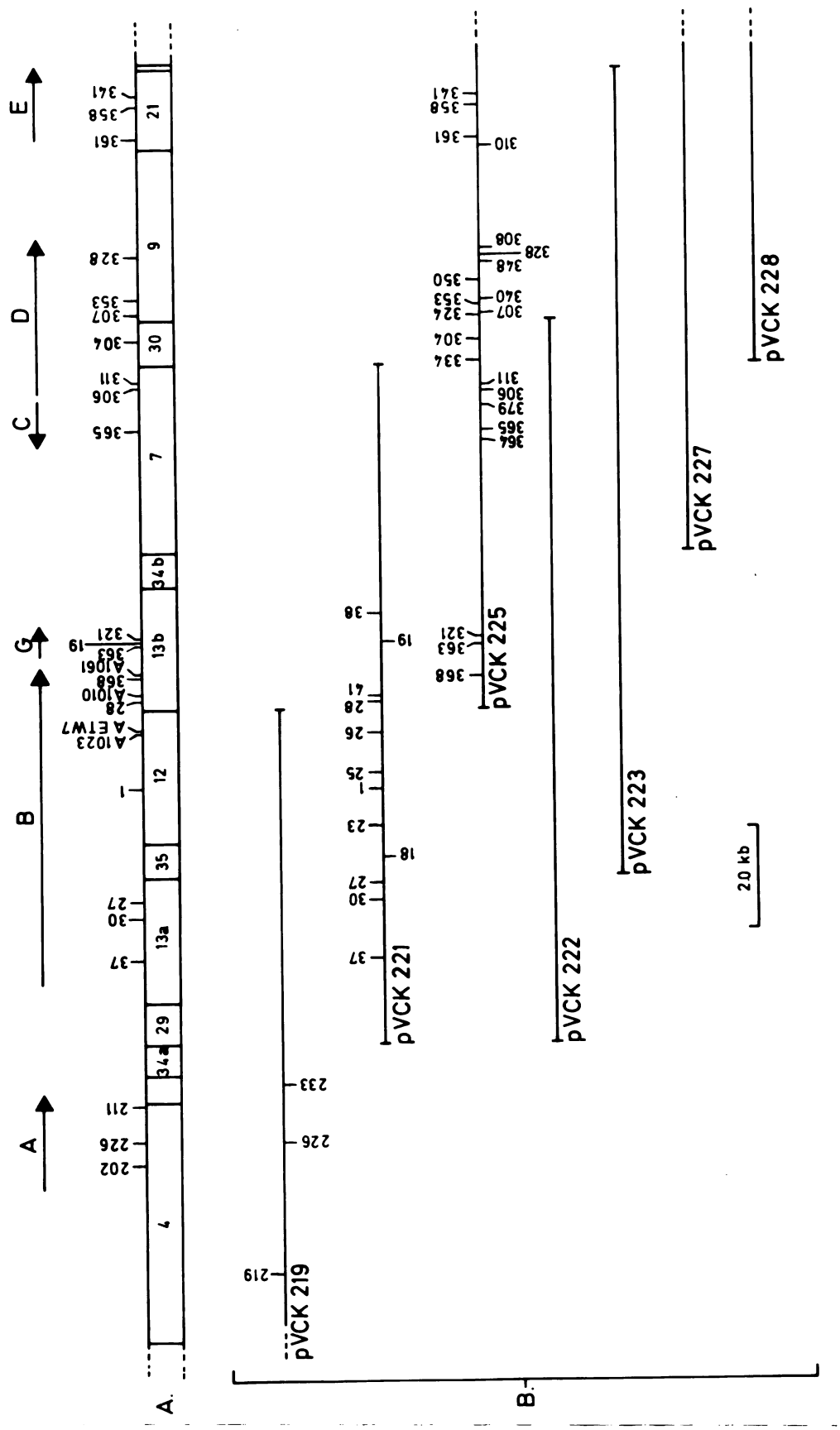


Figure 3.3. pTiA6 vir mutants and pVir cosmids used in the vir genetic complementation experiments of Table 3.1.

Legend to Figure 3.3.

A. The map positions and identifying numbers of the mutations of the tested pTiA6 *vir* mutants are indicated above the *Sal*I restriction map of the *vir* region.

B. pVir cosmids used to complement the pTiA6 *vir* mutants. Several of these cosmids are pSM(*vir*::*lac*) insertion plasmids and carry Tn*3*-HoHo1 insertions. The position of an insertion is given by the vertical line next to its identifying number. Those insertions whose Tn*3*-HoHo1 *lac* sequences are oriented leftward to rightward are indicated above, and those of the opposite orientation are indicated below, the horizontal lines that represent the pTiA6 *vir* sequences carried by the parental pVCK cosmid clones.

Table 3.1. Genetic complementation of pTiA6 vir mutants.

| Mutant | Merodiploid pTiA6/pVir cosmid | Virulent phenotype |
|---|---|---|
| VirA | 202 pSM219 | + |
| | 226 pSM226 | - |
| | 211 pSM233 | + |
| VirB | A1023 pSM19 | + |
| | AETW7 pSM23 | - |
| | pSM25 | - |
| | pSM26 | - |
| | pSM28 | - |
| | pSM41 | + |
| | 37 pVCK222 | + |
| | 27 pVCK223 | - |
| | 1 pSM37 | - |
| | 28 pSM27 | - |
| | pSM26 | - |
| | pSM28 | - |
| | 38 pVCK222 | + |
| | A1010 pVCK223 | - |
| | A1061 pSM18 | - |
| | pSM19 | + |
| | 30 pVCK219 | - |
| | 37 pSM41 | + |
| | 368 pVCK222 | + |
| | pVCK223 | - |
| | pSM28 | - |
| | pSM363 | - |
| VirG | 363 pVCK222 | + |
| | 19 pVCK223 | + |
| | 321 pSM28 | + |
| | pSM19 | - |
| | pSM304 | + |
| | pSM321 | - |
| | pSM368 | + |
| VirD | 306 pSM306 | - |
| | 353 pSM334 | - |
| | pSM340 | - |
| | pSM350 | - |
| | pSM348 | - |
| | pSM308 | - |
| | pSM358 | + |
| | 307 pSM365 | + |
| | 311 pSM311 | - |
| | 328 pSM324 | + |
| | pSM307 | - |
| | pSM328 | - |
| | 328 pVCK227 | + |
| | pVKC228 | - |
| | 311/pVCK222 | - |
| | pVCK223 | + |
| | 304 pVCK222 | + |
| | pVCK223 | + |
| | pVCK227 | + |
| | pVCK228 | - |
| | pSM365 | + |
| | pSM311 | - |
| | pSM324 | + |
| | pSM307 | + |
| | pSM328 | + |
| | 306 pSM304 | - |
| | 311 | |
| | 304 | |
| | 307 pSM304 | + |
| | 353 | |
| | 328 | |
| | 358 pSM311 | + |
| | pSM304 | + |
| | pSM307 | + |
| | pSM328 | + |

Table 3.1. (cont'd)

| Mutant | Merodiploid pTiA6/pVir cosmid | Virulent phenotype | Mutant | Merodiploid pTiA6/pVir cosmid | Virulent phenotype |
|---|---|---|---|---|---|
| VirC | 365 pSM364 | -/+ | VirE | 358 pSM361 | -/+ |
| | pSM311 | + | | 341 pSM358 | - |
| | pSM304 | + | | pSM341 | - |
| | pSM307 | + | | | |
| | pSM324 | + | | 361 pSM310 | + |
| | pSM328 | + | | pSM361 | -/+ |
| | pSM19 | + | | pSM358 | -/+ |
| | pSM379 | -/+ | | pSM341 | -/+ |
| | pSM365 | -/+ | | | |
| | | | | 358 pVCK223 | - |
| | | | | pVCK225 | + |

One-hundred and fifty-seven merodiploid strains were constructed and assayed for their ability to induce tumors on Kalanchoe daigremontiana leaf wounds. Each strain contains two plasmids, a pTiA6 derivative that is mutant for a vir locus (mutant pTiA6), and a complementing cosmid that carries a portion of the pTiA6 vir region (pVir cosmid). The pTiA6 vir mutants and pVir cosmids used are diagrammed in Figure 3.3. The experimental results are presented in sets as 2 x 2 matrixes, and indicate that each pTiA6 mutant (noted to the left of the vertical lines) in the set has been tested with each pVir cosmid (noted to the right of the vertical lines). For example, for virA, 3 pTiA6 virA mutants were tested with 3 pVir cosmids, and thus 9 independent merodiploid strains were constructed and assayed : pTi202(pSM219), pTi206(pSM219), and pTi211 (pSM219) are virulent; pTi202 (pSM226), pTi206(pSM226), and pTi211(pSM226) are avirulent; and pTi202 (pSM233), pTi206(pSM233), and pTi211(pSM233) are virulent. -, avirulent; +, virulent; -/+, partially virulent.

e) Constitutive and plant-induced vir expression

The set of 124 pTiA6(pSMvir::lac) merodiploid strains was used to identify constitutively expressed and plant-inducible vir::lac insertions (Figure 3.2). Each of these merodiploid strains carries a wild-type copy of the vir region on pTiA6. Two experimental values were determined for each strain, its vegetative and plant-induced β-galactosidase activities. Representative numerical data are presented in Table 3.2. As described below, these data identify (i) the vir loci which are constitutively expressed; (ii) the vir loci which are plant-inducible, including loci not required for Agrobacterium pathogenicity; and (iii) the respective transcriptional orientation of each expressed locus. These results, in total, allow the generation of a map of the transcriptional organization of the pTiA6 vir region, schematically represented by the arrows drawn at the top of Figure 3.2.

(i) Constitutively expressed vir loci

Vegetative β-galactosidase activity was measured following growth in minimal medium. Of the 124 insertions tested, 117 give less than 10 units of uninduced activity, indicating that much of the pTiA6 vir region is not expressed, or is expressed at only low levels, during vegetative growth. In contrast, certain vir sequences appear to be transcribed at significant levels in vegetative Agrobacteria since 7 insertions give levels of uninduced activity in excess of 25 units. These insertions are indicated in Figure 3.2 by vertical arrows and include the virA::lac insertions 202 and 211,

the virG::lac insertions 363 and 321, the virulent insertions 326 and 38 which are located just rightward of virG, and the virE::lac insertion 358. Each of these constitutive insertions is oriented leftward to rightward (LR) with respect to the vir region; oppositely orientated RL insertions within virA, virG, and virE effect only low levels of vegetative β-galactosidase activity. Thus, virA, virG, and potentially virE, appear to be transcribed in a leftward to rightward direction during vegetative growth. However, of the 5 virE::lac LR insertions tested, only insertion 358 gives a high level of vegetative activity, so that this high level may be anomalous and virE is probably not vegetatively expressed.

In a previous analysis of pTiA6-encoded RNA, three areas of pTiA6 were shown to be transcribed at detectable levels in vegetative bacteria (Gelvin et al., 1981). These areas were identified as the T-DNA region, and two segments of the vir region whose map positions correspond to the genetic positions of virA and virG.

(ii) Plant-inducible vir loci

Plant-induced β-galactosidase activity was measured following 16 hours cocultivation of bacteria with Nicotiana tabacum suspension culture cells. [The assay procedure is schematically shown in Figure 3.4]. For each insertion the ratio of its plant-induced activity to its vegetative activity was determined, and it is designated plant-inducible or plant-repressible if this ratio is greater than 4 or less than 0.25, respectively. Forty-eight of the 124 insertions are plant-inducible, demonstrating that the expression of much of the vir region becomes activated when Agrobacterium is grown in the

presence of plant cells. The β-galactosidase activity displayed by each of the remaining insertions is not affected by cocultivation. Because no insertions are plant-repressible, the vir region does not appear to contain sequences whose expression is repressed during cocultivation.

Figure 3.4. Assay for plant-induced vir::lac expression.

In Figure 3.2 the inducible phenotype of each insertion is represented by the length of the line that indicates its position; insertions represented by long lines are plant-inducible, and by short lines are non-inducible. The inducible insertions are seen to be organized into six distinct regions that respectively correspond to the positions of virB, virG, virC, virD, virE, and the virulent plant-inducible locus pinF (discussed below). Except for virC, all inducible insertions are oriented left to right, indicating that virB, virG, virD, virE, and pinF are expressed in this direction. The inducible virC insertions 379 and 365 are oriented right to left, and thus virC is transcribed in the opposite direction than the other vir loci.

There are at least two distinct classes of non-inducible insertions, those within an inducible locus but oriented opposite to its direction of transcription, and those within a region in which insertions of either orientation are non-inducible. This second class identifies non-inducible vir region sequences. These sequences occur between the 3' end of pinF and the 5' end of virB; between the 3' end of virG and the 3' end of virC; and between the 3' end of virD and the 5' end of virE. Since no virA insertions are inducible indicates, in conjunction with the results presented in the previous section, that virA is non-inducible and constitutively expressed in a leftward to rightward direction.

Besides defining the plant inducibility and transcriptional orientation of each vir locus, the results of Table 3.2 and Figure 3.2 also illustrate several features of vir transcription and organization. For example, all insertions located within each

inducible locus and oriented in the direction of inducible transcription are plant-inducible (with the exception of the virE insertion 361, and the virB insertions 377 and 368, discussed below); furthermore, all oppositely oriented insertions are non-inducible. Thus, each inducible locus likely encodes a single transcript.

Insertion 361 is located at the 5' end of virE. Unlike other virE LR insertions, this insertion is non-inducible and produces an attenuated virulent phenotype on Kalanchoe. These observations suggest that insertion 361 might be located outside the transcribed region of virE and within, or closely adjacent to, its promoter. The virB LR insertions 377 and 368 are non-inducible when carried on pVCK225. This plasmid lacks the proximal region of virB, and thus, presumably, the virB promoter. As presented below and in Table 3.3, insertion 368 becomes inducible when recombined onto pTiA6.

Table 3.2 shows that the different inducible insertions exhibit different levels of induced β-galactosidase activity. Furthermore, the insertions can exhibit different levels of relative inducibility (Δ units, the ratio of induced to uninduced units). These results potentially indicate that particular vir loci are inducibly expressed at higher levels, and/or are more inducible, than other vir loci. However, because the differences between different loci are likely the result of several variables that include transcriptional and translational efficiency, as well as transcript and protein stability, it is difficult to fully assess the significance of the different results obtained with different insertions. Keeping this proviso in mind, the following observations are made.

First, of the 48 inducible insertions, the virC insertions

generally give the lowest levels of induced activity, while the virE insertion 358 gives the highest; these results suggest that virC and virE are the lowest and highest expressed inducible vir loci, respectively. Second, different insertions within a single locus can display different levels of inducibility, and generally, insertions located 5' are more inducible than those 3'. For instance, of the several LR insertions of virB and virD, those distally located are the least inducible. These results suggest that proximal plant-activated transcription in these loci might be greater than distal transcription.

The most highly inducible insertions are seen to encode chimeric vir::β-galactosidase fusion proteins (S.E. Stachel, unpublished data). For instance, the highly inducible insertions 219 (pinF), 304 (virD), and 358 (virE) have been shown in a preliminary report (chapter 2) to encode fusion proteins of 185,000, 195,000, and 156,000 D, respectively. The size of a fusion protein can be used to obtain insight into the structure of a genetic locus, specifically to identify the position of the translational start site of the cistron in which the insertion element is located. The lacZ gene of Tn3-HoHo1 encodes a β-galactosidase protein of size approximately 148,000 D (chapter 2). Thus, the vir::β-galactosidase fusion proteins encoded from insertions 219, 304, and 358 are initiated approximately 37 000 D (1.0 kb), 47 000 D (1.3 kb), and 8 000 D (250 bp) 5' (leftward), respectively, of the positions of their respective Tn3-HoHo1 insertions. The respective sites of initiation of the insertion 304 and insertion 358-encoded proteins correspond closely to the genetically determined 5' map positions of virD and virE

(Figure 3.2). Since pinF mutants have no discernible phenotype, the limits of pinF cannot be defined genetically; however, the site of initiation of the insertion 219 fusion protein potentially identifies the map position of the 5' end of pinF.

Table 3.2. Vegetative and plant-induced β-galactosidase activities of representative pTiA6(pSMvir::lac) strains.

| Locus/ orientation | Strain pTiA6(pSMvir::lac) | β-galactosidase units - induction | + induction | Δ units |
|---|---|---|---|---|
| A/LR | 202 | 48.4 | 55.3 | 1.13 |
| | 211 | 28.2 | 25.5 | 0.90 |
| A/RL | 240 | 3.10 | 2.45 | 0.79 |
| | 210 | 3.05 | 5.16 | 1.72 |
| B/LR | 243 | 3.24 | 283 | 87.3 |
| | 37 | 3.60 | 220 | 61.1 |
| | 30 | 9.53 | 548 | 57.5 |
| | 16 | 5.60 | 127 | 22.7 |
| | 1 | 7.20 | 589 | 81.8 |
| | 11 | 6.28 | 310 | 49.4 |
| | 26 | 5.32 | 89.6 | 16.8 |
| | 41 | 9.20 | 85.4 | 9.28 |
| B/RL | 217 | 3.18 | 2.75 | 0.87 |
| | 18 | 3.99 | 2.58 | 0.65 |
| | 40 | 3.53 | 3.43 | 0.97 |
| | 32 | 3.94 | 2.46 | 0.62 |
| G/LR | 363 | 64.8 | 879 | 13.6 |
| | 321 | 77.0 | 981 | 12.7 |
| G/RL | 19 | 3.68 | 3.26 | 0.89 |
| C/LR | 364 | 5.66 | 5.22 | 0.92 |
| C/RL | 379 | 3.42 | 61.5 | 17.9 |
| | 365 | 4.55 | 69.7 | 15.3 |

Table 3.2 (cont'd)

| Locus/ orientation | Strain pTiA6(*vir*::*lac*) | β-galactosidase units - induction | + induction | Δ units |
|---|---|---|---|---|
| D/LR | 335 | 3.55 | 70.8 | 19.9 |
| | 352 | 3.29 | 73.8 | 22.4 |
| | 304 | 5.91 | 624 | 106 |
| | 324 | 3.50 | 199 | 56.9 |
| | 353 | 3.49 | 59.6 | 17.1 |
| | 350 | 9.70 | 74.9 | 7.6 |
| | 370 | 3.87 | 36.30 | 9.38 |
| D/RL | 306 | 2.85 | 3.68 | 1.29 |
| | 307 | 3.12 | 2.78 | 0.89 |
| | 328 | 2.86 | 2.07 | 0.72 |
| E/LR | 333 | 3.59 | 45.4 | 12.6 |
| | 358 | 59.9 | 2941 | 49.1 |
| | 341 | 2.07 | 205 | 29.6 |
| E/RL | 362 | 2.48 | 1.9 | 0.77 |
| | 330 | 4.82 | 5.07 | 1.05 |
| *pinF*/LR | 231 | 6.14 | 174 | 28.3 |
| | 219 | 10.3 | 826 | 80.2 |
| | 208 | 4.24 | 69.4 | 16.4 |
| *pinF*/RL | 213 | 2.81 | 2.23 | 0.79 |
| | 222 | 2.63 | 2.55 | 0.97 |
| 3'G/LR | 38 | 26.1 | 156 | 5.98 |
| | 326 | 28.1 | 127 | 4.52 |

LR, the orientation of the *lac* sequences of the insertion is leftward to rightward, with regard to the pTiA6 *vir* region map of Figure 3.2. RL, the orientation of the *lac* sequences of the insertion are rightward to leftward. Δ units, the ratio of plant-induced activity to vegetative activity.

(iii) Plant-inducible sequences not required for virulence

Several insertions which do not affect virulence also give plant-inducible β-galactosidase activity. These insertions fall into two distinct classes, insertions located just 3' of plant inducible loci, and insertions located within the plant-inducible locus pinF. Strains carrying virulent LR insertions located just downstream of virG and virD express low levels of plant-inducible β-galactosidase activity. Specifically, insertions 326, 38, 373, and 375, which fall across a 1-kb region 3' of virG, give 4.5, 6.0, 7.9, and 7.4-fold plant-inducible activity, respectively; and insertion 359, which lies 3' of virD, gives 12-fold plant inducible activity. These results suggest that the termination of plant-inducible vir transcription in Agrobacterium might be inefficient.

Five LR-insertions located across a 2.2 kb region of the left end of the SalI fragment 4 give significant levels of plant-inducible β-galactosidase activity. Because the corresponding pTiA6 recombinants of these insertions are fully virulent, they define a plant-inducible locus whose product has no detectable role in Agrobacterium pathogenicity. This locus has been designated pinF. pinF has approximate maximum size 3.5 kb, and its maximum boundaries are marked by the noninducible LR insertions 230 and 206, which flank it. The function of pinF is unknown. Because its expression is under vir regulation (chapter 6), and is induced by specific plant cell metabolites (chapter 5), the pinF gene product(s) probably functions during the bacterial/plant interaction. Another plant-inducible region of pTiA6 not required for virulence is located

approximately 10 kb rightward of virE (P. Totten, and S.E. Stachel, unpublished observations).

(iv) virB is a single transcription unit

VirB appears to be a single transcription unit whose expression is driven from a plant-activated promoter located within SalI fragment 13a, since it complements as a single locus and is transcribed in a leftward to rightward direction. The results presented in Table 3.3 show that virB LR insertions are inducible only if linked to the 5'-terminal sequences of virB, and provide further evidence that virB encodes a single transcript.

virB LR insertions carried either on pVCK219, which lacks the 3' sequences of virB, or on pTiA6, are plant inducible; the strains pTiA6 (pSM243) and pTi243 (Figure 3.2) express approximately equivalent levels of induction. In contrast, the virB insertion 368 is inducible when located on pTiA6 (i.e., pTi368) and linked to 5' virB sequences, but not when located on pVCK225 (i.e., pSM368; Figure 3.2), which lacks these sequences. Thus, SalI fragment 13b does not carry an inducible vir promoter. It is noted that virG expression is independent of upstream virB expression, since the closely adjacent virG LR insertions 363 and 321 are inducible when carried on pVCK225 (Table 3.2).

Similar results are obtained for LR-insertions located internal to virB. pSM1 and pSM26 carry inducible virB insertions 1 and 26 within SalI fragment 12 (Figure 3.2). The Tn3-HoHo1::SalI insertion fragment from each of these plasmids was subcloned onto pVCK102 to generate the respective deletion derivation plasmids, pSM1cd and

pSM26cd. In these plasmids, the SalI insertion fragment is no longer linked to the proximal region of virB, and the strains pTiA6 (pSM1cd) and pTiA6(pSM26cd) do not display plant-inducible activity. Insertions 1 and 26 were also recombined from pSM1cd and pSM26cd onto pTiA6 to generate the respective recombinants, pTi1cd and pTi26cd. In these strains the proximal region of virB is restored to insertions 1 and 26, and consequently their inducibility is restored as well (Table 3.3). Thus, SalI fragment 12 does not carry an inducible vir promoter. These results, in total, demonstrate that greater than 50% of the distal portion of virB is dependent on proximal virB sequences for its regulated expression, and are further support that virB transcribes a single message from a promoter located within SalI fragment 13a.

Table 3.3. Dependence of the induced expression of 3' virB insertions on 5' virB sequences.

| Strain | β-galactosidase units | | Δ units |
|---|---|---|---|
| | - induction | + induction | |
| pTiA6/pSM243 | 4.14 | 291 | 70.3 |
| pTi243 | 1.92 | 84.7 | 44.1 |
| pTiA6/pSM368 | 6.01 | 9.72 | 1.62 |
| pTi368 | 3.27 | 50.3 | 15.4 |
| pTiA6/pSM1 | 7.20 | 589 | 81.8 |
| pTi1 | 2.51 | 212 | 84.5 |
| pTiA6/pSM1cd | 4.98 | 6.52 | 1.31 |
| pTi1cd | 2.06 | 176 | 85.4 |

Table 3.3 (cont'd)

| Strain | β-galactosidase units - induction | β-galactosidase units + induction | Δ units |
|---|---|---|---|
| pTiA6/pSM26 | 5.32 | 89.6 | 16.8 |
| pTi26 | 1.64 | 41.2 | 25.1 |
| pTiA6/pSM26cd | 3.40 | 3.02 | 1.26 |
| pTi26cd | 1.71 | 35.9 | 21.0 |

The vegetative (- induction) and plant-induced (+ induction) β-galactosidase activities of different pTiA6(pSM*vir*::*lac*) merodiploid strains and their corresponding pTi*vir*::*lac* recombinant strains were measured, and the inducibility (Δ units) of each strain was calculated. The insertion plasmids used to construct these strains are given in Figure 3.2 and in the text. It is noted that both the basal and induced units effected by an insertion are typically approximately 3 to 4-fold greater when it is located on its insertion plasmid, than when on its pTiA6 recombinant. This affect reflects the higher copy number of the insertion plasmid than the Ti plasmid in *Agrobacterium*.

