OPINES IN CROWN GALL AND HAIRY ROOT DISEASES

by

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TABLE OF CONTENTS

	Page
SUMMARY	ii
STATEMENT	vi
ACKNOWLEDGEMENTS	vii
ABBREVIATIONS	viii
CHAPTER 1. INTRODUCTION	1
CHAPTER 2. MATERIALS AND METHODS	16
CHAPTER 3. THE STRUCTURE OF AGROCINOPINE A	20
CHAPTER 4. AGROPINE BIOSYNTHESIS	40
CHAPTER 5. AGROCINOPINES AND HAIRY ROOT STRAINS	60
CHAPTER 6. THE ORIGINAL SOURCES OF HAIRY ROOT ISOLATES AND THEIR IMPLICATIONS FOR COMPARATIVE STUDIES	66
CHAPTER 7. THE OPINES AND T-DNA OF CUCUMBER HAIRY ROOT STRAINS	73
CHAPTER 8. VIRULENCE PROPERTIES OF STRAINS OF AGROBACTERIUM ON THE APICAL AND BASAL SURFACES OF CARROT ROOT DISCS	85
CHAPTER 9. GENERAL DISCUSSION	108
APPENDICES	
A. CULTURE MEDIA	117
B. SOLUTIONS FOR PLASMID ISOLATION AND RESTRICTION ANALYSIS	122
C. COLORIMETRIC PENTOSE DETERMINATION	123
D. PUBLICATIONS	124
BIBLIOGRAPHY	125

SUMMARY

The project was a study of the chemistry and biology of some recently reported and previously unreported opines, which are substances characteristic of crown gall and hairy root tissues and are not found elsewhere in the plant kingdom. During the study, it was noticed that strains of Agrobacterium vary in their ability to induce disease on the basal (facing the shoot) surface of carrot root discs. This was also investigated.

This thesis is divided into two parts. The chemical aspects of the study are presented in section A and the biological aspects in section B.

A.

1. The structure of agrocinopine A: Agrocinopines are phosphorylated sugar opines which were discovered through their interaction with agrocin 84 (Ellis and Murphy, 1981). In the present study, agrocinopine A was extracted from nopaline type crown gall tumours and isolated as an analytically pure sodium salt. It was shown to consist of sucrose, L-arabinose and inorganic phosphate and shown to contain no nitrogen. Its properties are those of a phosphodiester and ^{13}C n.m.r. studies show that the phosphodiester linkage is from carbon-4 of the fructosyl moiety of sucrose to carbon-2 of L-arabinose. The linkage to the 2-position of L-arabinose was confirmed by ^{13}C n.m.r. study of synthetic L-arabinose-2-phosphate. Agrocinopine A is reducible by sodium borohydride to the corresponding arabinitol phosphodiester which is no longer active in the agrocinopine bioassay. Thus the arabinose moiety is required for induction or recognition of the permease for agrocinopine A uptake.

Agrocinopine B is also a phosphodiester, related to agrocinopine A by loss of the D-glucose portion of the molecule.

2. Agropine biosynthesis: Two new key intermediates in the pathway of agropine biosynthesis, 1-deoxy-D-fructosyl-L-glutamine (dFruGln) and 1-deoxy-D-fructosyl-L-glutamate (dFruGlu), have been isolated from B6 (octopine) tumours and characterized. In the proposed three-step pathway to agropine, dFruGln and dFruGlu are precursors which are reduced by a reductase enzyme to the opines N^2-(1-deoxy-D-mannityl)-L-glutamine (dManlGln) and N^2-(1-deoxy-D-mannityl)-L-glutamate (dManlGlu) respectively. Agropine is then formed by cyclization of dManlGln.

Tumours of nopaline strain IIBV7 are unusual in that they contain large amounts of dFruGlu and dFruGln, but not the reduced compounds dManlGlu or dManlGln. Agropin-1'-ene, a new opine which can be chemically converted to agropine by catalytic hydrogenation, is also present in these tumours. The opines of strain IIBV7 appear to be synthesized via a pathway which resembles the agropine biosynthetic pathway but lacks the reductase.

B.

