3.

AN INVESTIGATION OF THE MOLECULAR BIOLOGY AND GENETICS OF WITCHES' BROOM DISEASE OF *PROTEA CYNAROIDES*

ROSEMARY ANN DORRINGTON

A DISSERTATION SUBMITTED IN FULFILMENT
OF REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN THE FACULTY OF SCIENCE
UNIVERSITY OF CAPE TOWN

CAPE TOWN MARCH 1987

The University of Cape Town has been given the right to reproduce this thesis in whole or in part. Copyright is held by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

CONTENTS

PAGE

ACKNOWLEDGEMENTS 2

ABBREVIATIONS 3

ABSTRACT 4

CHAPTER 1 **GENERAL INTRODUCTION** 6

CHAPTER 2 **GENERAL INVESTIGATION OF THE CAUSE OF WITCHES' BROOM DISEASE OF *P. CYNAROIDES*** 46

CHAPTER 3 **INVESTIGATION OF THE GENETICS OF WITCHES' BROOM DISEASE OF *P. CYNAROIDES*** 66

CHAPTER 4 **GENERAL CONCLUSIONS** 90

APPENDIX 1 **GENERAL TECHNIQUES** 95

APPENDIX 2 **MEDIA, BUFFERS AND SOLUTIONS** 100

APPENDIX 3 **REFERENCES** 102

ACKNOWLEDGEMENTS

I acknowledge with thanks the financial support extended to me by the South African Council for Scientific and Industrial Research and Mr Douglas Jooste, who in part, funded this research.

For his unflagging interest, encouragement and constructive critique, I thank my supervisor, Prof Dave Woods. I also wish to thank the other members of staff, and especially Drs Doug Rawlings and Ralph Kirby for their advice and interest. I am indebted to all my collegues and friends for advice, encouragement and discussion; to Dr Karen Usdin for advice on Southern blotting techniques, to Alex Hartley for help with the electron microscopy, to Dr Jaacov Ben Jaacov for advice and help with tissue culture techniques and to Dr Stephen Farrand for advice and assistance. To those who helped with the proof-reading of this manuscript, my appreciation and heartfelt thanks.

My profound gratitude goes to my parents for their unfailing patience and encouragement during the course of this research.

ABBREVIATIONS

Ap^R	Ampicillin resistance
bp	base pairs
cpm	counts per minute
CsCl	caesium chloride
dieca	sodium diethyldithiocarbamate
ds	double stranded
h	hours
IPTG	isopropyl-B-D-thio-galactopyranoside
kb	kilobase pairs
min	minutes
NAA	napthalacetic acid
Na_2.EDTA	ethylenediaminetetra-acetic acid disodium salt
R.E.	restriction endonuclease
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
sec	seconds
Tn	transposon
ug	microgram
ul	microlitre
UV	ultraviolet light
V	volts
X-GAL	5-bromo-4-chloro-3-indolyl-B-galactoside

ABSTRACT

An investigation was undertaken into the biology and genetics of Witches' Broom disease on Protea cynaroides. The investigation was approached in two ways: firstly, from a physiological and pathological angle and secondly at the genetic level.

As very little is known about the causes of Witches' Broom disease on P. cynaroides, an attempt was made to identify a pathogen which could be held reponsible for the disease. A number of plants were studied in the field and from these samples were taken and cultured on culture medium. Healthy P. cynaroides tissue was not established in tissue culture, while more success was obtained with teratoma tissue. Attempts were made to transmit the disease but these were unsuccessful. Four strains of Agrobacterium tumefaciens were unable to induce tumours on P. cynaroides seedlings. Sections of vascular tissue from teratoma and healthy tissue were viewed under the electron microscope but revealed no pathogen.

Nothing is known about the genetics of Witches' Broom induction of P. cynaroides. The best-studied genetic system of tumourigenesis is that of A. tumefaciens which transfers T-DNA to host plant cells. T-DNA genes have been shown to be used by other bacteria for tumorigenesis (Agrobacterium

CHAPTER 1: **INTRODUCTION**

1.1 **General Introduction**

The Proteaceae consist of 61 genera and about 400 species which are fairly widespread throughout the Southern Hemisphere. The majority of species are indigenous to South Africa and Australia and the remainder are found in South America, tropical Africa, Malaysia, New Zealand and the Pacific Islands. In Africa, there are thirteen indigenous Proteaceae genera and close on 400 species. With the exception of a few species belonging to the genera *Protea* and *Faurea* which are found as far north as Central Africa, the vast majority of species grow in the south-western Cape Province of South Africa. The evolution and classification of the family has been described by Johnson and Biggs (1975).

The genus *Protea* L., with more than 100 species, is the largest in South Africa. Members of this genus bear the largest and most beautiful blooms and consequently are commercially important in the cut-flower industry (Roussouw, 1970). The commercial cultivation of Proteaceae in South Africa is a relatively young industry, with the first cropping of wild plants starting in 1974 and large-scale

plantations being established in 1978. Blooms are exported to both Europe and the United States of America. The cultivation of Proteaceae has been found to give the highest financial return per hectare of all agricultural products in South Africa.

P. cynaroides, the national flower of South Africa, was first described in 1698 by Thomas Petivar. The name is derived from the latin meaning cyanara-like which refers to the resemblance the flower bears to an artichoke. For many years the plant was known as Africa's Tree Artichoke. A detailed botanical description was made by Rourke (1976). The shrubs are evergreen and upright with a symmetrical growth habit and reach about one metre in height. Large pink to deep pink flowers of up to 120mm in diameter are borne in spring and early summer.

The only genetic classification of Genus Protea has been the determination of the chromosome number and karyotypes. The haploid chromosome numbers vary from 11 to 13 (Johnson and Biggs, 1975). P. cynaroides has 12 small haploid chromosomes (De Vos, 1943).

1.2 **Witches' Broom Disease**

1.2.1 Witches' Broom disease of P. cynaroides

Witches' Broom disease is a term used to describe a condition in which normally dormant axial buds begin to proliferate and form a bushy growth consisting of a multiplicity of short, malformed shoots and leaves.

Almost all Protea species, with the exception of P. repens which appears to be resistant, are affected by Witches' Broom disease (Rust and Myburgh, 1976). The disease appears to be species-specific and does not spread from one species to another (Meyer, 1981).

Witches' Broom disease in P. cynaroides manifests itself as a vigorous, teratoma-like growth in the otherwise dormant axial buds of the stems and lignotubers of the plant. The teratoma consists of a proliferation of malformed shoots and leaves. The disease causes severe stunting of normal tissue on affected plants and a marked reduction in flower production.

To date, no pathogen has been positively identified as the causal agent of the disease. The spread of Witches' Broom disease appears to be associated with the presence of a mite, Acarea protea, which lives beneath the bud scales of

the growth points of the plant (Meyer, 1981). Due to their microscopic size, (the females average 230μ in length and males 180fm) very little is known about these organisms. However, it has been noted that *A. protea* shows some specificity for its host species (Coetzee, 1982). Rust and Meyer (1976), speculated that the mites may not be directly responsible for Witches' Broom disease, but may be vectors of the real pathogen involved. This is supported by the fact that many members of the family to which *A. protea* belongs, the Eriophyoideae, are known to be vectors of various plant viruses and procaryotic pathogens (Coetzee, 1982). The causal agent of Witches' Broom disease in *Protea* species remains a mystery.

1.2.2 Witches' Broom disease of other plants

The condition has been observed on a wide variety of dicotyledonous plants and also on some monocotyledonous plants. Among plants affected are deciduous trees such as the walnut, (Seliskar, 1976) the plum, (Dreger-Jauffret, 1979) and the larch, *Larix decidua* (Nienhaus *et al.*, 1976). The condition is also found in annuals such as the tomato and the potato (Shekhawat *et al.*, 1981), in succulents such as *Opuntia tuna*, a cactus (Casper *et al.*, 1970) and in other plants such as cocoa (Evans, 1980).

A number of pathogens have been found to be associated with Witches' Broom disease. Mycoplasma-like organisms (MLO's) have been found in the phloem cells of affected walnut trees (Seliskar, 1976), pear trees (Seemuller, 1976) and in potato and tomato plants with Witches' Broom tumours (Shekhawat *et al.*, 1981). In *O. tuna*, MLO's were associated with a number of different viruses (Casper *et al.*, 1970). Witches' Broom of Bermudagrass has been associated with the presence of a spiroplasma (Raju *et al.*, 1980). In *L. decidua*, a Rickettsia-like organism has been shown to cause Witches' Broom tumours (Nienhaus *et al.*, 1976) and in Witches' Broom of cocoa, *Theobroma cacao*, the disease is caused by a basidiomycete, *Crinipellis perniciosa* (Evans, 1980).

1.3 Other Tumourous Conditions of Plants

1.3.1 Crown gall disease and *A. tumefaciens*

Crown gall disease has been the subject of intensive study for more than 70 years and is thus probably one of the best understood neoplastic transformations of both plants and animals. The study of this disease constitutes a major contribution to the understanding of fundamental plant molecular biology. The long history of crown gall research has been reviewed by Braun (1982).

For many years it was known that tumours were induced at wound sites by the gram-negative soil bacterium, A. tumefaciens. The disease was observed on over 600 dicotyledonous plant species. Many Gymnosperms and some monocotyledonous species are also affected (DeCleene and DeLey, 1976). For a considerable time, very little progress was made in understanding the mechanism of tumourigenesis. For reviews, see Braun (1978) and Zambryski et al. (1983b).

Zaenen et al., (1974) were the first to report the presence of large plasmids in several pathogenic Agrobacterium strains, but not in non-pathogenic strains. It was subsequently shown that these plasmids were essential for tumourigenesis (Van Larebeke et al., 1974; Watson et al., 1975) and they were named the Tumour-inducing or Ti plasmids. Ti plasmids were shown to carry conjugative functions (Genetello et al., 1977) and conjugation between a virulent and avirulent, plasmidless strain could confer virulence upon the latter after acquisition of the Ti plasmid (Bomhoff et al., 1976).

Early DNA homology studies showed that Ti plasmids from different A. tumefaciens strains were only distantly related (Currier and Nester, 1976) suggesting that only a small fraction of the plasmid was necessary for tumour induction. The tumours were shown to produce novel arginine derivatives

known as opines even after being cured of bacteria by antibiotics. These opines were not produced in non-transformed tissues (Kemp, 1977) and the type of opine produced was determined by the bacterial strain involved (Montoya *et al.*, 1977). Tumour cells can be cultivated *in vitro* in the absence of bacteria and exogenous plant growth regulators (auxins and cytokinins), retaining their tumourigenic properties, while untransformed tissue is unable to grow under similar conditions (Braun, 1958).

In the light of the above evidence Chilton *et al.* (1977) reasoned that the bacterium must transfer some of its genes to the plant cell during tumourigenesis in order to effect heritable tumour characteristics. They showed that a fragment of the Ti plasmid was present in the genome of the tumour cells and this fragment, the Transfer red or T-DNA was later found to be transcribed in transformed plant cells (Drummond *et al.*, 1977; Gurley *et al.*, 1979). The T-DNA was shown to be present in the nuclei of transformed cells (Chilton *et al.*, 1980; Willmitzer *et al.*, 1980) and was organised in nucleosomes (Schafer *et al.*, 1984). Ti plasmids transferred to *Escherichia coli* did not allow these bacteria to produce tumours on plants (Holsters *et al.*, 1978) suggesting that there may be *A. tumefaciens* chromosomal loci involved in tumourigenesis.

The DNA sequences of Ti plasmids from the various *A. tumefaciens* strains can vary considerably (Currier and Nester, 1976; Genetello *et al.*, 1977). The plasmids are grouped according to the type of opine they specify and have been classified as follows; (1) Octopine Ti plasmids which code for octopine and related opines such as octopinic acid, lysopine and histopine and also agropine metabolism (Firmin and Fenwick, 1978). Wild-type octopine plasmids induce tumours which are undifferentiated, but on certain test plants may form adventitious roots (Zambryski *et al.*, 1983b); (2) Nopaline Ti plasmids which code for nopaline, ornaline and succinopine (Kemp *et al.*, 1979; Chilton *et al.*, 1984) and for agrocinopine (Ellis and Murphy, 1981). Nopaline tumours may differentiate into leaf-like structures that can develop into abnormally structured shoots. These tumours are called teratomas (Zambryski *et al.*, 1983). If a teratoma is grafted onto normal tissue, it is possible to obtain revertants (Braun and Wood, 1976) that still retain T-DNA inserts (Lemmers *et al.*, 1980). (3) Agropine Ti plasmids which are related to octopine plasmids and code for agropine (Guyon *et al.*, 1980)

1.3.1A Genetic and functional organisation of octopine and nopaline Ti plasmids

Functions encoded by Ti plasmids were first identified by comparing plasmid-bearing *A. tumefaciens* strains with non-

plasmid-bearing strains. Plasmids were shown to encode the following functions; crown gall tumour induction, opine synthesis and catabolism, agrocin sensitivity, conjugative transfer of Ti plasmids and arginine and ornithine metabolism (Van Larebeke et al., 1974; Engler et al., 1975; Watson et al., 1975; Bomhoff et al., 1976; Genetello et al., 1976; Firmin and Fenwick, 1978; Klapwijk et al., 1978; Guyon et al., 1980).

Transposon mutagenesis was used to map these functions more accurately. Holsters et al. (1980) used Tn1 and Tn7 to map the nopaline plasmid pTiC58 while Tn904 and Tn5 were used to map the octopine plasmids pTiAch5 and pTiA6 (Ooms et al., 1980; Garfinkel and Nester, 1980; Klee et al., 1982). The plasmids have also been mapped (Fig. 1.1) using deletion mutations (Depicker et al., 1980).

Approximately half of the Ti plasmids which are about 130 kilobases (kb) in size, encodes genes involved in tumourigenesis (Koekmans et al., 1979). Heteroduplex studies showed that octopine and nopaline plasmids have four blocks of homologous sequences which comprise 30% of the total Ti plasmid sequence (Engler et al., 1981). Depicker et al. (1978) showed by means of Southern hybridisation (Southern, 1975) that sequences conserved between different Ti plasmids were essential for tumour induction.

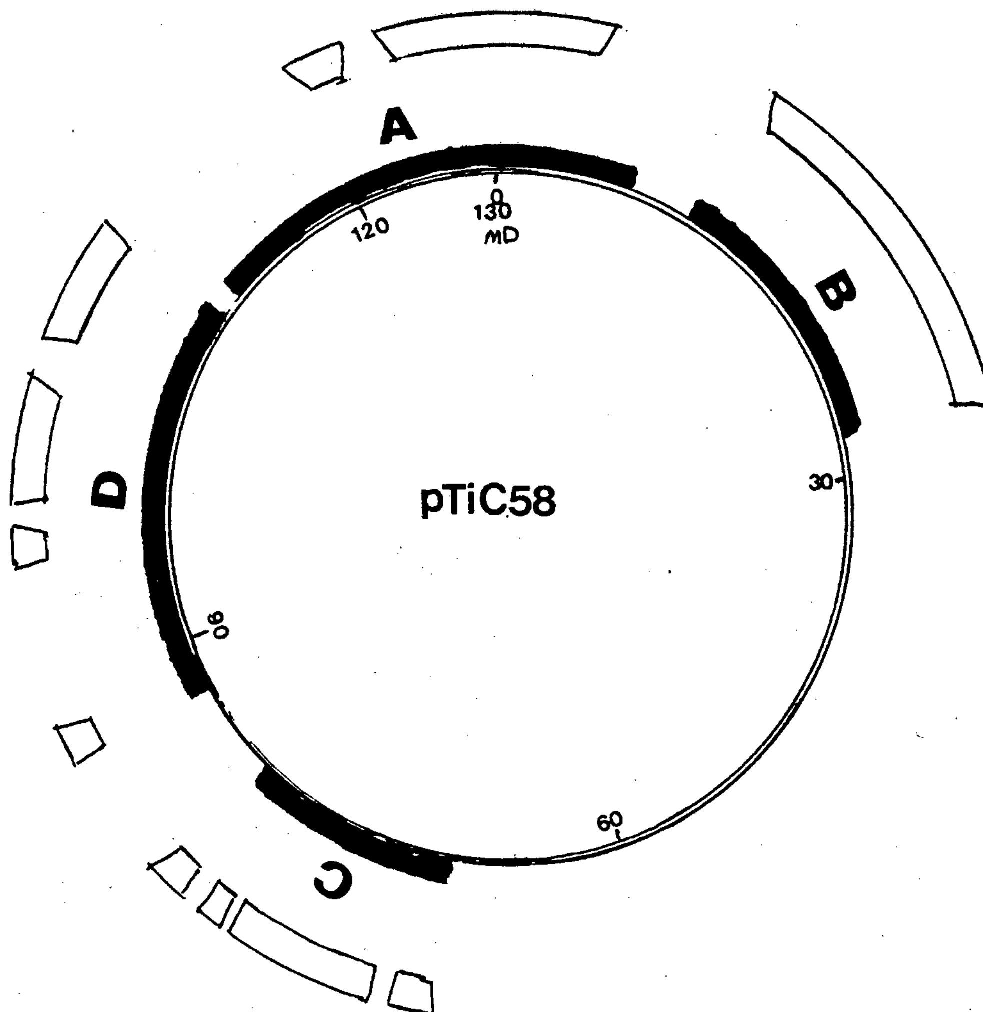


Fig. 1.1: **Functional map of a nopaline Ti plasmid, pTiC58** (Holsters et al., 1980). A. T-DNA region; B. Tra functions, opine metabolism; C. Tra functions; D. vir region. Open blocks denote sequences conserved between the nopaline and octopine Ti plasmids

The four conserved regions consist firstly of the area in and around the T-DNA which encodes functions affecting tumourigenesis , opine production and host range; secondly, the virulence region, to the left of the T-DNA, encoding functions involved in transfer of T-DNA from the bacterium to the plant cell. Both these regions will be discussed in detail later. The two other conserved regions carry the transfer functions which are involved in Ti-mediated conjugation (Genetello *et al.*, 1977), the origin of replication These genes enable the bacterium to utilise the opines as their sole carbon and nitrogen source (Lippincott *et al.*, 1976).

1.3.1B The mechanism of T-DNA transfer

The infection and transformation of plant cells by *A. tumefaciens* involves a number of steps and these are controlled by genes carried on the bacterial chromosome and also on the Ti plasmid. The bacterium must recognize susceptible plant cells, attach to them and process the T-DNA from the Ti plasmid into the plant cells. The T-DNA must then be integrated into the plant chromosome and from there it must direct the production of plant growth factors which are necessary for maintenance of the transformed state of the plant cells and opines.

The virulence (vir) region is essential for the transfer of T-DNA and maps to the left of the T-DNA sequence on the Ti plasmid (Klee et al.,1983). In addition, there are loci on the bacterial chromosome,chvA and chvB which are also involved in virulence (Douglas et al., 1985; Close et al., 1985). Klee et al. (1983) identified five separate loci on an approximately 35 kilobase (kb) fragment within the virulence region which are essential for virulence using transposon mutagenesis. The loci were named virA, virB, virC, virD, and virE. A further vir gene, virF was mapped to the left of the virA locus (Okker et al., 1984). Hille et al. (1984) mapped an octopine specific gene, virO in the virulence region (Fig. 1.2).

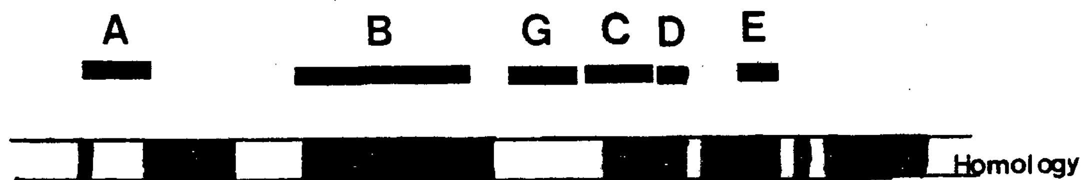


Fig. 1.2; **Functional map of the vir region of the octopine Ti plasmid** (Hille et al., 1984) Shown are the virA-E loci as well as the virG locus. The virF locus (not shown) maps to the left of virA. The blacked out areas below denote sequences of homology between octopine and nopaline Ti plasmids.