## Discussion

We have analyzed the *vir* region of the A6 Ti plasmid using the Tn*3*-*lac* transposon, Tn*3*-HoHo1 (chapter 2). This element is both a polar mutagen and a reporter for the expression of the sequences into which it has inserted. The results of the present analysis allow the determination of the genetic and transcriptional organization of the pTiA6 *vir* region, and provide a foundation for understanding how the genes carried within this region function during the T-DNA transfer process.

We have used 124 Tn*3*-HoHo1 insertions across the 40-kb pTiA6 *vir* region to define the structural limits and respective mutant phenotypes of six *vir* complementation groups, *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, and *vir*G. Mutations in these loci eliminate (*vir*A, *vir*B, *vir*D, and *vir*G) or restrict (*vir*C and *vir*E) the ability of *Agrobacterium* to infect plant cells. We note that while we have retained the nomenclature (i.e. *vir*A, *vir*B, ...) previously used to designate the pTiA6 *vir* loci (Klee *et al.*, 1983), our assignments for the location and structural limits of each of the *vir* complementation groups are different than those reported earlier. Also, *vir*G has not been previously described.

Since Tn*3*-HoHo1 contains the coding sequences of the *E. coli* *lac*Z gene, the regulated expression of the pTiA6 *vir* region sequences was characterized by measuring the levels of β-galactosidase (the *lac*Z gene product) expressed from each of the *vir*::Tn*3*-HoHo1 insertions. For an insertion which has generated a chimeric *vir*::*lac* gene, the production of β-galactosidase is controlled by the

promoter of the vir locus to which lacZ has been fused. The expression of each insertion was monitored under two bacterial growth conditions, vegetative growth and cocultivation with plant cells. The results of these experiments define the vir transcription units, their respective orientations, and levels of expression as a function of growth condition. Each of the genetically defined vir loci is shown to correspond to a defined vir transcription unit. VirB, virC, virD, and virE are plant-inducible and are significantly expressed only in plant-induced bacteria; virA is constitutively expressed in vegetative and plant-induced cells, and is non-inducible; and virG is both constitutively expressed and plant-inducible. The pTiA6 vir region also carries another plant-inducible locus, pinF. In contrast to the vir loci, mutations in pinF have no effect on virulence. The map positions of the 5' termini of the vir transcription units have been precisely localized in recent S1 nuclease protection studies (chapter 6; Das et al., 1986).

At least two vir loci, virB and virD, likely encode polycistronic messages. VirB is 9 kb in length and complements as a single locus. We have shown that more than 50% of the distal portion of virB is dependent on proximal virB sequences for its regulated expression. The isolation of a wild-type virB insertion (insertion 41) also suggests that virB is polycistronic. Furthermore, recent analysis of vir-specific proteins in Agrobacterium has demonstrated that virB encodes several protein species (Engström, P., Zambryski, P., Van Montagu, M., and Stachel, S., in preparation). virD is 4.5 kb in size, and with the exception of the insertion mutant pTi304, virD mutations complement as a single locus. We have

also isolated a wild-type insertion (insertion 324) in virD; since this insertion bisects the virD locus, the virD message possibly encodes at least two cistrons, respectively located to the left and the right of the map position of insertion 324.

To obtain a detailed understanding of the T-DNA transfer process it is necessary to determine the specific functions encoded by the vir loci. The mutant phenotype, size, and relative expressibility, of a vir locus (as defined here) likely reflects the particular function that it specifies. For example, since mutations in different vir loci result in two distinct phenotypes, avirulence (virA, virB, virD, virE, virG) or attenuated virulence (virC, virE), the loci responsible for these phenotypes respectively encode functions that are absolutely essential for, or increase the efficiency of, plant transformation by Agrobacterium. Essential functions might include those which regulate vir induction, or are responsible for the synthesis, transfer and integration of a T-DNA intermediate molecule; accessory functions might include bacterial production of phytohormones or plant cell wall degradation enzymes, whose actions on the plant cell could increase its susceptibility to Agrobacterium, and thus the efficiency of T-DNA transfer. A large polycystronic locus (i.e. virB or virD) might specify a set of proteins that in concert mediate a complex step in the transformation process, such as the synthesis of the T-DNA intermediate molecule or the transfer of this molecule to the plant cell. Also, the highly expressed vir loci (i.e. virB, virE, virG, pinF) might specify proteins required in high concentration, such as strutural proteins, while the less expressed loci (virA, virC, virD) might specify enzy-

matic functions.

A number of recent studies have begun to give some indication of the functions encoded by particular vir loci. With regard to the accessory vir loci, virC has been implicated in specifying, in part, the plant host range of different species of Agrobacterium (Yanofsky et al., 1985). VirE mutant strains, but not other vir strains, can be extracellularly complemented by coinfection with bacteria that carry a wild-type vir region (Otten et al., 1984; D. Corbin and S.E. Stachel, unpublished results). Thus, virE might specify the production of a protein or compound that functions extracellularly or within the target plant cell, during infection. With regard to the essential vir loci, virA and virG have recently been demonstrated to encode trans-acting functions that are essential for the induction of vir expression in response to the plant signal molecule (chapter 6); these loci comprise the vir regulatory system. Therefore, of the essential vir loci, virB and virD represent the only candidates for loci which specify the machinery that directly mediates the T-DNA transfer process. It is also possible that these functions are not encoded by vir genes, but instead by the chv genes, or other as yet unidentified genes.

Recent results have shown that the expression of the inducible vir and pin loci is stimulated by specific phenolic compounds produced by plant cells susceptible to Agrobacterium infection (chapter 5); these data, along with the present analysis, indicate the vir loci are organized as a complex regulon. It is interesting to note that this regulon is located in a single region of the Ti plasmid, and contains at least 6 distinct transcription units that are

regulated coordinately. Why is the transcription of the vir region organized in this fashion ? Since the vir loci are coordinately expressed and closely linked, they might have been expected to be organized as a single transcription unit analogous to the early or late transcripts of bacteriophage lambda. Perhaps the independent transcription of the separate vir loci provides Agrobacterium with a more flexible system to control the relative levels of expression of loci whose products are required in different amounts for different functions. Future studies should determine whether there is a relationship between the transcriptional organization and functional organization of the vir region loci.

## Materials and Methods

### a) Reagents and general procedures

Restriction and DNA modification enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. X-gal was from Bethesda Research Laboratories. ONPG, and all antibiotics were from Sigma. Plasmid DNA transformation was by the method of Cohen et al. (1982), plasmid conjugal transfer by the method of Ditta et al. (1980), and plasmid isolation by the method of Birnboim and Doly (1979). Standard recombinant DNA procedures were as described (Maniatis et al., 1982).

b) Construction of plasmids

pVCK102, pVCK219, pVCK221, and pVCK225 have been described (Knauf and Nester, 1982). Briefly, pVCK219, pVCK221, and pVCK225 are recombinant derivatives of pVCK102, a broad-host range cloning vector, and carry overlapping segments of the pTiA6 vir region; these recombinants were constructed by cloning a SalI partial digest of pTiA6 into the single SalI site of pVCK102. Here, pVCK219, pVCK221, and pVCK225 were mutagenized in E. coli with the Tn3-lac transposon, Tn3-HoHo1 (chapter 2), to generate a set of pSM(vir::Tn3-HoHo1) insertion plasmids. The position and orientation of the Tn3-HoHo1 insertion within each insertion plasmid was determined by EcoRI, BamHI, and SalI restriction analysis of plasmid DNA. In cases of ambiguity, HindIII, SstI, and KpnI digests were also analyzed. The map positions of each insertion was determined to an accuracy greater than ± 0.20 kb. Because pVCK102 and Tn3-HoHo1 confer resistance to kanamycin and carbenicillin, respectively, the insertion plasmids confer resistance to both these antibiotics.

Tn3-HoHo1 is not cleaved by SalI, and thus the SalI::Tn3-HoHo1 fragment carried by an insertion plasmid can be independently isolated and cloned, to produce a SalI deletion derivative of the insertion plasmid. The deletion derivative of a pSM(vir::Tn3-HoHo1) insertion plasmid is constructed using the following procedure. The insertion plasmid is digested with SalI, ligated, and E. coli transformants are selected on kanamycin and carbenicillin. Plasmid DNA from each of these transformants is subsequently screened with SalI to identify pVCK102 clones that only carry the SalI::Tn3-HoHo1

fragment. The deletion derivative of an insertion plasmid is designated pSM(X)cd, where X corresponds to the assigned number of the insertion of the parent plasmid.

c) Agrobacterium strains

Each pSM(vir::Tn3-HoHo1) insertion plasmid was conjugally transferred to Agrobacterium strain A348 (Garfinkel et al., 1981), which carries pTiA6, to generate a corresponding A348(pSMvir::Tn3-HoHo1) merodiploid strain. These strains are resistant to carbenicillin and kanamycin. Using the procedure of Garfinkel et al. (1981), the Tn3-HoHo1 insertion carried on an insertion plasmid was transferred onto pTiA6 by homologous recombination to generate a corresponding pTiA6:: Tn3-HoHo1 recombinant strain. Each recombinant strain carries a pTiA6:: Tn3-HoHo1 recombinant, and pPHIJ1 which confers resistance to gentamicin (Hirsch and Beringer, 1983), and was used to select for the recombination event. These strains are resistant to carbenicillin and gentamicin, and sensitive to kanamycin.

The avirulent Agrobacterium strains A1010, A1023, and A1061 are described by Garfinkel and Nester (1980). The position of the respective Tn5 insertion mutation of A1010 and A1061 was determined from HpaI restriction analysis (D. Garfinkel, personal communication), and of A1023 by BamHI, EcoRI, and HpaI restriction analysis (S.E. Stachel, unpublished results). Agrobacterium strain AETW7 was constructed as follows. The EcoRI fragment 21 of pTiA6 (Figure 3.2) was subcloned onto pBR325. The resultant plasmid clone was opened at the BamHI site of EcoRI fragment 21, treated with Klenow to blunt-

end fill this site, and ligated, to produce a plasmid carrying a 4-bp insertion mutation at the BamHI site of EcoRI fragment 21 (S.E. Stachel, unpublished results). This (potential frameshift) mutation was subsequently recombined onto the Ti plasmid of the virulent Tn5 strain A1085 (Garfinkel and Nester, 1980) to give the avirulent strain AETW7.

d) Bacterial and plant culture conditions

E. coli (pSMvir::Tn3-HoHo1) strains were grown at 37°C on L-agar and LB liquid media (Miller, 1972) containing 100 μg/ml each of carbenicillin and kanamycin. Agrobacteria were grown at 28°C on AB minimal agar, and in Mg/L and AB/glucose liquid media (Chilton et al., 1974). For the pTiA6(pSMvir::Tn3-HoHo1) merodiploid strains, 100 μg/ml each of carbenicillin and kanamycin were included in the growth media; and for the pTiA6::Tn3-HoHo1 recombinant strains, 100 μg/ml each of carbenicillin and gentamicin were included in the solid medium, and 100 μg/ml carbenicillin in the liquid media. Also, 0.1% X-gal was typically included in the AB agar to qualitatively screen bacterial β-galactosidase activity under these growth conditions.

Cultures of a Nicotiana tabacum callus suspension cell line, designated NT1, were grown in 50 ml of MS medium supplemented with 0.2 mg/ml 2,4-dichlorophenoxyacetic acid (Sigma) in 250-ml flasks at 28°C and 120 rpm, as previously described (chapter 4).

e) Determination of virulent phenotype

Each pTiA6::Tn*3*-HoHo1 recombinant strain was inoculated on *Kalanchoe daigremontiana* leaf wounds as described by Garfinkel and Nester (1980). The test leaves were also independently inoculated with the wild-type virulent parent A348(pPH1JI) and the Ti plasmid-less A136(pPH1JI) control strains. Except for the presence of pTiA6, these latter two strains are isogenic, and except for the presence of Tn*3*-HoHo1, all the recombinant strains are isogenic with A348(pPH1JI). A348 induces undifferentiated tumors on *Kalanchoe* leaf wounds that are apparent within 10 days post-inoculation, and large tumors that cover the complete wound site are seen by 30 days. A136 is completely avirulent. Virulent phenotype was scored 15 days and 60 days post-inoculation. Several strains were also tested on *Nicotiana glauca* stem wounds (Garfinkel and Nester, 1980), and with *Nicotiana tabacum* SR1 leaf discs (Horsch *et al.*, 1985).

f) Determination of vegetative and plant-induced β-galactosidase activity

Specific units of β-galactosidase activity were determined as previously described (chapter 2), and are reported as U per bacterium. Vegetative and plant-induced activities were assayed as previously described (chapter 4). Briefly, vegetative activity was determined following overnight growth in AB/ glucose liquid minimal medium. For plant-induced activity, an overnight bacterial culture was pelleted, resuspended in one-tenth volume Murashige and Skoog

plant medium at an absorbance of 0.1 O.D. units per ml at 600 nm/cm, and used to inoculate 2 ml of 2-day NT1 plant cell culture. Cocultivations were carried out at 28°C in 60-mm petri plates. Following 16 hours, bacteria were separated from the plant cells by passing the cocultivation mixture through Miracloth® (Calbiochem), and their plant-induced activity was determined. The vegetative and plant-induced activities of each strain were measured in duplicate.

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# CHAPTER 4

## A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression


### Abstract

The virulence genes of *Agrobacterium* are required for this organism to genetically transform plant cells. We show that *vir* gene expression is specifically induced by a small molecular weight (< 1000 Da) diffusible plant cell metabolite present in limiting quantities in the exudates of a variety of plant cell cultures. Active plant cell metabolism is required for the synthesis of the *vir* inducing factor, and the presence of bacteria does not stimulate this production. *Vir*-inducing factor is (i) heat and cold stable, (ii) pH-stable, although *vir* induction with factor is sensitive above pH 6.0, and (iii) partially hydrophobic. Induction of *vir* gene expression was assayed by monitoring β-galactosidase activity in *Agrobacterium* strains that carry gene fusions between each of the *vir* loci and the *lacZ* gene of *E. coli*. *Vir*-inducing factor (partially purified on a C-18 column) induces both the expression in *Agrobacterium* of six distinct loci, and the production of T-DNA circular molecules which are thought to be involved in the transformation process. *Vir*-inducing factor potentially represents the signal that *Agrobacterium* recognizes in nature as a plant cell susceptible to transformation.

## Introduction

Many species of soil bacteria form specialized symbiotic and parasitic interactions with plant cells (1). To initiate an interaction a bacterium must first recognize its appropriate susceptible plant cell. This recognition can be used by the bacterium to activate bacterial genes whose products mediate the development and/or maintenance of the interaction. But how does the bacterium recognize a susceptible plant cell; what are the signals and how are they detected ? Here we begin to characterize bacterial-plant cell recognition by studying the early interaction that occurs between the phytopathogen *Agrobacterium tumefaciens* and plant cells.

*A. tumefaciens* causes crown gall, a neoplastic disease of dicotyledonous plants, by transferring a specific segment of DNA, the T-DNA, from its large (200 kb) Ti plasmid to plant cells, where it becomes integrated into the plant nuclear genome and expressed (reviewed in 2). Although the mechanism of T-DNA transfer and integration into the plant cell DNA has not been elucidated, the Ti plasmid genes required for these events have been identified. These genes are not contained within the T-DNA but are located within the approximately 40-kb virulence (*vir*) region (3-5). Genetic analysis of this region has shown that it encodes six separate *vir* complementation groups (Figure 4.1; chapter 3). The proteins encoded by these genes likely mediate specialized functions, and thus *vir* expression might be limited to when the bacterium is in the presence of plant cells susceptible to transformation. Such regulation could be accomplished if *Agrobacterium* has the ability to

recognize a specific molecule(s) produced by these cells. Recognition could then trigger vir gene expression to initiate the steps of T-DNA transfer and integration.

In this paper we show that vir gene expression in Agrobacterium is indeed specifically stimulated by dicotyledonous plants, demonstrating that Agrobacterium recognizes and responds to plant cells. We further show that vir induction is mediated by one or more low molecular weight partially hydrophobic molecules found in the exudates of metabolically active plant cells We propose that this factor is the signal that Agrobacterium identifies in nature as a plant cell susceptible to transformation.

## Materials and methods

### a) Plant cell cultures

Nicotiana tabacum cv. xanthi and cv. W38 plants were used to prepare leaf disc cultures according to (6). Root cultures of N. glauca (untransformed) and N. tabacum transformed with strain A. rhizogenes A15834 (gift of J.-P. Hernalsteens) were grown in liquid Murashige and Skoog (MS) medium (7) (containing MS salts, 3% sucrose, 0.01% inositol, 0.0001% biotin, and 0.018% $KH_2PO_4$). Roots were grown in 100 ml of medium in 500-ml flasks at 26°C and 90 rpm; medium was changed weekly and the root mass was divided every 2 weeks to give an inoculum of approximately 10 g. Callus suspension cell lines of Vinca rosea and N. tabacum were grown in 50 ml of MS medium supplemented with 0.2 mg/ml 2,4-dichlorophenoxyacetic acid (2,4-D) in

250-ml flasks at 28°C and 120 rpm. Every 7 days, 2 ml of the N. tabacum suspension culture was subcultured into 50 ml of fresh culture medium; under these growth conditions this culture saturates in 7 days. Vinca rosea was subcultured weekly at a 20% inoculation. Mesophyll protoplasts were prepared from N. tabacum leaves (8) and regenerated for 72 hr prior to use.

b) Preparation of conditioned medium

Forty-eight to 72 hr after subculture of N. tabacum roots into fresh medium, the conditioned medium was removed, filtered through 0.22 μm nitrocellulose, and stored at -20°C; this material was designated cmr (conditioned medium roots).

c) Vir expression assays

All bacterial strains and plasmids are described in Figure 4.1 and Table 4.1. The plasmids used confer resistance to carbenicillin and kanamycin, and bacterial cultures were grown in YEB liquid medium (9) supplemented with 100 μg/ml each of these antibiotics. Overnight cultures were pelleted and resuspended in one-tenth volume of MS plant medium. Material to be tested for vir inducing activity was inoculated with bacteria at an absorbance of 0.1 OD units per ml at 600 nm ($cm^{-1}$). Specific units of β-galactosidase activity in Agrobacterium were determined as previously described (chapter 2) and are reported as units per bacterial cell.

## Results

### a) Lac fusions as probes for vir expression

Vir expression was monitored using Agrobacterium strains that carry gene fusions between the pTiA6 vir loci and lacZ. These fusions are described in detail elsewhere (chapters 2 and 3), and are schematically shown, along with the genetic map of the pTiA6 vir region, in Figure 4.1. In brief, they were constructed by randomly inserting the Tn3-lacZ transposon, Tn3-HoHo1 (chapter 2), into plasmid clones of the pTiA6 vir region (10). When this element inserts into a genetic locus such that the transcriptional orientation of the locus and the promoterless E. coli lac operon carried by the element are in register, the production of the lacZ gene product, β-galactosidase, is placed under the control of the locus. The relative level of expression of a particular vir locus in Agrobacterium can thus be easily and quantitatively monitored by measuring the amount of β-galactosidase activity present in a cell carrying a lac fusion to the locus.

The Agrobacterium vir::lac strains provide a bioassay for the identification of conditions that stimulate vir expression. To control that such conditions are specific for vir induction, an Agrobacterium strain that carries lacZ fused to the pTiA6 octopine catabolism (occ) locus was also employed (chapter 2). Occ is genetically and functionally distinct from vir, and expression of occ (11), and not vir (chapter 2), is specifically induced by octopine [$^{2}$N-(D-1-carboxyethyl)-L-arginine]. Thus occ expression should not be affected by conditions that induce vir.

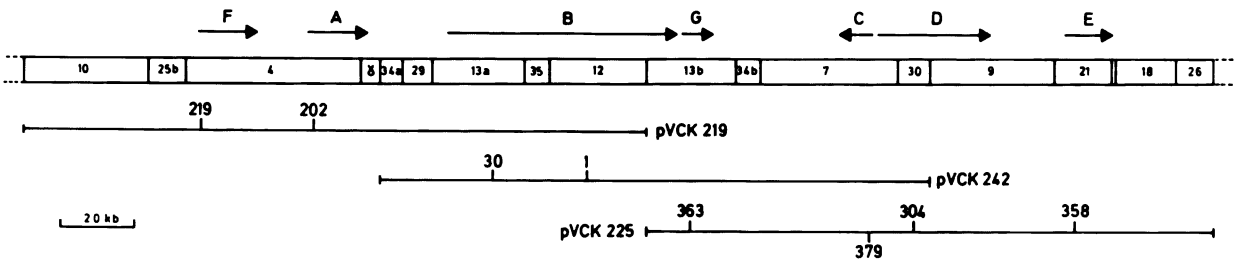


Figure 4.1. pTiA6 vir::lac gene fusions.

The map positions of virA through virG, and of the pil F locus (chapter 3), are indicated by the arrows drawn above the SalI restriction map of the pTiA6 vir region. pVCK219, pVCK242, and pVCK225, three cosmid clones (10) that contain pTiA6 vir region sequences, are shown below as horizontal lines. These clones were mutagenized with the Tn3-lacZ transposon (chapter 2), Tn3-HoHo1, to generate plasmids carrying vir::lacZ gene fusions. The map positions within these clones of eight vir::lacZ fusions are indicated by the vertical lines. The numbers designate the pSM(vir::lac) plasmids which carry the respective fusions. pSM219 and pSM202 were derived from pVCK219 and carry pilF:: lac and virA::lac fusions, respectively; pSM30 and pSM1 were derived from pVCK242 and carry separate virB:: lac fusions; and pSM363, pSM379, pSM304 and pSM358 were derived from pVCK225 and carry lac gene fusions to virG, virC, virD and virE, respectively. It is noted that each of the vir::lacZ insertions has been recombined onto pTiA6 to assess its effect on virulence (chapter 3). All these insertions produce an avirulent phenotype except for 219 and 379. The 379 insertion causes an attenuated virulence phenotype, while the 219 insertion does not affect virulence. The F locus in pTiA6 has been defined by its plant-cell inducibility and

Legend to Figure 4.1. (cont'd)

is designated pin (plant-inducible locus) F. The transcriptional orientation of each of these fusions is leftward to rightward, except for the virC::lac fusion of pSM379, whose orientation is rightward to leftward.] The eight pSM(vir::lac) plasmids were placed into A. tumefaciens strain A348 to give eight A348(pSMvir::lac) merodiploid strains. A348 contains the wild-type A6 Ti plasmid.

b) Plant cells induce vir expression

The induction of vir gene expression by plant cells was initially established using regenerating mesophyll protoplasts of N. tabacum. These cells are susceptible to high efficiency transformation by Agrobacterium (8,12). During cocultivation of these cells with Agrobacterium carrying wild-type pTiA6 and vir::lacZ fusion plasmids, the levels of β-galactosidase activity in the bacteria greatly increased (up to 100 fold) for fusions to all the vir loci, with the exception of virA, whose expression is constitutive (chapter 3) (Table 4.1). This induction is vir-specific as cocultivation has no effect on occ expression; β-galactosidase activity in the occ::lac strain A348(pSM102) increased only if octopine was added to the cocultivation. We note that vir induction is, at least in part, an Agrobacterium specific phenomenon : induction of β-galactosidase expression does not occur in E. coli that harbor our vir::lacZ plasmids (data not shown). Octopine induction of occ expression,

however, occurs in both Agrobacterium and E. coli (chapter 2).

To further study vir induction a more convenient source of plant material was desired. Vir induction was seen to occur during cocultivation with several plant culture systems, including leaf discs of N. xanthi and N. tabacum, roots of N. glauca and N. tabacum, and suspension callus cell lines of V. rosea and N. tabacum. A particular N. tabacum callus suspension cell line, designated NT1, which consistenly stimulates vir expression and can be directly transformed by Agrobacterium (G. An, personal communication), was chosen to characterize the phenomenon further.