1. Agrocinopines and hairy root disease: Strains of A.rhizogenes were tested for their ability to catabolize agrocinopines. The results are consistent with the agropine hairy root strains being of the agrocinopine A type and the dManlGln (also known as mannopine) hairy root strains being of the agrocinopine C type.

2. The original sources of dMan1Gln type hairy root isolates: The dMan1Gln group of hairy root strains comprises those designated TR7, 8196, TR101 and 11325. Strains TR7 and 8196 are known to be derived from the same single-cell isolate. Restriction endonuclease digestion of plasmids from dMan1Gln strains revealed identical patterns for 8196, TR101 and 11325 using both BamHI and EcoRI. Despite the common origin of TR7 and 8196, the size of their second-largest EcoRI plasmid restriction fragment differed by 3Md.

3. The opines and T-DNA of cucumber hairy root strains: The cucumber hairy root strains, which are now classified as A.tumefaciens (formerly known as biotype 1), form a distinct group of hairy root strains as they are unable to catabolize opines of the octopine, nopaline, agropine and agrocinopine families. A compound behaving like an opine in biological utilization studies has been found in root tissue induced by these strains. This opine has been given the trivial name cucumopine and has been partly characterized. Evidence suggests that it is an imino acid and that it contains an imidazole or modified imidazole group. The T-DNA of cucumber strain 2659 was found by probing 2659 hairy root DNA with the T-DNA of hairy root strain 8196, a strain belonging to the dMan1Gln group of A.rhizogenes (formerly biotype 2).

4. Virulence properties of strains of Agrobacterium on carrot root discs: Most pathogenic strains of Agrobacterium, when inoculated on carrot root discs, are able to transform both the apical tissues (facing the root tip) and the basal tissues (facing the shoot). These are referred to as Bas^{+} strains. There are however some wild type strains which form few or no tumours on the basal surface and are called basal attenuated (Bas^{att}).

Callus formation on uninoculated carrot discs occurs on the vascular cambium of the apical but not the basal surface. Of 22 wild type tumour-inducing strains tested, only one (TR104) was Bas^{att}. Tumour-inducing strains with mutations in genes 1 and 2 of the shoot inhibition region of the TL-DNA are also Bas^{att}. The axenic carrot tumour line of TL-DNA gene 1 mutant tms-328 is auxin dependent. Complementation between a strain with a mutation in gene 1 and a strain with a mutation in gene 2, when inoculated together on the basal surface of carrot root discs, resulted in restoration of virulence. Attenuation of virulence on the basal surface can be explained in terms of an auxin deficiency at that surface resulting from unidirectional auxin transport to the apical surface. Evidence suggests auxin has its effect in stimulating tumour growth after transformation (i.e. T-DNA transfer to the plant cell) has occurred.

Hairy root strains were more commonly Bas^{att} than Bas^{+}. The only factor investigated which altered the Bas^{att} phenotype of hairy root strain TR7 was auxin. Naphthalene acetic acid application with TR7 basal inoculation induced growth of transformed roots on the basal surface in approximately 1/3 of carrots tested. An explanation of the Bas^{att} phenotype of hairy root strains awaits a detailed study of Ri plasmid functions.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text.

M.H.Ryder

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ABBREVIATIONS AND SYMBOLS

ABTS	2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulphonate
ATCC	American Type Culture Collection
Bam HI	restriction enzyme ex *Bacillus amyloliquefaciens* H
c	concentration
DEAE-	diethylaminoethyl-
Eco RI	restriction enzyme ex *Escherichia coli* RY13
EDTA	ethylenediaminetetraacetate
dFruGlu	deoxyfructosyl glutamate
dFruGln	deoxyfructosyl glutamine
dFru-5-oxo-Pro	deoxyfructosyl-5-oxoproline
dGlc1Glu	deoxyglucityl glutamate
dGlc1Gln	deoxyglucityl glutamine
dGlc1-5-oxo-Pro	deoxyglucityl-5-oxoproline
Hind III	restriction enzyme ex *Haemophilus influenzae* Rd
HVPE	High Voltage Paper Electrophoresis
ICPB	International Collection of Phytopathogenic Bacteria
i.r.	infrared
kbp	kilobase pairs
Kpn I	restriction enzyme ex *Klebsiella pneumoniae* OK8
LTE	low concentration Tris-EDTA buffer
dMan1Glu	deoxymannityl glutamate
dMan1Gln	deoxymannityl glutamine
dMan1-5-oxo-Pro	deoxymannityl-5-oxoproline
Md	megadaltons
Me_4Si	tetramethylsilane