Inactivation of the _virO_ (also known as _virG_) locus causes avirulence on octopine Ti plasmids. _VirO_ is a;so carried by nopaline Ti plasmids. Okker _et al_. (1984) have shown that the _virC_ promotor is induced by an unidentified plant product. The mode of action of the plant product was thought to be indirect as it was too large to pass through the pores of the bacterial outer membrane.

Acetosyringone (AS) and a-hydroxyacetosyringone (OH-AS) were positively identified as molecules produced by wounded plant cells which activate the whole _vir_ region (Stachel _et al_., 1985). Induction of the _vir_ region of pTiA6 is attenuated by a mutation in the _virA_ locus and a mutation in the _virG_ locus renders the plasmid avirulent (Stachel and Nester, 1986). A tentative regulatory function has been assigned to _virG_ as the amino acid sequence of the _virG_ protein shows a striking resemblance to those of _E. coli_ regulatory proteins (Stachel _et al_., 1985). Stachel _et al_., (1986) proposed that the _virA_ protein functions in the initial recognition and/or transport of AS and OH-AS and that the signal molecules then allosterically activate the _virG_ locus, the product of which interacts with the other loci in the _vir_ region. Bolton _et al_. (1986) have shown that catechol, gallic acid, pyrogallic acid, p-hydroxybenzoic acid and vanillin were able to induce expression of the _vir_ region.

These molecules were able to induce expression of each of the *vir* loci. One can speculate that each host plant produces a different molecule on wounding and that whether the virulence region is induced by this molecule or not, determines whether the bacterium is virulent or not.

Attachment of *A. tumefaciens* cells to plant cells is a required early step in tumour formation, but the mechanism of attachment is still not fully understood. Tumour induction by virulent strains may be inhibited by preinoculation with certain avirulent strains, suggesting that attachment sites on plant cells are blocked by avirulent bacteria (Lippincott and Lippincott, 1969; Lippincott *et al.*, 1977). Thermodynamic studies with strains B6 on potato discs have revealed that the attachment sites on the plant cell wall may be non-specific (Kleupfel and Peupke, 1985). In some strains of *A. tumefaciens*, the functions for attachment are carried on the Ti plasmids, but in most cases, the chromosomal loci control attachment to the plant cell wall prior to transformation (Douglas *et al.*, 1986). Cellulose fibrils produced by *A. tumefaciens* have been shown to attach the bacteria to plant cells and also to each other but mutants deficient in these fibrils are still virulent and capable of attachment (Matthyse, 1983). Lipopolysaccharides (LPSs) isolated from *A. tumefaciens* have been shown to block attachment of bacteria to tissue

cultured carrot cells (Puvanesrajah et al., 1986) but not on potato discs (Peupke and Benny, 1983). This can probably be explained in terms of the host range specificity displayed by the different species of Agrobacterium.

By examining cell wall extracts of virulent and avirulent mutant A. tumefaciens strains, Puvanesrajah et al., (1985) showed that a 2-linked B-D-glucan was produced in virulent strains but not in attachment-deficient mutants. This is therefore probably one of the compounds involved in attachment. The locus involved in B-D-glucan production was mapped on the chromosome and designated chvA. These loci had previously been shown by means of transposon mutagenesis to be involved in virulence (Douglas et al., 1985). The chvA and chvB loci are found within an 11kb portion of the chromosome and are separated from each other by a 4kb intergenic region.

During transfer, the T-DNA acts solely as a structural element because its internal sequences can be fully deleted without affecting transfer (Zambryski et al., 1983a). The single, defined T-DNA segment of the nopaline Ti plasmid, pTiC58 (Zambryski et al., 1982), is flanked by two imperfect 25 base pair (bp) direct repeat sequences (Yadav et al., 1982; Zambryski et al., 1982). Analysis of the T-DNA/plant DNA integration events has shown that the junction typically

occurs internal or within 100 bp of these sequences (Zambryski *et al*., 1982). Deletion of the left 25 bp repeat does not affect tumourigenesis (Joos *et al*., 1983) whereas removal of the right repeat sequence eliminates virulence. Replacement of this sequence restores virulence (Wang *et al*., 1984) but only if the repeat is inserted in the orientation in which it normally occurs in the Ti plasmid. Wang *et al*. (1984) suggested that the right repeat sequence mediates the oriented transfer of sequences to the left of itself. In pTiT37, a deletion of the right border does not result in loss of virulence. If the border repeat sequence is placed in an abnormal position in the T-DNA, only part of the T-DNA is transferred (Caplan *et al*., 1985).

The T-DNA may be deleted from the Ti plasmid and maintained on a separate replicon without affecting virulence (Hoekema *et al*., 1983). This is known as a binary vector system. Using this system, Jen and Chilton (1986) constructed a series of plasmids, Mini T plasmids, which contained various permutations of the border repeats from a nopaline Ti plasmid. Mini T plasmids with both or only the right border sequence were equally virulent while those with just the left border sequence were severely attenuated for virulence. Plasmids without border sequences were avirulent. Analysis

of integration events revealed that the border sequences delimitted the integrated DNA. When only one border sequence was present, the other border of the integrated DNA was mapped in the vector sequences of the Mini T plasmid.

It had previously been demonstrated that if the right border sequence of the nopaline plasmid, pTiT37, was deleted, the resulting mutant was attenuated but not avirulent (Hepburn and White, 1985). This anomaly was ascribed to the presence just leftward of the right border repeat of a pseudorepeat which was thought to serve the function of the right border. However, subsequent analysis of the borders of integrated T-DNA resulting from transformations using the right border-deficient pTiT37, showed that this event was not due to the transforming ability of the pseudorepeat, but due to activity of the left border (Jen and Chilton, 1986).

The octopine Ti plasmid, pTiA6 has two discrete T-DNA sequences (Thomashow et al., 1980a and consequently four border sequences (Fig. 1.3). The left T-DNA (T_L-DNA) encodes the genes responsible for tumourigenesis while the right T-DNA (T_R-DNA) has no oncogenic function (Ooms et al., 1982). The T_R-DNA is frequently not found in octopine crown gall tumours (Thomashow et al., 1980a). The four imperfect 24bp direct repeat sequences which flank the T-DNAs of the octopine plasmids are highly homologous to their

counterparts in the nopaline Ti plasmids (Holsters et al., 1983). T-DNA/plant junctions have been shown to occur within 100 bp of repeats A and B (Holsters et al., 1983) and deletions leftward of the T_R-DNA, removing repeats D, C and B severely attenuate tumourigenesis (Ooms et al., 1982). Loss of repeats B and C does not affect virulence (Hille et al., 1983).

Rubin (1986) used a binary vector system to construct Mini T plasmids carrying various combinations of the border sequences of the octopine Ti plasmid pTiAch5. A single copy of repeats B and D was able to confer virulence on the constructs while a single copy of repeat A was unable to effect Mini T plasmid transfer to plant cells. Rubin (1986) suggested that repeat A was transfer-defective. He also showed that when the virulent construct was at least 40 kb in size, transfer was independent of the orientation and position of the repeat sequence. Constructs of 200 kb, however, showed dependence on the orientation and the position of the repeat. Rubin (1986) concluded that both border sequences were required for efficient transfer of constructs, but where the constructs were small, one repeat could effect transfer alone. He proposed a model in which the leftward and rightward repeats deliminate the sequences which are to be transferred and that transfer begins from the rightward repeat. Transfer efficiency decreases with increase in size of the constructs with one repeat sequence

and Rubin (1986) therefore postulated that where a single repeat is present, it may act as both a rightward and leftward border if the construct is not too large.

There are several differences between the T-DNA border repeats which may be responsible for polarity in transformation activity. (1) The sequence of the right and left repeats differ from each other; in the nopaline plasmid the right border differs by four base pairs from the left hand counterpart (Yadav et al., 1982). (2) Orientation of the repeats relative to the T-DNA is opposite for left and right borders (Wang et al., 1984). (3) Sequences flanking the border repeats are different for each border (Yadav et al., 1982).

Peralta and Ream (1985) showed that a sequence to the right of the right border of the octopine T_L-DNA of pTiAch5 enhanced transformation. This sequence, named Overdrive, functions in either orientation with respect to the adjacent repeat (Peralta et al., 1986). Comparison with other Ti plasmids shows a highly homologous eight base pair core and a less homologous 17 bp area. This suggests that there is a general feature of right borders on Ti plasmids and that this may explain the polarity of the left and right borders (Peralta et al., 1986).

When A. tumefaciens is co-cultivated with plant cells, circular T-DNA molecules with a single 25bp repeat can be isolated by transformation into E. coli and by cosmid packaging (Koukolikova-Nicola et al., 1985). It was suggested that the T-DNA was transferred via a circular, double-stranded intermediate and that the intermediate was formed by homologous recombination between the two repeats flanking the T-DNA. However, the ability of single flanking regions to effect transfer of T-DNA and the very low frequency of circular T-DNA molecules found per individual induced bacterial cell (10^{-5} molecules per induced cell) argue against this proposal (Jen and Chilton, 1986; Rubin, 1986; Stachel et al., 1986).

Single stranded, linear T-DNA molecules have been identified in bacterial cells that have been induced with AS (Stachel et al., 1986) and these T-strands were present as a single copy per induced cell. In addition, these workers were able to show single stranded nicks occurring in the repeat sequences after induction by AS. Analysis of the Ti plasmid revealed structural changes in the T-DNA area which affected the recognition of sites by restriction enzymes. Stachel et al (1986) proposed a model for the transfer of T-DNA which involves the nicking of border sequences, the subsequent synthesis of a DNA strand complementary to the top strand starting at the right border sequence and running 5' to 3' leftward. DNA synthesis terminates at the left border,

freeing the lower, linear strand, the T-strand, which is transferred possibly via a similar mechanism as in bacterial conjugation systems. The virC/D gene products have been implicated in this transfer process (Alt-Moerbe et al., 1986; Horsche et al., 1986).

The T-strand is probably carried across the bacterial and plant cell walls in a complex with proteins encoded by the vir region. Experimental evidence suggests that the virE gene product is secreted across the cell wall of the induced bacterium. By using the Ti plasmid to transmit Potato Spindle Tuber Virus (PSTV), and combining the virus with different permutations and orientation of the border sequences of a nopaline Ti plasmid, and use of various vir mutants, Gardiner and Nester (1986) showed that PSTV could be transmitted in the absence of the virE locus. Constructs were transferable when only one border sequence was present. Since PSTV does not require integration into the host chromosome during the infection process, it is possible that the virE locus encodes a protein involved in the integration of the T-strand into the plant chromosome.

1.3.1C Expression of T-DNA and tumourigenesis

After transfer and integration of the T-DNA into the plant chromosome, transcription of T-DNA genes is induced and these gene products are responsible for opine production, tumourigenesis, control of tumour morphology and host range.

The T-DNA is approximately 23 kb in size (Lemmers *et al.*, 1980; Thomashow *et al.*, 1980a) and Southern blotting has shown that approximately 8-9 kb are found common to both octopine and nopaline Ti plasmids (Depicker *et al.*, 1978; Engler *et al.*, 1981). The functions of the nopaline Ti plasmid have been mapped using Tn1 and Tn7 (Holsters *et al.*, 1980) and those of the octopine Ti plasmid using Tn5 mutagenesis (Garfinkel *et al.*, 1981; Leemans *et al.*, 1982). The T-DNA regions of several octopine and nopaline Ti plasmids have been partially and completely sequenced (Fig.1.3) (Barker *et al.*, 1983; Heidekamp *et al.*, 1983; Gielen *et al.*, 1984; Klee *et al.*, 1984; Lichtenstein *et al.*, 1984; Sciaky and Thomashow, 1984; Vanderleyden *et al.*, 1986).

Expression of T-DNA from the octopine Ti plasmid pTiAch5 has been studied in *E. coli* (Schroder *et al.*, 1981) and four proteins were identified. However, because T-DNA expression is plant specific, the use of this technique is limited as

the T-DNA promotors would have to be of a eucaryotic nature in order to regulate expression in the plant. Consequently, northern hybridisation techniques were used in subsequent studies on T-DNA expression in the plant cells. Transcription of T-DNA in plant cells is dependent on host RNA polymerases (Willmitzer et al., 1981). In octopine crown gall tumours, eight defined transcripts have been mapped on the T_L-DNA (Willmitzer et al ., 1982; Gielen et al., 1984) while five transcripts have been identified as originating from the T_R-DNA (Winter et al., 1984). The T_R-DNA is not required for tumourigenesis (Salomon et al., 1984). Two T_R-DNA transcripts are required for mannopine synthesis while a third is responsible for the conversion of mannopine to agropine (Salomon et al., 1984; Konro et al., 1985).

The eight transcripts of the T_L-DNA differ markedly in abundance and vary in size from 2700 bp to 670 bp (Willmitzer et al., 1982). The transcripts appear to be read from separate, eucaryotic promotor sequences (Gielen et al., 1984). Willmitzer et al. (1982) suggested that negative control of shoot formation could be assigned to transcripts 1, 2 and possibly 5 and negative control of root formation to transcript 4. Transcript 3 was shown to correspond to a protein responsible for octopine synthesis, lysopine dehydrogenase. No function has to date been assigned to

transcript 7. Transcripts 3 and 7, the most abundant transcripts, are read from a dual promotor which can induce transcription in either orientation (Velten *et al.*, 1984).

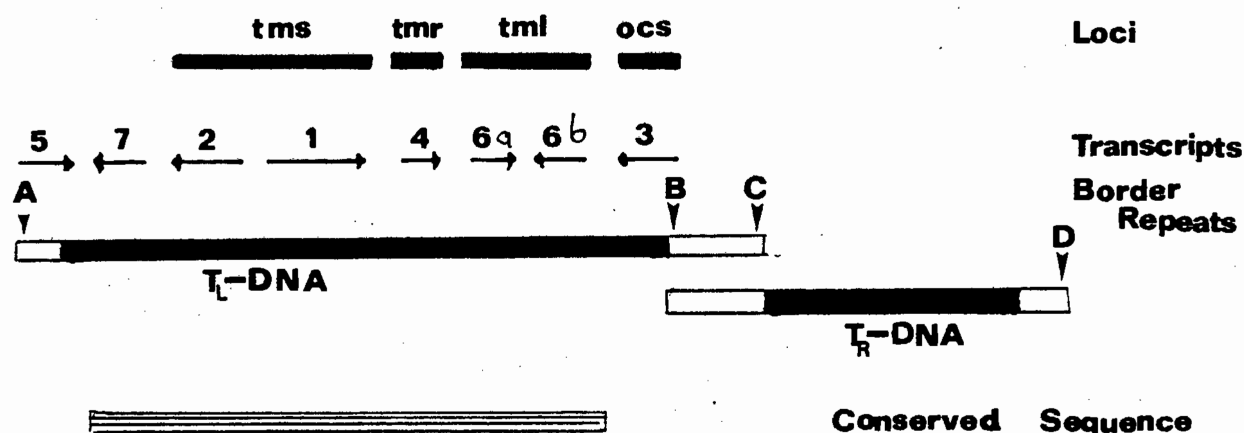


Fig. 1.3: **Functional map of the T-DNA regions of an octopine Ti plasmid** (Joos *et al.*, 1983; Willmitzer *et al.*, 1982; Willmitzer *et al.*, 1983). In the diagram are shown the transcripts read from the T-DNA with the loci which have been assigned to some of them, also the border sequences of the T_L-DNA and T_R-DNA as well as the sequences conserved between the octopine and nopaline Ti plasmid.

Thirteen distinct transcripts have been detected in nopaline crown gall tumours (Willmitzer *et al.*, 1983)and these are read on each DNA strands from independent promotors of eucaryotic origin. Transcripts 6a,6b,2 and 4 are highly homologous to their counterparts on the octopine Ti plasmid and map at the same relative position on their respective T-DNAs (Fig. 1.3). Transcripts 5 of both plasmids also show DNA homology while transcript 1 which in both types of

tumours is produced in very low amounts, is also homologous to its counterpart on the octopine plasmid. Due to the high degree of conservation between these transcripts, Willmitzer *et al*. (1983) suggested that they were involved in tumourigenesis.

At least three genes of the common DNA are directly involved in tumour induction. Mutations in genes 1 and 2, the *tms*$_1$ and *tms*$_2$ loci,result in the differentiation of normally unorganised tumours into a proliferation of shoots while a mutation in gene 4, the *tmr* locus results in the formation of roots (Garfinkel *et al*., 1981; Ooms *et al*., 1981; Lemmers *et al*., 1982; Joos *et al*., 1983).

Root and shoot formation in plants is controlled by the concentrations of the growth regulators, auxin and cytokinin relative to each other. A high cytokinin to auxin ratio leads to shoot formation, a condition similar to that of plant cells transformed with T-DNA bearing mutants in the *tms* loci. The combined effect of the *tms* loci is "auxin-like" (Ooms *et al*., 1981; Liu *et al*., 1982) and these genes have been shown to be involved in the production of the auxin, indole-3-acetic acid (Inze *et al*., 1984; Schroder *et al*., 1984; Thomashow *et al*., 1984). The *tms*$_2$ locus has been shown to encode the enzyme indole-3- acetamide (IAM) hydrolase which converts IAM into the biologically active

IAA and this enzyme is also able to hydrolyse other plant metabolites to produce active auxin (Kemper *et al.*, 1985). Thomashow *et al.* (1986) proposed that crown gall auxin biosynthesis involved the conversion of tryptophan to IAM, possibly by the product of *tms*$_1$ and the subsequent conversion of IAM to IAA by the product of *tms*$_2$, IAM hydrolase (Fig. 1.4). At present, this biosynthetic pathway has not been detected in plants.

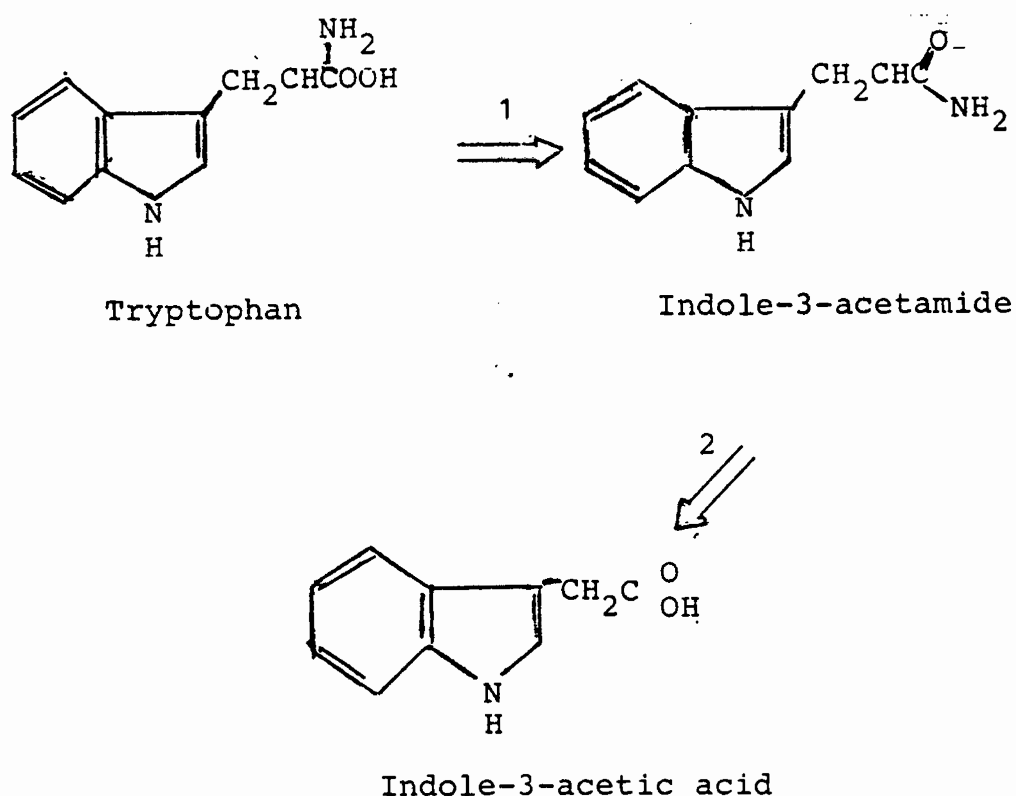


Fig. 1.4: **Pathway for auxin biosynthesis in crown gall tumours** (Akiyoshi et al., 1984)

The response of different plant species to tms_1 and tms_2 can vary. A Ti plasmid with a non-functional tms_2 gene cannot transform N. tabacum to auxin independence, but can transform N. glutinosa to auxin autonomy (Garfinkel et al., 1981; Ooms et al., 1982).