Table 4.1. Vir induction by protoplasts, cmr, and C-18 factor

| | | β-galactosidase activity | | | |
|---|---|---|---|---|---|
| Strain | Locus | MS⁻ | protoplast | cmr | C-18 factor |
| A348/pSM219 | virF | 10.3 | 726.0 | 100.0 | 1289.0 |
| A348/pSM202 | virA | 51.9 | 55.3 | N.D. | 61.7 |
| A348/pSM30 | virB | 10.1 | 634.0 | 383.0 | 1124.0 |
| A348/pSM1 | virB | 13.6 | 792.0 | 133.0 | 919.0 |
| A348/pSM363 | virG | 72.6 | 941.0 | 277.0 | 1022.0 |
| A348/pSM379 | virC | 3.9 | 58.2 | 5.6 | 105.0 |
| A348/pSM304 | virD | 8.2 | 763.0 | 51.9 | 983.0 |
| A348/pSM358 | virE | 56.7 | 2980.0 | 630.0 | 4034.0 |
| A348/pSM102 | occ | 2.6 | 3.0 | 2.7 | N.D. |
| A348/pSM102* | occ | 84.4 | 72.9 | 61.8 | N.D. |

The β-galactosidase activity present in bacteria after incubation in MS medium, protoplast cultivation, cmr, and C-18 factor was deter-

Legend to Table 4.1. (cont'd)

mined for the eight A348(pSMvir::lac) strains shown in Figure 4.1, and the occ::lac strain A348(pSM102). A348(pSM102) incubations were carried out in the absence and presence (*) of 100 μg/ml octopine (obtained from Dr. R. Jensen, and from Sigma). Conditions of protoplast cocultivation were as previously reported (chapter 2) and β-galactosidase activity was assayed following 16-hr cocultivations. For the MS, cmr and C-18 factor experiments, 1.5 ml of the respective material in 17 x 100 mm Falcon tubes was inoculated with bacteria, and β-galactosidase activities were determined following 12 hr incubation at 28°C and 200 rpm, and are expressed as activity units/bacterium.

c) Small diffusible factor mediates induction

While NT1 culture stimulates high levels of vir induction, the conditioned cell-free medium from this culture stimulates only low-levels (from zero to six-fold) of induction (Table 4.2). Because bacteria both grow and exhibit normal levels of octopine-induced occ expression in this medium (Table 4.2), we inferred that the NT1 cells must be present for efficient vir induction to occur. We tested three possible explanations for this requirement : either physical contact between bacteria and plant cells is required; a vir inducing factor(s) produced by plant cells is only produced when bacteria are present; or the inducing factor is constitutively produced by the NT1 cells and is present in their exudate in quanti-

ties limiting for vir induction.

First, we determined whether physically blocking plant-bacterial contact during cocultivation affects vir induction (Table 4.3). Bacteria were enclosed within a dialysis bag prior to incubation with NT1 culture. Parallel incubations contained bacteria in contact with NT1 cells, or bacteria both inside and outside a dialysis bag in an NT1 culture. After 16 hours, the level of induced β-galactosidase activity in the bacteria inside the bag was approximately equivalent to that in the bacteria outside the bag. Also, the presence or absence of bacteria outside the dialysis bag had no significant effect on the induction levels of bacteria inside the bag.

The above data demonstrate that the stimulation of vir expression by NT1 cells does not require plant-bacterial contact and is mediated by a soluble factor. We estimate the upper limit of its size to be 1,000 Da, the exclusion limit of the dialysis membrane used in these experiments.

Table 4.2. NT1 plant metabolism affects the level of vir induction

| A348(pSM) strains | 1 | 30 | 358 | 102/-oct | 102/+oct |
|---|---|---|---|---|---|
| Treatments | | β-galactosidase units | | | |
| (a) fm | 14.0 | 10.2 | 59.4 | 2.55 | 84.4 |
| (b) 2d | 1007.0 | 767.0 | 1431.0 | 2.92 | 116.0 |
| (c) 2d-5 ml | 240.0 | 254.0 | 649.0 | 3.12 | 111.0 |
| (d) 2d-boil | 68.0 | 84.1 | 88.5 | 2.34 | 91.8 |
| (e) 2d/cx | 163.0 | 301.0 | 403.0 | 1.94 | 86.2 |
| (f) 2d/fm | 410.0 | 410.0 | N.D. | 3.02 | 47.0 |
| (g) 2d/fm+cx | 74.2 | 137.0 | N.D. | 2.63 | 76.1 |
| (h) 6d | 40.4 | 108.0 | N.D. | 2.58 | 82.6 |
| (i) 6d/fm | 379.0 | 473.0 | N.D. | 2.18 | 109.0 |
| (j) 6d/fm+cx | 35.6 | 24.2 | N.D. | 1.84 | 87.2 |
| (k) 2dcm | 41.7 | 68.8 | 106.0 | 2.79 | 99.9 |
| (l) 2dcm-cc | 27.6 | 32.4 | 74.4 | N.D. | N.D. |

Agrobacterium lac fusion strains A348(pSM1/virB), A348(pSM30/virB), A348(pSM358/virE) and A348(pSM102/occ) were used to assess the vir-inducing activity of NT1 culture subjected to different treatments, and of conditioned medium from NT1 culture. Incubations were carried out in 2 ml volumes, unless otherwise indicated, for 16 hr at 28°C in 60 mm petri dishes, and the units of β-galactosidase activity in the bacteria were then determined. A348(pSM102) incubations were carried out in the absence (-oct) and presence (+oct) of 100μg/ml octopine. The materials tested were : a) fm (fresh medium), MS medium supplemented with 0.2 mg/ml 2,4-D; b) 2d (2-day NT1 cul-

Legend to Table 4.2. (cont'd)

ture), NT1 suspension culture 2 days post-subculture; c) 2d-5 ml, 5 ml of the 2-day NT1 culture; d) 2d-boil, 2-day NT1 culture boiled for 15 minutes directly prior to cocultivation with bacteria; e) 2d/cx, 2-day NT1 culture simultaneously inoculated with bacteria and 20 μm cycloheximide; f) 2d/fm, the NT1 suspension cells of the 2-day culture were separated by centrifugation from the culture medium and resuspended in an equivalent volume of fresh medium, and immediately inoculated with bacteria; g) 2d/fm+cx, as in f), except that the fresh medium contained 20 μm cycloheximide; h) 6d (6-day NTI culture), NTI suspension culture 6 days post-subculture; i) 6d/fm, the NT1 suspension cells of the 6-day culture were separated by centrifugation from the culture medium and resuspended in fresh medium to a density corresponding to that of the 2-day culture; j) 6d/fm+cx, as in h) except that the fresh medium contained 20 μm cycloheximide; k) 2dcm (2-day conditioned medium), the cell-free conditioned culture medium from 2-day NT1 culture; l) 2dcm-cc (2-day conditioned-medium cocultivation), the cell-free conditioned medium from a 16-hr cocultivation of 2-day NT1 culture and A348(pSM30) bacteria.

Table 4.3. Effect of dialysis membrane on *vir* induction

| Strain A348(pSM) | Factor source | | β-galactosidase activity in bacteria | |
|---|---|---|---|---|
| | | | Outside | Inside |
| 358 | NT1 culture | a) | 1850 | 1362 |
| | | b) | 1721 | -- |
| | | c) | -- | 1412 |
| 30 | NT1 culture | a) | 692 | 381 |
| | | b) | 515 | -- |
| | | c) | -- | 451 |
| 102 | NT1 culture + 100 µg/ml octopine | a) | 143 | 102 |
| | | b) | 129 | -- |
| | | c) | -- | 96 |
| 30 | cmr | a) | 237 | 197 |
| | | b) | 220 | -- |
| | | c) | -- | 189 |

MS medium was separately inoculated with *Agrobacterium* strains A348(pSM358/*virE*), A348(PSM30/*virB*) and A348(pSM102/*occ*). 5 ml of each inoculated medium was enclosed in Spectrapor 6 dialysis membrane (VWR), with an exclusion limit of 1000 Da (Stokes radius). In parallel experiments 5 ml of uninoculated MS medium was enclosed in the membrane. The dialysis bags were incubated in 250-ml flasks with 50 ml of 2-day NT1 culture inoculated with bacteria. Dialysis bags were also incubated in uninoculated NT1 culture. In a parallel set of experiments *cmr* was used in place of NT1 culture. Three experiments were set up for each bacterial strain : (a) bacteria both inside and outside the dialysis bag; (b) bacteria only outside the dialysis bag; and (c) bacteria only inside the dialysis bag. Following 16 hours incubation at 28°C and 120 rpm, the β-galactosidase activity of each population of bacteria was determined. In the A348(pSM102) experiments 100 µg/ml octopine was added to the NT1 culture. The molecular weight of octopine is 246 D.

d) Efficient induction requires plant metabolism

In our standard NT1 induction assay a fixed number of bacteria are cocultivated for 16 hr with 2 ml of suspension culture in 60-cm petri plates. Using these conditions, a 2-day subculture induced β-galactosidase activity 70-fold in the virB::lac strain A348(pSM1). Lower levels of induction were obtained in parallel cocultivations in which NT1 cell metabolism was restricted. For example, induction was 12-fold when cycloheximide, an inhibitor of eukaryotic translation, was added, and 5-fold when the NT1 culture was boiled prior to cocultivation. Restricted culture aeration also reduced induction : when the volume of 2-day culture was increased from 2 ml to 5 ml in the cocultivation, A348(pSM1) induction decreased to 17-fold (Table 4.2). Also, in large-volume cocultivations, efficient vir induction occurs in shaking, but not stationary, NT1 suspension cultures (data not shown).

The level of vir induction is also affected by the age of the NT1 culture (Table 4.2). A 6-day culture induced A348(pSM1) only 3-fold; however, when the NT1 cells from this culture were subcultured in fresh medium prior to cocultivation, 27-fold induction was obtained. Furthermore, cycloheximide blocked this effect. We note that the levels of octopine-induced occ expression were not significantly affected by any of these treatments.

The above results demonstrate that continual NT1 cell metabolism during cocultivation is required to produce vir-inducing factor in sufficient quantity for efficient vir induction. This production is likely constitutive and not a response to the presence

of Agrobacterium, as conditioned medium from a cocultivation induced vir no better than conditioned medium from an NT1 culture which had never seen bacteria (Table 4.2). Thus the factor is likely a plant cell metabolite.

e) Physical properties of factor

To characterize vir-inducing molecules it is necessary to have a culture system that produces factor in sufficient quantities to allow detection by bioassay in the absence of plant cells. Conditioned media from N. glauca and N. tabacum root cultures, designated cmr (conditioned medium roots), were found to contain detectable activity in the absence of root cells.

Several experiments were carried out to characterize the N. tabacum cmr factor. Essentially identical results have also been obtained with the N. glauca cmr (data not shown). The cmr factor is soluble and small, since it passes through dialysis membrane with an exclusion limit of 1,000 Da (Table 4.3). The cmr factor is heat- and cold-stable : when cmr was either boiled for 15 min or frozen for several months at -20°C prior to bioassay, its original inducing activity was fully retained. The cmr factor is pH-stable : when cmr was adjusted to pH 1.0 or pH 11.0, incubated at 28°C for 15 min, and readjusted back to pH 5.5, vir-inducing activity was fully retained. Conversely, vir induction by cmr is pH-sensitive : induction of the virB::lac strain A348(pSM30) by cmr that had been pH-adjusted to cover a range of 5.0 to 7.0, dropped from 40-fold at pH 5.5 to zero-fold at pH 6.0 and above (Figure 4 2). This effect is vir-

specific, because the pH of cmr had no effect on octopine induction of occ. The sensitivity of vir induction to pH might result from an alteration in conformation of the inducing molecule, or of a bacterial component of vir induction.

Figure 4.3 presents the induction kinetics of the β-galactosidase activity in the virD::lac strain A348(pSM304) during incubation with N. tabacum root culture, and with cmr obtained from this culture. At 10 hours the level of induction stimulated by cmr was 6-fold less than that stimulated by root culture. Cmr did not further stimulate induction after this time, whereas induction continued to increase steadily in the presence of roots. Thus, the inducing activity in cmr is limiting and the presence of roots is required for high induction levels. This limitation can be overcome if fresh cmr is added at various times to the bacteria/cmr culture. After 30 hr incubation, the level of vir induction of twice-refreshed bacteria was 46% that of bacteria cocultivated with roots.

Results of similar experiments employing other lac fusion strains indicate that different vir loci are differentially induced by cmr (Table 4.1). The induction of the virB::lac strain A348(pSM30) by plant cell culture and by nonrefreshed cmr was 63-fold and 38-fold, respectively. Conversely, virC::lac strains that are induced approximately 15-fold by plant cell culture exhibited less than 2-fold stimulation by cmr. The inducibility by cmr of the other vir loci falls between that of virB and of virC. Thus, the cmr factor might induce the expression of some, but not all, vir loci. Conversely, this factor might induce all of vir but greater amounts are required to stimulate virC than virB.

These questions were resolved using concentrated cmr factor. To this end different gel chromatography matrices were tested for their ability to retain the cmr factor. Silica C-18 was found to retain the vir-inducing activity quantitatively, indicating that it has some hydrophobic character. 150 ml of cmr was passed through a rapid sample Sep-Pak C-18 cartridge (Waters Associates, Inc.). The cartridge was step eluted with 10 ml each of $H_2O$, and 20%, 40%, and 90% $CH_3OH/H_2O$ (v/v). Following lyophilization, each elution sample was resuspended in 1.5 ml MS medium containing 12.5 mM sodium phosphate, pH 5.5 (phosphate was added to aid bacterial growth). These 4 samples, along with the cartridge flow-through material, were bioassayed for vir-inducing activity. Activity was only found in the 40% $CH_3OH$-eluted fraction; this material, designated C-18 factor, was stored at -20°C.

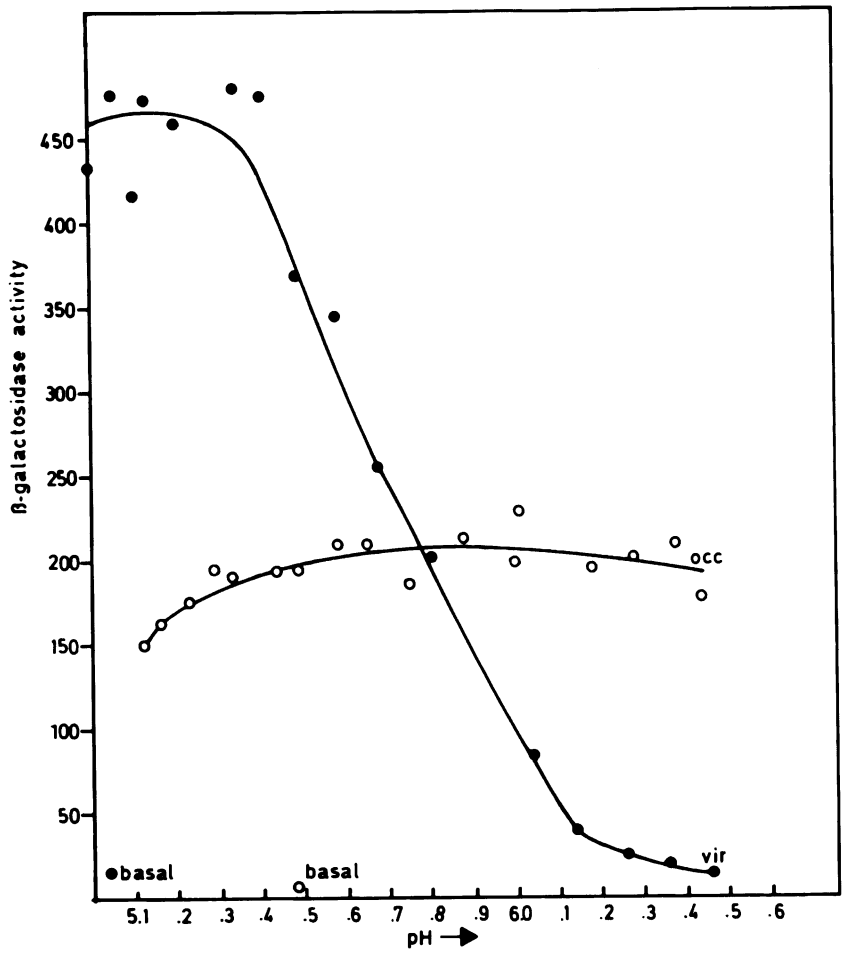


Figure 4.2. Effect of pH on vir induction by cmr.

Agrobacterium virB::lac strain A348(pSM30) (o) or occ::lac strain A348(pSM102) (o) were incubated in 2 ml samples of cmr which were adjusted with sodium phosphate to a final concentration of 12.5 mM to cover a range of pHs between pH 5.0 and 7.0. The starting pH of the cmr was 5.5. For the occ::lac incubations, 100 μg/ml octopine was included in the medium. All incubations were for 8 hours at 28°C and 200 rpm. The pH of each cmr sample was determined directly prior to assay of the β-galactosidase activity of the bacteria in the sample. The basal β-galactosidase activities of A348(pSM30) and A348(pSM102) after growth in MS medium, 12.5 mM sodium phosphate, pH 5.5, were 10.1 and 2.69 units, respectively.

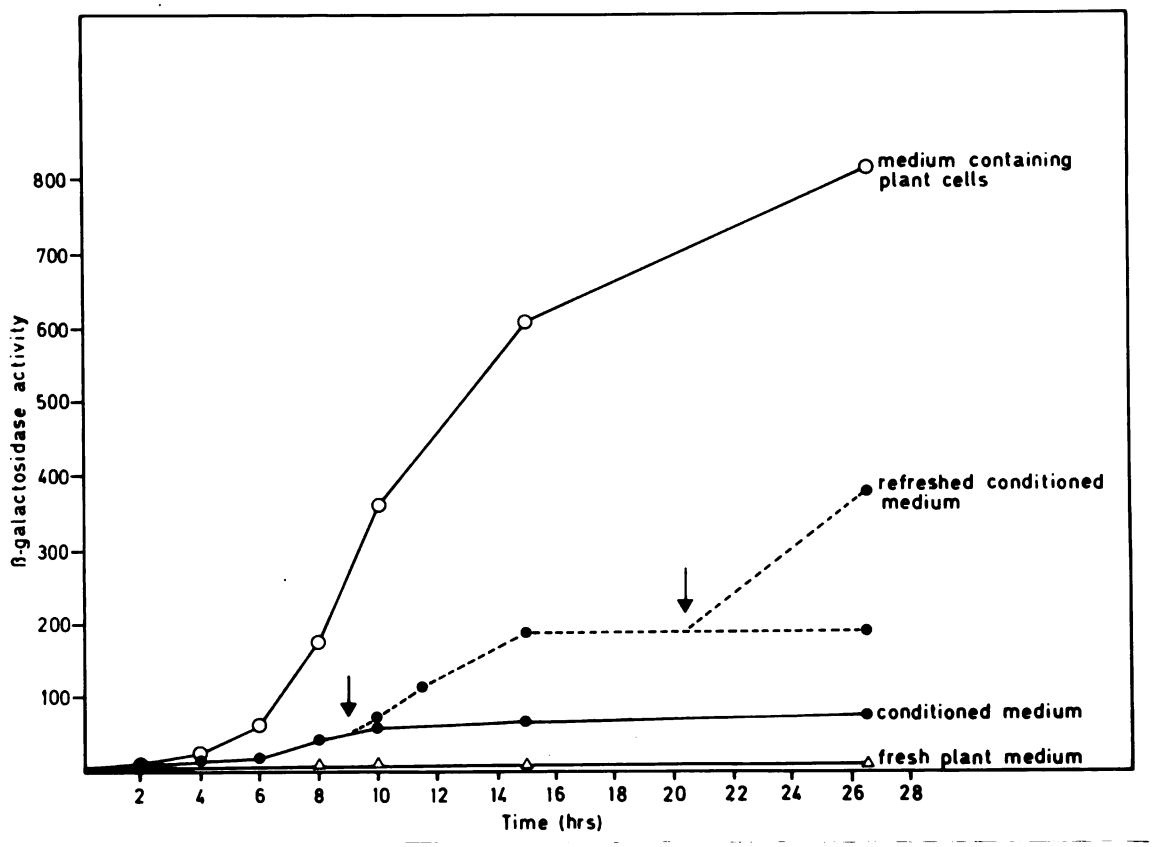


Figure 4.3. Plant factor is limiting for vir induction. Agrobacterium virD::lac strain A348(pSM304) was separately inoculated into 50 ml of N. tabacum-rhizogenes-transformed root culture (o), the conditioned-medium derived from this culture (cmr) (o), and unconditioned MS medium (Δ). Incubations were carried out in 250-ml flasks at 28°C and 120 rpm. At 2-hr intervals 2 ml aliquots were removed and β-galactosidase activity of the bacteria was determined. At 9 hr and 20 hr the cmr culture was refreshed with an equivalent volume of fresh cmr as indicated in the figure by the arrows.

f) Biological activity of C-18 factor

Two tests confirmed the biological activity of the C-18-concentrated factor. First, the inducibility of several vir::lac strains with factor (corresponding to a 100-fold concentration of the original cmr) was determined. Induction of all the inducible vir loci was obtained (Table 4.1), and the levels of induction were equivalent to, or higher than, those obtained with regenerating protoplasts or roots. Even the virC::lac strain A348(pSM379), which was not significantly induced by cmr, was induced 35-fold in the presence of the C-18 factor, suggesting that virC might require higher levels of factor than other vir loci for its induction.

Second, we tested whether the concentrated factor could induce T-DNA-associated molecular events which have been identified to occur within Agrobacterium during cocultivation with mesophyll protoplasts. In the Ti plasmid, the T-DNA is defined and bounded by identical 25-bp direct-repeats; only DNA between these T-DNA borders is seen to be transferred to the plant genome (reviewed in 13). During cocultivation with plant cells, independent T-DNA circles are formed in Agrobacterium by a specific recombination between the 25-bp sequences at the ends of the T-DNA (14).

We assayed whether C-18 factor could induce T-DNA circles in Agrobacterium carrying Ti plasmid pGV3850 (15). The T-DNA of pGV3850 consists of the cloning vehicle pBR322 flanked by the left and right T-DNA border regions, and T-DNA circular intermediates can be isolated by transforming Escherichia coli with DNA isolated from pGV3850 and selecting for the carbenicillin resistance marker of the

pBR322 portion of the pGV3850 T-DNA (14). Total DNA prepared from 5 independent incubations (2 experiments for 12 hr, and 3 experiments for 24 hr) of Agrobacterium containing pGV3850 in C-18 factor gave 20 and 33, and 44, 70, and 86 T-DNA circular intermediates per μg DNA, respectively. In comparison, total Agrobacterium DNA prepared following 3 independent cocultivations (for 48 hr) of Agrobacterium containing pGV3850 with regenerating protoplasts gave 36, 40, and 48 T-DNA circular intermediates per μg DNA. Uninduced Agrobacterium containing pGV3850 never produces these intermediates. The transformation efficiency in all these experiments was equivalent and determined to be $6 \times 10^6$ transformants/μg with supercoiled pBR322 DNA. These data confirm that concentrated cmr factor induces biologically significant events, associated with plant cell transformation, in Agrobacterium.

## Discussion

The phytopathogen Agrobacterium tumefaciens is able to genetically transform plant cells and this process is mediated, in part, by the gene products of the Ti plasmid vir genes. We show that the expression of the vir genes in Agrobacterium is induced by a variety of dicotyledonous plants and by several types of plant cells. We have investigated this phenomenon with regard to the production and properties of the plant cell factor which mediates vir induction.

Vir-inducing activity is produced in quantities limiting for vir induction, and active plant cell metabolism is required for this production. Also, production of inducing activity is not significant-

ly affected by the presence of bacteria, suggesting that it is not regulated by Agrobacterium. The vir-inducing activity is a diffusible molecule(s) present in plant cell exudates. Semi-purified and concentrated exudate induces the expression of each of the inducible vir loci to levels equivalent to, or greater than, those induced by cocultivation.

Our results indicate that plant cell induction of the vir gene expression in Agrobacterium is a vir- and Agrobacterium-specific phenomenon that is mediated by an inducing factor composed of one or more small, stable, plant cell metabolites. These findings are not in agreement with a recent report that a heat-labile proteinaceous factor of size greater than 7,000 Da induces vir expression both in E. coli and Agrobacterium (16); a single vir::lac gene fusion was employed to assess vir induction which is reported as qualitative changes in β-galactosidase activity. In the present work vir expression is quantitatively monitored using gene fusions between each of the vir loci and lacZ, and non-specific metabolic effects are controlled for by using a lacZ fusion to the metabolic occ locus. We have also directly monitored vir induction by measuring vir-encoded RNA in uninduced and factor-induced bacteria (chapter 6), and by measuring a functional product of vir induction, T-DNA intermediates.