$M_{O.G}$	electrophoretic mobility relative to Orange G
m.p.	melting point
M_R	electrophoretic mobility relative to ribose
NAA	naphthalene acetic acid
NCIB	National Collection of Industrial Bacteria
NCPPB	National Collection of Plant Pathogenic Bacteria
n.m.r.	nuclear magnetic resonance
ppm	parts per million
R_f	chromatographic mobility relative to the solvent front
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
t.l.c.	thin layer chromatography
TE	Tris-EDTA buffer
TSP	3-(trimethylsilyl)propionic acid
UV	ultraviolet
2,4-D	2,4-dinitrophenylacetic acid
$[\alpha]$	specific optical rotation
δ	chemical shift

to Kathryn Naomi

CHAPTER 1

INTRODUCTION

Crown gall and hairy root diseases of plants are neoplastic disorders caused by soil bacteria of the genus Agrobacterium. Crown gall is characterized by the proliferation of unorganized tissue to form tumours whereas hairy root disease results in the proliferation of roots, often emerging from a tumorous growth. The host range of the two diseases is confined to dicotyledons but within this group a large number of species can act as hosts (De Cleene and De Ley, 1976; 1981).

The diseases have been subjects of considerable interest and study both because they cause economic losses in orchards and nurseries in many parts of the world and because of the unique nature of the parasite-host relationship. The diseases are rare examples of natural genetic manipulation of a eukaryote by a prokaryote. Recent advances have in fact opened the way for artificial genetic manipulation of plants, an area of great significance and potential for research.

A large number of reviews on crown gall disease have been published, especially in recent years, dealing with various aspects of the disease. Lippincott and Lippincott (1975) cover much of the earlier literature. More recent general reviews include those of Schell et al. (1979), Tempé et al. (1980) and Roberts (1982). Reviews concentrating more on the tumour-inducing (Ti) and root-inducing (Ri) plasmids and their transferred DNA (T-DNA) are those of Nester and Kosuge (1981), Bevan and Chilton (1982), Caplan et al. (1983) and Zambryski et al. (1983).

Aetiology and the taxonomy of Agrobacterium

The bacterial aetiology of crown gall disease has been known since early this century (Smith and Townsend, 1907). Hairy root was believed to be another form of the same disease (Smith et al, 1911) and was called the "apple strain" of Phytomonas tumefaciens. Riker et al. (1928), Riker et al. (1930) and Suit (1933) differentiated between the crown gall- and hairy root-inducing bacteria and believed that the two were distinct. Both types of bacteria are Gram-negative rods which occur in soil.

The crown gall and hairy root-inducing organisms are now placed in the genus Agrobacterium (Conn, 1942) belonging to the family Rhizobiaceae (Allen and Holding, 1974).

The taxonomy within the genus Agrobacterium has been confused. In the past, distinctions between species have been made on the basis of pathogenicity, i.e. between crown gall-, hairy root-, cane gall-inducing and non-pathogenic types. However, the discovery that pathogenicity functions reside on large plasmids which can move between bacteria by conjugation both in vivo (Kerr, 1969) and in vitro (Van Larebeke et al., 1975; Watson et al., 1975; Kerr et al., 1977) has meant that these functions are unsuitable for taxonomic purposes. This difficulty is overcome by naming species on the basis of chromosomally-determined characters. Such a system, based on the three biotypes of Agrobacterium (Keane et al., 1970; Kerr and Panagopoulos, 1977) has been proposed by Holmes and Roberts (1981). Biotypes 1, 2 and 3 have been given the specific epithets A.tumefaciens, A.rhizogenes and A.rubi respectively, regardless of pathogenicity. This system is used throughout this thesis, with tumour-inducing and hairy root-inducing strains referred to as pTi and pRi strains respectively, to indicate whether they contain a Ti- or an Ri-plasmid.