A low cytokinin to auxin ratio leads to root formation, as happens when plant cells are transformed with T-DNA with a non-functional tmr locus which exhibits a "cytokinin- like" function (Akiyoshi et al., 1983). The tmr locus has been shown to code for an enzyme involved in the synthesis of cytokinin (Akiyoshi et al., 1983).

Letham et al. (1982) as quoted in Beaty et al. (1986) speculated on the biosynthetic pathway of cytokinin in plants. They proposed that dimethylallylpyrophosphate (DMAPP) is converted to give iso-pentenyladenosine monophosphate (iPMP) (Fig 1.5). The enzyme, DMAPP transferase, converts DMAPP to iPMP and this enzyme is produced by the tmr locus (Buchmann et al., 1985). The next step in the cytokinin biosynthetic pathway involves three hydroxylations; hydroxylation of iPMP to trans-ribosylzeatin (ZMP), hydroxylation of isopentenyladenosine to yield trans-zeatin (ZR) and hydroxylation of iso-pentanyladenine (iP) to yield zeatin(Z). The tzs locus, which shows considerable homology to the tmr gene has been identified outside the T-DNA in the vir region. The tzs gene product shows DMA transferase activity, however, expression of the gene in E. coli and in A. tumefaciens, results in the production of ZR (Beaty et al., 1986).

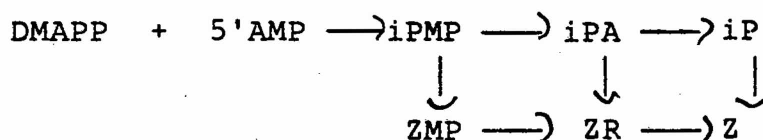


Fig. 1.5; **Diagrammatic representation of the biosynthetic pathway for the cytokinin, zeatin** (Beaty et al., 1986)

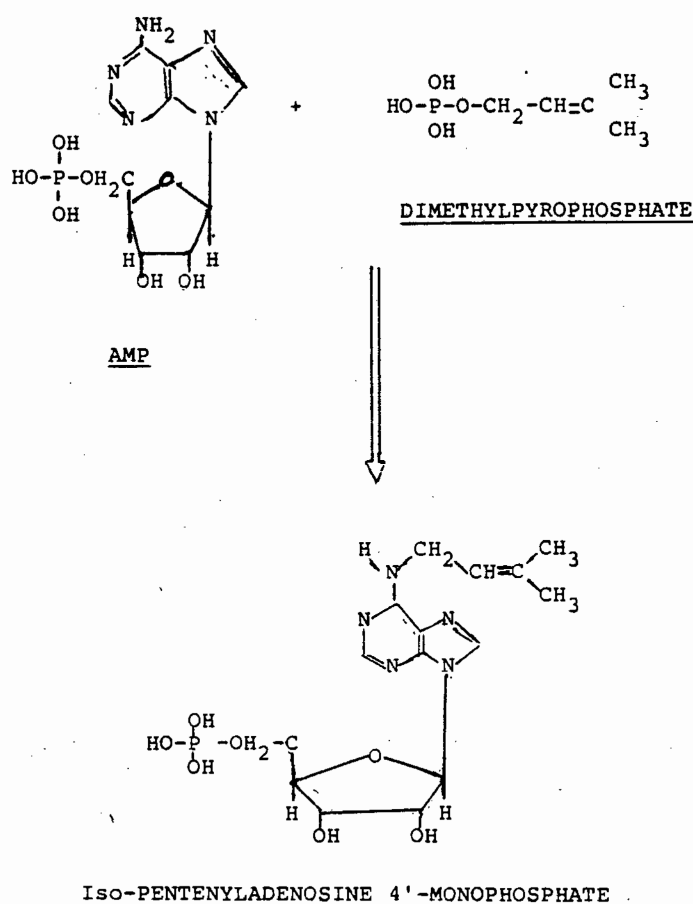


Fig. 1.6: **Chemical conversion of DMAPP to iPMP by the enzyme DMAPP transferase** (Buchmann et al., 1985)

Inactivation of the tml locus which is located between the tmr and ocs loci on the octopine Ti plasmid, pTiA6NC, results in large tumours on Kalanchoe leaves and stems (Garfinkel et al., 1981). The tml region encodes two transcripts of 900 nucleotides in length named transcripts 6a (the most abundant) and 6b (far less abundant) (Willmitzer et al., 1983). This region has been sequenced (Vanderleyden et al., 1986). Transcript 6a appears to code for an enzyme which is involved in the active secretion of opines across the plant cell wall (Messens et al., 1985) while the function of transcript 6b remains unclear. Thetml phenotype appears to be plant species-dependent (Vanderleyden et al., 1986) and there is also variation in the phenotype carried by different Ti plasmids (Joos et al., 1983). Comparison of this locus with those of various other Ti plasmids reveals sequence divergence which might account for differences in phenotype (Vanderleyden et al., 1986).

The host range of any A. tumefaciens strain can vary and this appears to be dependent on the type of Ti plasmid carried by the particular strain (Thomashow et al., 1980b) Buchholz and Thomashow (1984a,b) compared a wide host range Ti plasmid, pTiA6 with a limited host range Ti plasmid, pTiAg63 and found that host range was determined by the T-DNA loci differed between the two plasmids. A fragment from pTiA6 which carried genes 4, the tmr locus,6a and 6b could

extend the host range of pTiAg63 if it was present in the T-DNA. However, the host range was not as wide as that of pTiA6. Similar results were obtained by Hoekema et al. (1984) who showed that it was the tmr locus which was involved in determining the host range. The virA and virc loci have also been shown to be involved in the control of host range (Yanofsky et al., 1986).

A new class of limited host range Ti plasmids has been identified. Bacteria which carry these plasmids are unable to catabolise octopine, nopaline, agropine or mannopine. The T-DNA bears no homology to the tms_1, tms_2 or tmr genes of T-DNA from a wide host range Ti plasmid although there is homology with the virB, virC, virD and virG loci (Unger et al., 1985).

1.3.2 Hairy root disease and A. rhizogenes

A. rhizogenes induces hairy root tumours when inoculated onto host plants. Virulence is encoded by a large plasmid, the Root-inducing or Ri plasmid which is about 400 kb in size (White et al., 1982). The tumourigenic traits may also be carried on a smaller (250 kb) plasmid which results from the dissociation of the larger Ri plasmid. Chilton et al. (1982) and others (Spano et al., 1982; White et al., 1982) demonstrated independently that tumourigenesis involved the transfer of T-DNA from the Ri plasmid to transformed cells. The T-DNA was stably integrated into the plant chromosome

and was passed from one generation to the next (David *et al.*, 1984; Constantino *et al.*, 1984). The transformed cells produced opines such as mannopine (Spano *et al.*, 1982) and agropine (White *et al.*, 1982).

A study of T-DNA structure in the transformed plant cells reveals multiple copies, each with a different plant DNA-T-DNA junction, which presumably arises from separate transformation events. Fragments varied in length from 19 to 36 kb, depending on the Ri plasmid involved. The T-DNA was flanked by borders in a similar manner to that of Ti T-DNA in plants (Byrne *et al.*, 1983).

Homology studies between Ti and Ri plasmids showed strong homology between the virulence region of the Ti plasmid and sequences of the Ri plasmid just leftward of the Ri T-DNA (Jouanin, 1984). Clones bearing the pTiC58 $\underline{tms_1}$ and $\underline{tms_2}$ genes hybridised to areas on the Ri T-DNA. In addition areas of the Ti plasmids carrying genes for agropine synthesis and metabolism as well as the origin of replication have homologous sequences on the Ri plasmid (Jouanin, 1984). No homology has been shown between the *tmr* gene of the Ti plasmid and the Ri plasmid sequences (Willmitzer *et al.*, 1982; Huffman *et al.*, 1984).

A. rhizogenes transfers two discrete segments of T-DNA to transformed plant cells, the rightward T_R-DNA and the leftward T_L-DNA, each of 15-20 kb in size. The T_R-DNA and T_L-DNA are separated from each other by about 15 kb of unintegrated DNA (Fig. 1.7) (White *et al.*, 1985). The tms_1 and tms_2 loci have been mapped on the T_R-DNA by hybridisation with the auxin biosynthesis genes of the Ti plasmid, while a portion of the T_L-DNA has been found homologous to a genetically undefined region mapping near the left border of nopaline Ti plasmids (Huffman *et al.*, 1984).

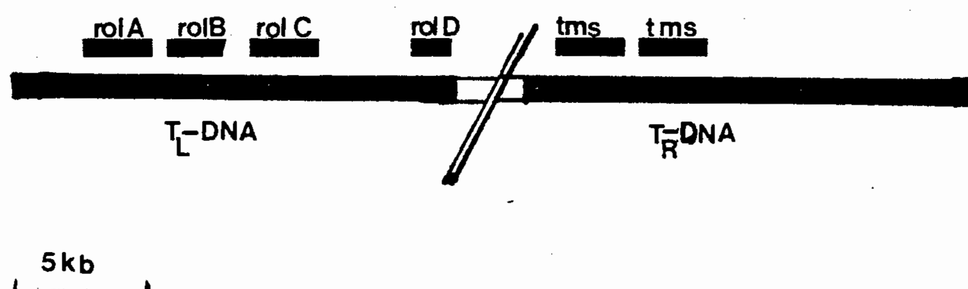


Fig. 1.7: **Functional map of the Ri plasmid T-region** (Taylor *et al.*, 1985; White *et al.*, 1985). The diaqgram shows the *rol* loci as well as the two *tms* loci

White et al. (1985) showed that the Ri tms_1 and tms_2 genes could compliment the auxin biosynthesis genes of the Ti plasmid and that they have a similar function. At least six transcripts have been defined on the TR-DNA of which two are at the tms_1 and tms_2 loci. Five transcripts have been mapped on the TL-DNA among which are the rolA-D loci (Taylor et al., 1985). The rolA-D loci individually are not essential for tumourigenesis (White et al., 1985; Boulanger et al., 1986) but deletion of the TL -DNA results in loss of virulence. White et al. (1985) speculated that these genes are involved in cytokinin biosynthesis, although they show no homology to the tmr gene of the Ti plasmid. Boulanger et al. (1986) suggested that these loci are involved rather in making cells more sensitive to auxin or that their gene products stimulate the uptake of the hormone. It has recently been shown that the expression of some of the Ri T-DNA loci is regulated by the plant cell and that expression may be organ-specific (Durand-Tardif et al., 1986).

Sequences in the genome of N. glauca have been detected which show homology to the T_L-DNA of the A. rhizogenes Ri plasmid (White et al., 1983). These sequences have been cloned and characterised and contain an inverted repeat carrying the rol A-D loci, which are potentially capable of expression. Some, but not all other species of Nicotiana

carry these sequences in their genome and it is postulated that an A. rhizogenes transformation occurred somewhere in the evolution of the species (Furner et al., 1986).

1.3.3 Wound tumour disease

Wound tumour disease was first described by Black (1945) who showed that the disease was caused by a virus which he called the Wound Tumour Virus (WTV). For a review, see Nuss (1984). WTV is transmitted by the leafhopper, Agallosis novella. Tumours are initiated at wound sites (Black, 1945). Plants infected with the virus are also able to form tumours when treated with auxin (Black and Lee, 1957).

WTV is a double stranded (ds) RNA virus (Ganatos and Tamm, 1963) consisting of twelve components (Kalmakoff et al., 1969) which have similar electrophoretic mobilities to the twelve components of Rice Dwarf Virus (RDV) (Reddy et al., 1974). However, RDV does not cause tumours on host plants. RDV, therefore, may have lost its tumour inducing capability due to a mutation in its genome. The combined molecular mass of the WTV genome is approximately $16x10^6$ (Reddy and Black, 1973). Each component of the genome encodes a single mRNA transcript and the genome has been cloned and sequenced (Asamizu et al., 1985).

Tissue cultured tumours can be maintained in the absence of exogenous growth factors and these appear to retain their tumourigenic properties in the absence of WTV (Black, 1957). Very little progress has been made in understanding the mechanisms of tumour induction by WTV.

1.3.4 Genetic tumours

It has been observed that interspecific hybrids between members of the genus *Nicotiana* may produce spontaneous tumours throughout the plant without the presence of any external factors (Smith, 1972). The same phenomenon has also been observed between hybrids of the genus *Brassica* (Kajanus, 1917) and also in the genus *Datura* (Satima *et al.*, 1951).

Tumour formation in *Nicotiana* interspecific hybrids is a function of specific loci on the chromosomes (Hess *et al.*, 1976) and tumourigenesis is not dependent on which species is used as the male or female parent, eliminating the possibility of the involvement of cytoplasmic factors (Bayer, 1982). It is thought that the combination of genes from the species involved in the formation of the hybrid, serve to upset the normal regulation of genes involved in

the control of cell division, growth and development (Braun, 1972). Plant growth factors such as auxin and cytokinin appear to be involved in the formation of genetic tumours (Schaeffer,1961), although the mechanism is still poorly understood.

White _et al_. (1983) discovered regions in healthy _N. glauca_ plant DNA which were 90% homologous to the T-DNA of pRiA4b, the root-inducing plasmid of _A. rhizogenes_. Furner _et al_. (1986) cloned and sequenced these plant DNA homologues and found that they contained a large, inverted repeat sequence which was homologous to the T_L-DNA of pRiA4b. The inverted repeat contained open reading frames which corresponded to similar open reading frames on the Ri plasmid which encodes tumourigenic functions. Furner _et al_. (1986) also found that these T-DNA-homologues were present in some but not in all other species of _Nicotiana_. They were unable to detect any mRNA homologous to these sequences and concluded that the sequences were probably not transcribed in the plants.

Since _N. glauca_ is one of the species which, after being crossed with another _Nicotiana_ species, produces hybrids with a tendency for developing spontaneous tumours, one may speculate that these silent T-DNA genes may be involved in the tumourigenic process. The hybrid genomes may in some way cause the transcription of these genes.

1.3.5 Habituation of cultured plant cells

When plant cells and tissues are propagated by means of tissue culture,they sometimes lose their requirement for exogenous growth factors and may therefore be cultured in the absence of added hormones. This heritable alteration is known as habituation and the phenomenon has been reviewed by Meins (1982). The change from growth dependent on exogenous growth factors to autotrophic growth is gradual and degrees of habituation may vary from cell line to cell line (Binns and Meins, 1973). The process involves changes in the cell's production of auxin and other cell division factors such as cytokinin (Binns and Meins, 1979). Habituation appears to be due to the expression of genes normally silent in cultured plant cells. Whether this is caused by a genetic mutation or an epigenetic change is not clear, although most evidence argues in favour of the latter. Meins (1982) defines epigenetic changes as changes in the cell phenotype occurring at a very high frequency, which is far higher than the natural mutation rate. These changes are readily reversible at a rate which is also far higher than the natural back mutation rate. The mechanism of these changes is unknown although one can speculate that as in the case of genetic tumours, habituation could result from the activation of otherwise silent oncogenes present in the genome of apparently healthy cells. Arguments in favour of this proposal are the detection of silent <u>A. rhizogenes</u> Ri

sequences in *N. glauca* (Furner *et al*., 1986) and data presented by Van Slogteren *et al*. (1984) shows that T-DNA genes that are otherwise silent in transformed plants, can be activated by grafting and treatment with 5-azacytidine, a hypomethylating agent.

1.4 Discussion

Plant tumours can be caused by a number of factors. Among pathogenic causes are viruses, bacteria and MLO's. However, tumours may also arise spontaneously as a result of changes in the genome of the cell by mutation, or as a result of changes in the regulation of genes involved in normal growth and differentiation.

Apart from the identification of pathogens associated with Witches' Broom disease, little or nothing is known about the mechanism by which teratoma formation is induced. No pathogen has as yet been identified as being involved in the formation of Witches' Broom teratomas on members of the genus *Protea*.

This study of Witches' Broom disease of *P. cynaroides* was approached from two angles. The first was an attempt to identify the pathological cause of the disease and also to

discover what physiological factors contributed to the formation of the teratomas. The second approach was to attempt to show a genetic basis for the disease by analysing the genome of the plant.

Chapter 2 details field observations on the epidemiology of the condition, attempts to cultivate both healthy and teratoma material on tissue culture media and a fluorescent and electron microscopic study of affected tissue. Attempts were also made to inoculate *P. cynaroides* plants with various strains of *A tumefaciens*. Chapter 3 describes the development of a reliable method for the isolation of DNA from *P. cynaroides* and the subsequent probing of the genome for sequences which could be responsible for the formation of tumours using a clone of the *Agrobacterium* T-DNA oncogene complement.

CHAPTER 2: **GENERAL INVESTIGATION OF THE CAUSE OF WITCHES' BROOM DISEASE OF P. CYNAROIDES**

2.1 **Abstract**

As very little is known about the causes of Witches' Broom disease on *P. cynaroides*, an attempt was made to identify a pathogen which could be held responsible for the disease. A number of plants were studied in the field and from these samples were taken and cultured on culture medium. Healthy *P. cynaroides* tissue was not established in tissue culture, while more success was obtained with teratoma tissue. Attempts were made to transmit the disease but these were unsuccessful. Four strains of *A. tumefaciens* were unable to induce tumours on *P. cynaroides* seedlings. Sections of vascular tissue from teratoma and healthy tissue viewed under the electron microscope revealed no identifyable pathogen.

2.2 **Introduction**

Very little is known about the cause, epidemiology and physiology of Witches' Broom disease on *P. cynaroides* and on other species of *Protea*. The disease has been described by Rust and Myburgh (1976). To date no pathogen has been identified as being the causal agent for Witches' Broom

disease on *P. cynaroides* or on any other *Protea* species. Rust (1981) speculated that the disease may result from a mycoplasmal infection of the plants and Meyer (1981) proposed the involvement of the mite *A. protea* either as causal agent or in the spread of the disease.

Knowledge of the physiological basis for teratoma formation is scant. Witches' Broom teratoma cuttings have shown a very high rooting efficiency and root readily without treatment with the rooting hormone, auxin. Plants regenerated from Witches' Broom cuttings do not revert to a normal growth habit (Dr J. Ben Jaacov, Dept of Floriculture, Vulcani Institute, P.O. Box 6, Beit Dagan 65250, Israel, personal communication).

This chapter describes various field observations, experiments to identify and isolate a pathogen and experiments to culture *P. cynaroides* on tissue culture medium.

2.3 Methods and Materials

2.3.1 Plant material and bacterial strains

P. cynaroides plants studied in the field were cultivated in the Kirstenbosch Botanical Gardens, Cape Town. Seedlings

used were cultivated from seed collected from the plants studied at the Kirstenbosch Botanical Gardens. Healthy tissue was collected from plants infected with Witches' Broom disease. A. tumefaciens strains C58, A892, A281 and 15955 were a gift from Dr Stephen Ferrand, Dept of Plant Pathology, College of Agriculture, N-5190 Turner Hall,1102 South Goodwin Avenue, Urbana, Illinois 61801, USA.

2.3.2 Tissue culture of healthy and Witches' Broom-infected tissue

Healthy and infected material was collected from actively growing shoots and tumours. Leaves were cut off leaving 1-2 mm petioles and the stems cut into 6-7 cm pieces before being surface sterilised by stirring continuously in a 2% NaOCl solution containing a few drops of Teepol for 20 min. After three successive washes in sterile, distilled water, the sections were cut into segments with one healthy axial bud each and placed on a modified version of Anderson's medium (Anderson, 1975) which was poured as 10 ml slants in standard containers. The rubber seals were removed from the lids of the standard containers. Four phytohormone combinations were used (Table 2.1).