How the vir factor mediates induction is not known. It might act at the surface of the bacterial cell to trigger a secondary messenger system, or directly within the cell; that the factor is small and diffusible, suggests that it might enter the bacterium. The pTiA6 vir region is seen to function as a single regulon whose

induction is attenuated in virA, and does not occur in virG, mutant bacteria (chapter 6). Conceivably, the virA and virG gene products might function in Agrobacterium as the receptor and the effector molecules for the vir-inducing factor.

It is not known whether the vir gene loci are transferred to and expressed in the plant cell during the transformation process. These sequences are not found integrated in the plant genome, and our data demonstrate that all the vir loci are expressed in a regulated fashion in the bacterium. Thus, if vir-encoded proteins function in the plant cell during transformation, they are likely first synthesized in the bacterium and then transferred to the plant cell, perhaps as part of a T-DNA/protein complex.

In nature only wounded plant cells are susceptible to transformation by Agrobacterium (17). A primary step in transformation should thus be the detection and recognition by the bacterium of such plant cells. The vir inducing factor that we have begun to characterize could be the signal that Agrobacterium recognizes in nature as a wounded plant cell. We note that all the plant cultures determined to produce this factor contain, to some degree, mechanically damaged cells.

The present analysis offers insight into how a bacterium recognizes a plant cell. This work should provide a basis for the study of other bacterial-plant interactions, and may have practical application for the promotion of useful, and the prevention of harmful, bacterial/plant cell interactions.

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Note added in proof : We have recently identified the cmr vir-inducing activity (chapter 5) to be composed of at least two derivatives of acetophenone that each separately fully activates the vir regulon.

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# CHAPTER 5

## Identification of the signal molecules produced by wounded plant cells that activate the T-DNA transfer process in *Agrobacterium tumefaciens*


## Abstract

*Agrobacterium tumefaciens* genetically transforms wounded plant cells. The *Agrobacterium* virulence genes mediate this interaction. *Vir* gene expression is activated by plant cell molecules. We purify these molecules and identify them to be acetosyringone (AS) and α-hydroxyacetosyringone (OH-AS) [4-acetyl-2,6-dimethoxyphenol, and 4-(2-hydroxyacetyl)-2,6-dimethoxyphenol, respectively]. These molecules can induce in *Agrobacterium* the entire *vir* regulon as well as the formation of T-DNA intermediate molecules. This induction is a highly specific process. AS and OH-AS are specifically found in exudates of wounded and metabolically active plant cells and thus likely allow *Agrobacterium* to recognize susceptible cells in nature.


## Introduction

Soil bacteria often form specialized interactions with plant cells. Usually a particular bacterium interacts only with a limited number of species and/or types of plant cells (1-2). A primary step in the formation of a bacterial/plant interaction is thus the detec-

tion by the bacterium of the appropriate susceptible plant cell. This recognition subsequently triggers the activation of the bacterial genes whose products direct the development and/or maintenance of the interaction. The soil is a complex biological and chemical environment, and the signals that mediate the detection of a specific plant cell by a bacterium are unknown. Here, we investigate such signals by purifying and identifying the plant molecules that initiate the interaction between Agrobacterium tumefaciens and wounded plant cells.

Agrobacterium tumefaciens is a soil phytopathogen that genetically transforms plant cells to cause crown gall, a neoplastic disease of dicotyledonous plants (3-4). Only cells that have been wounded are seen to be susceptible to crown gall (5-6). In the transformation process the bacterium transfers a specific segment of DNA, the T-DNA, from its large (> 200 kb) Ti plasmid to the susceptible plant cell, where it becomes integrated into the nuclear genome (7-8). In the Ti plasmid, the T-DNA is defined and bounded by identical 25-bp direct-repeats; only DNA between these T-DNA borders is seen to be transferred to the plant genome (9-12). During cocultivation with plant cells, independent T-DNA circles are formed in Agrobacterium; these molecules arise by a specific recombination between the 25-bp sequences at the ends of the T-DNA, and are potential intermediates in the transfer of the T-DNA from Agrobacterium to the plant cell (13).

The Ti plasmid genes required for plant transformation are not contained within the T-DNA but are located within the approximately 40-kb virulence (vir) region (14-16). Genetic analysis of the vir

region of the A6 Ti plasmid has shown that it encodes at least seven separate complementation groups, virA, B, C, D, E, and G, and pinF that are organized as a single regulon (chapters 3 and 4). In the vegetative bacterium only virA and virG, the vir regulatory genes, are significantly expressed; however, when Agrobacterium is cocultivated with plant cells the expression of virB, C, D, E, G and pinF become induced to high levels (chapters 2 and 4). This activation of vir expression by plant cells likely initiates the steps of T-DNA transfer and integration into the plant cell genome. We have shown that vir induction, along with the production of T-DNA circles, is mediated by a small diffusible factor produced by actively growing plant cells [17]. Here, we purify and establish the chemical identity of this factor, and demonstrate that its production is related to plant cell wounding and that its recognition by the bacterium is a highly specific process. This factor is likely to be the signal that allows Agrobacterium to recognize in nature a plant cell susceptible to transformation.

## Results

### a) Purification of signal molecules

To purify the plant molecule(s) that specifically signals Agrobacterium to initiate its interaction with plant cells, requires (i) an efficient and quantitative bioassay for a primary event in this transformation process, specifically the induction of vir gene expression, and (ii) a starting source of the signal molecule(s).

We have previously described an assay for vir induction in Agrobacterium that employs gene fusions between the pTiA6 vir loci and the E. coli lacZ gene (chapters 2 and 4). Briefly, in a bacterium carrying a vir::lac gene fusion, the production of β-galactosidase, the lacZ gene product, is controlled by the vir locus to which lacZ has been fused. Thus, the state of expression of the locus can be monitored by measuring the β-galactosidase activity present in the bacterium; increased activity indicates increased vir expression. Furthermore, the relative amount of induced activity reflects the relative amount of vir-inducing activity to which the cell has been exposed. Here we use Agrobacterium strain A348(pSM30) (chapter 4) to detect and measure vir-inducing activity. This strain contains wild-type pTiA6 and pSM30, a virB::lac fusion plasmid, and gives high levels (up to approximately 100-fold of basal activity) of induced β-galactosidase activity (chapter 4).

Induction of vir expression occurs during cocultivation of Agrobacterium with plant cells, and during incubation of bacteria in plant cell exudates. We have shown that the medium in which Nicotiana tabacum root culture has grown (designated cmr, conditioned medium roots) contains substantial amounts of a vir-inducing activity (chapter 4). This activity stimulates the expression of each of the inducible pTiA6 vir loci, and also the formation of T-DNA circular intermediates, indicating that it triggers in Agrobacterium the initiation of the events of plant cell transformation. This activity has relative molecular mass less than 1000 D; is stable to boiling, freezing, lyophylization, and high and low pH; and is partially hydrophobic, as it is retained by silica C-18 and com-

pletely elutes from this matrix with 40% $CH_3OH$ (chapter 4). Here we purify and identify this cmr activity.

The above properties suggest that the cmr vir-inducing activity is composed of one or more small organic molecules; that it completely partitions into the organic solvent chloroform (Figure 5.1) confirms this identity, and provided a basis for its purification. The cmr vir-inducing activity was fractionated by reverse phase chromatography (RPC) using a high-resolution fast-performance liquid chromatography (FPLC) system. [Briefly, cmr was extracted with chloroform and the chloroform phase was dried and resuspended in 20% $CH_3OH$, 0.1% $CH_3COOH$. This material was loaded onto a silica C-2/C-18 RPC column preequilibrated with 10% $CH_3OH$, 0.1% $CH_3COOH$, and the column was eluted with a linear gradient of 10% to 60% $CH_3OH$, 0.1% $CH_3COOH$. The column fractions were monitored at 280 nm, and bioassayed for vir-inducing activity (Figure 5.1A)]. Activity was localized to two major and two minor peaks that had eluted, respectively, with 18% (peak A) and 27.5% (peak B), and 32% (peak C), and 42% (peak D) $CH_3OH$ (Figure 5.1A). Thus, the cmr vir-inducing activity is represented by at least four distinct compounds. When less material is loaded onto the FPLC column (Figure 5.1B), peak B is seen to be the major component of the organic fraction of cmr. Because of their low abundance, the peak C and peak D activities were not further analyzed. The signal molecules in peaks A and B were purified to homogeneity by further RPC fractionation, and analyzed by gas chromatography/mass spectroscopy (GC/MS) (Figure 5.2), and U.V. absorption spectroscopy (Figure 5.3).

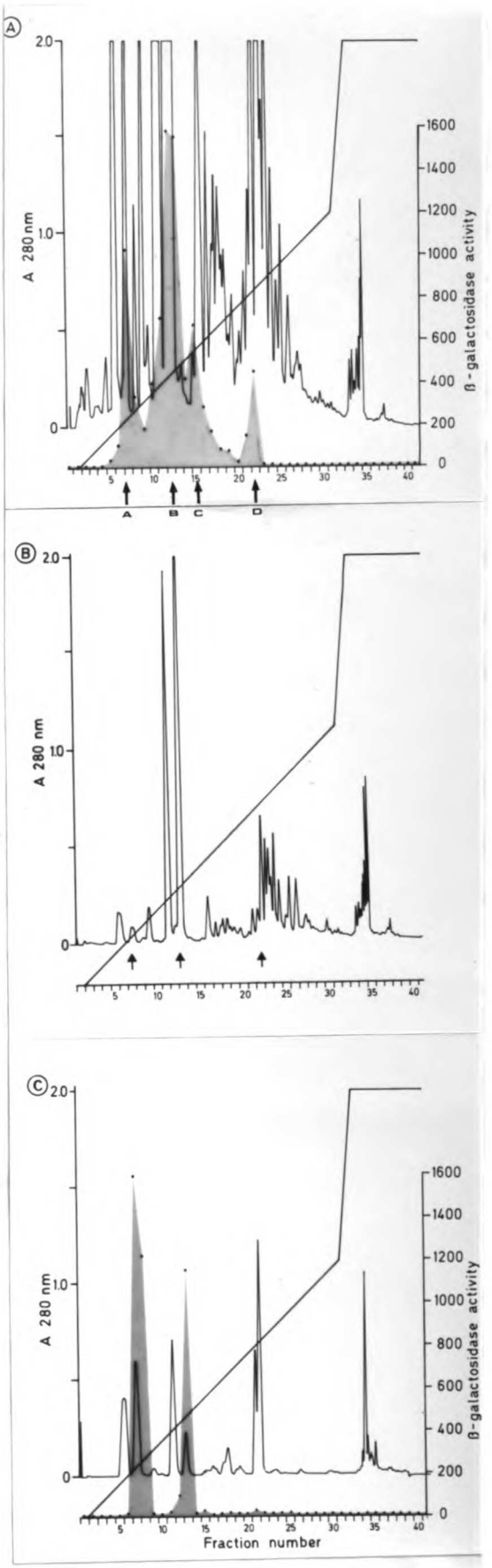



**Figure 5.1.**
**RPC/FPLC fractionation of the vir-inducing activity in plant cell exudates.**

Legend to Figure 5.1.

The organic compounds present in plant cell medium conditioned with root culture (cmr; panels A and B), or with leaf discs (cml; panel C), were prepared by chloroform extraction and analyzed by RPC/FPLC. The material analyzed in panels A, B, and C was prepared from 2 l cmr, 40 ml cmr, and 50 ml cml, respectively. Each sample was fractionated on a C-2/C-18 RPC column eluted with a linear methanol gradient. In panels A and C the column fractions were bioassayed for vir-inducing activity in Agrobacterium. In each panel the diagonal line represents the elution gradient, and the solid line indicates the UV absorbance measured at 280 nm of the column fractions; in panels A and C the shaded curves indicate relative specific units of β-galactosidase activity induced in the virB::lacZ tester strain A348(pSM30) by the column fractions. The major peaks of vir-inducing activity in panel A are indicated by arrows and the letters A, B, C and D. In panel B the arrows indicate peaks corresponding to peaks A, B, and D, of panel A.

Methods : Preparation of conditioned medium – Root culture of Nicotiana tabacum transformed with Agrobacterium rhizogenes A15834 was grown and maintained as previously described (chapter 4). Transformed roots were used because they are easy to propagate. Every 72 hours the conditioned medium (cmr) was removed and stored at -20°C. Leaf discs were prepared from 6-week-old untransformed N. tabacum SR1 plants. Two-g samples of 1.5-cm diameter discs were incubated in 50 ml MS medium (Murashige and Skoog (26) salts, 3% sucrose, supplemented with 0.018% $K_2HPO_4$, 0.01% inositol, 0.0001%

Legend to Figure 5.1. (cont'd)

biotin, pH 5.5) in 150-mm petri dishes. Following 72 hours, the conditioned medium (cml) was removed and stored at -20°C.

Organic extraction - The conditioned medium (cmr and cml) was filtered through 0.22 μm nitrocellulose. The filtrate was extracted twice with a 25% volume of chloroform and the pooled chloroform phase was back-extracted with one-volume MS medium, rotary-evaporated to dryness, and resuspended in 500 μl 20% $CH_3OH$, 0.1% $CH_3COOH$ for analysis by RPC/FPLC. Note that initial solvent extraction experiments were performed to determine the solvent partitioning character of the cmr and cml vir-inducing activities. In these experiments 50 ml of the respective conditioned-medium was extracted with chloroform; the interphase and chloroform phase were lyophilized and each resuspended in 2 ml MSSP [ref.17] (MS medium supplemented with 12.5 mM sodium phosphate, pH 5.5), and the aqueous phase was blown with a stream of air to remove traces of chloroform. Each of these samples was bioassayed for vir-inducing activity (see below), and, for both cmr and cml, only the chloroform phase material contained this activity.

RPC/FPLC analysis - A Pep RPC pre-packed 5 mm x 50 mm column HR5/5 containing 6 μm silica particles with C-2 and C-18 alkyl side chains (Pharmacia) was preequilibrated with 10% $CH_3OH$, 0.1% $CH_3COOH$. The column was run at a flow rate of 0.7 $ml.min^{-1}$ using a Pharmacia FPLC system equipped with the LCC500 chromatographic programmer. A single-path UV monitor was used to monitor absorbance at 280 nm. The column was eluted with a linear gradient of 10% to 60% $CH_3OH:H_2O$

Legend to Figure 5.1. (cont'd)

(v:v), 0.1% $CH_3COOH$. Forty 1.4-ml (2-minute) fractions were collected; 140 µl aliquots (in A) or the entire fractions (in C) were lyophilized, resuspended in 1.5 ml MSSP and bioassayed for vir-inducing activity.

Vir induction assays – Overnight cultures of strain A348(pSM30) were centrifuged and resuspended in MSSP medium. Material to be tested for vir-inducing activity was inoculated with bacteria at an absorbance of 0.1 O.D. units per ml at 600 nm ($cm^{-1}$). Incubations were for 10 hours at 28°C and 200 rpm. Specific units of β-galactosidase activity were determined as previously described (chapters 2 and 4) and are expressed as units per bacterial cell.

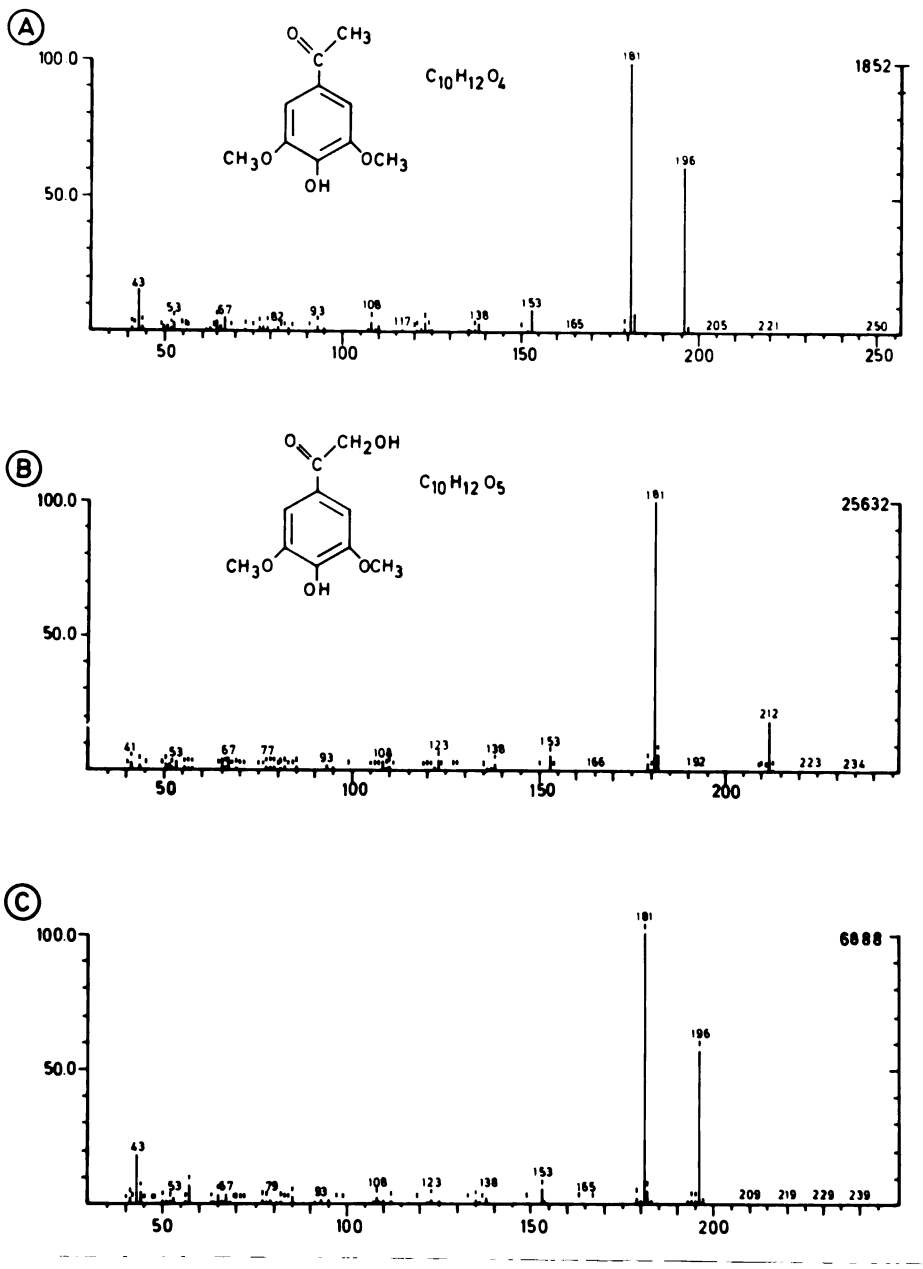



Figure 5.2. Mass spectra of vir-inducing molecules.

The mass spectra of purified peak B compound (Figure 5.1), purified peak A compound (Figure 1) and authentic acetosyringone (Janssen Chimica), are shown in A, B, and C, respectively. In each spectrum, the vertical axis indicates the relative intensity of the fragment

Legend to Figure 5.2. (cont'd)

ions, and the horizontal axis the mass/charge ratio of the fragment ions. The determined chemical formulas and structures of peak B and peak A are indicated in A and B, respectively.

Methods : Purification of cmr vir-inducing molecules – The biologically active compounds present in the peak A and B fractions were purified to homogeneity by RPC/FPLC; fractionation conditions were identical to those described in Figure 5.1, except for the column solvent. The fractions containing peak A or peak B (from Figure 5.1A) were pooled, lyophilized, resuspended in 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) and injected onto the C-2/C-18 column equilibrated with this buffer. The column was eluted with a linear gradient of 5% to 30% acetonitrile, 0.1% TFA, and monitored for UV absorbance at 280 nm and biological activity. For each sample a single peak was resolved from other minor UV-absorbing compounds, and a portion of the peak fraction was lyophilized, dissolved in 500 µl chloroform, and evaporated to 40 µl under a nitrogen stream for GC/MS analysis. Separate portions of each sample were dissolved in $CH_3OH$ for UV adsorption analysis (Figure 5.3), and in MSSP for anaysis of biological activity. GC/MS – 0.5 µl of sample was injected into a 0.32 mm x 25 m Carlo Erba (HRGC) gas chromatography column SF-52, directly coupled to a Finnigan 4000 mass spectrometer. GC fractionation was with a 50°-250° gradient, 5° $min^{-1}$. Data was collected and processed on a Nova 3 computer (Data General).

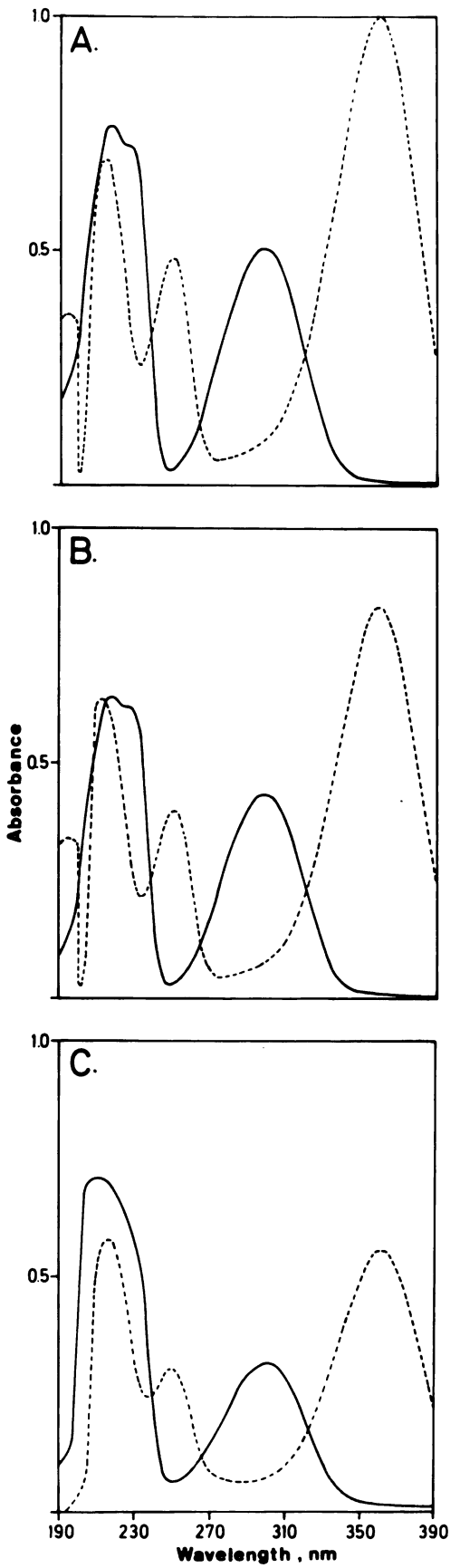



Figure 5.3.
UV absorption spectroscopy of vir-inducing compounds purified from cmr.

Legend to Figure 5.3.

Spectra were determined both in $CH_3OH$ (solid line) and $CH_3OH$/NaOH (dashed line). A, spectra of peak B compound; B, spectra of commercially obtained acetosyringone; C, spectra of peak A compound. Methods : Absorption spectra were measured in a Beckman DU-6 spectrophotometer scanning at 60 nm/min. For each compound dried material was resuspended in $CH_3OH$ (HPLC grade, Rathburn Chemicals Ltd.) to an approximate concentration of 50 μM, and scanned versus $CH_3OH$ as a blank solution. The pH of the sample was subsequently adjusted to basic pH by the addition of 1 M NaOH to 20 mM and the sample was scanned against $CH_3OH$/ 20 mM NaOH. Note that when the base-adjusted sample of each compound is readjusted to acid pH by adding 1 M HCl to 40 mM, the determined spectrum is seen to be equivalent to the original $CH_3OH$ spectrum of the compound (data not shown). Thus, the base-induced red-shift exhibited by each compound is fully reversible under the conditions used.

b) Identification of *vir* inducers

The peak B compound, the major *cmr* *vir*-inducing molecule, is 4-acetyl-2,6-dimethoxyphenol. This identification is based on several experimental observations :

(1) the GC/MS spectrum of the peak B compound (Figure 5.2A) indicates a molecule of relative molecular mass ($M_r$) 196, chemical composition $C_{10}H_{12}O_4$, and general structure acetyl-dimethoxyphenol.