Tumour and hairy root induction

Apart from requiring pathogenic strains of Agrobacterium and a susceptible plant host, other requirements must also be met for tumour or hairy root formation to occur. Plant tissue must normally be wounded to enable the bacteria to gain access to the plant cells (Riker et al, 1946). Temperature is also critical to tumour induction. At temperatures above 30°C, tumour and hairy root induction is prevented even though the bacteria and plant cells are able to grow at those temperatures (Braun, 1958; Moore et al., 1979; Rogler, 1981). Bacterial attachment to the plant cell walls is considered important in pathogenicity (Lippincott and Lippincott, 1980).

The introduction of successful plant tissue culture methods enabled White and Braun (1941) to culture crown gall tumours in the absence of the inciting bacteria. Bacteria-free secondary tumours from sunflower stems in culture continued to proliferate in the absence of plant growth substances, whereas control non-tumorous tissue grew only poorly. This led to the proposal that a hypothetical factor, later called the "tumour-inducing principle" or T.I.P. (Braun, 1947) was involved in the disease-causing mechanism, and that once this T.I.P. had been acquired by the plant cells, the bacteria were no longer necessary for tumour growth.

Octopine and nopaline

Crown gall tumours contain unusual chemicals, now called opines, which are not found in normal plant tissues. The first group of these compounds discovered were the imino acids lysopine, or N^2-(1-D-carboxyethyl)-L-lysine, (Lioret, 1956, 1957), octopine, or N^2-(1-D-carboxyethyl)-L-

arginine, (Menagé and Morel, 1964) and nopaline, or N^2-(1,3-D-dicarboxypropyl)-L-arginine (Goldmann et al., 1969). These compounds are all conjugates of L-amino acids, with D-carboxylic acids (Fig. 1-1).

Petit et al. (1970) showed that the type of imino acid (octopine or nopaline) found in the tumour was dependent upon the inducing strain of bacteria and not on the host plant. Furthermore, they found that the bacteria inducing octopine tumours were able to grow on octopine and those inducing nopaline tumours grew on nopaline as growth substrate. These discoveries shed new light on the relationship between Agrobacterium strains and their plant hosts; an exchange of genetic material during tumour induction was suggested by Petit et al. (1970). Pathogenic agrobacteria came to be classified as octopine, nopaline or "null type" strains.

The Ti plasmid and its functions

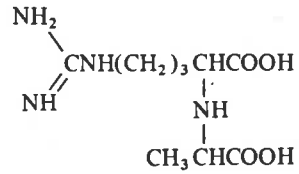
Transfer of virulence in vivo from pathogenic to non-pathogenic strains of Agrobacterium was reported by Kerr (1969). The search for the movable element culminated in the discovery of the tumour-inducing (Ti) plasmids (Zaenen et al., 1974). These plasmids are large covalently closed circular molecules of extrachromosomal DNA. Proof that the Ti plasmids are responsible for the tumour-inducing properties of pathogenic agrobacteria came from plasmid transfer studies in which virulence and the Ti plasmid were simultaneously transferred to a non-pathogenic recipient (Van Larebeke et al., 1975; Watson et al., 1975); in addition, loss of the Ti plasmid by curing at 37°C resulted in loss of virulence (Van Larebeke et al., 1974).

The Ti plasmids are large, ranging in size from 120 Md to 150 Md ,

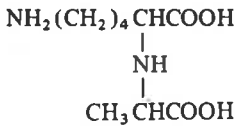
FIGURE 1-1

Chemical structures of opines of the octopine, nopaline and agropine families (from Petit *et al.*, 1983).