Table 2.1: **Phytohormone concentrations used in tissue culture experiments**

Treatment no	Phytohormone	Concentration (ug/ml)
1	-	-
2	NAA	0.2
3	kinitin	2
4	NAA kinitin	0.2 2

Cultures were incubated at 25°C with a 16 h photoperiod with cool white light.

2.3.3 A. tumefaciens inoculations

Agrobacterium strains were grown overnight in 10 ml Luria broth at 28°C. Twelve three-month old P. cynaroides seedlings and two N. tabacum cv. Soulouk plants were each inoculated with each of the four A. tumefaciens strains by first wounding the stems with a sterile syringe needle and then inoculating with 100 ul of the overnight A. tumefaciens culture. The N. tabacum plants and two P. cynaroides plants were, in addition, inoculated with sterile Luria broth. The plants were grown in the laboratory for three months.

2.3.4 Electron Microscopy

Immediately after collection, samples of approximately 0.3 cm^3 in size, were immersed in a cold (4°C) solution of 4% glyceraldehyde in a 199 mM phosphate buffer at pH 7.3. The samples were left overnight and washed twice for 10 min in 0.1 M phosphate buffer, pH 7.3, after which they underwent secondary fixation in osmium tetraoxide for 2 h. The samples were washed twice for 10 min in phosphate buffer. During fixation, care was taken to ensure that the samples were completely submerged under fixative at all times. The samples were dehydrated and embedded in resin (Table 2.2).

Table 2.2: **Dehydration and embedding of samples for electron microscopy**

Solution	Time
50% ethanol	5 min
70% ethanol	5 min
80% ethanol	5 min
90% ethanol	5 min
absolute ethanol	5 min
propylene oxide	15 min
propylene oxide	15 min
75:25 propylene oxide:resin	12 h
50:50 propylene oxide:resin	12 h
25:75 propylene oxide:resin	12 h
100% resin	12 h

Samples were left in resin in moulds at 60°C for 36 h after which sections were cut with a glass knife and stained for 30 min in uranyl acetate and then in lead citrate for 5 min.

Thick sections of each block were examined under the light microscope to ensure that thin sections were of the vascular tissue. Sections were viewed with a Zeiss EM109 Transmission Electron Microscope.

2.4 Results and Discussion

2.4.1 Field observations

Witches' Broom disease of P. cynaroides is characterised by a bushy proliferation of short, malformed shoots (Fig. 2.1) which form in the leaf axes of affected plants and a number of teratomas can develop on the same plant over a period of at least two years. Removal of the teratomas did not result in a reduction in tumour formation. Tumour induction was especially high during the spring growth period of the plants. Teratoma shoots vary in length from a few centimetres to 30 cm in length. Stems seem to elongate with age. One teratoma was observed to be almost normal in phenotype with stem nodes elongated and leaves almost the same size as normal leaves (Fig. 2.2). Tumours were observed to die back after about a year (Fig. 2.3) and new teratomas would sprout from elsewhere on the plant. Growth of the teratomas resulted in severe stunting of the plants and greatly reduced flower production.

A.

B.

Fig. 2.1: **A.** Healthy P. cynaroides plant. B. infected P. cynaroides PC3 which has Witches' Broom disease

Fig. 2.2: **Healthy and teratoma tissue from P. cynaroides PC3**. From left to right, young teratomas, teratoma shoot showing almost normal phenotype and a healthy shoot

Fig. 2.3: **Old teratomas on P. cynaroides PC4**

The P. cynaroides population of the Kirstenbosch Botanical gardens in Cape Town was divided into individual blocks. Each block consisted of plants which were cultivated from seed collected from plants growing in a specific locality in the wild. There are various phenotypic differences between

blocks, with, for example, variations in leaf shape and shape and colour of blooms. This would indicate genetic variability between the plants from different areas and each block could be considered as a variety.

It was apparent that certain blocks were heavily infected with Witches' Broom disease (Block 14, which contained the diseased plants used in this study had 114 out of 120 plants with teratomas), while others showed no sign of the infection. Seed from diseased plants was prone to developing the disease within a few months of germination and the teratomas would rapidly smother the healthy tissue and eventually result in death of the seedling. These observations may indicate either that certain varieties of P. cynaroides are genetically more susceptible to Witches' Broom disease or alternatively, that seed collected from symptomless plants were infected by the pathogen which is thus seed-transmissable.

Attempts were made to infect healthy plants by rubbing the sap of infected plants in wound sites on the stems of healthy plants. These were unsuccessful and could be due to one of the following reasons. The method used to transmit the disease was unsuitable, with the pathogen not surviving transmission or alternatively needing to pass though an intermediate host (A. protea) as part of its life cycle. Another possible explanation for the lack of success may be

~~that~~ there is no pathogen in the teratoma tissue. Other methods of transmission would have to be employed before any conclusions can be made.

MLO's have been transferred from infected plants to healthy plants using the plant parasite, dodder, as an intermediate host (Dr H-J Su, Dept of Plant Pathology, National Taiwan University, Taipei, Taiwan, Republic of China, personal communication). A disease-free dodder plant is allowed to parasitise a MLO-infected plant, acquiring the MLO's from the host's vascular tissue. The dodder is then transferred to a healthy plant to which it transfers the MLO's. However, at this stage no similar parasitic dodder-like infection has yet been found on P. cynaroides. It was therefore not possible to transfer the disease in this way. If A. protea is the vector of the disease, it would be interesting to develop a method for raising "clean" insects, allowing them to feed on infected plants and subsequently transferring them to healthy plants in an attempt at transfer of the disease.

Sap from infected plants was plated onto Luria plates to identify bacteria and fungi present. Of the 12 cultures obtained most were fungi and none were able to induce the disease when inoculated onto the plants. The cultures

isolated were not identified. As no plant MLO or Ricketsia-like organism has to date been successfully cultured on agar or in medium, this approach was not pursued any further.

2.3.2 Tissue culture

Attempts to culture healthy tissue were unsuccessful. No callus was produced and the tissue sprouted one or two leaves from the axial bud which did not elongate beyond about 1 cm (Fig. 2.4A). Response to the different treatments was not uniform and it was difficult to choose a representative sample from each treatment. After a few months in culture, the plant tissue slowly blackened and died. A publication subsequent to this work has shown that shoot elongation of cultured healthy *P. cynaroides* tissue is dependent on the presence of gibberellic acid in the growth medium (Ben Jaacov and Jacobs, 1986)

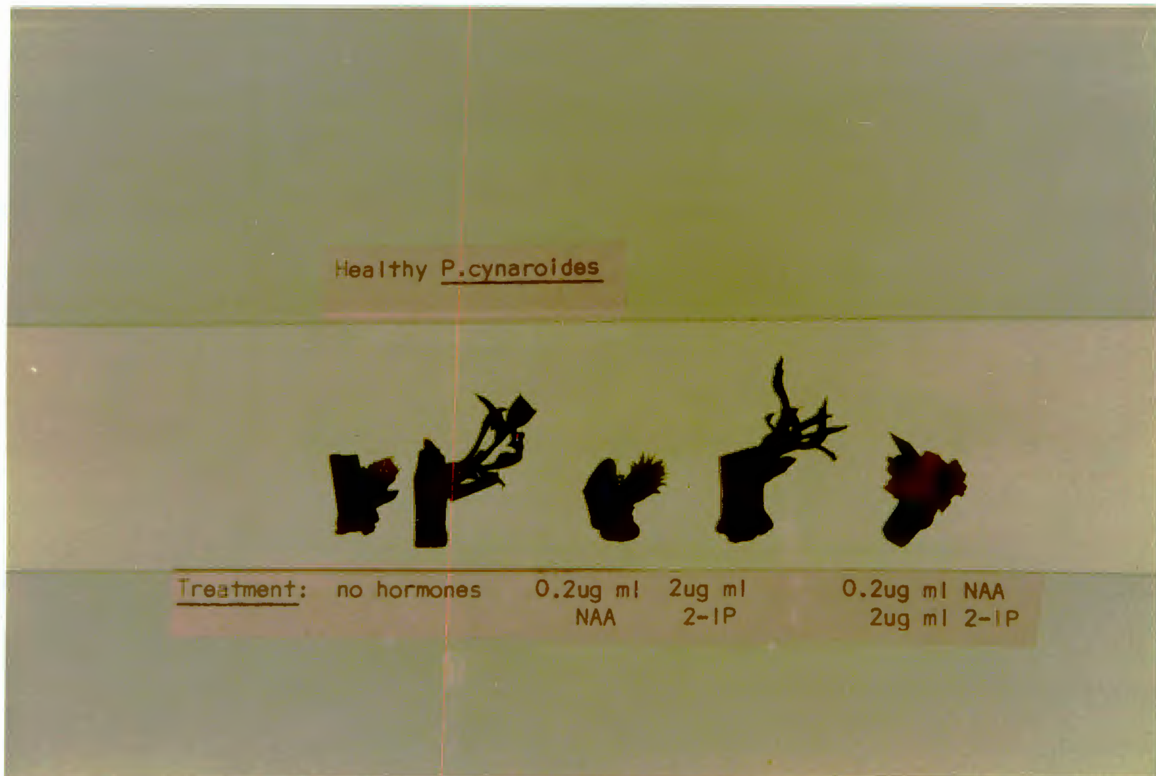
More success was achieved with Witches' Broom tissue which continued to proliferate on culture medium (Fig. 2.4B). Very little difference was noted in response to different hormone treatments, although some explants treated solely with auxin tended to produce callus tissue and not shoots. Tissues cultured in media containing no growth regulators proliferated as well and in some cases even better than their counterparts in media containing growth regulators. Some cultures have been maintained on media containing no

exogenous growth regulators for two years without dying and have continued to proliferate, although the growth rate was greatly reduced.

A number of factors could be involved in determining the response of healthy and teratoma tissue. Firstly, as axenic sections were used to initiate cultures, initial response of the tissue could be governed by endogenous growth regulators already present in the tissue. This could explain the behaviour of healthy tissue on the culture media. However, the persistent, although slow proliferation of teratoma tissue indicated that there is a difference in the hormone status of teratoma and healthy tissue.

The very slow growth rate of the tissue was probably due to either the presence of growth inhibitors in the culture medium or a sub-optimum culture medium. The lack of definitive differences in response to growth regulator combination could be due to one of two reasons. Firstly, neither auxin nor cytokinin may be involved in the formation of Witches' Broom teratomas. Alternatively, the concentration of auxin and cytokinin may have been insufficient to elicit a response from the tissue.

A.



B.

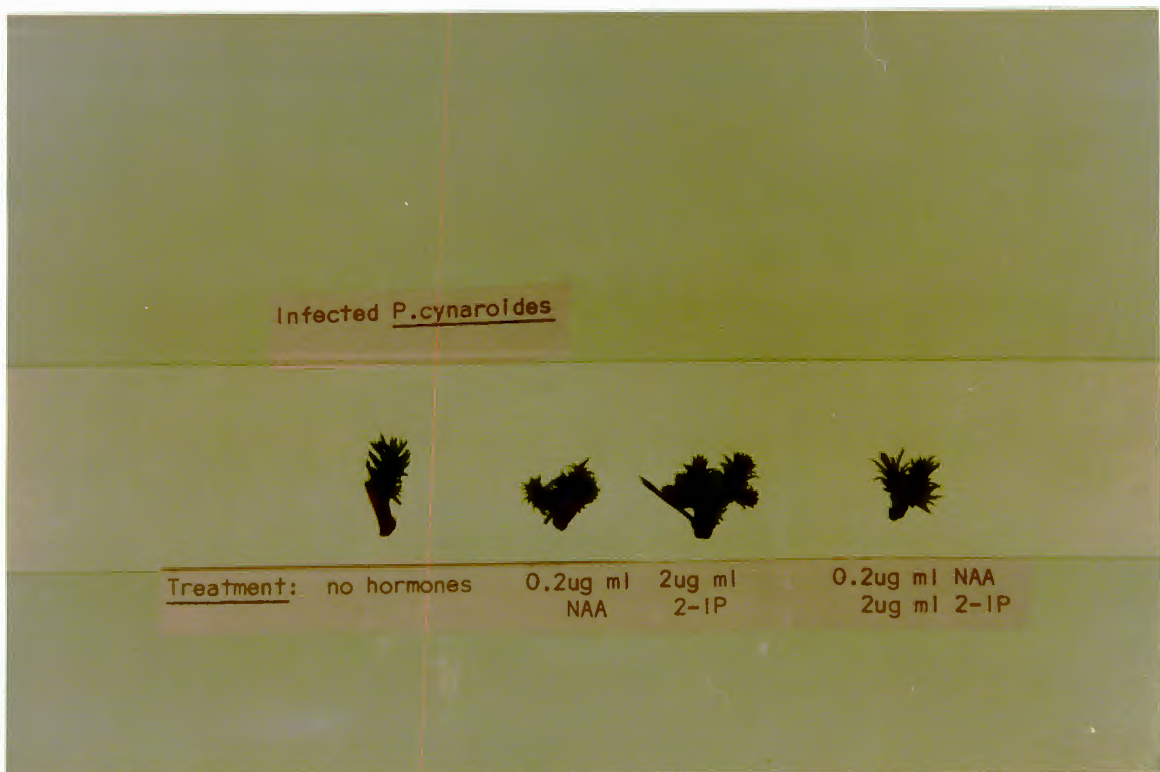


Fig. 2.4: **Tissue cultured explants from *P. cynaroides***

It has been shown in the field that healthy (symptomless) plants treated with a foliar application of cytokinin, developed shooty growths at the growth tips of the treated plants which closely resemble Witches' Broom teratomas (Dr J. Ben Jaacov, Dept of Floriculture, Vulcani Centre, P. O. Box 6, Beit Dagan 65250, Israel, personal communication). Furthermore, mutations in the tms loci of the T-DNA of A. tumefaciens caused a shift in the hormone balance in favour of cytokinin. Transformation of plant cells with these mutants resulted in "shooty" tumours (Akiyoshi et al., 1984). It is therefore likely that cytokinin is involved in the production of Witches' Broom teratomas and that the auxin and cytokinin concentrations and ratios used in these experiments were not high enough. This is supported by the response of teratoma tissue to treatment with auxin, where callus tissue developed, as referred to earlier.

2.3.3 Infection of P. cynaroides with A. tumefaciens

A possible cause of Witches' Broom disease could be infection of the plants by A. tumefaciens, even though the tumours bear little resemblance to classic crown gall tumours (Fig. 2.5). Attempts were made to infect P. cynaroides seedlings with four A. tumefaciens strains. Strain C58 has a wide host range and carries the nopaline plasmid pTiC58. Strain A892 is an agrocinopine C NT.1

showed no homology to that of octopine and nopaline Ti plasmids (Unger *et al.*, 1985). The host range of this strain is limited to cucurbits and lupin. Strain A281 is also a NT.1 strain which contains an agropine-type Ti plasmid which confers a wide host range and renders the bacterium supervirulent. Strain 15955 contains an octopine-type Ti plasmid and has a wide host range (Dr Stephen Ferrand, personal communication).

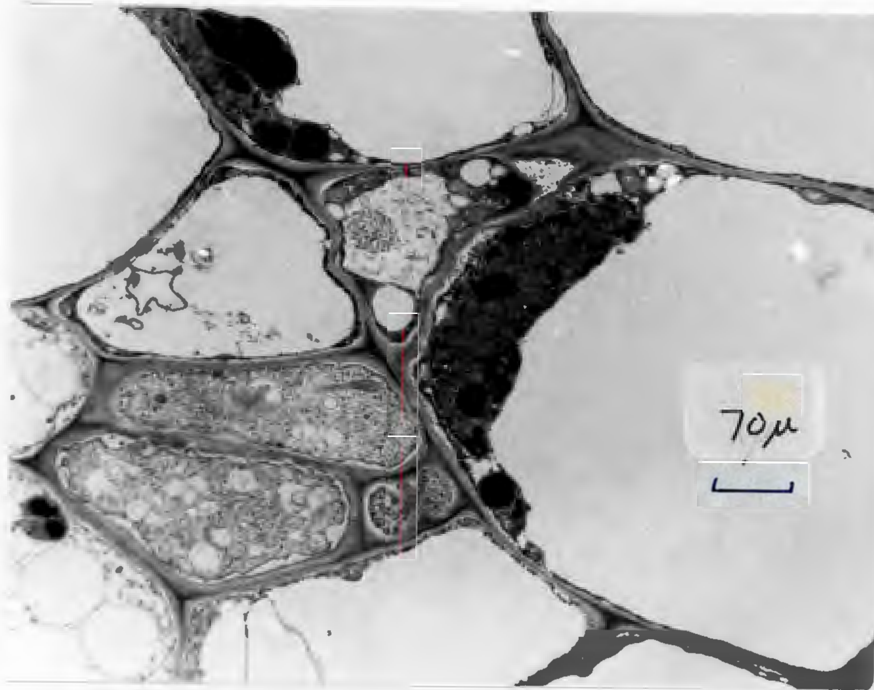
After 3 months, no tumours were observed on *P. cynaroides* seedlings. Control tobacco plants formed tumours when inoculated with strains C58, A281 and 15955, therefore lack of tumours on the *P. cynaroides* plants was not due to loss of virulence by the cultures used for inoculation. Virulence of strain A892 was not tested on host plants and absence of virulence on the *P. cynaroides* could be due to avirulence of the culture used. However, as other *A. tumefaciens* strains tested were avirulent on *P. cynaroides* it is most probable that strain ~~15955~~ A892 is also unable to induce tumours on these plants.

A.

B.

Fig. 2.5: **Comparison of Witches' Broom tumours of P. cynaroides with a crown gall tumour on N. tabacum.** A. Witches' Broom tumour on P. cynaroides; B. Crown gall tumour on N. tabacum induced by A. tumefaciens strain C58

A.



B

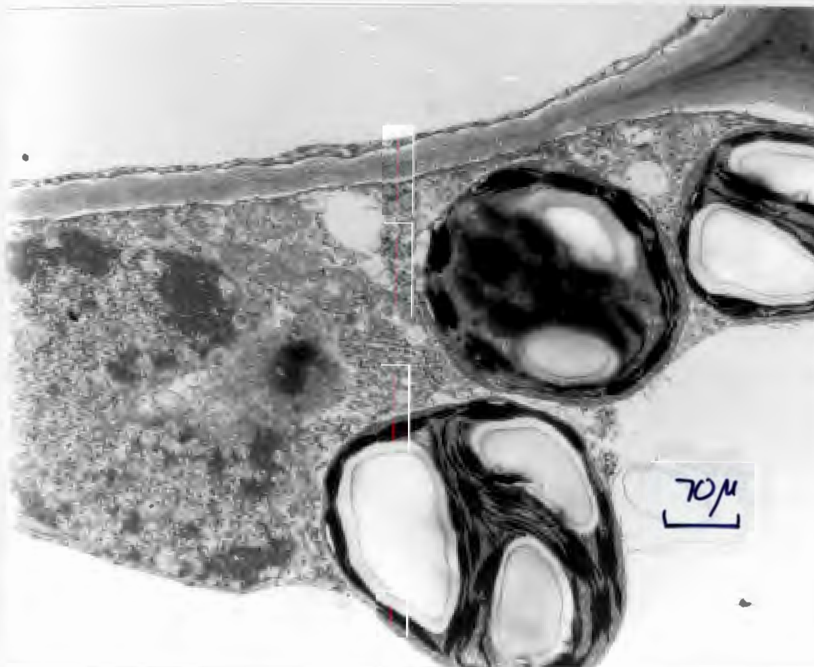


Fig. 2.6A-B: (legend on following page)

both teratomas and healthy tissue on each plant. A control plant, PC7, was chosen from a block in which no plant showed symptoms of the disease.

Results (Fig. 2.6) show no apparent vascular infection. The inability to visualise a pathogen could be due to the following reasons. Firstly, the sections may have been incorrectly processed and the pathogen destroyed in the process. This is unlikely in view of the fact that normal cellular organelles such as mitochondria, chloroplasts and endoplasmic reticulum are clearly visible and apparently intact.