(2) Comparison of the ultraviolet absorption spectrum of the peak B compound in $CH_3OH$ with its spectrum in $CH_3OH$/NaOH (Figure 5.3A) indicates that this molecule exhibits a strong absorption shift to longer wavelengths in the presence of NaOH; this base-induced red-shift is diagnostic of phenolic compounds in which the phenolic hydroxyl group is para (but not ortho or meta) to a conjugated ring substituent, such as a ketone or allyl group (17).

(3) The p-hydroxyl derivative, 4-acetyl-2,6-dimethoxyphenol, commonly designated acetosyringone (AS), is commercially available and greatly stimulates *vir* expression in *Agrobacterium*. AS and the purified peak B compound co-elute on RPC/FPLC (data not shown). Their respective GC/MS spectra are indistinguishable (Figure 5.2); [subtraction of these spectra yields a flat line]. Furthermore, their respective ultraviolet spectra, both in $CH_3OH$ and in $CH_3OH$/NaOH, are identical with respect to the shape, wavelength and relative intensity of each of the absorption maxima (Figure 5.3A-3B).

(4) The quantitative vir-inducing activities of authentic AS and the peak B compound are equivalent. In the experiment described in Figure 5.4, the units of induced β-galactosidase activity as a function of concentration (of inducing compound) were determined for each compound at several concentrations; the respective activity/concentration curves are identical.

The peak A compound, the second major cmr vir-inducing compound, is 4-(2-hydroxyacetyl)-2,6-dimethoxyphenol, called α-hydroxy acetosyringone (OH-AS), a close analogue of AS. This identification is based on the following observations :

(1) the GC/MS spectrum of the peak A compound (Figure 5.2C) indicates a molecule of mass 212, whose only signigicant fragmentation product has mass 181. The GC/MS spectrum of compound A following trimethylsilylation indicates a mass increase corresponding to two trimethylsilyl groups (data not shown). Thus, compound A contains two hydroxyl groups, and has general structure of either (3-propanol)dimethoxyphenol ($C_{11}H_{14}O_4$) or (2-hydroxyacetyl)dimethoxyphenol ($C_{10}H_{12}O_5$). Only this latter structure is consistent with the observation that the peak A compound is more polar than AS, as it elutes prior to AS on RPC/FPLC (Figure 5.1).

(2) Comparison of the ultraviolet absorption spectrum of the peak A compound in $CH_3OH$ with its spectrum in $CH_3OH$/NaOH indicates that, similarly to AS, this molecule exhibits a strong base-induced red-shift (Figure 5.3C). Thus, compound A contains a phenolic hydroxyl group para to a conjugated ring substituent.

Furthermore, the absorption spectra of compound A are almost identical to the equivalent spectra of AS; the respective absorption maxima occur at identical wavelengths (i.e., the largest maxima occur at 298 nm in $CH_3OH$ and 355 nm in $CH_3OH/NaOH$), although their relative extinction coefficients are different. These similarities strongly indicate that the peak A compound is very closely related to AS.

(3) The quantitative vir-inducing activity of the peak A compound is approximately equivalent to that of AS (Figure 5.1). As described below, the structure/activity specificity of vir-induction indicates that only molecules of structure closely related to AS induce vir expression to high levels (Table 5.1).

In the above analyses plant molecules that induce vir expression are chemically identified. The experiments described below are aimed at providing insight into the relationship between these molecules and Agrobacterium in nature. For the Agrobacterium/plant transformation system to be most efficient its activation should be limited to when Agrobacterium is in the presence of susceptible plant cells. This could be achieved if this activation is signalled only by molecules specific to these cells. For instance, such molecules should (i) be produced by different types of plant cells, as Agrobacterium can transform several different cell types (18); (ii) be specifically synthesized by susceptible plant cells, such as wounded cells; and (iii) be available to the bacterium in quantities sufficient for efficient activation. The following experiments demonstrate that AS has these properties, and that activation of Agrobacterium is a highly specific process.

c) Different plant tissues produce AS/OH-AS

To determine whether AS and OH-AS are specific to roots or whether they are also produced by other plant tissues, we purified and identified the vir-inducing activity produced by leaf cells (Figure 5.1). Medium in which N. tabacum leaves that have been cut into discs have been cultured (cml, conditioned medium leaves) contains substantial amounts of inducing activity which completely partitions into chloroform. The RPC/FPLC profile and corresponding bioassay profile of this material (Figure 5.1C) show that the cml activity fractionates into two peaks that respectively coelute with OH-AS and AS. Subsequent purification and GC/MS and UV spectrophotometric analysis of the active molecules in these peaks confirmed that the major vir-inducing activity in cml is composed of OH-AS and AS (data not shown). Thus, these two compounds are present in the exudates of at least two different plant tissues.

d) AS and OH-AS are exudate-specific

In the soil Agrobacterium probably detects plant cells through their exudates. To determine whether AS and OH-AS are exudate-specific compounds we assessed their relative concentrations within the N. tabacum leaf discs of Figure 5.1C. These discs were extracted with chloroform and the extract was analyzed by RPC/FPLC fractionation and corresponding bioassay. The relative concentrations of AS and OH-AS are less than 0.5% of the organic compounds present in the total leaf discs (data not shown). In comparison, the relative

concentrations of these compounds in the cml extract is approximately 25% and greater (Figures 5.1C, 5.5A). Thus AS and OH-AS probably do not leak out of damaged plant cells and are exudate-specific compounds.

e) Concentration of vir inducers in cmr/cml

Figure 5.4 presents the relationship between the concentration of AS (both commercial and cmr-purified) and induction of virB expression as measured in units of β-galactosidase activity. Under the conditions used, AS at 10 μM or greater, and AS at 1.5 μM, induce maximal (1200 units) and half-maximal (600 units) expression, respectively. We note that AS induction does not significantly affect cell viability (i.e., induction is a non-lethal event), and that concentrations of AS in excess of 200 μM are not significantly toxic to the bacteria. Using the concentration/response curve of Figure 5.4, the concentration of vir-inducing compounds in plant cell exudates relative to AS can be estimated. Our starting cmr and cml preparations typically induce between 250 and 500 units β-galactosidase activity in A348(pSM30); this activity approximately corresponds to between 0.5 and 1 μM AS.

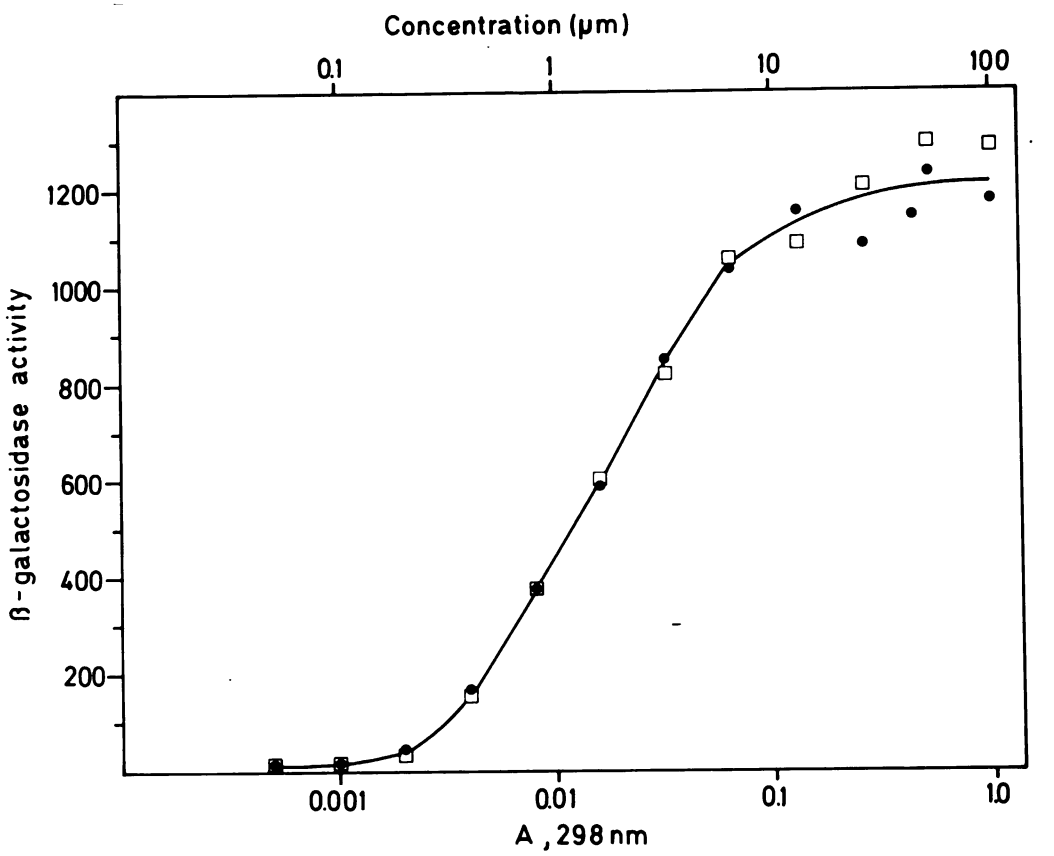



Figure 5.4. Comparison of vir-inducing activity of purified peak B compound (Figure 5.2) and commercially obtained acetosyringone.

The vertical axis indicates the β-galactosidase activity (units/bacterium) in the Agrobacterium virB::lacZ strain A348(pSM30). The lower horizontal axis indicates the relative concentration of purified peak B compound (Figure 5.2) or authentic AS (Janssen Chimica) in the inducing medium, measured as absorbance at 298 nm ($cm^{-1}$). The extinction coefficient of AS at 298 nm is 10,300 (27); this value was used to calculate the concentration of inducer compound in the inducing medium indicated by the upper horizontal axis.

Methods : purified peak B compound and authentic AS were separately dissolved in MSSP to an absorbance of 1.0 OD units per ml at 298 nm ($cm^{-1}$), measured against fresh MSSP. Each solution was serially diluted and the resultant samples were inoculated with bacterium at an absorbance of 0.09 OD units/ml at 600 nm ($cm^{-1}$). Samples were incubated for 14 hours at 28°C, 200 rpm, and the β-galactosidase activity of the bacteria in each sample was determined.

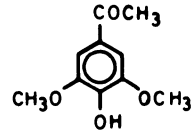
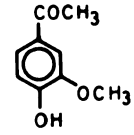
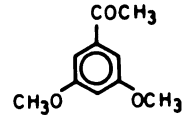
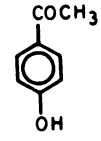
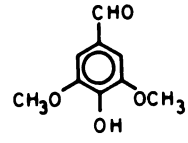
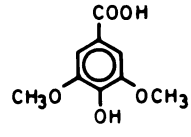
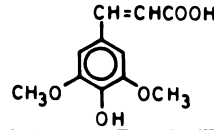
f) Biological activity of acetosyringone

The primary molecular signal for the initiation in Agrobacterium of the events of plant cell transformation should induce in the bacterium both the entire vir regulon and the initial steps of T-DNA transfer. We determined that AS has these activities. Several vir::lac and pin::lac Agrobacterium strains were incubated in 20 μM AS (both commercial and cmr-purified) , and assayed for β-galactosidase activity. Induction of all the previously identified inducible vir loci (B, C, D, E, G) and pinF was obtained, and the levels of induction were at least 20–50% higher than those stimulated during cocultivation with plant cell cultures or incubation with cmr (chapter 4) (data not shown). AS also induces the production of T-DNA intermediates : when bacteria are incubated with 20 μM AS for 12–18 hours, T-DNA circles (chapter 4) are found at a frequency 2–5-fold greater than that obtained following 48-hour cocultivation with protoplasts [17] (data not shown). We have also seen that AS induces the appearance and/or disappearance of several major proteins in Agrobacterium (P. Engström, P. Zambryski, and S. Stachel, in preparation). Thus, a single compound is sufficient to trigger the complete activation of vir and the initial events of T-DNA transfer; and AS (and likely OH-AS as well) is a primary signal for plant cell transformation by Agrobacterium.

g) Molecular specificity of *vir* induction

Table 5.1 shows the respective *vir*-inducing activities of AS (a) and analogues of AS (b-g). These results indicate that *Agrobacterium* *vir* expression is efficiently induced by molecules that conform to a structure best represented by AS itself, and suggest that *Agrobacterium* has evolved to recognize and respond to AS as a specific signal for the activation of plant cell transformation. For instance, AS minus one methoxy group (b) has greatly attenuated *vir*-inducing activity, while AS minus both methoxy groups (d) or its hydroxyl group (c) is inactive. Furthermore, the acetyl substituent of AS is important for activity. The formyl (e) and carboxylic acid (f) analogues of AS have attenuated activity; on the other hand, the cinnamic acid analogue of AS, sinapinic acid (g), has approximately equivalent activity as AS. This latter result is interesting in that sinapinic acid is a precursor of lignin, an integral cell-wall constituent of all vascular plants (see Discussion).

Table 5.1. Biological activity of derivatives of acetosyringone

| Compound | β-galactosidase activity Δ concentration (μM) 200 | 50 | 5 | 0.5 |
|---|---|---|---|---|
| a) acetosyringone  | 110 | 109 | 93 | 29 |
| b) acetovanillone  | 80 | 33 | 1.9 | 1.6 |
| c) 3,5-dimethoxy acetophenone  | N.D. | 1.0 | N.D. | N.D. |
| d) 4-hydroxy acetophenone  | N.D. | 1.1 | N.D | N.D. |
| e) syringaldehyde  | 86 | 51 | 2.2 | N.D. |
| f) syringic acid  | 21 | 8.6 | 1.7 | N.D. |
| g) sinapinic acid  | 104 | 98 | 68 | 18 |

Acetosyringone (a) and six related compounds, acetovanillone (b), 3,5-dimethoxy acteophenone (c), 4-hydroxy acetophenone (d), syringaldehyde (e), syringic acid (f), and sinapinic acid (g), were tested for their ability to induce β-galactosidase in the Agrobacterium

Legend to Table 5.1. (cont'd)

virB::lacZ strain A348(pSM30). All compounds were purchased from Janssen Chemica, and prepared as 0.1 M solutions in dimethyl sulfoxide. Each compound was serially diluted into MSSP medium to 200, 50, 5, and 0.5 μM (a, b, e, f, g) or 50 μM (c, d). These solutions were inoculated with bacteria at 0.05 O.D. units/ml at 600 nm/cm, and incubated at 28°C, 200 rpm. Following 12 hours, the bacterial β-galactosidase activity in each sample was determined. Here the data are expressed as activity of the bacteria incubated in the presence of a compound relative to the basal activity present in bacteria incubated without added compound. The basal activity for the pSM30 strain is 10 units. N.D., not determined.

h) Wounding stimulates AS/OH-AS production

While a wide range of dicotyledonous plant cells can be transformed by Agrobacterium, only cells that have been wounded or traumatized are specifically seen to be susceptible. Thus we tested whether the production of AS and OH-AS is stimulated by plant cell wounding. Figure 5.5 displays the RPC/FPLC profiles of the organic extracts of cml produced by equivalent amounts of N. tabacum leaf discs (wounded cells; Figure 5.5A), or intact leaves (unwounded; Figure 5.5B). Comparison of these profiles indicates that the "wounded" cml contains greater than 10-fold more AS and OH-AS than the "unwounded" cml, demonstrating that wounding stimulates the appearance of these molecules in cell exudate. The low levels of AS and OH-AS in the "unwounded" leaf exudate are potentially due to the cut stem surfaces of the leaves resulting from their excision from the plant.

These results, do not define which cells of the wounded tissue produce AS and OH-AS. For example, damaged or dead cells could release AS, although such cells are not good targets for Agrobacterium infection. We tested whether active plant cell metabolism is required for the production of AS, by analyzing the cml produced by leaf discs incubated in the presence of cycloheximide. This material does not contain AS and OH-AS (Figure 5.5C). These results confirm and extend our previous observation that in plant/Agrobacterium cocultivations only actively growing plant cell cultures are able to stimulate efficient vir gene expression (chapter 4). Our data concerning the production of AS and OH-AS in total suggest that these compounds are specifically synthetized and exuded by metabolically active wounded cells.

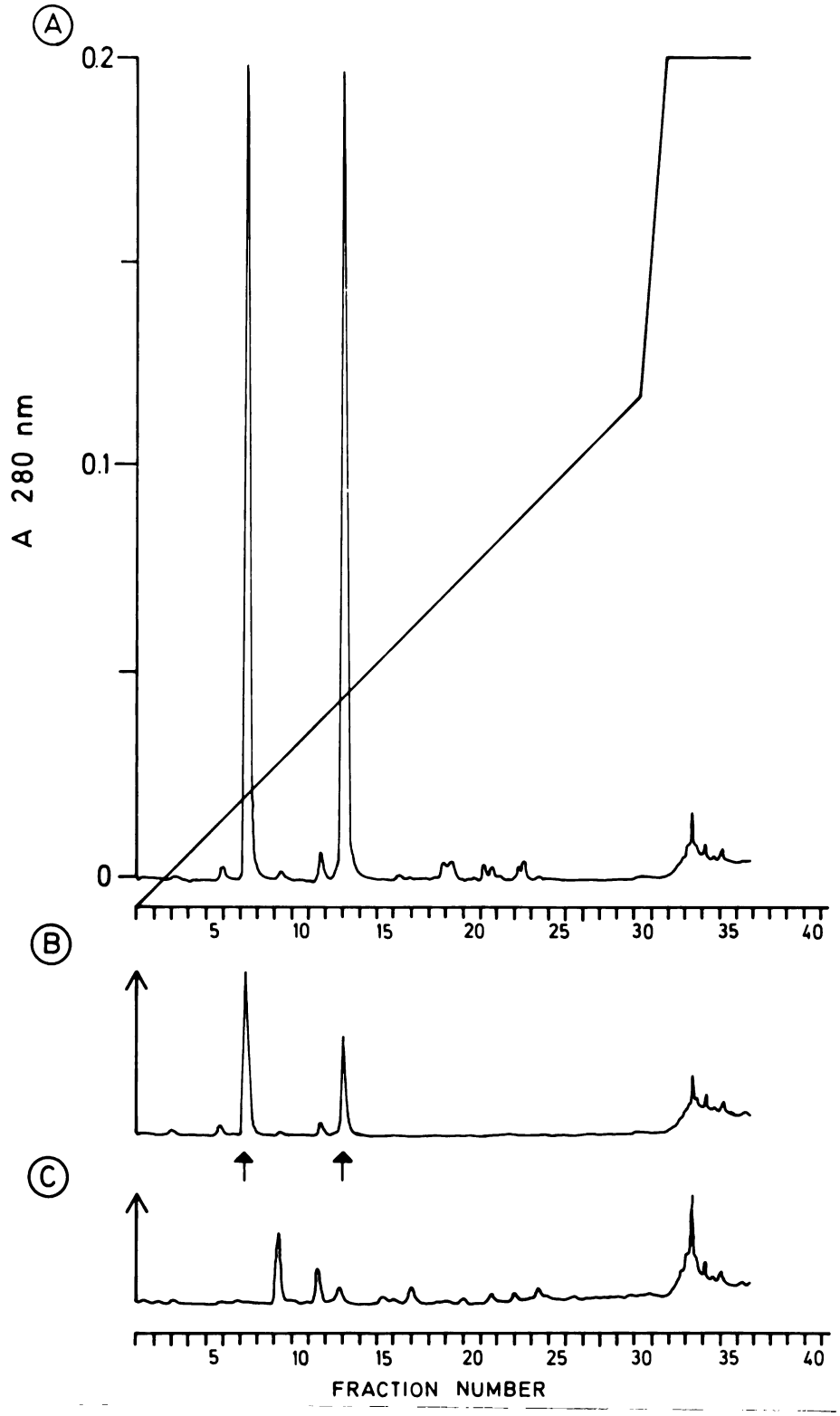



Figure 5.5. Effect of wounding and inhibition of plant cell metabolism on production of AS and OH-AS by plant cells.

Legend to Figure 5.5.

RPC/FPLC fractionations of equivalent amounts of medium conditioned with (A) 1.5-cm diameter leaf discs; (B) intact leaves; and (C) leaf discs in the presence of cycloheximide. The vertical axes are drawn to the same scale in all 3 panels. Arrows represent the elution positions of OH-AS (fraction 7), and AS (fraction 13).

Methods : 4 g of leaves excised from 6-week-old N. tabacum SR1 plants were treated as described below, and incubated in 50 ml MS medium in a 150-ml petri dish. Care was taken to use visually equivalent leaves in each experiment. The treatments were (A) leaf discs, 5–6 1-$cm^2$ discs were obtained per leaf; (B) intact leaves; (C) leaf discs as in (A) but with 5 ppm (12.8 μM) cycloheximide (Actidion, 99%; Aldrich Chemicals) added to the medium. Following 72 hours incubation, 40 ml of each respective conditioned medium was recovered. Thirty-five ml was extracted with chloroform as described (Figure 5.1) and the organic pellet was resuspended in 400 μl 10% $CH_3OH$, 0.1% $CH_3COOH$. Hundred μl of this sample was analyzed by RPC/FPLC using the conditions described in Figure 5.1. The remaining 5 ml of conditioned medium was bioassayed for vir-inducing activity; the units of β-galactosidase activity induced by the conditioned media of A, B, and C, were 565, 135 and 20, respectively.

## Discussion

The molecules that we have purified signal *Agrobacterium* to activate the expression of its virulence genes, setting in motion a series of molecular events ultimately resulting in the transfer of T-DNA from the bacterium to the plant cell.

Wounded cells are known to be susceptible to *Agrobacterium* infection, perhaps because the intact cell walls of undamaged cells restrict T-DNA transfer, or because T-DNA integration is dependent on host-cell replication and wounding stimulates this replication. Because *Agrobacterium* responds to AS and OH-AS to initiate the steps leading to plant cell transformation, these molecules potentially represent the signal that *Agrobacterium* detects and recognizes in the biologically complex soil environment as susceptible plant cells. That AS and OH-AS are specifically present in the exudates of wounded, but actively metabolizing plant tissues, supports our hypothesis.

The activation of *Agrobacterium* *vir* expression by plant signal molecules likely involves at least two steps, extracellular recognition and intracellular response. The former step might depend on the signal molecule acting as a chemical attractant and/or nutritive source for the bacterium. The latter step must depend on the bacterium being able to convey the information of the signal from outside to inside the cell and to activate *vir* expression. The mechanism of these events is unknown. Induction of the pTiA6 *vir* region is attenuated in *virA*, and does not occur in *virG*, mutant bacteria (chpater 3). Also, the amino acid sequence of the *virG* gene product

is highly homologous to several positive regulatory proteins of E. coli (19). Because AS can cause the induction of the complete vir regulon, we suggest that this compound might act to allosterically activate the virG protein which then activates vir transcription by directly interacting with vir gene promoter sequences. The virA protein potentially functions in the initial extracellular/intracellular recognition and/or intracellular transport of the signal molecule.

An extensive search of the literature reveals that AS and OH-AS have not previously been identified as natural components of plant cells, suggesting that the appearance of these molecules in nature is not widespread. Thus, these molecules potentially represent to Agrobacterium only cells which are its desired targets. In addition, these or related molecules might also serve to initiate other bacterial/plant interactions in the soil. The observed resistance of most monocotyledonous plants to Agrobacterium might result because these plants do not produce, or only produce in low quantities, vir signal molecules. AS and OH-AS might be useful for obtaining Agrobacterium transformation of plants species previously seen to be resistant to Agrobacterium, and also for the analysis of the initial molecular steps of the T-DNA transfer process.

Although it is important for Agrobacterium to detect and respond to such molecules, it is equally important not to respond to closely related but functionally different molecules; we have shown that vir induction is most efficiently stimulated by AS, and by the lignin precursor, sinapinic acid. This latter compound is not present in detectable quantities in our exudate preparations; how-

ever, sinapinic acid could be present in the soil in exudates of wounded cells in the process of rebuilding their cell walls.

It is interesting to speculate on the function of AS and OH-AS in plants. These compounds are likely products of the shikimic acid biosynthetic pathway that provides the plant cell with the precursors to a broad spectrum of molecules, including the flavonoids, and lignins (20-22). These classes of compounds are important to a plant subjected to stress or injury. For example, many flavonoid-derived phytoalexins are potent inhibitors of the growth of invading pathogens (23), while lignin, a major component of the cell wall, provides a physical barrier to invasion (24). Thus, AS and OH-AS might be part of the wound response (25) of plant cells. Potentially these compounds are toxic to other soil pathogens, and Agrobacterium has evolved to be resistant to AS and OH-AS and to use them to recognize wounded cells. Alternatively, these compounds might be products related to lignin repair in damaged cells.