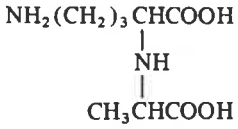
Octopine family



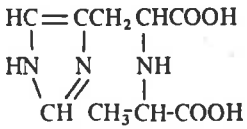
Octopine



Lysopine

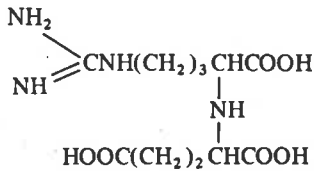


Octopinic acid

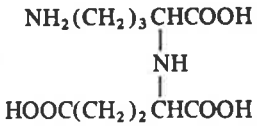


Histopine

Nopaline family

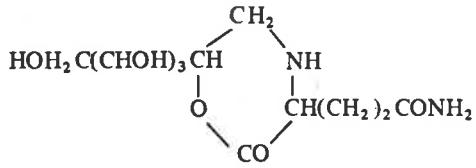


Nopaline

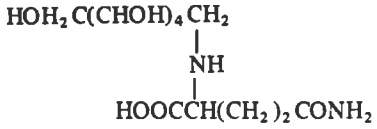


Nopalinic acid

Agropine family

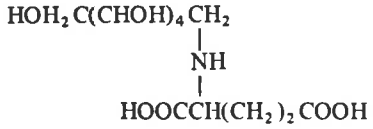


Agropine



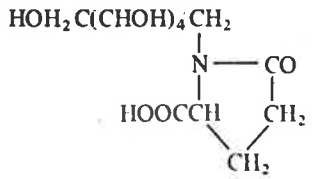
Mannopine

(N^2-(1'-deoxymannityl)-L-glutamine)



Mannopinic acid

(N^2-(1'-deoxymannityl)-L-glutamic acid)



Agropinic acid

i.e. 180 kbp to 225 kbp (Currier and Nester, 1976). A range of functions has been mapped on Ti plasmids (Holsters et al., 1982). Ti plasmids code for the utilization of opines (Bomhoff et al., 1976; Kerr and Roberts, 1976; Montoya et al., 1977) and are classified according to opine type (Table 1-1). The best-understood are the octopine and nopaline Ti plasmids. The portion of the Ti plasmid which is transferred to the plant, the T-DNA, will be discussed in a separate section. Mutations in several regions of the Ti plasmid affect virulence. The largest of these, the so-called virulence (Vir) region, is not transferred to the plant DNA but is essential for tumour induction. The Vir region of octopine and nopaline strains show a large degree of homology (Holsters et al., 1982). Other Ti plasmid regions which show such homology are the replicator region, plasmid transfer functions, the plasmid incompatibility functions and the "common" or "core" T-DNA (Holsters et al., 1982).

In addition to the Ti plasmid, strains of Agrobacterium may or may not contain other plasmids (Currier and Nester, 1976). Some have known functions, e.g. the small (30 Md) bacteriocinogenic plasmid which codes for agrocin 84 production in non-pathogenic strain K84 (Ellis et al., 1979; Slota and Farrand 1982). Other large plasmids remain cryptic, e.g. the very large (>214 Md) plasmid of strain C58 (Casse et al., 1979).

The opine concept

The central importance of octopine and nopaline in the biology of octopine and nopaline tumours respectively was stressed by Tempé and Schell (1977), Petit et al. (1978) and Schell et al. (1979). Tempé and Schell (1977) coined the term "opine" for these compounds and the opine concept was stated in the following terms by Petit et al. (1978):

Opines are "substances synthesized by the cells of the host plant in response to a stimulus of the pathogen. Their presence creates a favourable environment for the pathogen and contributes to its dissemination."

The term "genetic colonization" was used by Schell et al. (1979) to describe the action of pathogenic agrobacteria in the induction of plant tumours in which opines are synthesized as a supply of nutrients for the inciting bacteria.

The finding that some opines are able to induce conjugal transfer of Ti plasmids (reviewed by Kerr and Ellis, 1982) further strengthens the opine concept. The so-called conjugative opines are able to contribute to the dissemination of Ti plasmids in a population of agrobacteria in two ways: by acting as a nutrient source and by induction of plasmid transfer.

The octopine and nopaline families of opines are shown in Fig. 1-1. Several related compounds occur in each family, and can be catabolized by octopine and nopaline strains respectively. The octopine family are L-amino acid conjugates of D-pyruvate and the nopaline family consists of L-amino acid conjugates of 2-ketoglutarate.