Another reason for the absence of visible pathogen may simply be that there is none present in the samples. In Witches' broom diseases of other plants, however, it has been relatively easy to identify the pathogens in the vascular tissue of infected plants (Nisu et al., 1970; Shekhawat et al., 1981). Thus it may be possible that there was no pathogen present in the samples. This could either be due to the absence of pathogen in the areas from which the sections were taken, or tumour formation is not dependent on the presence of a pathogen in the vascular bundles.

CHAPTER 3: INVESTIGATION OF THE GENETICS OF WITCHES' BROOM DISEASE OF P. CYNAROIDES

3.1 Abstract

Nothing is known about the genetics of Witches' Broom induction of P. cynaroides. The best-studied genetic system of tumourigenesis is that of A. tumefaciens which transfers T-DNA to host plant cells. T-DNA genes have been shown to be used by other bacteria for tumorigenesis (A. rhizogenes and P. savastanoi). P. cynaroides genomic DNA (from healthy and teratoma tissue) was probed with plasmids containing DNA fragments coding for these conserved T-DNA sequences. Homologous DNA fragments were detected. When the genomic DNA was probed with vector sequences and E. coli chromosomal DNA, these fragments were also detected indicating that they were homologous to contaminating E. coli chromosomal and vector DNA sequences. There was therefore no T-DNA present in the Witches' Broom-infected P. cynaroides genome.

3.2 Introduction

Witches' Broom disease of P. cynaroides, of other Protea species and of other plants is characterised by a bushy outgrowth of shoots in the axes of the leaf buds of Protea

(as shown in Chapter 2 and also on the stems of other affected plant genera and species. The teratomas on P. cynaroides have been shown to have an altered growth regulator status (Ben Jaacov and Jacobs, 1985; this study) and it is possible that this altered growth regulator balance is due either to a transformation by some pathogen, alteration due to alteration in the regulation of the plant loci responsible for growth regulator production.

In olives and oleander, a disease characterised by galls on leaves and stems is induced by systemic invasion of the plants by P. syringae subsp. savastanoi (Wilson et al., 1964; Wilson, 1965). Tumours are not caused by transformation of infected plant but rather as a result of the production of the auxin, indole-3-acetic acid which induces the tumours (Smidt and Kosuge, 1978). Auxin production in P. savastanoi is via the same pathway as that used by A. tumefaciens (Hutzinger and Kosuge, 1968). In the P. savastanoi strains which infect oleanders, the genes coding for auxin production are carried on a large plasmid, pIAA, while in strains infecting olive, the genes are carried on the chromosome (Yamada et al., 1985). The two P. savastanoi loci responsible for auxin production have been shown to complement the two genes involved in auxin

synthesis in A. tumefaciens (Follin et al., 1985) and the Pseudomonas auxin genes show DNA homology to their counterparts on the Agrobacterium Ti plasmid (Yamada et al., 1985).

The T-DNA oncogenes and in particular, tms_1 and tms_2 which are involved in indole-3-acetic acid production, are conserved between A. tumefaciens and A. rhizogenes. This evidence suggests that the oncogenes responsible for tumour formation in a number of different diseases are conserved in pathogens belonging to different genera. It is therefore possible that the pathogen causing Witches' Broom disease on P. cynaroides may harbour genes which have a similar function and show DNA homology to their counterparts in Agrobacterium.

Described in this chapter is the development of an efficient and quick method for the isolation of high molecular mass chromosomal DNA from P. cynaroides and the subsequent probing of the genomic DNA with clones containing the conserved region of the A. tumefaciens T-DNA in pBR322 and phage M13, with pBR322 and also with E. coli chromosomal DNA.

3.3 **Methods and Materials**

3.3.1 Plant material, bacterial and plasmid strains

P. cynaroides leaf material was collected from plants PC3, PC4 and PC6 in block 14 at Kirstenbosch Botanical Gardens, Cape Town. All other plant material was obtained from plants grown under controlled conditions in the Department of Microbiology, University of Cape Town. Plasmid DNA was prepared from E. coli strain K514 and chromosomal DNA was isolated from E. coli HB101. pGV0319 was a gift from Dr Marc van Montagu, Laboratorium voor Genetika, Rijksuniversiteit Gent, Belgium (Fig. 3.2).

3.3.2 Isolation of bacterial and plasmid DNA

Bacterial and plasmid DNA were isolated according to the methods outlined in Appendix 1. All E. coli strains were grown in Luria broth and the strain harbouring pGV0319 was plated onto Luria agar containing Ap (100 ug/ml) or grown in Luria broth containing Ap (75 ug/ml).

3.3.3 Isolation of plant DNA

Initial attempts to isolate DNA from P. cynaroides utilised the methods described by Heyn et al. (1974), Lemmers et al. (1980), Hamilton et al. (1982), Dellaporta et al. (1983) and

Ketterman and Shattuck (1983). However, these methods proved to be unsatisfactory for the isolation of chromosomal DNA from P. cynaroides. The following procedure was developed from the method of Ketterman and Shattuck (1983) (Appendix 1).

Plant material was collected early in the morning when the ambient temperature was low and leaves were processed as quickly as possible after collection. Leaves were deveined and cut directly into liquid nitrogen. The frozen tissue was ground to a fine powder in a Bosch coffee grinder. The still frozen material was suspended at a concentration of 10 ml/g fresh weight in Buffer I and stirred on ice for 5 min (Buffer I: 1 M glucose, 0.1 M citric acid, pH 5.0, 0.5% Triton X-100). Prior to centrifugation in a Sorval GSA rotor at 5000 rpm for 7 min at 4°C, the suspension was passed through muslin cloth. After centrifugation the pellet was resuspended in an equal volume of Buffer I and centrifuged at 5000 rpm for 5 min at 4°C. This step was repeated until the pellet was cream in colour and all traces of chlorophyll had disappeared. The pellet was washed by resuspending it in an equal volume of Buffer II (Buffer II: 0.5 M glucose, 0.05 M citric acid, pH 5.0) and the centrifugation step repeated. The washing step was repeated twice. Buffers I and II were kept at 4°C at all times.

The washed pellet was resuspended in 1 ml Buffer III per gram fresh material (Buffer III: 200 mM Tris-HCl, pH 8.0, 200 mM Na_2.EDTA, 200 mM NaCl, 200 mM Dieca) an equal volume of 2% SDS was added and the solution incubated on ice for 30-60 min, shaking gently. The lysate was centrifuged in a Sorval SS34 rotor at 4°C and 10000 rpm for 15 min. Two volumes of 96% ethanol were added to the supernatant and after careful mixing, the solution was left to stand at room temperature for 5 min to precipitate the DNA. Chromosomal DNA was removed from the tube with a sterile glass rod and resuspended in 5-10 ml TE buffer. The DNA was then subjected to caesium chloride density ultracentrifugation as described in Appendix 1.

3.3.4 Agarose gel electrophoresis and hybridisation conditions

Restriction enzyme digestion conditions are described in Appendix 1. Chromosomal DNA (10 ug) was digested with the appropriate enzymes and electrophoresed in 0.8% agarose in TBE buffer for 16 h. The DNA was visualised by staining with ethidium bromide under ultraviolet light. The DNA was transferred to Genescreen membranes by a modified method of Smith and Summers (1981). The gel was denatured by incubation in two volumes of 0.5 M NaOH and 1.5 M NaCl for 15 min and twice in two volumes 1 M NH_4CH_3COO and 0.02 M NaOH for 30 min to neutralise the gel. The filters were

presoaked in neutralisation buffer and the DNA was allowed to transfer for 2 to 3 h. The filter was then baked for 2 h under vacuum at 80°C and stored in a plastic bag at -20°C. Probes were labelled with ^{32}P (Appendix 1) to a specific activity of 1-3 x 10^8 cpm/ug and 5 x 10^7 cpm of labelled probe was added per filter. Positive controls and lambda standards were hybridised apart from genomic blots. Filters were prehybridised at 60°C for 2 to 4 h in 10 x Denhardts solution (Maniatis et al., 1982), 0.5% SDS, 6 x SSPE and 100 ug/ml denatured salmon sperm DNA. Hybridisation was carried out in the same buffer (after the addition of the probe) at 60°C for 16 h in a shaking incubator. After hybridisation, the filters were washed twice for 30 min at 60°C in 2 x SSPE; 0.1% SDS. The wet filter was then sealed in a plastic bag and Kodak XAR film was exposed to it under intensifying screens at -70°C for 2 to 3 weeks.

If filters were to be reprobed, they were stripped by washing for 2 to 3 min in two volumes of 0.1% SDS which had been heated to boiling point. Filters were then prehybridised or air-dried and stored at -70°C in an evacuated plastic bag.

3.3.5 Subcloning of pGV0219

SmaI fragments of plasmid pGV0319 were subcloned into the SmaI site of phage M13 mp9 (Appendix 1). Recombinants were

selected on H-top Agar plates containing IPTG, X-gal and ampicillin (Appendix 2) as white plaques and the DNA was prepared using the method of Ish-Horowicz and Burke (1981). Relevant subclones were characterised by their restriction maps and these selections confirmed by Southern hybridisation with nick-translated pGV0319.

3.4 **Results and Discussion**

3.4.1 Development of a method for the isolation of DNA from P. cynaroides

Conventional methods of DNA extraction proved to be unsatisfactory. Members of the genus Protea possess a very high concentration of cellular phenolic compounds which oxidise rapidly when the tissue is damaged. Polyphenolics appear to bind irreversibly to the DNA and neither repeated phenol extractions nor caesium chloride density ultracentrifugation removed these compounds. DNA isolated by most of the conventional methods was red-brown in colour and resisted digestion with restriction endonuleases. DNA yields were low and the DNA was badly sheared.

The method of Ketterman and Shattuck (1983) proved to be more satisfactory in combatting the formation of polyphenolics. However, there were a number of

disadvantages. Firstly, grinding the frozen material with a pestle and mortar did not result in a powder fine enough for lysis and reduced DNA yields were obtained. The lysis buffer did not prevent polyphenol formation and the DNA became red-brown in colour. Ethanol precipitation of the DNA by incubation at -70°C resulted in the co-precipitation of SDS and the DNA yield dropped because the SDS reduced the solubility of the DNA. Finally, the DNA was sheared. It was therefore essential to develop an efficient method for isolating high quality DNA from _P. cynaroides_.

P. cynaroides and other _Protea_ species have very hard, woody leaves and it was very difficult to grind the leaf material finely enough to optimise DNA yields. In addition, the rapid oxidation of tissues upon exposure to air necessitated the pulverisation of leaf material under liquid nitrogen and the shorter the time between cutting the material and suspension in an anti-oxidant buffer, the less polyphenol formation occurred. The quickest and cheapest way to accomplish this was by grinding material frozen in liquid nitrogen in a coffee grinder to a fine powder for 30 to 45 s and then suspending the frozen material in Buffer I using a magnetic stirrer. The beaker containing the suspension was kept in ethanol-ice to keep the temperature low.

At pH 5.0 the nuclear membrane is impervious to lysis with Triton X-100 (Ketterman and Shattuck, 1983). Buffer I differentially lyses the plant cell membrane and also those of the mitochondria and chloroplasts but leaves the nuclei intact. To check that the nuclei were still intact after treatment with Buffer I, a sample of the pellet was stained with analine blue (a dye which stains nucleic acids) and examined under ultraviolet light (Hiruki et al., 1974).

After lysis, nuclei and other cell debris were pelletted by centrifugation. The degree of lysis was monitored by noting the disappearance of chlorophyll (an indication of chloroplast lysis) from the pellet. Repeated washes with Buffer II removes residual Triton X-100 as well as cytoplasmic nucleic acids.

The high NaCl and Na_2.EDTA concentrations in Buffer III inhibited nuclease activity and the high concentration of Dieca inhibited phenolic oxidation. Omission of the Dieca resulted in a brown DNA pellet. The concentration of Tris-HCl had to be raised to 200 mM in order to ensure that the pH of the solution after addition of Buffer III was raised to pH 8.0.

Concentration of DNA by ethanol precipitation at -70°C proved unsatisfactory due to the co-precipitation of insoluble SDS. The precipitation of the DNA was done at

room temperature which prevented SDS precipitation and also reduced the amount of protein which co-precipitated with the DNA. Centrifugation to pellet precipitated DNA caused shearing and the DNA was therefore removed with a glass rod as it either floated on the surface of the solution or precipitated as a large conglomerate. Although this resulted in reduced yield, high molecular mass DNA was obtained. Addition of caesium chloride caused the precipitation of the remainder of the soluble proteins and these were removed by a clearing spin. Yields varied between 10 and 20 ug of DNA per gram fresh material and were largely dependent on the degree to which the tissue was pulverised. The softer the leaf tissue, the higher was the yield of DNA. Control extractions of tobacco leaves yielded 10 ug/g fresh weight but the yield of DNA from monocotyledenous plants such as sugarcane and barley was very low (1-2 ug/g fresh weight). Possibly, Buffer I lyses the nuclei of these plants.

The big advantage of this procedure is that it is a rapid method. From the time of collection of the plant material to the beginning of ultracentrifugation took approximately 2.5 h. This was considerably faster than other conventional DNA extraction methods. The DNA was of uniformly high molecular mass and was easily digested with restriction R.E.s (Fig. 3.1).

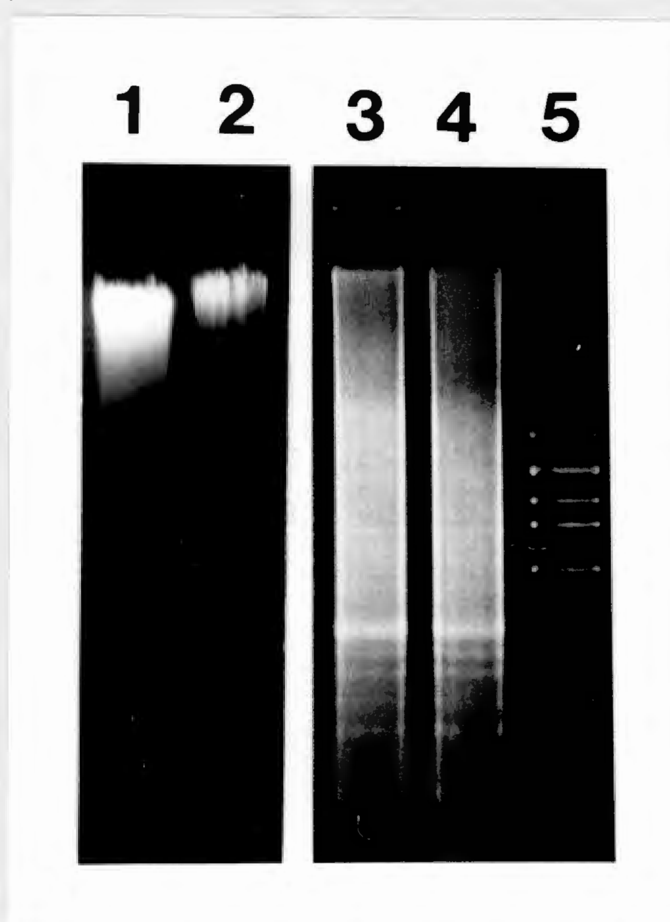


Fig. 3.1: **Ethidium bromide visualisation of an agarose gel containing P. cynaroides and N. tabacum genomic DNA.** Lane 1 and lane 2 contain unrestricted P. cynaroides and N. tabacum genomic DNA respectively. Lanes 3,4 contains P. cynaroides healthy and teratoma DNA restricted with HindIII R.E.

3.3.2 Hybridisation of T-DNA probes to the P. cynaroides genomic DNA.

DNA was isolated from both healthy leaves and infected tissue on P. cynaroides PC3, an infected plant, restricted with BglII R.E., subjected to gel electrophoresis and Southern blotting. The filters were probed with pGV0319

(Joos et al., 1983), a HindIII R.E. partial clone of the A. tumefaciens Ti plasmid pTiC58 in pBR322 (Fig. 3.2). The plasmid carries the T-DNA region which is conserved between most Ti plasmids except those belonging to the group characterised by the mega Ti plasmid AB2/73 (Unger et al., 1985). The clone includes areas which encode the tms_1, tms_2, and tmr genes.

The results (Fig. 3.3) indicate that there were sequences present in the genome of P. cynaroides which hybridised to the probe. Homologous DNA fragments of 4.8, 3.6, 3.35, 3.05, 1.6 and 1.35 kb were detected in infected tissue. However, these fragments were also apparent in healthy tissue. The appearance of these fragments in both healthy and infected tissue suggested that either they are not involved in Witches' Broom tumour induction or that the healthy tissue from which DNA had been isolated was infected with the Witches' Broom pathogen.

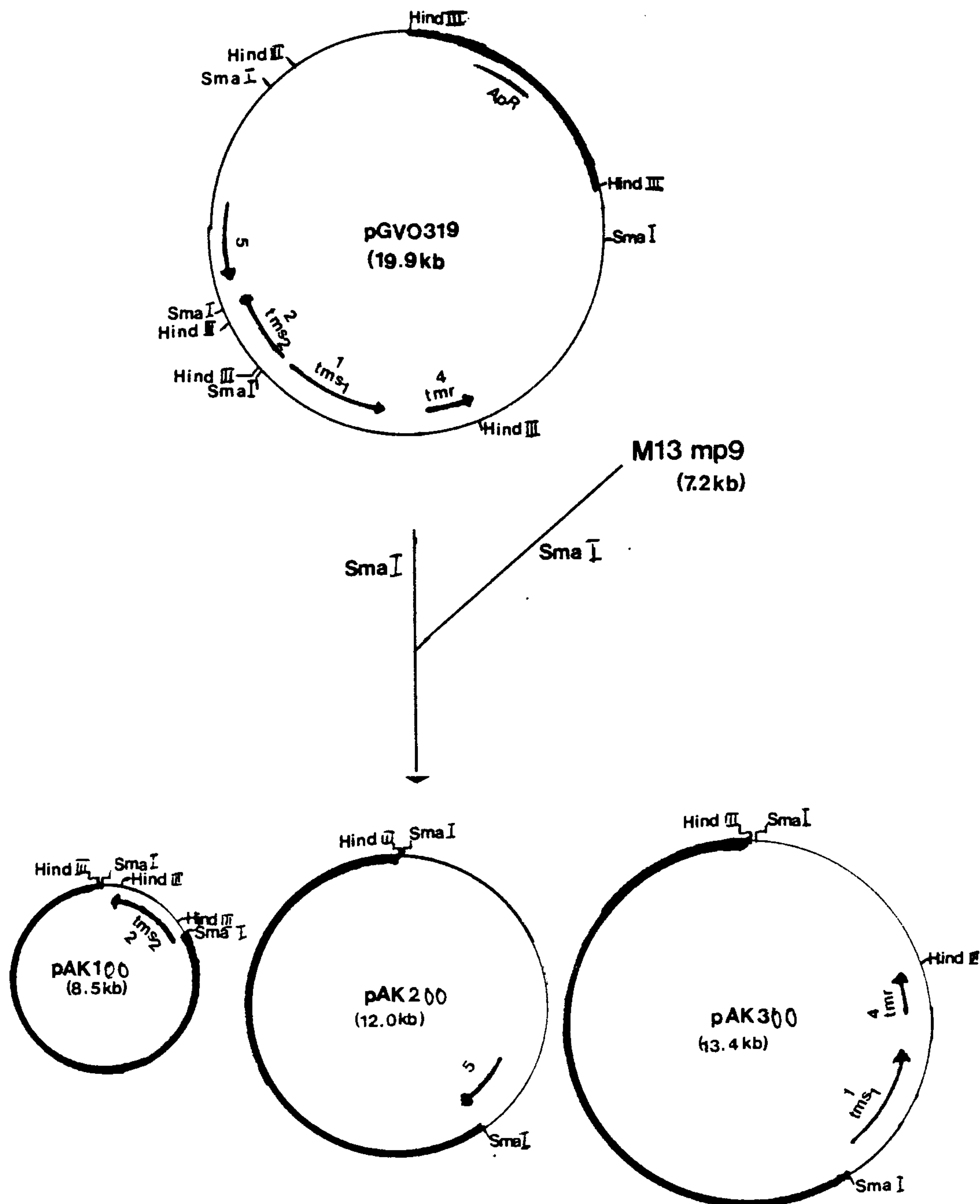


Fig. 3.2: **Restriction and functional maps of plasmids used as probes in Southern blotting** (Joos et al., 1983) Shown also is the strategy for the subcloning of the conserved T-DNA region of pGV0319 into M13 mp9

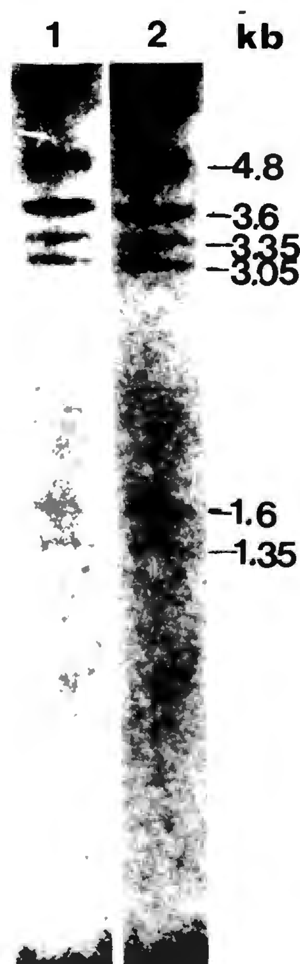


Fig. 3.2: **Autoradiograph of healthy and infected P. cynaroides DNA probed with pGV0319.** Lanes 1 and 2 each contain healthy and teratoma P. cynaroides DNA restricted with BglII R.E.