We propose the following hypothesis for the role of AS and OH-AS in the Agrobacterium/plant interaction in nature. There must be a continual excretion of wound-related phenolics during growth due to abrasion from the soil. Agrobacterium may be attracted to the vicinity of plants based on the recognition of these compounds. However, significant levels of vir induction and the events of T-DNA transfer will only occur if signal molecules are present in sufficient concentrations. Since the highest levels will be found at wound sites, Agrobacterium tumefaciens effectively infects these sites.

Acknowledgements

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# CHAPTER 6

## The plant-induced activation of the T-DNA transfer process in *Agrobacterium tumefaciens* is regulated by *virA* and *virG*


### Abstract

The Ti plasmid *vir* loci of *Agrobacterium tumefaciens* are transcriptionally activated in response to signal molecules produced by plant cells susceptible to *Agrobacterium* transformation, and this activation initiates the T-DNA transfer process. The present study shows that the *vir* loci of the A6 Ti plasmid are organized as a single regulon, and that two *vir* loci encode trans-acting functions which mediate the induction of this regulon by plant cells. Mutations in *virA* result in greatly attenuated induction. This locus is constitutively transcribed and non-inducible. Mutations in *virG* totally eliminate *vir* induction. This locus is constitutively transcribed, inducible by plant cells, self-regulated in a complex fashion, and produces two differentially regulated transcripts that differ at their 5' termini. *VirA* and *virG* are proposed to encode, respectively, a transport protein for the plant signal molecule, and a positive regulatory protein that in conjunction with the signal molecule activates the expression of the *vir* regulon.

## Introduction

The soil pathogen, Agrobacterium tumefaciens, genetically transforms plant cells by transferring a specific segment of DNA, the T-DNA, from its large (> 200 b) Ti plasmid to the plant cell genome (Caplan et al., 1983; Nester et al., 1984; Gheysen et al., 1985). The Agrobacterium system is the only known example of the directed insertion of prokaryotic DNA into a eukaryotic genome. The molecular details of the T-DNA transfer process are not yet understood; however, a primary event of this process has been identified. This event is the transcriptional activation within the bacterium of the virulence vir loci of the Ti plasmid (chapter 4). This induction occurs in response to plant cells susceptible to Agrobacterium transformation (chapter 3). The vir genes are absolutely essential to the transfer process, and their induction directly results in the activation in the bacterium of specific T-DNA-associated molecular events (Koukolíková-Nicola et al., 1985; chapter 4), which have been proposed to represent early steps of T-DNA transfer.

The plant/bacterial interaction that leads to the induction of the vir loci has at least two components – an inducing factor synthesized by susceptible plant cells, and a bacterial regulatory system that allows the bacterium to recognize and respond to this factor. For Nicotiana tabacum, the plant signal has recently been purified and characterized; this signal is composed of the phenolic compounds, acetosyringone and α-hydroxyacetosyringone, which are specifically produced in the exudates of wounded and metabolically

active plant cells (chapter 5). Here, the vir regulatory system that these molecules act through is characterized.

The vir regulatory system likely carries out at least two distinct steps to mediate vir induction. First, the presence of the plant signal molecules must be transduced from outside to inside the bacterial cell. This step can be direct, the cell can take up the signal through a permease or porin function; or indirect, the signal can activate a secondary messenger system present at the bacterial cell surface. Because the signal is a small organic molecule, it likely enters and functions within the bacterium. Second, the signal, once internalized, must be converted into the transcriptional activation of several separate genetic loci. This activation can be mediated through one or more positive or negative regulatory proteins that interact with vir promoter sequences to control their transcription, or through the Agrobacterium RNA polymerase protein itself. The vir induction process might also involve other potential steps; for example, modification of the signal molecule to a more active form. Furthermore, the inactivation of the signal molecule might also play an important role in the control of vir induction. It is not unlikely that the vir regulatory system is directed by the products of several Agrobacterium genes.

A bacterium in which a gene that regulates vir expression is mutant, will likely be avirulent, or only partially virulent, on plants. Two such classes of mutations have been isolated and identified in Agrobacterium, those within the Ti plasmid vir loci (Garfinkel and Nester, 1980; Klee et al., 1983; Hille et al., 1984;

chapter 3), and those within the chromosomal virulence loci, chvA and chvB (Garfinkel and Nester, 1980; Douglas et al., 1985). The chv and vir loci encode trans-acting products, since they are expressed in Agrobacterium and all mutations in these loci are transcomplementable (Douglas et al., 1985; chapter 3). Thus, these loci are good candidates for genes that encode functions involved in the regulation of vir expression. The specific vir expression phenotype displayed by a bacterium mutant for such a gene can provide insight into the function encoded by the gene. For example, increased vegetative expression of plant-activated vir loci identifies a repressor function, loss of plant inducibility identifies an activator function, and decreased inducibility suggests a receptor function. Furthermore, superinduction suggests a function involved in the inactivation of the inducer molecule.

In the present study, virA and virG are identified as genetic components of the vir regulatory system. The effect on vir expression of mutations in each of these genes is determined. Furthermore, the regulated expression of virA and virG is characterized. The results of this study provide a foundation for understanding how the virA and virG gene products function together with the plant signal to mediate vir induction and activate the T-DNA transfer process.

## Results

### a) Strategy for identification of genes involved in vir expression

The vir region of the A6 Ti plasmid is presented in Figure 6.1. Genetic analysis has shown this region to be organized into six vir complementation groups, virA, virB, virC, virD, virE, and virG (chapter 3). Transposon insertion mutations in these loci eliminate (virA, virB, virD, and virG) or restrict (virC and virE) the ability of Agrobacterium to infect plant cells. The expression of the pTiA6 vir loci has been characterized using a set of gene fusions between each of these loci and the lacZ gene of E. coli. In a bacterium carrying a chimeric vir::lac gene the production of β-galactosidase, the lacZ gene product, is under the control of the promoter of the vir locus to which lacZ has been fused; and the level of β-galactosidase activity in the bacterium therefore directly reflects the state of expression of the locus. Results obtained with the vir::lac gene fusions have shown that virB, virC, virD, and virE are plant-inducible and are significantly expressed only in plant-induced Agrobacteria; virA is constitutively expressed in vegetative and plant-induced cells and is non-inducible; and virG is both constitutively expressed and plant-inducible. The pTiA6 vir region also carries another plant-inducible locus, pinF. In contrast to the vir loci, mutations in pinF have no effect on virulence.

The previously constructed vir::lac gene fusions provide an assay system for the identification of genes which are involved in the regulation of vir expression. When a fusion is carried on a

plasmid that replicates in Agrobacterium and can coexist with the Ti plasmid, the vegetative and plant-induced β-galactosidase activity expressed by the fusion can be readily assessed in different Agrobacterium genetic backgrounds, including mutant backgrounds previously identified to affect virulence on plants. Furthermore, by using lac fusions to each of the vir loci, potential differences in the expression and regulation of different loci can be obtained, as well as an overall picture of vir expression. [It is noted that the N. tabacum NT1 plant culture is used as the source of the inducer molecule throughout the present study; essentially identical results are obtained when acetosyringone, the chemically pure inducer, is directly used to stimulate vir induction.]

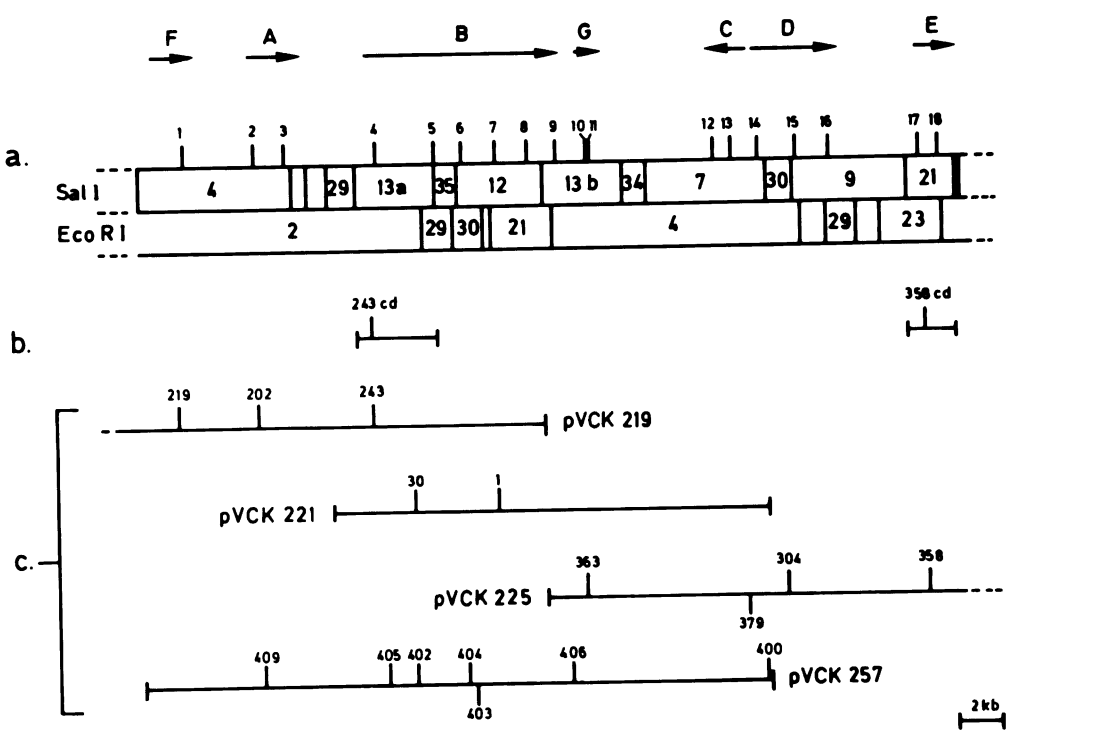


Figure 6.1. pTiA6 vir mutant strains and p(vir::lac) plasmids used to construct the experimental strains of Tables 6.1, 6.2, and 6.3.

Legend to Figure 6.1.

A. Eighteen pTiA6 vir strains that each carries a transposon insertion mutation are shown. The position of the mutation carried by a mutant is represented by a vertical line drawn above the SalI and EcoRI restriction maps of the pTiA6 vir region. The number adjacent to a line corresponds to the mutant key given in Table 6.2. The genetic and transcriptional organization of the pTiA6 vir region (chapter 3) are represented by the arrows at the top of the figure.

B. The deletion derivative plasmids pSM243cd and pSM358cd are shown. These molecules are derived from pSM243 and pSM358, respectively.

C. Sixteen pSM(vir::lac) plasmids are shown, along with the parental vir cosmid clones pVCK219, pVCK221, pVCK225, and pVCK257.

In B and C, the horizontal lines represent the vir region sequences cloned into pVCK102, and the vertical lines represent the positions of the Tn3-HoHo1 insertions which have generated the vir::lac fusion genes carried by the pSM(vir::lac) molecules. The orientation of the lac sequences of an insertion are represented by whether it is drawn above or below a horizontal line; those insertions represented by upper lines are oriented left to right, and those by lower lines are oriented right to left.

b) pTiA6 carries positive vir regulatory elements

To begin to determine whether pTiA6, and particularly the pTiA6 vir region, carries genes which positively and/or negatively regulate the induction of vir expression by plant cells, the levels of vegetative and plant-induced β-galactosidase activity produced by several pSMvir::lac constructs (Figure 6.1), previously determined to give high levels of inducible β-galactosidase activity in Agrobacterium strain A348, which carries the wild-type A6 Ti plasmid, were measured in Agrobacterium strain A136. This strain is isogenic to A348, except that it lacks pTiA6 (Garfinkel et al., 1981). The results of these experiments indicate that the Ti plasmid does not encode a function that represses vir expression in the absence of plant cells; the A136 and A348 derivatives of each vir::lac plasmid display essentially identical units of vegetative activity (data not shown). On the other hand, the Ti plasmid does encode a trans-acting positive regulatory element(s) required for vir induction by plant cells; efficient induction is not obtained for any of the A136(pSMvir::lac) strains (Table 6.1). This effect is specific for vir induction (and the particular vir::lac plasmids tested here, see section (c)), as the induction of another cloned pTiA6 regulon efficiently occurs in A136. For example, the expression of the pTiA6 octopine catabolism (occ) locus is induced by octopine, and a plasmid clone that carries an occ::lac fusion gene gives equivalent levels of octopine-induced β-galactosidase activity in both A136 and A348 (chapter 2; Table 6.1).

Table 6.1 further shows that the vir::lac plasmids display two different induction phenotypes in A136, no induction and greatly attenuated induction. The induction phenotype obtained is not a function of the vir locus to which lac is fused, but of the presence or absence of other vir sequences on the particular vir plasmid which carries the fusion gene. For example, pSM243, a virB:: lac derivative of pVCK219 is noninducible in A136, while pSM1 and pSM30, virB::lac derivatives of pVCK221, are partially inducible. Furthermore, all derivatives of pVCK219 are noninducible, while all derivatives of pVCK221 and pVCK225 are partially inducible. pVCK219 carries pinF, virA, and the leftward 8 kb of virB; pVCK221 carries virB, virG, and the leftward (5') 0.5 kb of virD; and pVCK225 carries the rightward (3') 1-kb of virB, virG, virC, virD, and virE. The results obtained with these plasmids indicate that pTiA6 encodes at least two distinct positive regulatory elements which mediate different steps in the vir induction process. Both pVCK221 and pVCK225, and not pVCK219, appear to carry an element that is absolutely essential for vir induction to occur. Since pVCK221 and pVCK225 share the sequences from SalI fragment 13b through SalI fragment 30 (Figure 6.1), this essential positive regulatory element is likely encoded within this region. A second element must be located elsewhere on pTiA6; this element is not absolutely essential for vir induction to occur, but instead is required for this induction to be efficient.

Table 6.1. pTiA6 carries genes required for *vir* induction

| | locus | Induction, Δ units | |
|---|---|---|---|
| | | - pTiA6 | + pTiA6 |
| pSM219 | *pinF* | 0.74 | 40 |
| pSM202 | *virA* | 1.1 | 0.84 |
| pSM243 | *virB* | 1.0 | 87 |
| pSM30 | *virB* | 3.0 | 73 |
| pSM1 | *virB* | 4.4 | 68 |
| pSM363 | *virG* | 5.1 | 15 |
| pSM379 | *virC* | 2.9 | 18 |
| pSM304 | *virD* | 3.9 | 72 |
| pSM358 | *virE* | 4.0 | 36 |
| pSM243cd | *virB* | 0.81 | 86 |
| pSM358cd | *virE* | 1.1 | 34 |
| pSM102* | *occ* | 42 | 40 |

Eleven pSM(*vir*::*lac*) constructs shown in Figure 6.1 and one pSM(*occ*::*lac*) construct, were placed in A136(-pTiA6) and A348 (+ pTiA6) to give 24 strains. The units of vegetative and plant-induced β-galactosidase activity expressed by each strain were measured and its inducibility (induction, Δ units) calculated. *, the cocultivations of the *occ*::*lac* strains A136(pSM102) and A348 (pSM102) were carried out in the presence of 100 μg/ml L-octopine (Sigma); pSM102 effects octopine-inducible, but not plant-inducible, β-galactosidase activity (chapters 2 and 4), and is used in these experiments as a control. The pSM202 strains do not display inducible β-galactosidase activity, since *virA* expression is not plant-inducible (chapter 3; Figure 6.2).

c) virA and virG are essential for induction

To identify vir regulatory loci, the plant inducibility of vir expression in 18 different pTiA6 vir and 2 different chv mutant strains was surveyed (Figure 6.1A; Table 6.2). Specifically, the inducibility of three vir::lac tester was measured in each of these strains to identify mutations that affect the induction of virB, virE, and pinF expression. The tester plasmids that were used are pSM243cd, pSM358cd, and pSM219. pSM243cd and pSM358cd respectively carry a virB::lac and a virE::lac fusion gene in the absence of other vir sequences (Figure 6.1B), and these plasmids display the same levels of plant-inducible β-galactosidase activity in A348 as do pSM243 and pSM358, from which they were derived (Table 6.1). pSM219 carries a pinF::lac fusion, virA, and the proximal (5') 8 kb of the virB operon (Figure 6.1C).

Table 6.2 shows that mutations in virA and virG block the induction of pSM243cd and pSM358cd by plant cells; and mutations in virG block the induction of pSM219. As discussed in detail below, these results, in conjunction with other data, indicate that virA and virG encode functions essential to the vir induction process. The results in Table 6.2 also show that pinF, virB, virC, virD, virE, chvA, and chvB are fully dispensible for virB and virE induction, since mutations in these loci have no significant effect on the induction of pSM243cd and pSM358cd. Furthermore, none of the gene products of the vir and chv loci appear to negatively regulate vegetative virB or virE expression, nor to inactivate the signal molecule, since elevated levels of vegetative or plant-induced vir

expression are not observed in any of the mutant strains. Similar results were also obtained for pSM219; however, because this plasmid carries a wild-type copy of virA and the proximal 8-kb of virB, the requirement of virA and virB for the induction of pinF expression is not assessed in these experiments.

Both pSM243cd and pSM358cd are only weakly inducible in the virA mutant strains pTi240 and pTi237. This attenuated vir induction phenotype is also observed for the virG::lac plasmids pSM363cd and pSM321cd in the virA mutant strain A1016 (Figure 6.2; Table 6.4), and the virC::lac plasmid pSM379 and the virD::lac plasmid pSM304 (Figure 6.1) in both A1016 and pTi240 (data not shown). The pinF::lac plasmid pSM219 is fully inducible in virA mutant strains (Table 6.2); however, pSM219 carries its own wild-type copy of virA. These results, in total, demonstrate that only low levels of induction occur in the absence of virA. Thus, the virA gene product is essential for the efficient induction of vir expression in response to the plant cell signal molecule.

pSM243cd, pSM358cd, and pSM219 are noninducible in the virG mutant strain pTi19 (Table 6.2). The corresponding derivatives of another virG mutant strain, pTi363, display low levels of inducible β-galactosidase activity; however, this activity is due to the induced expression of the virG::lac gene of pTi363 instead of the vir::lac gene of the tester insertion plasmid, since pTi363 itself displays essentially the same low-level inducibility (Table 6.4) as its insertion plasmid derivatives (Table 6.2). Thus, pSM243cd, pSM358cd, and pSM219 are also noninducible in pTi363. These results, in total, demonstrate that the virG gene product is absolutely

essential for pinF, virB, and virE induction. That pTi363 is partially inducible suggests that this requirement does not fully hold for virG itself. This observation is further analyzed in a later section.

In the present experiments, the requirement of virA for pinF induction and of virG for virC and virD induction is not examined. However, the data presented, in conjunction with the observation that the plant metabolite acetosyringone induces the expression of each of the plant-inducible vir loci of pTiA6 (chapter 5), strongly indicate that the vir and pin loci are organized as a single regulatory unit, designated the vir regulon, whose inducible expression is positively controlled by the virA and virG gene products.

Table 6.2. Effect of *vir* and *chv* mutations on *vir* induction

| Mutant strain | Locus | Reference | Induction, Δ units | | |
|---|---|---|---|---|---|
| | | | pSM243cd | pSM358cd | pSM219 |
| 1) pTi222 | *pinF* | d | 84 | 39 | 42 |
| 2) pTi240 | *virA* | d | 3.7 | 3.4 | 50 |
| 3) pTi237 | *virA* | d | 3.3 | 2.8 | 47 |
| 4) A2005 | *virB* | b | 90 | 30 | 39 |
| 5) A2019 | *virB* | b | 72 | 24 | 42 |
| 6) A2002 | *virB* | b | 82 | 36 | 55 |
| 7) A2024 | *virB* | b | 72 | 27 | 38 |
| 8) AETW7 | *virB* | d | 104 | 30 | 38 |
| 9) A1061 | *virB* | a,d | 69 | 36 | 47 |
| 10) pTi363 | *virG* | d | 5.1 | 2.0 | 3.8 |
| 11) pTi19 | *virG* | d | 0.75 | 0.88 | 0.81 |
| 12) pTi364 | *virC* | d | 94 | 28 | 36 |
| 13) A1034 | *virC* | a | 73 | 33 | 45 |
| 14) pTi311 | *virD* | d | 82 | 29 | 41 |
| 15) pTi307 | *virD* | d | 79 | 31 | 42 |
| 16) pTi348 | *virD* | d | 82 | 26 | 48 |
| 17) pTi361 | *virE* | d | 81 | 39 | 40 |
| 18) pTi330 | *virE* | d | 99 | 33 | 40 |
| A2505 | *chvA* | c | 87 | 41 | 47 |
| A1045 | *chvB* | a | 79 | 34 | 44 |

pSM243cd(*virB*::*lac*), pSM358cd(*virE*::*lac*), and pSM219(*pinF*::*lac*) were placed in 18 *vir* mutants and 2 *chv* mutants to generate 60 strains, and the plant inducibility of the β-galactosidase activity of each strain was determined (induction, Δ units). The plasmids and *vir* mutants used in the construction of these strains are shown in Figure 6.1. With the exception of pTi363 (see Table 6.4) none of the *vir* mutant strains express either a significant level of vegetative

Legend to Table 6.2. (cont'd)

β-galactosidase activity (>2 units), or any plant-inducible activity, when not carrying a vir:: lac plasmid. a, Garfinkel and Nester (1980); b, Klee et al. (1983); c, Douglas et al. (1985); d, chapter 3.

d) virA and virG are the only pTiA6 vir regulatory loci

If virA and virG are the only pTiA6 loci required for regulated vir expression in Agrobacterium, then a vir::lac insertion plasmid that carries both virA and virG should be equally inducible for β-galactosidase activity in A136 and A348. To this end, pVCK257, a plasmid which carries pTiA6 vir region sequences from SalI fragment 4 through SalI fragment 7 (Knauf and Nester, 1982) was mutagenized with the Tn3-lac element, Tn3-HoHo1 (chapter 2), to generate derivatives of pVCK257 which carry plant-inducible vir::lac gene fusions. The 7 pVCK257::Tn3-HoHo1 derivatives shown in Figure 6.1C were transferred to A136 and A348, and the respective inducibility of each of the 14 resultant strains was determined.

Table 6.3 shows that each pVCK257::Tn3-HoHo1 construct which is inducible for lac expression in A348, is equally as inducible in A136. This result is obtained for pSM400, which carries a virD::lac gene fusion, and pSM402, pSM404, and pSM405, which carry 3 independent virB::lac fusions. Thus, pVCK257 contains all the necessary genetic elements required for efficient plant induction of Agrobacterium vir expression; the results presented in the previous section identify these elements as virA and virG.

Table 6.3. Plasmids that contain virA and virG are fully inducible in the absence of pTiA6

| | Induction, Δ units | |
|---|---|---|
| | - pTiA6 | + pTiA6 |
| pSM400 | 20 | 23 |
| pSM402 | 28 | 36 |
| pSM403 | 1.2 | 1.1 |
| pSM404 | 22 | 24 |
| pSM405 | 39 | 44 |
| pSM406 | 0.91 | 1.0 |
| pSM409 | 0.94 | 1.2 |

The 7 pVCK257:: Tn3-HoHo1 derivatives shown in Figure 6.1C were placed in A136(-pTiA6) and A348(+pTiA6). For each resultant strain, its units of vegetative and plant-induced β-galactosidase activity were measured, and its inducibility (induction, Δ units) calculated. The pSM403, pSM406, and pSM409 strains do not display plant-inducible β-galactosidase activity, since pSM403 carries a virB::lac insertion oriented in the direction opposite that of virB transcription; pSM406 (which is observed to complement virB and virG mutants for virulence) carries a wild-type insertion in noninducible sequences located between virB and virG; and pSM409 carries a virA::lac insertion.

e) virA and virG equivalent loci in other Ti plasmids

Since the vir regions of different Ti plasmids share considerable DNA sequence homology (Engler et al., 1981; Thomashow et al., 1981), their vir loci probably encode analogous functions, including those that regulate vir expression. To this end the p(vir::lac) system was used to determine whether Ti plasmids other than the wide host-range (WHR) A6 Ti plasmid carry genes that are functionally equivalent to virA and virG. The quantitative inducibility of the virB::lac and virE::lac deletion derivative plasmids, pSM243cd and pSM358cd (Figure 6.1) was measured in Agrobacterium strains carrying either the WHR nopaline-type plasmid pTiC58 (Holsters et al., 1980), the WHR agropine-type plasmid pTiBo542 (Hood et al., 1984), or the limited host-range (LHR) plasmid pTiAg162 (Knauf et al., 1985). Except for Ti plasmid content, each of these strains is isogenic with the pTiA6 strain A348. The vir::lac plasmids are as inducible against pTiC58 or pTiBo542, as against pTiA6 (data not shown). Thus, these Ti plasmids carry vir regulatory loci that are functionally equivalent to virA and virG of pTiA6, and the vir regulatory system defined here for pTiA6 is generalizable to other Ti plasmids.

On the other hand, the vir::lac plasmids are only partially inducible against pTiAg162. For example, pSM243cd which gives approximately 80-fold and 3-fold levels of induction against pTiA6 and the pTiA6 virA mutant pTi222, respectively (Tables 6.1 and 6.2), gives an 11-fold level of induction against pTiAg162. This attenuated induction is related to virA, since pSM243, which carries the virB::lac fusion of pSM243cd and also a copy of the pTiA6 virA locus

(Figure 6.1), is fully inducible (74-fold) against pTiAg162. Thus, pTiAg162 appears to carry a gene functionally equivalent to virG, but not to virA. Instead, pTiAg162 must carry a locus whose product has partial virA activity. It is noted that Agrobacteria that carry pTiAg162 infect only a limited number of plant species, and the addition of the pTiA6 virA gene to these cells has been observed to increase, in part, their infective host-range (Yanofsky et al., 1985). The data presented here suggests that this increase might be because the addition of virA allows the bacteria to better recognize and respond to plants that they normally are unable to infect.

f) S1 analysis of vir transcripts

Expression data previously obtained with the different vir::lac gene fusions has indicated that virA and virG, the vir regulatory loci, and the other vir loci are differentially expressed; while pinF, virB, virC, virD, and virE become expressed only upon plant activation, virA is constitutively expressed, and virG is both constitutively expressed and plant-inducible (chapter 3). To further assess this differential expression, and to gain additional insight into vir regulation, the transcription of virA, virB, and virG was directly examined under two conditions, vegetative growth and cocultivation. Specifically, the S1 nuclease mapping technique was used to define the 5' termini of the virA, virB, and virG transcripts, and to assess the presence of these transcripts in RNA prepared from bacteria grown in minimal medium (vegetative RNA) and cocultivated with plant cells (induced RNA).

The approximate locations of the virA, virB, and virG promoter regions were determined from the results of the transposon mutagenesis analysis of the pTiA6 vir region (chapter 3), and 5' end-labelled DNA fragments corresponding to these regions were prepared as vir promoter probes (Figure 6.2A). Each of these probes was independently hybridized to the vegetative and induced RNA, the RNA::DNA hybrids were digested with S1 nuclease, and the resultant S1-resistant DNA fragments were analyzed by gel electrophoresis and autoradiography. The results of these experiments are presented in Figure 6.2.

(i) VirA is constitutively transcribed

An approximately 540-bp HindIII/DdeI DNA restriction fragment 5' end-labelled at the DdeI site was used to map the 5' terminus of the virA transcript. A 183-base segment (± 2 bases) of this probe is protected from S1 digestion by both the vegetative and induced RNA (Figure 6.2B). Thus, the 5' terminus of the virA transcript is located approximately 2.05 kb leftward of the rightend of SalI fragment 4 (Figures 6.1 and 6.2A). The presence of this message in both vegetative and induced bacteria demonstrates that virA is constitutively transcribed. The relative intensity of the protected band obtained with the vegetative RNA is approximately equivalent to that obtained with the induced RNA (Figure 6.2B). Since the probe was used in large excess of the homologous RNA in these experiments, this result suggests that virA transcription is not significantly

affected by plant induction of the vir regulon. Also, it is noted that the presence or absence of virG has no effect on virA expression (Table 6.1).

(ii) VirB transcription is limited to induced cells

A 324-bp AvaII/HinfI DNA restriction fragment 5' end-labelled at the HinfI site was used to map the 5' terminus of the virB transcript. No protection of this probe is observed with the vegetative RNA. In contrast, fragments between 216 to 219 bases (± 2 bases) of the virB probe are protected by the induced RNA (Figure 6.2B). Thus, the virB message begins approximately 0.63-kb rightward of the left end of SalI fragment 13a (Figures 6.1 and 6.2A), and significant transcription of virB only occurs in plant-induced bacteria.

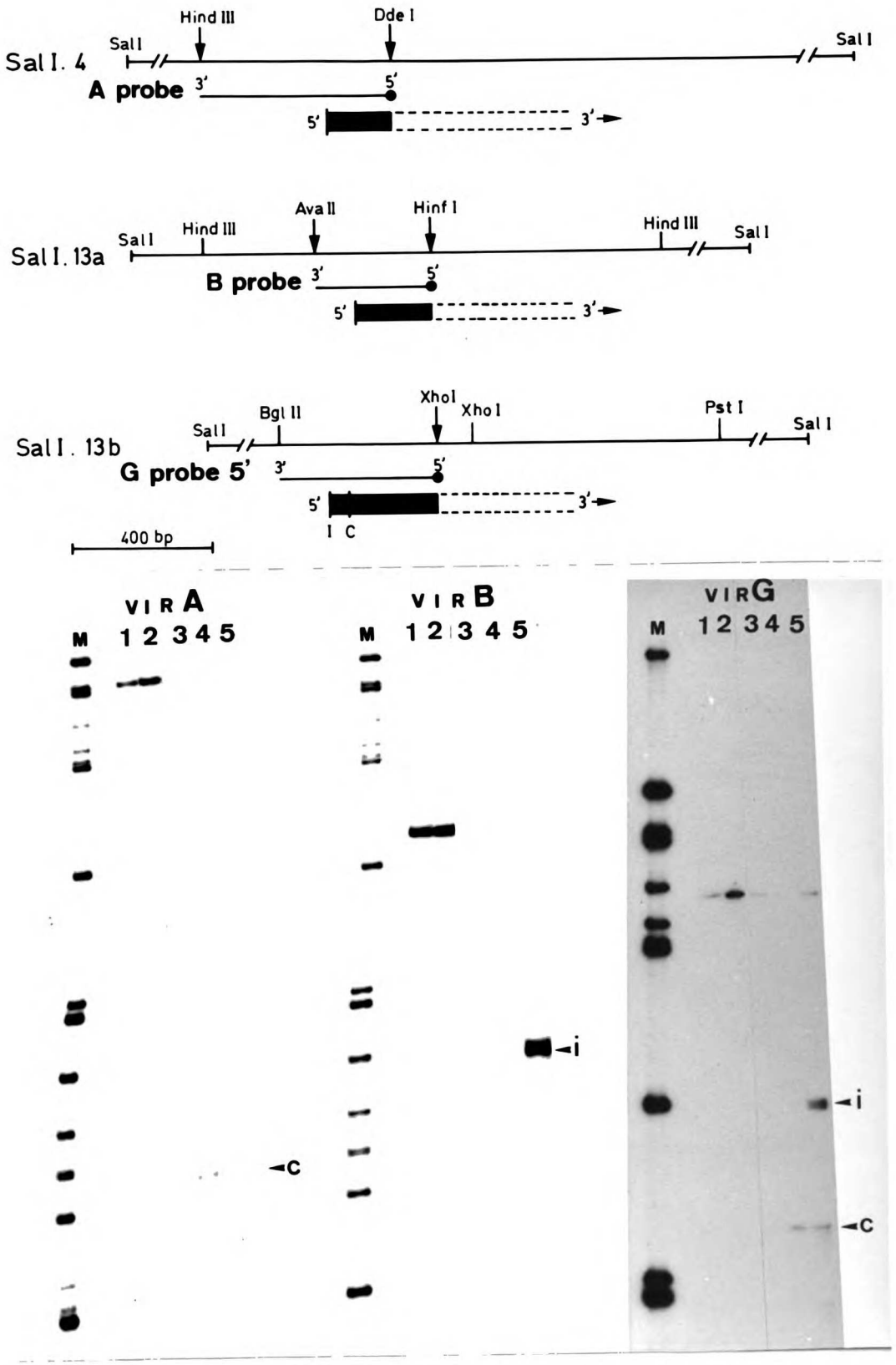


Figure 6.2. S1 nuclease protection of virA, virB, and virG promoter probes by RNA prepared from vegetative and plant-induced Agrobacteria.

Legend to Figure 6.2.

A. Vir promoter probes and protection results.

The SalI restriction fragments correspond to the SalI restriction map of the pTiA6 vir region (Figure 6.1). The shaded bars indicate the portion of the each probe protected from S1 nuclease digestion, and correspond to the data shown in (B). For virG, I and C represent the fragments protected by induced RNA, and by vegetative and induced RNA, respectively. The black dot indicates the position of $^{32}P$ 5' end-label.

B. Autoradiograms of S1 protection experiments.

Experimental lanes : 1), probe, no treatment; 2), probe hybridized with non-homologous RNA (E. coli total RNA), no digestion (mock experiment); 3), probe, non-homologous RNA, S1 digestion; 4), probe, vegetative RNA, S1; 5), probe, induced RNA, S1; m), size markers are a mixture of end-labelled DdeI and HpaII restriction digests of pBR322. For virA and virB, the sizes of the marker fragments from the top of the autoradiograms are (in bases) 623, 543, 541, 528, 466, 427, 410/405, 310, 243, 239, 218, 202, 191, 181, 167, 163, and 161; and for virG are 1653, 623, 543/541, 528, 466, 427, 410/405, 310, 243, and 239. I, fragments protected by induced DNA; C, fragments protected by vegetative and induced RNA.

(iii) VirG transcript has two 5' termini

A 452-bp BglII/XhoI restriction fragment 5' end-labelled at the XhoI site was used to determine the 5' terminus of the virG transcript. A 260-base fragment (± 2 bases) of this probe is protected from the S1 nuclease digestion by the vegetative RNA. This fragment and also a second fragment of length 310 bases (± 2 bases) are protected by the induced RNA (Figure 6.2B). The sizes of these protected fragments have been verified against a DNA sequencing ladder (data not shown). These results suggest that virG encodes two distinct transcripts whose respective 5' termini map approximately 2.0 and 2.05 kb leftward of the rightend of SalI fragment 13b (Figures 6.1 and 6.2A).

The production of the two virG messages appears to be differentially regulated. The longer transcript, designated the I (inducible)- message and represented by the 310-base protected fragment, is present only in induced bacteria, while the shorter transcript, designated the C (constitutive)-message, and represented by the 260-base protected fragment, is present in both vegetative and plant-induced bacteria. Thus, plant-activated expression of virG appears to be initiated from an upstream start site, designated the 5'-I site, to produce the I-message; and constitutive expression appears to be initiated from a downstream start site, designated the 5'-C site, to produce the C-message. Since the C-message is present in both preparations of RNA, vir induction does not preclude transcription initiated at the 5'-C site. In Figure 6.2B the relative intensity of the 310-base fragment protected by the induced RNA is significantly greater than the 260-base fragment protected by this

RNA. This result suggests that the level of the I-message is greater than that of the C-message in induced cells. An alternative hypothesis for the virG S1 results is the C-message potentially results from a specific cleavage of the I-message, which is absolute during vegetative growth and only partial during plant induction.

The BglII/PstI restriction fragment of SalI fragment 13b (Figure 6.4) contains the complete virG gene, since it complements both the avirulence and non-inducibility of virG mutants (Table 6.4). The DNA sequence of this fragment has been determined (Ebert et al., 1986), and is seen to contain a 267-codon open-reading frame (ORF) that likely codes for the virG protein. The sizes of different virG::β-galactosidase fusion proteins suggest that the translation of the virG protein, at least in induced bacteria, is initiated at the initial methionine codon present at the start of this 267 ORF (Ebert et al., 1986; S. Stachel, unpublished results). Figure 6.3 presents the DNA sequence of the amino terminus and promoter region of virG, and the corresponding position of the 2 presumptive start sites of virG transcription (indicated by the circled bases). This figure shows that while the virG I-mRNA has a significant non-translated leader (51 bases) 5' of the proposed initiation codon of the virG protein, the C-mRNA surprisingly begins immediately 5' of, and adjacent to this codon. We have not determined whether the C-message is translated in the cell to produce a functional and full-length virG protein, and the function of this message is presently unclear. In the following section the regulation of virG induction is shown to be complex; this complexity may in part be related to the two virG messages.

```
                          BglII                                        50
AGGACGTTTA  TGAAGTAGGC  GAGATCTGGC  TCGCGGCGGA  CGCACGACGC  CGGGGCGAGA

                                                 100
CCATAGGCGA  TCTCCTAAAT  CAATAGTAGC  TGTAACCTCG  AAGCGTTTCA  CTTGTAACAA

                                 150                  5'-I
CGATTGAGAA  TTTTTGTCAT  AAAATTGAAA  TACTTGGTTC  GCAT(T)TTGT CATCCGGGCA

                    200                  5'-C
GCCGCAATTC  TGACGAACTG  CCCATTTAGC  TGGA(G) ATG ATT GTA CAT CCT TCA CGT
                                              Met Ile Val His Pro Ser Arg

                                250
GAA AAT TTC TCA AGC GCT GTG AAC AAG GGT TCA GAT TTT AGA TTG AAA GGT GAG
Glu Asn Phe Ser Ser Ala Val Asn Lys Gly Ser Asp Phe Arg Leu Lys Gly Glu

                           300
CCG TTG AAA CAC GTT CTT CTT GTC GAT GAC GAC GTC GCT ATG CGG CAT CTT ...
Pro Leu Lys His Val Leu Leu Val Asp Asp Asp Val Ala Met Arg His Leu ...
```

Figure 6.3. DNA sequence of the virG promoter region and positions of the constitutive and plant-activated virG transcriptional start sites.

The DNA sequence of the promoter region and 5'-coding region of the virG locus is from Ebert et al. (1986). The circled bases represent the 5' termini of the plant-induced (5'-I) and constitutive (5'-C) virG transcripts, as determined from S1 nuclease mapping (Figure 6.2). The horizontal bars indicate that the accuracy of this analysis is ± 2 bases.

g) The regulation of virG induction

The experiments presented in Table 6.4 show that while an intact virG gene is essential for the induction of other inducible pTiA6 loci, its role in its own induction is complex. Before discussing these experiments, the molecules used to measure virG expression are described. pSM363 and pSM321 (Figure 6.4) carry independent plant-inducible virG::lac gene fusions that were generated by the insertion of the Tn3-lac transposon Tn3-HoHo1 into the virG coding sequence (chapter 3; Figure 6.4). These insertions disrupt virG coding sequences since their plant-induced expression has been previously shown to result in the production of chimeric virG::β-galactosidase fusion proteins (chapter 2) that have approximately 65 and 130 amino acids (25% and 50%) of the virG protein located at their respective amino termini (S. Stachel, unpublished observations). Since pTi363 and pTi321, the pTiA6 recombinant strains of insertions 363 and 321, are virG mutants (chapter 2), the virG::β-galactosidase proteins encoded by insertions 363 and 321 do not appear to have functional virG activity. Yet, as shown in Table 6.4, the vir:: lac fusions of both pTi363 and pTi321 are partially plant-inducible. Furthermore, strain A136(pSM363) which lacks pTiA6, and therefore does not carry a functional copy of virG, is also partially inducible for virG::lac expression (i.e. one third of the level observed for A348 (pSM363), which carries pTiA6) (Table 6.1). These results are surprising and suggest that the regulation of the induction of virG might in part be different than the other inducible vir and pin loci.

To further investigate the regulation of virG, the inducibility of the virG::lac insertions 363 and 321 was evaluated in a variety of genetic backgrounds (Figure 6.4; Table 6.4). First, these insertions were subcloned separately of other vir loci to give pSM363cd and pSM321cd (Figure 6.4); these smaller plasmids display essentially equivalent levels of constitutive and plant-induced levels of β-galactosidase activity in A348 as pSM363 and pSM321, respectively (Tables 6.1 and 6.4). The inducibility of each of these plasmids, and also the virB::lac plasmid pSM243cd, was then evaluated in A136, in the virA mutant A1016, and in the virG mutant pTi19. Table 6.4 shows that pSM243cd is partially inducible in A1016 and noninducible in A136 and pTi19. In contrast, both pSM363cd and pSM321cd are partially inducible in each of these three backgrounds. Thus, the control of the inducible expression of virB and virG is similar but different. VirA is essential for the efficient induction of both these loci. In contrast, while virB induction is absolutely dependent on intact virG, virG induction is not. Instead intact virG is necessary only for the efficient induction of its own gene.

The above results suggest that the virG gene product positively stimulates its own induction. This effect was further studied using the virG mutant strains pTi363 and pTi321 (Table 6.4). These strains have the virG::lac insertions 363 and 321 homologously recombined onto pTiA6. In response to plant cells both these strains display low levels of induced virG::lac expression. The addition of the vir plasmid clone pVCK221 (Figure 6.4) to these strains results in a large increase in their inducibility to levels approximately equivalent to those observed in A348(pSM363cd) and A348(pSM321cd). This

affect is directly due to the presence of a wild-type copy of virG on pVCK221, since it is not obtained with pSM19, a virG mutation derivative of pVCK221. Furthermore, the inducibility of virG::lac expression in pTi363 and pTi321 is similarly stimulated by pBgl/Pst, a plasmid clone of a 1.25-kb segment of SalI fragment 13b (Figure 6.4). pBgl/Pst contains the complete virG locus since it fully complements the avirulent phenotype of pTi363 and pTi321 (Table 6.4), and of pTi19. Analogous experiments were also carried out with the virB mutant strain pTi30, which carries an inducible virB::lac gene fusion recombined into pTiA6. Neither pVCK221 nor pSM19 significantly affects the level of induction of virB::lac expression in pTi30 (Table 6.4). Thus, an increase in the copy number of virG in a cell does not result in an observable increase in its quantitative induction of virB (i.e., compare pTi30 with pTi30 (pVCK221)), to suggest that the amount of virG protein present in the wild-type cell is above that required for maximal induction. A similar conclusion can also be made with regard to virA, since the addition of vir plasmids that carry a wild-type copy of virA to pTi30 have no affect on the quantitative level of induction of virB::lac expression of this strain (data not shown).

The above results indicate that the regulation of virG induction is more complex than the other inducible pTiA6 vir loci. The induction of these loci is absolutely dependent on virG and greatly stimulated by virA. In contrast, virG expression can be partially activated by plant cells in the absence of an intact virG locus, and this induction is potentiated both by virA and by virG itself. Thus, the virG locus appears in part to be positively autoregulated

by its own gene product, and possibly also under the control of an unidentified element not located on pTiA6. It is noted that chvA and chvB do not appear to be involved in the control of virG expression since pSM363cd and pSM321cd are both fully inducible in chvA and chvB mutant strains (data not shown).

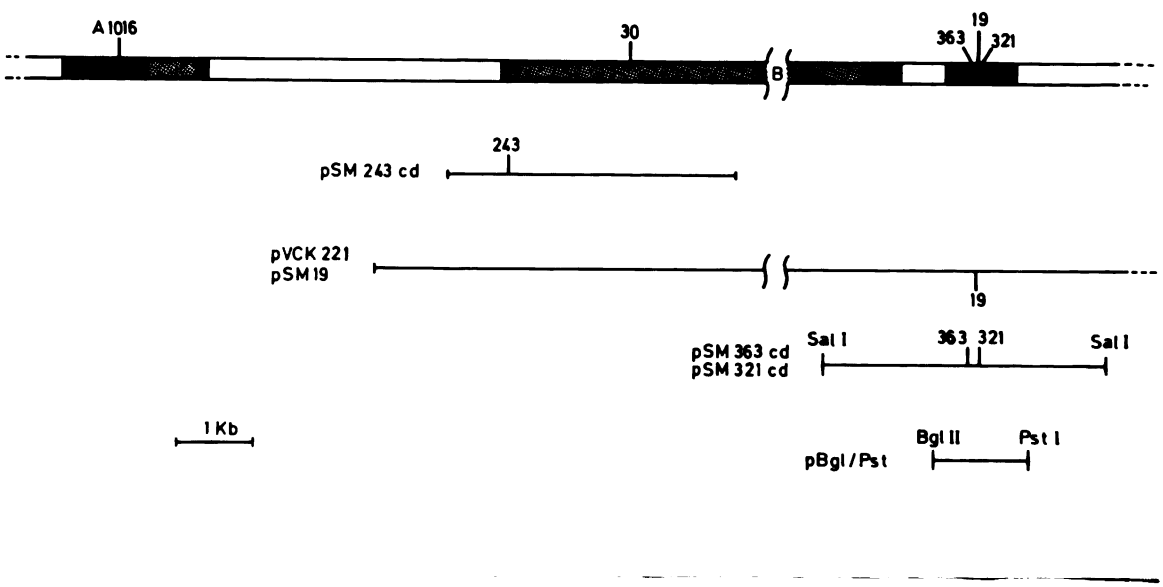


Figure 6.4. Vir mutant strains and vir::lac plasmids used to construct the experimental strains of Table 6.4.

Legend to Figure 6.4.

The positions of the mutations of the vir mutant strains A1016, pTi30, pTi363, pTi19, and pTi321 are shown. A1016 is from Garfinkel and Nester (1980); the other mutants are from chapter 3. pTi30 carries a virB::lac fusion gene recombined into pTiA6, and pTi363 and pTi321 carry two independent plant-inducible virG::lac genes on pTiA6. The virG::lac insertion of pTi19 (and of pSM19) is noninducible since it is orientated opposite to the direction of virG transcription. Six vir plasmids are shown below. The deletion derivative plasmids pSM243cd, pSM363cd and pSM321cd are derived from pSM243, pSM363, and pSM363 respectively (Figure 6.1). pSM19 is a virG insertion derivative of pVCK221. pBgl/Pst is a clone of the 1.25-kb BglII/PstI restriction fragment of SalI fragment 13a.

Table 6.4. The regulation of virG expression is complex

| pTi(pVir) | β-galactosidase units - induction | β-galactosidase units + induction | Δ units | Virulence |
|---|---|---|---|---|
| A348(pSM363cd) | 58.1 | 822 | 14 | + |
| A136(pSM363cd) | 55.5 | 330 | 6.6 | - |
| A1016(pSM363cd) | 43.6 | 346 | 7.9 | - |
| pTi19(pSM363cd) | 52.3 | 304 | 5.8 | - |
| pTi363 | 15.4 | 61.9 | 4.0 | - |
| pTi363(pVCK221) | 18.6 | 420 | 23 | + |
| pTi363(pSM19) | 19.4 | 80.8 | 4.2 | - |
| pTi363(pBgl/Pst) | 16.8 | 394 | 24 | + |
| A348(pSM321cd) | 68.1 | 1115 | 17 | + |
| A136(pSM321cd) | 72.4 | 433 | 6.0 | - |
| A1016(pSM321cd) | 65.6 | 412 | 6.3 | - |
| pTi19(pSM321cd) | 82.3 | 368 | 4.5 | - |
| pTi321 | 18.1 | 72.5 | 4.0 | - |
| pTi321(pVCK221) | 22.9 | 391 | 17 | + |
| pTi321(pSM19) | 23.3 | 82.1 | 3.5 | - |
| pTi321(pBgl/Pst) | 24.1 | 405 | 17 | + |
| A348(pSM243cd) | 4.26 | 401 | 94 | + |
| A136(pSM243cd) | 3.82 | 2.91 | 0.76 | - |
| A1016(pSM243cd) | 4.40 | 16.2 | 3.7 | - |
| pTi19(pSM243cd) | 5.43 | 5.90 | 1.1 | - |
| pTi30 | 3.28 | 330 | 101 | - |
| pTi30(pVCK221) | 3.44 | 253 | 74 | + |
| pTi30(pSM19) | 3.18 | 268 | 84 | + |

The units of vegetative and plant-induced β-galactosidase activity were measured for 23 different virB::lac and virG::lac Agrobacterium strains. The plasmids and mutants used to construct these strains

Legend to Table 6.4. (cont'd)

are shown in Figure 6.4. The inducibility (Δ units) of each strain was calculated, as well as its ability to form tumors on Kalanchoe diagremontiana leaf wounds, as previously described (chapter 3). +, virulent; -, avirulent. Note that the approximately 4-fold difference that is observed in the vegetative β-galactosidase activity effected by each vir::lac insertion in its A348(pSMvir::lac) strain and its pTiA6(vir:: lac) recombinant, reflects the difference in copy number between a pSM-plasmid (inc-P1; Ditta et al., 1980) and a Ti plasmid (inc-Rh-1; Koekman et al., 1980) in Agrobacterium.