Genomic DNA was subsequently isolated from healthy leaves on P. cynaroides PC3 and PC6, both infected plants, and probed with pGV0319 (Fig. 3.4). The same pattern of homologous BglII R.E. fragments was detected in both plants. Four

homologous HindIII R.E. fragments of 6.0, 5.5, 3.0,and 2.8 kb were detected. There appeared to be no difference between tissue from two different infected plants. In order to determine unequivocally that these fragments were not involved in tumour induction one would need to probe DNA restricted with a number of different enzymes to identify restriction polymorphisms. This avenue was not pursued because of results obtained in further hybridisation experiments.

As homologous fragments appeared in both healthy and infected tissue it was necessary to prove that these were homologous to T-DNA sequences on pGV0319 and not due to homology with vector or contaminating E. coli chromosomal DNA. The conserved T-DNA region was subcloned into M13 mp9 (Fig. 3.2). To confirm that the subclones contained the correct inserts and also that there was no homology between M13 and pBR322, the clones were Southern blotted and probed with pGV0319 (Fig. 3.5).

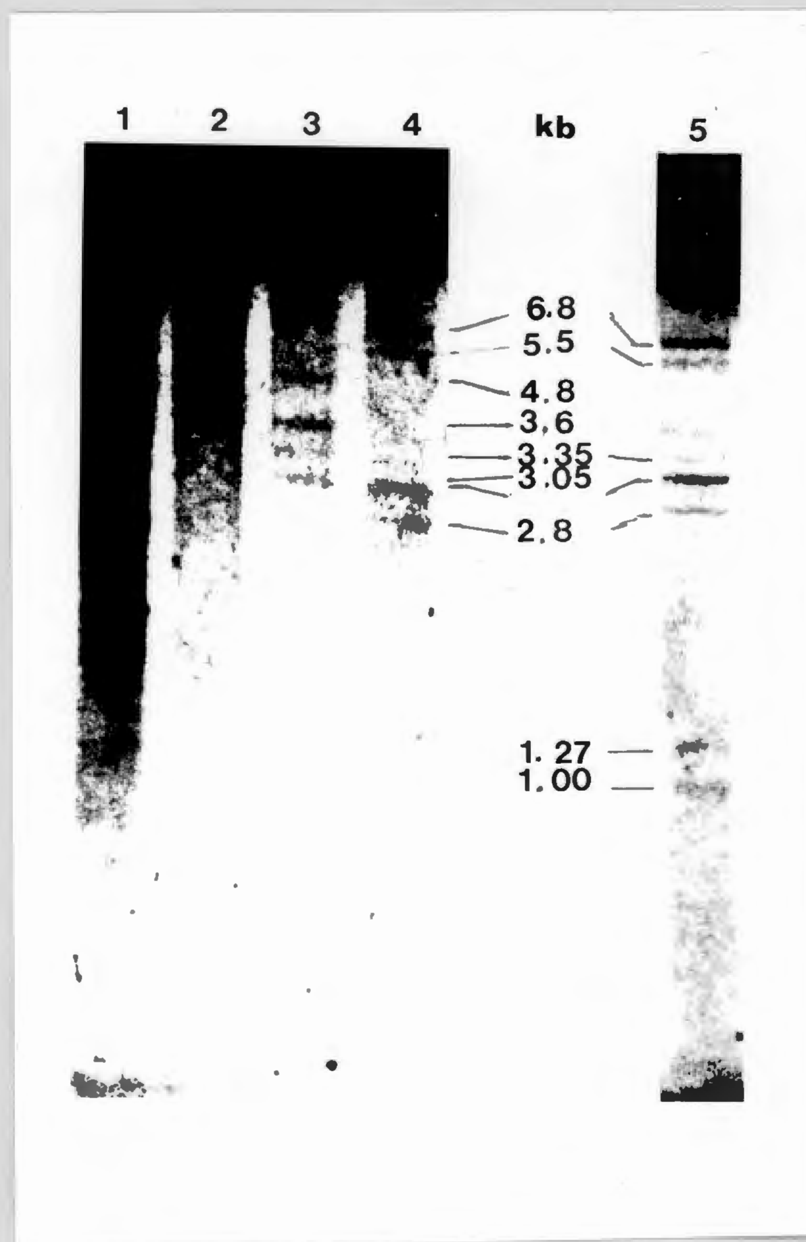


Fig 3.4: **Autoradiograph of healthy P. cynaroides DNA probed with pGV0319.** Lane 1 contains E. coli chromosomal DNA restricted with HindIII R.E.; lane 2 contains barley genomic DNA restricted with HindIII R.E.; lanes 3 and 4 contain P. cynaroides PC3 genomic DNA restricted with BglII R.E. and HindIII R.E. respectively; lane 5 contains healthy P. cynaroides PC6 genomic DNA restricted with HindIII R.E.. Specific activity of probe: 1.27×10^8 cpm/ug

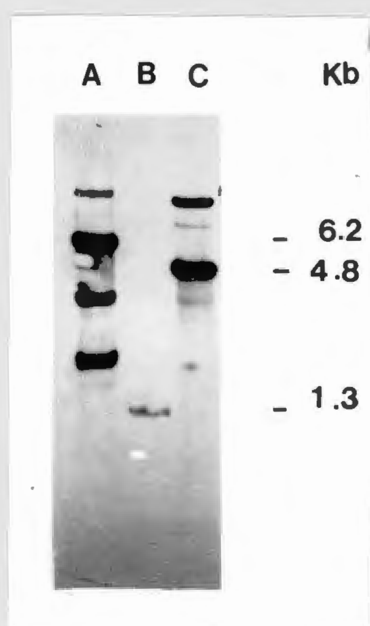


Fig. 3.5: **Autoradiograph showing hybridisation of DNA from M13 T-DNA subclones to pGV0319.** Lane 1, pAK300; 2, pAK100 and 3, pAK200 all restricted with SmaI R.E.

This experiment showed that the SmaI R.E. fragments subcloned into M13 came from pGV0319 and also that there was no homology with the M13 SmaI R.E. fragment (7.2 kb). The autoradiograph also showed that the insert in pAK300 was unstable and prone to deletions.

Genomic DNA from P. cynaroides PC3 was restricted with HindIII and BglII R.E.s. In addition, included on the gel were lanes containing genomic DNA (isolated concurrently with genomic DNA from P. cynaroides) from healthy barley plants and E. coli chromosomal DNA, both of which were restricted with BglII R.E.. The filter was probed first with a combined probe containing pAK100, pAK200 and pAK300; subsequently it was stripped and reprobed with pBR322 and E. coli chromosomal DNA.

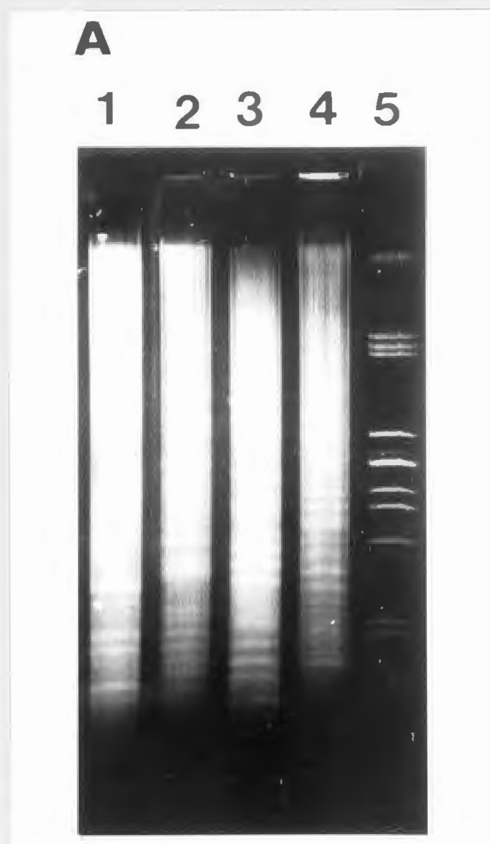


Fig. 3.6A: **Agarose gel of healthy P. cynaroides genomic DNA.** Lane 1 contains P. cynaroides genomic DNA restricted with HindIII R.E.; lane 2 contains P. cynaroides DNA restricted with BglII R.E.; lane 3 contains barley genomic DNA restricted with HindIII R.E.; lane 4 contains E. coli chromosomal DNA restricted with HindIII R.E.; lane 5 contains a lambda PstI R.E. molecular mass standard

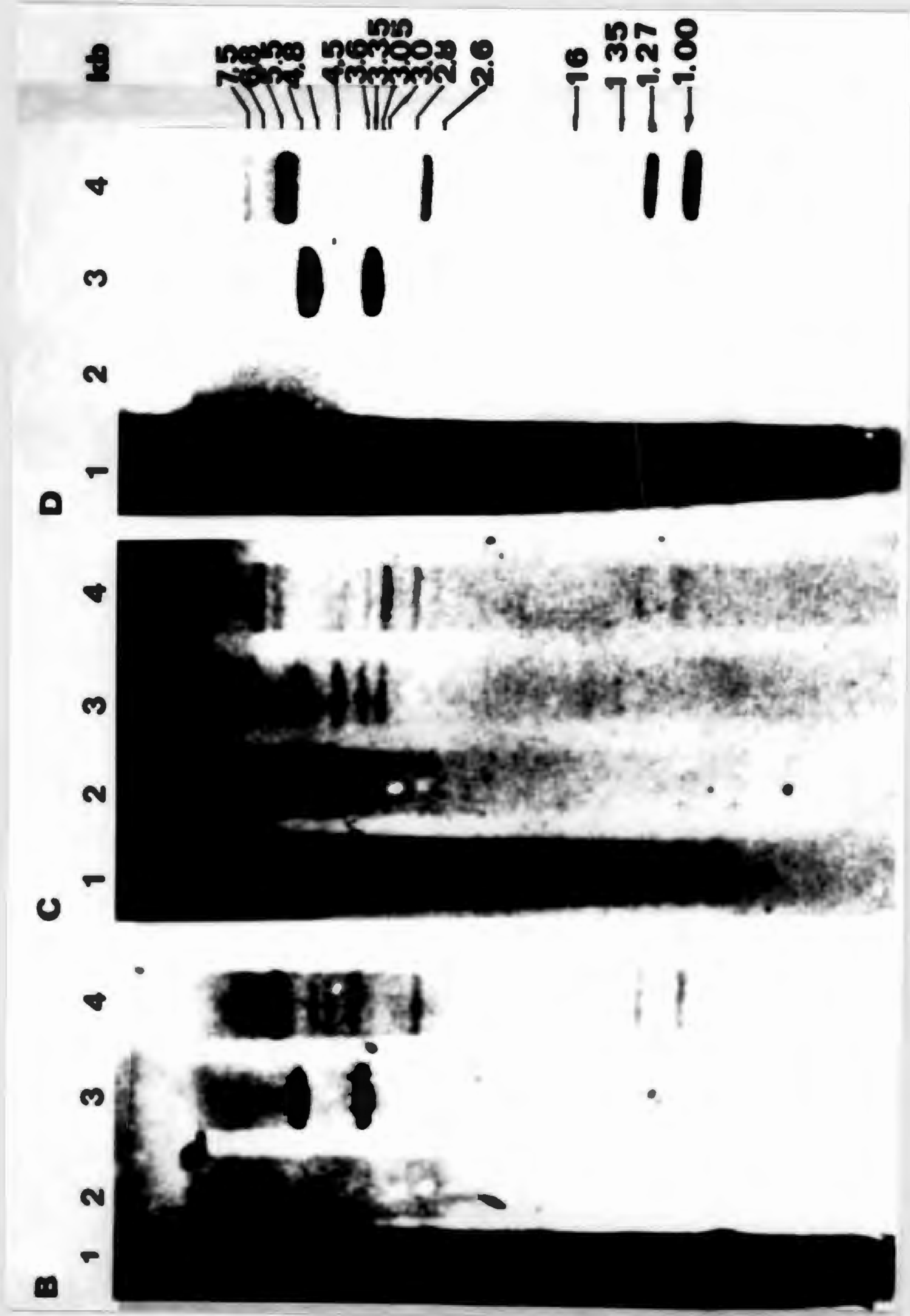


Fig. 3.6B-D: (Legend on following page)

Fig. 3.6B-D: (preceding page): **Autoradiograph of healthy P. cynaroides PC3 DNA probed with B: a combined probe containing pAK100, pAK200 and pAK300; C: pBR322 and D: E. coli chromosomal DNA.** Lane 1: E. coli chromosomal DNA restricted with HindIII R.E.; Lane 2: barley genomic DNA restricted with BglII R.E.; Lane 3: PC3 restricted with BglII R.E.; Lane 4 PC3 restricted with HindIII R.E.. . Specific activities of probes: 1-3x10^8 cpm/ug

P. cynaroides genomic DNA (Fig. 3.6A) when probed with T-DNA fragments cloned into M13 contained 6 homologous HindIII restriction R.E. fragments (Fig. 3.6B). The molecular masses of these fragments were 5.5, 4.5, 3.5 2.8, 1.27 and 1.0 kb each. The 5.5 and 2.8 kb fragments were also detected when the genomic DNA was probed with pGV0319 (Fig. 3.4). DNA restricted with BglII R.E. probed with the M13 clones showed fragments of 4.8 and 3.35 kb in size which corresponded to 2 of the 4 DNA fragments detected when pGV0319 was used as a probe. No homologous fragments were detected in the lane containing barley genomic DNA although there was some non-specific binding. A strong, but non-specific signal from the lane containing E. coli chromosomal DNA indicated that there was some chromosomal contamination of the probe.

When the gel was stripped and reprobed with nick-translated pBR322, all the fragments that had been detected with pGV0319 were present (Fig. 3.5C). In addition 2 additional HindIII R.E. fragments of 1.27 and 1.0 kb were detected which were also present when the gel was probed with pAK100-300. All 6 BglII R.E. fragments corresponded to those

detected when the DNA was probed with pGV0319. No fragments were detected in the lane containing barley DNA and there was a strong, non-specific signal from the E. coli chromosomal DNA.

The filter was subsequently stripped and reprobed with E. coli chromosomal DNA. The lane containing barley genomic DNA showed no homologous DNA fragments (Fig. 3.5D). There were 2 BglII R.E. P. cynaroides DNA fragments showing homology to E. coli chromosomal DNA. The fragments were the same size as those detected with the M13 probes. Six homologous HindIII R.E. fragments were detected and 4 were similar in size to those detected with the M13 probe and two 2 7.5 and 2.6 kb) were novel. Some but not all of these fragments were detected with pBR322 as a probe.

These results can be summarised as follows: 1) There appears to be no difference in hybridisation to pGV0319 between BglII R.E. DNA fragments of healthy and teratoma genomic DNA; 2) Both BglII and HindIII R.E. DNA fragments detected using pGV0319 as a probe were also detected when pBR322 was used as a probe; 3) When M13 was used as vector for the T-DNA fragments, some, but not all of the R.E. DNA fragments detected with pBR322 and pGV0319 were also detected. In addition, some novel HindIII R.E. DNA fragments were

detected; 4) E. coli chromosomal DNA showed homology to the same BglII fragments as those homologous to the M13 subclones and some HindIII R.E. DNA fragments were novel.

From these results it can be concluded that the DNA fragments identified when P. cynaroides DNA was probed with pGV0319 were not due to homology with the T-DNA fragments carried on the plasmid. Some fragments (the 6.8, 3.3 and 3.0 kb HindIII R.E. DNA fragments and the 3.6, 3.05, 1.6 and 1.35 kb BglII R.E. DNA fragments) were probably due to sequences homologous to pBR322 sequences while the rest were due to varying contamination of probes with E. coli chromosomal DNA. This contamination occurred despite purification of the plasmid DNA through two caesium chloride density ultracentrifugation steps.

These results indicate that the genes involved in the developments of Witches' Broom tumours are not homologous to the T-DNA genes of A. tumefaciens. Results presented in Chapter 2 indicated that the growth regulator cytokinin may be involved in the formation of Witches' Broom teratomas. In an article published subsequent to the start of this work, Hansen et al. (1986) present evidence for a gene from habituated tobacco cells which was able to complement a mutation in the tmr locus of the A. tumefaciens T-DNA. This implies that the plant gene is involved in the synthesis of cytokinin. However, there did not appear to be any homology

between the plant and *A. tumefaciens* genes. Furthermore, the *tmr* gene, unlike the *tms* loci, has not been shown as yet to have any homologues in other pathogens. It would therefore seem unlikely that the genes involved in Witches' Broom disease development would show homology to genes on the *A. tumefaciens* T-DNA.

CHAPTER 4: **GENERAL CONCLUSIONS**

This study was aimed at obtaining an understanding of the cause and development of Witches' Broom disease of P. cynaroides. This was approached in two ways: firstly, from a physiological and pathological angle and secondly the genetic level.

Observations of plants growing in the field and also of tissue cultured material indicated that the disease involved the altering of the growth regulator status of the plant. Teratoma tissue behaved differently in tissue culture from material cultured from healthy tissue on the same plant. This suggests that there has been some kind of transformation in the affected cells.

The cause of such a transformation could be either physiolgical or genetic. In the event of the former being the case, the cause could be an external factor such as a pathological infection. Among the pathogens that could be responsible are MLO's, P. savastanoi, and WTV.

The conventional method for the identification of mMLO's is by electron microscopy and this procedure was followed to

identify these organisms in the vascular tissues of infected plants. No such infection was identified. It is possible that the pathogen was present in the vascular bundles of infected plants, but were not detected.

It is unlikely that the disease could be caused by *P. savastanoi* as the symptoms of *P. savastanoi*-infected plants differ from those of Witches' Broom tumours. Furthermore, *P. savastanoi* was not isolated from diseased material and transmission of the disease was unsuccessful. However, attempts at culturing a possible pathogen were not extensive and this possibility cannot therefore be ruled out.

It is unlikely that WTV is responsible for Witches' Broom disease on *P. cynaroides* as the symptoms produced are different (galls as opposed to shooty teratomas). The virus affects a narrow host range and also passes through a leaf-hopper species as an intermediate host.