## Discussion

*Agrobacterium tumefaciens* transcriptionally activates the expression of its Ti plasmid *vir* and *pin* loci in response to specific phenolic compounds produced by plant cells susceptible to *Agrobacterium* transformation (chapter 5). We have investigated the regulation of this induction process for the A6 Ti plasmid. Our results show that the pTiA6 *vir* and *pin* loci belong to a single regulon, the *vir* regulon, whose transcriptional activation is positively controlled by the gene products of *virA* and *virG*. The other *vir* loci, and also the chromosomal *chv* loci, do not encode *vir* regulatory functions since mutations in these loci have no significant affect on *vir* expression in either vegetative or plant-induced bacteria. In addition to identifying *virA* and *virG* as *vir* regulatory loci, we have assessed the *vir* induction phenotype of *virA* and *virG* mutant cells, and have characterized different aspects of the regulated expression of *virA* and *virG*. As discussed below, these data provide insight into the functions mediated by the *virA* and *virG* gene products in the *vir* induction process.

A bacterium that is mutant for *virA* or *virG* is both avirulent and unable to efficiently activate *vir* expression in response to the plant signal molecule. *VirG* mutant cells are non-inducible for *vir* expression, while *virA* mutant cells are plant-inducible at a greatly reduced level in comparison to wild-type bacteria; thus, the *virG* gene product must mediate a step that is essential to the transcriptional activation of the inducible *vir* loci, while the *virA* gene product must mediate a step that is involved in the efficiency of

this activation. [Figure 6.5 presents two hypothetical views, simple and complex, of how the virA and virG gene products in conjunction with the plant signal molecule control the vir induction process.]

One hypothesis for the attenuated phenotype of virA mutant cells is the virA gene product is involved in the modification of the plant signal molecule; for example, the signal molecule in its initial form might have only low activity for vir induction, and require modification into a compound that is more effective in activating the vir induction machinery. An alternative hypothesis for virA function requires that the plant signal molecule functions intracellularly, and virA encodes the permease or porin protein that promotes its efficient uptake. Since low levels of vir induction can be stimulated in virA mutant cells, the signal molecule must also be able to non-specifically gain entry into the bacterium; however, in the absence of virA, the cell can only attain intracellular concentrations of inducer that are below those required for effective induction.

The virA locus is approximately 2 kb in size and probably unicistronic (chapter 3). Direct measurement of virA transcription by S1 nuclease protection analysis and of β-galactosidase activity expressed from virA::lac gene fusions indicate the expression of virA is constitutive and not influenced by plant cells, nor by virG. Since virA is constitutively expressed, the bacterium must always be ready to carry out the virA function, which probably mediates an early step in the vir induction process. The virA gene product appears to be present in only low amounts, since the quantitative levels of β-galactosidase activity expressed from virA::lac fusions

in vegetative and induced cells is low compared to the levels expressed by lac fusions to the inducible vir and pin loci in induced cells (chapter 3). Thus, virA might encode an enzymatic activity.

A simple hypothesis for the non-inducible phenotype of virG mutant cells is virG encodes a positive regulatory protein that directs the transcriptional activation of the plant-inducible vir and pin promoters. Such a protein might be a DNA-binding protein that interacts with specific vir promoter sequences to enhance their recognition by RNA polymerase, or a sigma-like factor that interacts with RNA polymerase to increase its specificity for vir promoters. Both of these possibilities predict that the inducible vir and pin promoters share a specific DNA sequence element that is responsible for their inducibility. Potentially vir induction results from a conformational shift in the virG protein that is directly controlled by the plant signal molecule.

The virG locus is approximately 1 kb in size and is unicistronic. The 267-amino acid ORF encoded by the virG gene has been shown to be closely related to the protein encoded by the ompR gene of E. coli (Ebert et al., 1986). This protein is required for the transcription of several E. coli loci, including ompC and ompF, and is thought to function by binding to the promoter regions of these loci to stimulate their transcription (Hall and Silhavy, 1981; Inokuchi et al., 1984). The close similarity of the virG protein to the ompR protein, suggests that it might similarly function to activate vir expression.

VirG is constitutively transcribed and also plant-inducible. As judged from virG::lac fusion data, the level of constitutive

expression is similar to that of virA (chapter 3), to suggest that a low amount of virG protein is maintained in the non-induced cell; however, we have not determined whether the constitutive expression of virG results in the production of functional virG protein. The levels of induced β-galactosidase activity expressed from a virG::lac fusion in a cell that carries wild-type virA and virG genes are high (chapter 3), and the virG::β-galactosidase fusion protein produced in these cells can be readily visualized by one-dimensional SDS polyacrylamide gel electrophoresis (chapter 2). If virG does encoded a classical positive regulatory protein this result is surprising, since such proteins typically are present and function at low intracellular concentration.

While one can presently envisage that virA and virG might encode a signal transport and a positive regulatory function [Figure 6.5, "simple" view], the control of the vir induction process must also include additional interacting components and mechanisms, since the regulated expression of virG itself is complex [Figure 6.5, "complex" view]. For example, two different messages are produced from the virG gene. The constitutive C-message is produced under both vegetative and plant-induced growth, while the induced I-message is produced only during plant induction; by S1 mapping analysis the C-message is shorter by 50 bases (± 4 bases) at its 5' terminus than the I-message. How these two transcripts are generated cannot be determined from our analysis; they might be transcribed from different start sites that are controlled by differentially regulated promoters, or alternatively they might result from a differentially regulated processing step of a primary virG tran-

script. The significance and functions of the two virG messages is also unclear; however, since the I-message is made only during plant induction, it likely is responsible for the production of the virG protein that mediates the induction of the vir regulon. Perhaps the C-message acts in some manner to repress the production of functional virG activity.

The respective 5' terminus of the I-message and the C-message have been mapped to the DNA sequence of the promoter region of the virG gene. While the I-mRNA has an untranslated leader, the C-mRNA does not, and instead begins (within the accuracy of our experimental determination) a single base 5' of the proposed virG initiation codon. Such a start has precedent in bacteriophage λ, whose CI repressor gene has two differentially regulated promoters, $P_{RE}$ and $P_{RM}$, that produce two different CI transcripts (Ptashne et al., 1976; Walz et al., 1976; Ptashne, 1978). The $P_{RE}$-message has a long untranslated leader, while the $P_{RM}$-message begins with the adenine residue of the CI initiation codon. This latter message is translated, albeit at low efficiency in comparison to the $P_{RE}$-message, to give functional repressor protein. Potentially the virG C- and I-messages might similarly function in virG expression. It will be interesting to determine which of these transcripts is produced from the low level induction of virG that occurs in the absence of virG, as well as whether the C-mRNA can be translated in the cell to give functional virG protein.

An understanding of virG expression is further complicated by our observations that the induction of virG by plant cells is apparently regulated at two distinct levels. One level is independent

of whether a wild-type copy of virG is present in the bacterium, and since the other inducible vir and pin loci are noninducible in the absence of intact virG, this virG-independent induction of virG likely occurs prior to and is essential for the activation of the vir regulon. How virG-independent induction of virG is mediated is unclear. Potentially, an unidentified element that is specific for regulating virG might be activated (or derepressed) by the plant signal molecule, or alternatively the signal molecule might directly interact with the virG promoter to stimulate its transcription. It is also possible that this effect is at the level of translation instead of transcription. The second level of virG induction is, like the other inducible loci, dependent on intact virG, and thus virG appears to positively autoregulate its own expression. The significance of the dual control of virG induction is presently unknown. It will be interesting to determine the relationship between this dual control and the two virG transcripts.

In conclusion, the results of this study provide initial insight into the vir regulatory system. These results also pose several questions about the vir induction process and its control. Future experiments will help to answer these questions and provide a more complete understanding of the detailed molecular events that control vir induction.

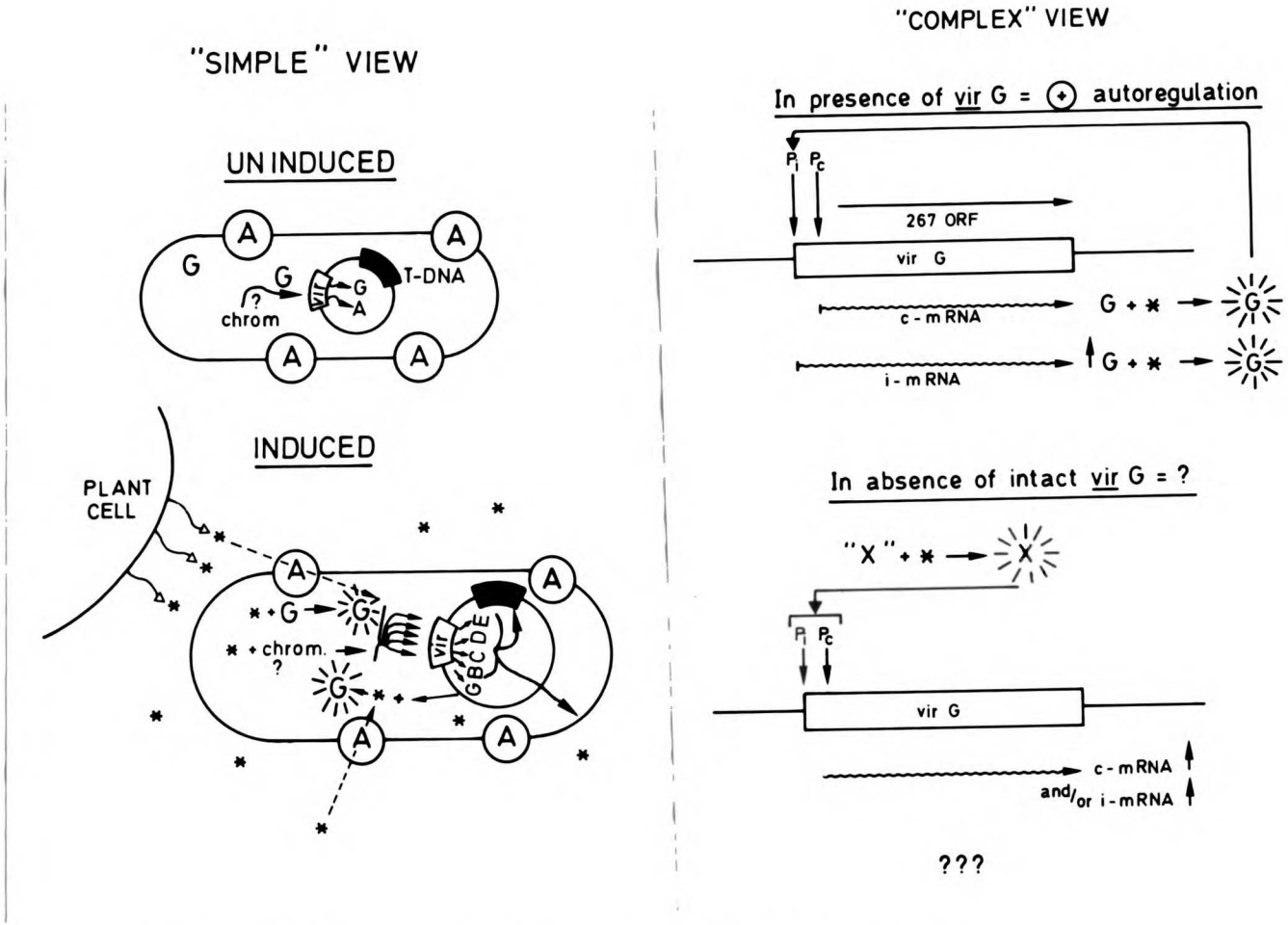


Figure 6.5. The control of the vir induction process by virA and virG.

*, plant signal molecule; chrom. and X, hypothetical chromosomal regulatory element; -G-, activated G-protein; -X-, activated X-protein; Pi, plant-inducible virG promoter; Pc, constitutive virG promoter.

## Materials and Methods

### a) Reagents and general procedures

Restriction and DNA modification enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. X-gal was from Bethesda Research Laboratories. ONPG, and all antibiotics were from Sigma. $\gamma$-$^{32}$P-ATP (5000 Ci/mMol) was purchased from New England Nuclear. Standard recombinant DNA procedures were as described (Maniatis et al., 1982).

### b) Plasmids

The pSM constructs, numbered between 1 and 379, are described in chapter 3. pSM400 series constructs were generated by Tn3-HoHo1 mutagenesis (chapter 2) of pVCK257, a recombinant derivative of the wide-host range cloning vector, pVCK102 (Knauf and Nester, 1982). pVCK257 carries pTiA6 vir region sequences from SalI fragment 4 through SalI fragment 30 (Figure 6.1). pSM243cd, pSM321cd, pSM363cd, and pSM358cd are SalI deletion derivatives of pSM243, pSM321, pSM363, and pSM358, respectively. Each of these molecules consists of the SalI::Tn3-HoHo1 insertion fragment of the parental plasmid cloned into pVCK102, and was constructed according to the procedure described in chapter 3. Specifically, pSM243cd, pSM363cd, pSM321cd, and pSM358cd contain the SalI fragments 13a, 13b, 13b, and 21, respectively of pSM243, pSM363, and pSM321, and pSM358. All of the above plasmids confer resistance to carbenicillin and kanamycin.

pBgl/Pst was constructed by cloning the 1.25-kb BglII-PstI restriction fragment of SalI fragment 13a into a BamHI-PstI restriction digest of the wide-host range cloning vector pRK404, Tn5 was subsequently inserted into the vector portion of this resultant plasmid to give pBgl/Pst (Ebert et al., 1986), which consequently confers kanamycin resistance.

c) Bacterial strains

Agrobacterium strains A136 and A348 are described by Garfinkel et al. (1981). These strains are isogenic except that A348 carries pTiA6. Agrobacterium vir and chv mutant strains are given in Figures 6.1 and 6.4, and in Table 6.2 and Table 6.4. Using the method described by Ditta et al. (1980), and Garfinkel et al. (1981), insertion plasmids were conjugally transferred from E. coli to Agrobacterium to generate the insertion plasmid strains given in Tables 6.1, 6.2, 6.3, and 6.4; each of these transconjugant strains is resistant to carbenicillin and kanamycin.

d) Determination of the plant inducibility

Units of vegetative and plant-induced β-galactosidase activity were measured as described previously (chapter 3). The plant inducibility of a strain is calculated as the ratio of its induced units to vegetative units.

e) Preparation of vegetative and induced Agrobacterium RNA

RNA was isolated by a modification of the procedure of Corbin et al. (1983). For induced RNA, 250 ml of 2-day NT1 culture was inoculated with A348 bacteria at an absorbance of 0.1 O.D. units at 600 nm, and incubated for 7.5 hours at 28°C, 200 rpm. Fifty ml of cold 100-mM $NaN_3$ was then added to the cocultivation, and this mixture was passed through Miracloth® to separate the induced bacteria from the plant cells. The bacteria were pelleted at 10,000 rpm, -5°C, for 5 minutes, and resus pended in 2 ml 20-mM NaOAc, 1 mM EDTA, pH 5.5; 0.5 ml 10% Na-Sarkosyl was added and the solution was placed at 100°C for 2 minutes; 0.3 ml 5-M NaCl was added and this solution was twice extracted with 60°C phenol saturated with 0.5 M NaCl, twice extracted with phenol:chloroform (1:1), and twice extracted with chloroform:isoamylalcohol (24:1). Two volumes EtOH, -20°C, was then added, and the DNA was removed by spooling onto a glass rod. The resultant RNA preparation was stored as an EtOH-precipitate at -70°C and its integrity was assessed by gel electrophoresis in 0.8% agarose containing formaldehyde (Maniatis et al., 1982). For vegetative RNA, 250 ml of a culture of A348 bacteria in AB/glucose liquid medium (Chilton et al., 1974), was grown to an $A_{600}$ of 0.4 O.D. units, and total RNA was prepared as described above.

f) S1 nuclease mapping of 5' ends of *vir*-encoded transcripts

Double-stranded DNA fragments labelled at a single 5' end were used as *vir* promoter probes. To prepare a probe, an appropriate restriction fragment was end-labelled with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP, digested with a second restriction enzyme, and the desired fragment was gel-purified.

S1 nuclease analysis was essentially as previously described (Berk and Sharp, 1977; Weaver and Weissman, 1979), and all buffers used are according to Maniatis *et al*. (1982). Briefly, 2.5 µg RNA and approximately 5 ng of promoter probe DNA were coprecipitated with EtOH, resuspended in 10 µl hybridization buffer, denatured at 85°C for 15 minutes, and then hybridized at 52°C for 8 hours. Following hybridization, 300 ml S1 digestion buffer was added, and the reaction was chilled to 0°C. Two-hundred fifty units of S1 nuclease were added, and digestion was carried out for 75 minutes at 18°C, and terminated by the addition of 75 µl termination buffer. The digestion temperature of 18°C was used to avoid artifacts caused by local DNA::RNA denaturation that can occur at higher temperatures. Then, 1.5 µl tRNA, 10 mg/ml, and 1 ml EtOH were added, and the precipitate was electrophoresed on an 8 M urea/5% polyacrylamide sequencing gel to identify and resolve S1-resistant DNA fragments. Gel markers were prepared by Klenow end-labelling *Dde*I and *Hpa*II restriction digests of pBR322 DNA.

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# CHAPTER 7

## Conclusions

The results described in chapters 2–6 demonstrate a general principal of nature's design – that biological events are efficient and make sense. It is clear that the initiation of the Agrobacterium/plant cell interaction is an active process dependent on both partners. The plant cell is not just a passive target of Agrobacterium transformation; rather it signals the activation of the transformation process by synthesizing phenolic molecules which trigger the expression of the bacterial genes that encode the T-DNA transfer machinery. Furthermore, Agrobacterium does not recognize and respond any small phenolic compound present in the soil environment, but only those molecules which best represent what it is after – a plant cell susceptible to Agrobacterium infection. Thus, the specificicty of the vir induction process ensures that Agrobacterium will only attempt to transform plant cells that can be transformed. This specificity undoubtedly greatly enhances the biological success of Agrobacterium in its natural habitat, the biologically and chemically complex rhizosphere environment.

The plant molecules identified to activate the Agrobacterium/plant cell interaction are the first such bacterial/plant signal molecules to be described. However, there is no doubt that the plant rhizosphere is composed of a large variety of specific plant/bacterial communication systems. One looks forward to the analysis

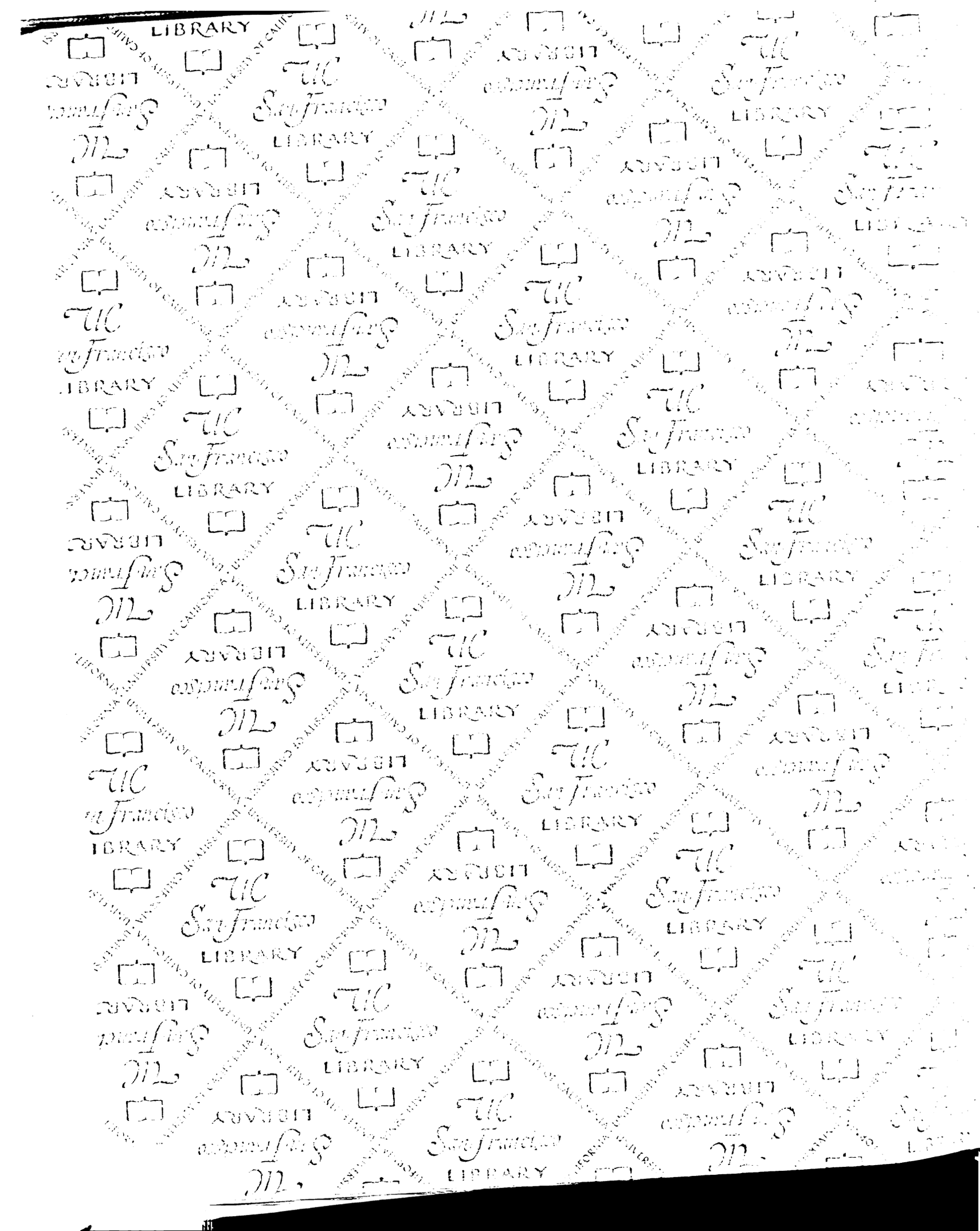
of these other systems to see whether they share common elements to their control.

Several questions concerning the biology of Agrobacterium remain to be resolved. For example, while the plant signal molecule and virA and virG have been identified as essential components of the vir induction process, the roles of each of these components are still unclear. Further analysis of the vir induction process will hopefully answer the following questions : (i) does the signal molecule act as an attractant for Agrobacterium; (ii) does this molecule enter the bacterial cell to mediate induction; (iii) what are the functions of the virA and virG proteins; (iv) are other vir regulatory elements (proposed as gene X in Figure 6.5) also involved in the control of the vir regulon; (v) what defines the structure of a plant-inducible vir promoter; and (vi) what is the meaning of the complexity of the expression of virG for the expression of the vir regulon, and how is this complexity mediated ?

The determination of the mechanisms of T-DNA transfer and integration remains as the last frontier of the Agrobacterium system. The genetic components (T-DNA borders, chv genes, vir genes) of this process are now known, but surprisingly little else. In this thesis the first step in the T-DNA transfer process has been identified as the transcriptional activation of the T-DNA transfer machinery by plant cells. It is hoped that this knowledge will help lead to a mechanistic understanding of T-DNA transfer. Since this process can now be activated in the absence of plant cells by using purified signal compound, it should be possible to biochemically identify (i) the molecular changes that occur to the T-DNA within the bacterium

during the early stages of T-DNA transfer; (ii) the specific proteins (and genes which encode them) which mediate these T-DNA-associated molecular events; and (iii) novel structures of the bacterial cell surface which are involved in the transfer of the T-DNA to the plant cell.

To this researcher one of the most intriguing issues of the Agrobacterium system is its evolutionary origins. While certain components of this system have prototypes in other prokaryotic species (i.e., the T-DNA phytohormone genes are found in certain Pseudomonads; the vir regulatory protein, virG, shares strong amino acid homology with several E. coli-positive regulatory proteins), the T-DNA transfer process at present appears to be limited to Agrobacterium. How this system evolved and whether any of its mechanistic components are found elsewhere in nature remains a mystery. These issues perhaps will be answered when the mechanisms of the T-DNA transfer become known, as well as when a more complete understanding of the rhizosphere and its myriad of different bacterial/ plant cell interactions becomes available.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