If the development of Witches' Broom disease is as a result of a genetic transformation of the affected plant cells, the most obvious pathogen would be *A. tumefaciens* or *A. rhizogenes*. The results of this study indicate that an *Agrobacterium* is not responsible for the disease. Firstly, no *A. tumefaciens* was isolated and no success was achieved with attempts to infect *P. cynaroides* seedlings with four representative *A. tumefaciens* strains. Secondly, no

sequences in the P. cynaroides genome hybridised with a probe containing the conserved area of the T-DNA which is present in all transformed cells. The experimental conditions were sensitive enough to detect A. tumefaciens T-DNA sequences in the plant genome because sequences homologous to E. coli chromosomal contamination were detected in the genome of the P. cynaroides plants as well as sequences which appeared to show homology to pBR322 sequences.

An A. tumefaciens strain with T-DNA which is not homologous to the T-DNA of octopine and nopaline Ti plasmids could alternatively be reponsible for the disease. No success was achieved in infecting a P. cynaroides plant with such a strain and therefore this possiblility seems remote.

What may be significant is the fact that E. coli chromosomal homologues were detected in P. cynaroides but not in barley from which the DNA was extracted concurrently with that of P. cynaroides. If the detection of these sequences was due to bacterial contamination of the plant material during processing, then both should have contained these sequences.

Another possible explanation for the detection of the E. coli-homologues in P. cynaroides is that the sequences do not originate from the genome of the plants, but from the chromosome of the pathogen present in the plant cells. A number of genes are conserved across most prokaryotes. In addition, the use of a radioactive DNA probe is far more sensitive for the detection of a bacterial pathogen than electron microscopy of infected tissue. Genomic DNA from healthy tissue on infected plants also contained the E. coli-homologous fragments. If the infection of the plant was a systemic one, as in the case of an MLO-infection, but not an A. tumefaciens-infection, healthy tissue on an infected plant would also contain the pathogens. Treatment of infected plants and tissue cultured teratoma tissue with antibiotics such as teracycline could indicate whether a bacterial infection is the cause of the diseas.

The results indicate that an A.t tumefaciens-type infection is not responsible for Witches' Broom disease of P. cynaroides, although no pathogen was positively identified as the causal agent of the condition.

APPENDIX

APPENDIX 1: **GENERAL TECHNIQUES.**

1 **Bacterial chromosomal DNA isolation**

E. coli was grown overnight at 37°C in 200ml Luria broth and the cells were pelletted by centrifugation at 7000 rpm for 5 min in a Sorval GSA rotor. Cells were resuspended in 1 ml lysis buffer per 50 ml overnight culture (Lysis buffer: 10 mM Tris pH 8.0; 100 mM Na_2.EDTA; 100 mM NaCl). The solution was made up to 1% (w/v) SDS from a 25% stock solution and left on ice for 10 to 20 min to lyse after which caesium chloride was added to an relative density of 0.384-0.396, Ethidium bromide (400 ug/ml) was added. The solution was centrifuged at 12000 rpm for 10 min in a Sorval SS34 rotor and centrifuged in a Beckman vertical Ti65 rotor at 55000 rpm for at least 8 h.

2 **Plasmid DNA isolation** (Ish-Horowicz and Burke, 1981)

A 200 ml overnight culture of the *E. coli* strain carrying the plasmid was centrifuged at 5000 rpm in a Sorval GSA rotor for 7 min and the pellet resuspended in 4 ml of Solution 1 (50 mM Glucose; 25 mM Tris pH 8.0;10 mM Na_2.EDTA) and incubated at room temperature for 5 min in a Sorval SS34 centrifuge tube. Eight ml of freshly made Solution 2 (0.2 M NaOH; 1% SDS) were added and the tube gently rolled to mix

throughly. The solution was incubated on ice for 5 min to lyse the cells after which 6 ml of ice cold Solution 3 (5 M KOAc, pH 4.8) was added mixed throughly and the tube returned to ice for a further 10 min. The lysate was centrifuged at 4°C and 10000 rpm for 10 min after which the DNA was precipitated at room temperature by the addition 2 volumes of 96% ethanol. Plasmid DNA was resuspended in 4 ml TE buffer and subjected to two cycles of ultracentrifugation as described above.

3 **Isolation of Plant DNA** (Ketterman and Shattuck, 1983)

Plants were ground under liquid nitrogen with a mortar and pestle until fine and then ground under buffer (1 M glucose: 0.1 M citric acid, pH 5.0; 0.5% Triton X-100) at 4°C after which the material was squeezed through a muslin cloth into a cold centrifuge tube. Material remaining in the muslin cloth was extracted a further two times by grinding under buffer and the combined filtrate was then centrifuged at 5000 rpm for 10 min at 2°C. The resulting pellet was resuspended in cold grinding buffer and recentrifuged. This was repeated until the pellet was pale cream in colour. The pellet was resuspended in a wash buffer (0.5 M glucose; 0.05 M citric acid, pH 5.0) and centrifuged as before. This step was repeated twice.

The pellet was subsequently lysed in a buffer containing 1% SDS; 0.14 M NaCl and 0.1 M Na_2.EDTA pH 8.3 at 50°C for 10 min after which cellular debris was pelleted by centrifugation at 5000 rpm for 10 min at 2°C. The resulting pellet was relysed twice. The combined supernatnats were precipitated by adding 2 volumes of ethanol and 1/9 volumes 3 M Na OAc pH 5.0 and incubating at -70°C for 30 min. The DNA precipitate was collected by centrifugation at 10000 rpm for 10 min at 0°C and the pellet resuspended in a small volume of TE buffer. DNA was removed by adding DNAse-free RNAse to a total concentration 50 ug/ml and incubating on ice for 30 min. The DNA was extracted with chloroform/isoamyl alcohol (24:1) and then concentrated by ethanol precipitation.

4 **Removal of ethidium bromide and caesium chloride**

Ethidium bromide was removed by repeated extractions with NaCl-saturated isopropanol (pH 8.3). The DNA was concentrated by diluting the solution with 2 volumes of distilled water and adding and equal volume of isopropanol. After centrifugation in an Eppindorf microfuge for 10 min, the pellet was resuspended in 400ul of TE buffer. The DNA is reprecipitated by the addition of 40 ul of 5M $NaClO_3$ and 1 ml of cold 96% ethanol, incubation on ice for at least 10 min and then centrifugation in an Eppindorf microfuge at 4°C

for at least 10 min. The pellet was washed twice with cold 70% ethanol and allowed to dry either in air for 20 min or under a vacuum for 5 min. The pellet was resuspended in 500 ul of TE buffrer and the DNA concentration estimated from the absorbance at 260 nm.

5 Restriction endonuclease digestion conditions

All digenstsions were done at 37°C with 2 units of enzyme added per ug of DNA. Buffers were made up as in Maniatis *et al.* (1982).

6 Cloning techniques

After R.E. digestion, the DNA was extracted twice with phenol (Maniatis *et al.*, 1982) and then twice with ether to remove residual phenol. The DNA was concentrated by ethanol precipitation and resuspended in TE buffer. Ligations were done at 4°C overnight (ligation mix: 10 mM ATP, 1x ligation buffer (Maniatis *et al.*, 1982), 1 unit T4 DNA ligase per 25 ul ligation mix) and both vector and insert DNA were at a concentration of 5 pM. Preparation of competent cells, transformations and preparation of plates was as described in the Amersham M13 cloning and sequencing handbook.

7 Nick-translation conditions

The Amersham nick-translation kit was used for labelling of probes with ^{32}P dCTP. The nick-translation mixture was made up as follows: 0.5 ug of DNA was labelled in 25 ul

containing 0.050 mCu ^{32}P-labelled dCTP, nick-translation buffer (5ul) and enzyme mix (2.5 ul). The mix was incubated at 15°C for 2 h after which the DNA was passed through a Sephdex G50 spin column (Maniatis et al., 1982).

APPENDIX 2: **MEDIA, BUFFERS AND SOLUTIONS**

1 **Media**

1.1 Anderson's Medium (Anderson, 1975) (100x stock solutions except for solution H which is a 2x solution)

A.	NH_4NO_3	40 ug/ml
	KNO_3	48 ug/ml
B.	$MgSO_4.7H_2O$	37 ug/ml
	$MnSO_4.H_2O$	1.69 ug/ml
	$ZnSO_4.7H_2O$	0.86 ug/ml
	$CuSO_4.5H_2O$	0.0025 ug/ml
C.	$CaCl_2.2H_2O$	44 ug/ml
	KI	0.03 ug/ml
	$CoCl_2.6H_2O$	0.0025 ug/ml
D.	H_3BO_3	0.62 ug/ml
	$Na_2MoO_4.2H_2O$	0.25 ug/ml
	$NaH_2PO_4.H_2O$	38 ug/ml
E.	$FeSO_4.7H_2O$	5.57 ug/ml
	$Na_2.EDTA$	7.45 ug/ml
F.	Thiamine.HCl	0.1 ug/ml
	Nicotinic acid	0.05 ug/ml
	Pyrodoxine.HCl	0.05 ug/ml
	glycine	0.20 ug/ml
G.	Myo-inositol	10 ug/ml
H.	1.8% H_2O agar (Oxoid Purified Agar)	
*I.	NAA	200 ug/ml
**J.	kinitin	2.00 mg/ml

* Dissolve in a few drops of 1N NaOH and make up to final volume with distilled H_2O. Make up fresh and filter sterilise.

** Dissollve in a few deops of 1N HCl and then make up to final volume with distilled H_2O. Filter sterilise and store at 4°C

Solutions A-H are sterilised by autoclaving and make up a 2x solution using stock solutions A-G, and adding an equal volume of H_2O agar, subsequently adding the relavant of solutions I and J. Ten ml slopes are poured in sterilestandard containers from which the rubber seals have been removed.

1.2 Luria Broth: (per litre)
10 g NaCl
10 g Tryptone
5 g Yeast extract

1.3 Luria Agar: 15 g bacto agar per liter Luria broth

1.4 2 x TY broth: (per litre)
16 g bacto tryptone
10 g yeast extract
5 g NaCl

1.5 2 x TY Agar: 15 g bacto agar per litre 2xTY broth

1.6 H-top agar: (per litre)
10 g bacto tryptone
5 g NaCl
8 g bacto agar

2 Buffers and Solutions

2.1 TE Buffer: 10 mM Tris.Cl (pH 8.0)
1 mM Na_2 EDTA

2.2 10x TBE Buffer: 89 mM Tris.OH (pH 8.3)
89 mM Boric acid
2.5 mM Na_2 EDTA

2.3 20 X SSPE: 174g NaCl (per litre)
27.6g $NaH_2PO_4.H_2O$ (pH 7.4 with NaOH)
7.4g Na_2 EDTA

2.4 Phosphate buffer: 0.05 M PO_4^{2-} (pH 7.4)

2.5 IPTG: (stock solution) 238 mg in 10 ml H_2O frozen at -70^oC

2.6 X-Gal: 2% X-Gal in dimethylformamide stored at -70^oC.

APPENDIX 3: **REFERENCES**

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

Alt-Moerbe, J, B Rak, and J Schroder. 1986. A 3.6-kbp segment from the vir region of the Ti plasmids contains genes responsible for border sequence-directed production of T region circles in E. coli. EMBO J. 5:1128-1135.

Anderson, W C,. 1975. Propogation of Rhododendrons by tissue culture: Part 1 development of a culture medium for multiplication of shoots. Int. Plant Propogation Society 25:129-135.

Asamizu, T, D Summers, M B Motika, J A Anzola, and D L Nuss. 1985. Molecular cloning and characterisation of the Wound Tumour Virus; A tumour inducing plant reovirus. Virology 144:398-409.

Barker, R F, K B Idler, D V Thompson, and J D Kemp. 1983. Nucleotide sequence of the T-DNA region from the Agrobacterium tumefaciens octopine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

Bayer, M H,. 1982. Genetic tumours: physiological aspects of tumor formation in interspecies hybrids. Molecular Biology of Plant Tumours. (Kahl, G and J Schell, eds) pp 33-69. Academic Press Inc, New York.

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

Ben Jaacov, J, G Jacobs. 1986. Establishing Protea Leucospermum and Serruria in vitro. Acta Horticulture 185:146-158.

Binns, A N, F Jr Meins. 1973. Evidence that habituation of tobacco pith cells for cell division-promoting factors is heritable and potentially reversible. Proc. Natl Acad. Sci. USA 70:2660-2662.

Binns, A N, F Jr Meins. 1979. Cold sensitive expression of cytokinin habituation by tobacco pith cell in culture. Planta 145:365-369.

Black, L M,. 1945. A virus tumor disease of plants. Am. J. Bot. 32:408-415.

Black, L M,. 1957. Viruses and other pathogenic agents in plant tissue culture. J. Natl. Cancer Inst. 19:663-685.

Black, L M, C L Lee. 1957. Interaction of growth regulating chemicals and tumefaciens virus on plant cells. Virology 3:146-159.

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

Bomhoff, G, P M Klapwijk, H C M Kester, R A Schilperoort, J P Hernalsteens, and J Schell. 1976. Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of Agrobacterium tumefaciens. Mol. Gen. Genet. 145:177-181.

Boulanger, F, A Berkaloff, and F Richaud. 1986. Identification of hairy root loci in the T-region of Agrobacterium rhizogenes plasmids. Plant Mol. Biol. 6:271-279.

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

Braun, A C,. 1972. The relevance of plant tumors in the understanding of the basic cellular mechanisms underlying tumorigenesis. Prog. Exp. Tumor Res. 15:165-187.

Braun, A C,. 1978. Crown gall disease. Biochem. Biophys. Acta 516:167-196.

Braun, A C,. 1982. A history of the crown gall problem. Molecular Biology of Plant Tumors (Kahl G and Schell J J eds.):pp 155-210. Academic Press Inc, New York.

Braun, A C, H N Wood. 1976. Suppression of the neoplastic state with the acquisition of specialised functions in cells, tissues and organs of crown gall teratomas of tomato. Proc. Natl. Acad. Sci. USA 75:496-500.

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

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

Buchmann, I, F J Marner, G Schroder, S Waffenschmidt, and J Schroder. 1985 . Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. EMBO J. 4:853-859.

Caplan, A B, M Van Montagu, and J Schell. 1985. Genetic analysis of integration mediated by single T-DNA borders. J. Bacteriol 161:1655-1664.

Casper, R, D Lesemann, and R Bartels. 1970. Mycoplasma-like bodies and viruses in Opuntia tuna Witches' Broom disease. Plant Disease Reporter 54:851-853.

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

Chilton, M-D, R S Sciaky, N Yadav, M P Gordon, and F Quetier. 1980. T-DNA from Agrobacterium Ti plasmid is in the nuclear DNA fraction of crown gall tumour cells. Proc. Natl. Sci. USA 77(7):4060-4064.

Chilton, M-D, D A Tepfer, A Petit, C David, and F Casse-Delbart. 1982. Agrobacterium rhizogenes inserts T-DNA into the genome of the host plant root cells. Nature (London) 295:432-435.

Chilton, S W, J Tempe, M Matzke, and M-D Chilton. 1984. Succinopine: a new crown gall opine. J. Bacteriol.:157-362.

Close, T J, R C Tait, and C I Kado. 1985. Regulation of Ti plasmid virulence genes by a chromosomal locus. J. Bacteriol. 164:774-781.

Coetzee, J F,. 1982. Anatomie en klassifikasie van Acarea met spesiale verwysing na Eriophyoideae (ook Biologie) Manuscript: Dept Entomologie, Universiteit Stellenbosch, Stellenbosch, 7600 :-.

Constantino, P, L Spano, N Pomponi, E Benvenuto, and G Ancora. 1984. The T-DNA of Agrobacterium rhizogenes is transmitted through meiosis to the progeny of hairy root plants. J. Mol. Appl. Genet. 2:465-470.

Currier, T C, E W Nester. 1976. Evidence for diverse types of large plasmids in tumor- inducing strains of Agrobacterium tumefaciens. J. Bacteriol. 126:157-165.

David, C, M-D Chilton, and J Tempe. 1984. Conservation of T-DNA in plants regenerated from hairy root cultures. Biotechn. Jan 1984:75-78.

De Vos, M P,. 1945. Cytogenetic studies in Genera of Proteaceae. SA J. Sci. 40:113-122.

DeCleene, M, J DeLey. 1976. The host range of crown gall. Bot. Rev. 42:389-446.

Dellaporta, S L, J Wood, and J B Hicks. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Reporter 1:19-21.

Depicker, A, G De Wilde, R De Vos, M De Vos, M Van Montagu, and J Schell. 1980. Molecular cloning of overlapping segments of the nopaline plasmid pTiC58 as a means to restriction endonuclease mapping. Plasmid 3:193-211.

Depicker, A, M Van Montagu, and J Schell. 1978. Homologous DNA sequences in different Ti-plasmids are essential for oncogenesis. Nature (London) 275:150-152.

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

Dreger-Jauffret, F,. 1980. Observations sur le developpement des bourgeons des balais de sorciere du Prunier. Can. J. Bot. 58:1341-1346.

Drummond, M H, M P Gordon, and E W Nester. 1977. Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumours. Nature (London) 269:535-536.

Durand-Tardif, M, R Broglie, J Sleighton, and D Tepfer. 1985. Structure and expression of Ri T-DNA from Agrobacterium rhizogenes in Nicotiana tabacum (organ and phenotypic specificity). J. Mol. Biol. 186:557-564.

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

Engler, G, A Depicker, R Maenhaut, R Villaroel-Mandiola, M Van Montagu, and J Schell. 1981. Physical mapping of DNA base sequence homology between an octopine and a nopaline Ti plasmid of Agrobacterium tumefaciens. J. Mol. Biol. 152:183-308.

Engler, G, M Holmes, M Van Montagu, J Schell, J P Hernalsteens, and R A Schilperoort. 1975. Agrocin sensitivity: a plasmid determined property in Agrobacterium tumefaciens. Mol. Gen. Genet. 138:345-349.

Evans, H C,. 1980. Pleomorphisms in Crinipellis perniciosa, causal agent of Witches' Broom disease of cocoa. B. Mycol. Soc. 74(3):515-523.

Firmin, L, R G Fenwick. 1978. Agropine, a new plasmid determined metabolite in crown gall tumours. Nature (London) 276:842-844.

Follin, A, D Inze, F Budar, C Genetello, M van Montagu, and J Schell. 1985. Genetic evidence that the tryptophan 2-monooxygenase gene of Pseudomonas savastanoi is functionally equivalent to one of the T-DNA genes involved in plant tumour formation by Agrobacterium tumefaciens. Mol. Gen. Genet. 201:178-185.

Furner, I J, G A Huffman, R M Amasino, D J Garfinkel, M P Gordon, and E W Nester. 1986. An Agrobacterium transformation in the evolution of the genus Nicotiana. Nature (London):319-422.

Ganatos, P J, I Tamm. 1963. Animal and plant viruses with double helical RNA. Proc. Natl. Acad. Sci. U.S.A. 50:878-885.

Gardner, R C, V C Knauf. 1986. Transfer of Agrobacterium DNA to plants requires a T-DNA border but not the virE locus. Science 211:725-727.

Garfinkel, D J, E W Nester. 1980. Agrobacterium tumefaciens mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. 144(2):732-743.

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

Genetello, C, N Van Larebeke, M Holsters, A Depicker, M Van Montagu, and J Schell. 1977. Ti plasmids of Agrobacterium tumefaciens as conjugation plasmids. Nature(London) 265:561-563.

Gielen, J C, M De Beuckeleer, J Seurinck, F Deboeck, H De Greve, M Lemmers, M Van Montagu, and J Schell. 1984. The complete nucleotide sequence of the T-DNA of the Agrobacterium tumefaciens plasmid pTiAch5. EMBO. J. 3:835-846.

Goodman, T, A L Montoya, S Williams, and M-D Chilton. 1986. Sustained ethylene production in Agrobacterium-transformed carrot disks caused by expression of the T-DNA tms gene products. J. Bacteriol. 167:387-388.

Gurley, W B, J D Kemp, M J Albert, D W Sutton, and J Callis. 1979. Transcription of Ti plasmid plasmid-derived sequences in three octopine-type crown gall rumor lines. Proc. Natl. Acad. Sci. 76:2828-2832.

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

Hamilton, W D O, D M Bisaro, and K W Buck. 1982. Identification of novel DNA forms in tomato golden mosaic virus infected tissue. Evidence for a two component viral genome. Nucl. Acids Res. 10:4901-4911.

Heidekamp, F, W G Dirkse, J Hille, and H Van Ormonde. 1984. Nucleotide sequence of the Agrobacterium tumefaciens octopine Ti plasmid-encoded tmr gene. Nucl. Acids Res. 11:6211-6223.

Hepburn, A G, J White. 1985. The effect of right terminal deletion on the oncogenicity of the T-region of pTiT37. Plant. Mol. Biol. 5:3-11.

Hess, D, G Schneider, H Lotz, and G Blaich. 1976. Investigation on the tumor induction in Nicotiana glauca by pollen transfer of DNA isolated from Nicotiana langsdorffii. Z. Pflanzenphysiol. 77:247-254.

Heyn, F, A Hermans, and R A Schilperoort. 1974. Rapid and efficient isolation of highly polymerized plant DNA. Plant Sci. Letters 2:73-78.

Hille, J, J Van Kan, and R A Schilperoort. 1984. Trans-acting virulence functions of the octopine Ti plasmid from Agrobacterium tumefaciens. J. Bacteriol. 158:754-756.

Hille, J, G Wullems, and R A Schilperoort. 1983. Non-oncogenic T-region mutants of Agrobacterium tumefaciens do transfer T-DNA into plant cells. Plant. Mol. Biol. 2:155-163.

Hiruki, K, J Gianotti, and J Dijkstra. 1974. Comparative study of fluorescence in stems of Vinca rosea infected with mycoplasmas of different plant origins. Neth. J. Pl. Path. 80:148-153.

Hoekema, A, P R Hirsch, P J J Hooykaas, and R A Schilperoort. 1983. A binary plant vector strategy based on separation of the vir and T-region of the Agrobacterium Ti plasmid. Nature (London) 303:179-181.

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

Holsters, G, R Villaroel, J Gielen, J Seurinck, H De Greve, M Van Montagu, and J Schell. 1983. An analysis of the boundaries of octopine TL-DNA in tumors induced by Agrobacterium tumefaciens. Mol. Gen. Genet. 190:35-41.

Holsters, M, B Silva, F Van Vliet, C Genetello, M De Block, P Daewe, A Depicker, I Inze, G Engler, R Villaroel, M Van Montagu, and J Schell. 1980. The functional organisation of the nopaline Agrobacterium tumefaciens plasmid pTiC58. Plasmid 3:212-230.

Horsch, R B, H J Klee, S E Stachel, S C Winans, E W Nester, S G Rogers, and R T Fraley. 1986. Analysis of Agrobacterium tumefaciens virulence mutants in leaf discs. Proc. Natl. Acad.Sci. USA 83:2571-2175.

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

Hutzinger, O, T Kosuge. 1968. Microbial synthesis and degradation of indole-3-acetic acid.III. The isolation and characterisation of indole-3-acetyl-E-L-lysine. Biochem. 7:601-605.

Inzé, D, A Follin, M Van Lijsbetten, C Simons, C Genetello, M Van Montagu, and J Schell. 1984. Genetic analysis of the individual T-DNA genes of Agrobacterium tumefaciens; further evidence that two genes are involved in indole acetic acid synthesis. Mol. Gen. Genet. 194:265-274.

Ish-Horowicz D and J F Burke.1981. Rapid and efficient cosmid cloning. Nucl. Acid Res. 9:2989-2998.

Jen, G, M-D Chilton. 1986. Activity of T-DNA borders in plant. Cell transformation by mini-T plasmids. J. Bacteriol. 166:491-499.

Johnson, L A S, B L Briggs. 1975. On the Proteaceae - the evolution and distribution of a Southern family. Bot. J. Linn. Soc. 83:182-.

Joos, H, D Inze, A Caplan, M Sormoans, M Van Montagu, and J Schell. 1983. Genetic analysis of the nopaline Ti plasmid 25 bp repeat. Cell 32:1057-1067.

Joos, H, B Timmerman, M Van Montagu, and J Schell. 1983. Genetic analysis of transfer and stability for Agrobacterium T-DNA in plant. Cells. EMBO. J. 2:2151-2160.

Jouanin, L,. 1984. Restriction map of an agropine type Ri plasmid and its homology with Ti plasmids. Plasmid 12:81-102.

Kajanus, B,. 1917. Bastardie rungen zwischen Brassica najus L. und Brassica rapa. L. Z. Pflanzensucht 5:265-332.

Kalmaloff, J, L J Lawandowski, and D R Blade. 1969. Composition of the ribonucleic acid subunits of reovirus, cytoplasmic polynecrosis virus and wound tumour virus. J. Virology 4:831-826.

Kemp, D,. 1977. A new amino acid derivative present in crown gall tumor tissue. Biochem. Biophys. Res. Commun. 74:862-848.

Kemp, D, D W Sutton, and E Hack. 1979. Purification and characterisation of the crown gall-specific enzyme nopaline synthetase. Biochem. 18:3755-3760.

Kemper, E, S W Waffenscmidt, E W Weiler, T Rausch, and J Schroder. 1985. T-DNA=encoded auxin formation in crown gall. Cells Planta 163:257-262.

Ketterman, J, S Shuttuck. 1983. A method for the extraction of nuclear DNA from plants with a high phenolic content Prep. Biochem. 13:347-359.

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

Klee, H, A L Montoya, F Horoyski, C Lichtenstein, D Garfinkel, C Fuller, C Flores, J Peschon, E W Nester, and M P Gordon. 1984. Nucleotine sequence of the tms genes of the pTiA6NC octopine Ti plasmid: Two gene products involved in plant tumorigenesis. Proc. Natl. Acad. Sci. 81:1728-1732.

Klee, H J, M P Gordon, and E W Nester. 1982. Complementation analysis of Agrobacterium tumefaciens Ti plasmid mutations affecting oncogenicity. J. Bacteriol. 150(1):327-331.

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

Kleupfel, D A, S G Puepke. 1985. Isotherm for adsorption of Agrobacterium tumefaciens to susceptible potato (Solanum tuberosum L.)tissue. J. Bacteriol. 49:1351-1355.

Koekmans, B P, G Ooms, P M Klapwijk, and R A Schilperoort. 1979. Genetic map of an octopine Ti plasmid. Plasmid 2:347-357.

Komro, C T, V J DiRita, S B Gelvin, and J D Kemp. 1985. Site-specific mutagenesis in the TR-DNA region of octopine-type Ti plasmids. Plant. Mol. Biol. 4:253-263.

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

Leemans, J, R Deblaere, L Willmitzer, H De Greve, J P Hernalsteens, M Van Montagu, and J Schell. 1982. Genetic identification of functions of t-DNA transcripts in octopine crown gall tumours. EMBO. J. 1:147-152.

Lemmers, M, M De Beuckeleer, M Holsters, P Zambryski, A Depicker, J P Hernalsteens, M Van Montagu, and J Schell. 1980. Integration, organisation,boundaries and integration of Ti plasmid DNA in nopaline crown gall tumors. J. Mol. Biol. 144:353-374.

Letham, D S, G Q Tao, and C W Parker. 1982. An overview of cytokinin metabolism. In: Plant growth substances. (Wareing, P F, ed) Academic Press, London.

Lichtenstein, C, H Klee, A Montoya, D Garfinkel, S Fuller, C Flores, E W Nester, and M P Gordon. 1984. Nucleotide sequence and transcript mapping of the tmr gene of the pTiA6NC octopine Ti plasmid: a bacterial gene involved in plant tumorigenesis. J.Mol.Appl.Genet. 2:354-362.

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

Lippincott, B B, M H Whatley, and J A Lippincott. 1977. Tumour induction by Agrobacterium tumefaciens involves attachment of the bacteria to a site on the host cell wall. Plant Physiol. 59:388-390.

Lippincott, J A, R Beidebeck, and B B Lippincott. 1973. Utilisation of octopine and nopaline by Agrobacterium species. J. Bacteriol. 116:378-383.

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

Maniatis, T, Frisch, E F, and J Sambrook. 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor laboratory Press, Cold Spring Harbor New York.

Matthyse, A G,. 1983. Role of. Cellulose fibrils in Agrobacterium tumefaciens infection. J. Bacteriol. 154:906-915.

Meins, F Jr,. 1982. Habituation of cultured plant cells Molecular Biology of Plant Tumors (Kahl G and Schell J J eds.):pp 3-33.

Messens, E, A Lenaerts, M Van Montagu, and J Schell. 1985. Genetic basis for opine secretion from crown gall tumor cells. Mol. Gen. Genet. 199:344-348.

Meyer, M K P,. 1981. Mytplae in landbougewasse in Suid-Afrika FFTRI Scientific Bulletin 397:-.

Montoya, A L, M-D Chilton, M P Gordon, D Sciaky, and E W Nester. 1977. Octopine and nopaline metabolism in Agrobacterium tumefaciens and crown gall tumor cells: role of plasmid genes. J. Bacteriol. 129:101-109.

Nasu, S, D D Jensen, and J Richardson. 1970. Electron microscopy of mycoplasma-like bodies associated with insect and plant hosts of peach western X-disease. Virology 41:583-595.

Nienhaus, F, H Brussel, and U Schinzer. 1976. Soil-borne transmission of Rickettsia-like organisms found in stunted and witches' broom diseased larch trees (Larix decidua). Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 83:309-316.

Nuss, D L,. 1984. Molecular Biology of Wound Tumor Virus. Adv. Virus Res. 29:57-93.

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

Ooms, G, P J Hooykaas, G Moleman, and R A Schilperoort. 1981. Crown gall plant tumours of abnormal morphology induced by Agrobacterium tumefaciens carrying mutated octopine Ti plasmids; analysis of T-DNA functions. Gene 14:35-50.

Ooms, G, P J J Hooykaas, D P J M Van Veen, P Van Beelen, A J Regenburg-Tuinck, and R A Schilperoort. 1982. Octopine Ti plasmid deletion mutants of Agrobacterium tumefaciens with emphasis on the right side of the T-DNA. Plasmid 7:15-29.

Ooms, G, P Klapwijk, J A Poulis, and R A Schilperoort. 1980. Characterisation of Tn904 insertions in octopine Ti plasmid mutants of Agrobacterium tumefaciens. J. Bacteriol. 144(1):82-91.

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

Peralta, E G, L W Ream. 1985. T-DNA base sequences required for crown gall tumorigenesis. Proc. Natl. Acad. Sci. USA 82:5112-5116.

Puepke, S G, U K Benny. 1983. , Agrobacterium tumefaciens tumorigenesis in potato: effect of Agrobacterium lipopolysaccharides on the degree of attachment. Physiol. Plant Path.-23.

Puvanesarajah schell, F M, G Stacey, C J Douglas, and E W Nester. 1985. Role for 2-linked-B-D-glucan in the virulence of Agrobacterium tumefaciens. J. Bacteriol. 164:102-106.

Raju, B C,. 1980. Effect of pH, osmotic pressure, temperature serum co Z. Pflanzenkr. Pflanzenschutz 87(4):200-209.

Reddy, D V R, L M Black. 1873. Electrophoretic separation of all components of the double stranded RNA of Wound Tumour Virus. Virology 54:557-562.

Reddy, D V R, I Kimura, L Black, and M. 1974. Co-expression of dsRNA from Wound Tumour Virus and Rice Dwarf Virus. Virology 60:283-296.

Reddy, D V R, D P Rhodes, J A Lesnaw, R MacLeod, A K Banerjee, and L M Black. 1977. In vitro transcription of Wound Tumour Virus(WTV) by virus-associated RNA transcripts. Virology 80:336-361.

Rourke, J P,. 1980. The Proteaceae of Southern Africa Purnell and Sons(SA) Cape Town

Roussouw, F,. 1970. The Proteaceae of Southern Africa Purnell and Sons(SA) Pty Ltd Cape Town:-.

Rubin, R A,. 1986. Genetic studies on the role of octopine T-DNA border regions in crown gall tumor formation. Mol. Gen. Genet. 202:312-320.

Rust, D J,. 1981. Heksebesem by Proteas FFTRI Scientific Bulletin

Rust, D J, J Myburgh. 1976. Heksebesem by Proteas Veld en Flora 62:21-22.

Salomon, F, R Deblaere, J Leemans, J P Hernalsteens, M Van Montagu, and J Schell. 1984. Genetic identification of functions of TR-DNA transcripts in octopine crown galls. EMBO. J. 3:141-146.

Satima, S, J Rappaport, and A F Blakeslee. 1951. Ovular tumor connected with incompatable crosses in Datura. Am. J. Bot. 37:576-586.

Schaeffer, G W,. 1962. Tumor induction by an indolyl-3-acetic acid-kinetin-interaction in a Nicotiana hybrid. Nature (London) 196:1322-1327.

Schafer, W, K Weising, and G Kahl. 1984. T-DNA of a crown gall tumour is organised in nucleosomes. EMBO. J. 3(2):373-376.

Schröder, G, S Waffenschmidt, E W Weiler, and J Schröder. 1984. The T-region of Ti plasmids codes for and enzyme synthesizing indole-3-acetic acid. Eur. J. Biochem. 138:391-387.

Schröder, J, A Hillebrand, W Klipp, and A Puhler. 1981. Expression of plant tumor-specific proteins in minicells of Escherichia coli: a fusion protein of lysopine dehydrogenase with chloramphenicol acetyltransferase. Nucl. Acids Res. 9:5187-5202.

Sciaky, D, M F Thomashow. 1984. The sequence of the tms transcript 2 locus of the Agrobacterium tumefaciens plasmid pTiA6 and characterisation of the mutation in pTiA66 that is responsible for auxin attenuation. Nucl. Acids Res. 12:1447-1461.

Seemüller, E,. 1976. Fluoreszenzoptischer direktnachweis von mykoplasmaahnlichen organismen im phloem pear-decline-und triebesuchtkranker baume. Phytopath. Z. 85:368-372.

Seliskar, C E,. 1875. Mycoplasma-like organisms found in the phloem of bunch-diseased walnuts. Forest Science 22:144-148.

Shaw, C H, M D Watson, G H Carter, and C H Shaw. 1984. The right hand copy of the nopaline Ti plasmid 25bp repeat is required for tumor function. Nucl. Acids Res. 138:6031-6041.

Shekhawat, G S, M P Khurana, and B B Nagaich. 1981. Fluorescence microscopy of potato and tomato plants infected with three mycoplasmal diseases. J. Plant Disease and Protection 87(2/3):148-151.

Smidt, M, T Kosuge. 1978. The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of Pseudomonas savastanoi in gall formation on oleanders. Physiol. Plant Pathol. 13:203-214.

Smith, H,. 1972.. Plant genetic tumours. Prog. Exp. Tumor Res. 15:138-159.

Southern, E,. 1975. Detection of specific sequences among DNA fragments separated by electrophoresis. J. Mol. Biol. 98:503-528.

Spano, L, M Pomponi, P Constantino, G M S Van Slogteren, and J Tempe. 1982. Identification of T-DNA in the root-inducing plasmid of the agropine type Agrobacterium rhizogenes. Plant Mol. Biol. 1:291-300.

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

Stachel, S E, E W Nester. 1986. The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of Agrobacterium tumefaciens. EMBO. J. 5:1445-1454.

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

Taylor, B H, F F White, E W Nester, and M P Gordon. 1985. Transcription of Agrobacterium rhizogenes A4 T-DNA. Mol. Gen. Genet. 201:546-553.

Thomashow, L S, S Reeves, and M F Thomashow. 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the Agrobacterium Ti plasmid pTiS6 encodes an enzyme that catalyzes synthesis of indole-3-acetic acid. Proc. Natl. Acad. Sci. USA 81:5071-5075.

Thomashow, M F, S Hughly, W G Buchholz, and L S Thomashow. 1986. Molecular basis for the auxin-independent phenotype of crown gall tumor tissue. Science 231:616-618.

Thomashow, M F, R Nutter, A L Montoya, M P Gordon, and E W Nester. 1980a. Integration and organisation of Ti plasmid sequences in crown gall tumors. Cell 19:729-739.

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

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

Van Larebeke, N, G Engler, G Holsters, S Van Den Elsack, I Zaenen, and R A Schilperoort. 1974. Large plasmids in Agrobacterium tumefaciens essential for crown gall-inducing ability. Nature (London) 252:169-170.

Van Slogteren, G M S, P J J Hooykaas, and R A Schilperoort. 1984. Silent T-DNA genes transformed by Agrobacterium tumefaciens are activated by grafting and by 5-azacytidine treatment. Plant. Mol. Biol. 3:333-336.

Vanderleyden, J, J Desair, C De Meirsman, K Miechiels, A Van Gool, G Jen, and M-D Chilton. 1986. Nucleotide sequence of the T-DNA region encoding transcripts 6a and 6b of the pTiT37 nopaline Ti plasmid. Plant. Mol. Biol. 7:33-41.

Velten, J, L Velten, R Hain, and J Schell. 1984. Isolation of a dual plant promoter fragment from the Ti plasmid of Agrobacterium tumefaciens. EMBO. J. 3:2723-2730.

Wang, K, L Herrera-Estrella, M Van Montagu, and P Zambryski. 1984. Right 25bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from Agrobacterium to the plant genome. Cell 38:455-462.

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

White, F F, G Ghidossi, M P Gordon, and E W Nester. 1982. Tumor induction by Agrobacterium rhizogenes involves the transfer of plasmid DNA to the plant genome. Proc. Natl Acad. Sci. USA:19-3193.

White, F F, B H Taylor, G A Huffman, M P Gordon, and E W Nester. 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of Agrobacterium rhizogenes. J. Bacteriol. 154:33-44.

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

Willmitzer, L, P Dhaese, P H Schreier, W Schmalenbach, M Van Montagu, and J Schell. 1983. Size, location and polarity of T-DNA-encoded transcripts in nopaline crown gall tumors: common transcripts in octopine and nopaline tumors. Cell 32:1045-1056.

Willmitzer, L, J Sanchez-Serrano, E Buschfeld, and J Schell. 1982. DNA from Agrobacterium rhizogenes is transferred to and expressed in axenic hairy root plant tissues. Mol. Gen. Genet. 186:16-22.

Willmitzer, L, W Schmalenbach, and J Schell. 1981. Transcription of T-DNA in octopine and nopaline crown gall tumours is inhibited by low concentrations of a-amanitin. Nucl. Acids. Res. 9:4801-4812.

Willmitzer, L, G Simons, and J Schell. 1982. The TL-DNA in octopine crown-gall tumours codes for seven well-defined polyadenylated transcripts. EMBO. J. 1:139-146.

Wilson, E,. 1965. Patholgica histogenesis in oleander tumors by Pseudomonas savastanoi. Phytopathology 55:1244-1249.

Wilson, E E, A R Magie. 1964. Systemic invasion of the host plant by the tumor-inducing bacterium, Pseudomonas savastanoi. Phytopathology 54:576-579.

Winter, J A, R L Wright, and W B Gurley. 1984. Map locations of five transcripts homologous to TR-DNA in tobacco and sunflower tumors. Nucl. Acids. Res. 12:2391-2406.

Yadav, N S, J Vanderleyden, D R Bennett, W M Barnes, and M-D Chilton. 1982. Short direct repeats flank the T-DNA on nopaline Ti plasmid. Proc. Natl. Acad. Sci. USA 79:6322-6326.

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

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

Zaenen, E, N Van Larebeke, H Teuchy, M Van Montagu, and J Schell. 1974. Supercoiled circular DNA in crown gall inducing Agrobacterium strains. J. Mol. Biol. 86:109-127.

Zambryski, P, A Depicker, K Kruger, and H M Goodman. 1982. Tumor induction by Agrobacterium tumefaciens analysis of the boundaries of T-DNA. J. Mol. Appl. Genet. 1(4):362-370.

Zambryski, P, H M Goodman, M Van Montagu, and J Schell. 1983a. Agrobacterium tumor induction mobile genetic elements (J.Shapiro ed.):506-535. Academic Press, New York.

Zambryski, P, H Joos, C Genetello, J Lemmers, M Van Montagu, and J Schell. 1983b. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO. J. 2:2143-2150.

18 JUN 1987