Other opines have been found since the discovery of octopine and nopaline. The prediction (Guyon et al., 1980) that null type strains, inducing tumours with no known opines, should be found to contain previously unidentified opines, has been validated. Agropine, found in octopine tumours by Firmin and Fenwick (1978), was identified in null type tumours of strains Bo542 and A281 by Guyon et al. (1980). Tumours of most strains are now known to contain more than one family of opines and agrobacteria are correspondingly able to utilize more than one family of

opines. The agropine family has been extended (Dahl et al., 1983) to include N^2-(1'-deoxymannityl)-L-glutamine (also known as mannopine or dManlGln) and N^2-(1'-deoxymannityl)-L-glutamic acid (also known as mannopinic acid or dManlGlu). Agrocinopines, which are phosphorylated sugars, represent another new family of opines (Ellis and Murphy, 1981). Nopaline tumours usually contain agrocinopines A and B and agropine tumours usually contain agrocinopines C and D. Agrocinopines are also conjugative opines (Ellis et al., 1982).

Opine catabolic genes have been mapped for octopine and agropine catabolism for octopine strains and for nopaline and agrocinopine catabolism in nopaline strains (Holsters et al., 1982). These genes are found on the Ti plasmids and map outside the T-DNA region.

Opines are produced by axenic tumours, i.e. in the absence of the inciting bacteria (Lioret, 1957; Menagé and Morel, 1964, 1965; Ellis and Murphy, 1981), and thus are neither products of bacterial metabolism nor plant products produced as a direct response to the presence of the bacteria.

Table 1-1 lists the known types of Agrobacterium strains as classified by opine type. The recent reports of leucinopine (pTi strains 542, 398, A2 and AT1; Chang et al., 1983) and glutaminopine (pTi strains 181 and EU6; Chang and Chen, 1983) are not included in Table 1-1 since no biological utilization studies have been carried out with those compounds to test whether they are growth substrates for the corresponding bacterial strains. Succinamopine (Chilton et al., 1984), also called asparaginopine (Chang and Chen, 1983), has been included in Table 1-1 as it is specifically utilized by strains harbouring the 181 and EU6 Ti plasmids.

Opines of three different groups have now been reported to occur in tumours of Bo542. These opines are agropine (Guyon et al., 1980),

TABLE 1-1

Opine types found among naturally occurring strains of Agrobacterium[a].

STRAIN TYPE	EXAMPLES	OPINES IN TUMOURS
A.tumefaciens		
(biotype 1)		
pTi octopine	A6, B6	octopine, agropine, dMan1Glu/dMan1Gln
pTi nopaline	C58, T37	nopaline, agrocinopine A
pTi agropine	Bo542	agropine, dMan1Glu/dMan1Gln, agrocinopine C
pTi	EU6, 181	succinamopine (= asparaginopine)
pRi null type	2659	
A.rhizogenes		
(biotype 2)		
pTi nopaline	K108, K27	nopaline, agrocinopine A
pTi agropine	A4, TR105	agropine, dMan1Glu/dMan1Gln
pRi dMan1Gln	TR7, 8196	dMan1Glu/dMan1Gln
A.rubi		
(biotype 3)		
pTi octopine	K305, K308	octopine

[a] Only compounds shown to act as a nutrient source for the inciting strain are included in the Table.

agrocinopine C (Ellis and Murphy, 1981) and leucinopine (Chang et al., 1983).

The T-DNA and its functions

The discovery of the Ti plasmids soon stimulated efforts to find DNA sequences homologous to Ti plasmid DNA in tumour tissue. Prior to knowledge of the existence of the Ti plasmids, attempts to find Agrobacterium DNA in axenic tumours had been unsuccessful (reviewed by Chilton, 1982). Chilton et al. (1977, 1978) demonstrated by DNA reassociation experiments that strain B6 Ti plasmid sequences were present in an axenic B6 (octopine) tobacco tumour. The transferred DNA was referred to as T-DNA. The T-DNA is in the plant cell nucleus (Chilton et al., 1980; Willmitzer et al., 1980) and is covalently attached to plant DNA (Yadav et al., 1980). The T-DNAs of octopine and nopaline strains are the best known at this stage. The T-DNA represents only a small portion, ca. 23 kbp, of the Ti plasmid. The T-DNA of nopaline strains is transferred as a single segment of uniform size, whereas the transfer of the octopine T-DNA is more variable. The left hand octopine T-DNA, called the TL-DNA (approx. 12 kbp), is always present, but the right hand portion, the TR-DNA, is sometimes absent from transformed plant tissue (Thomashow et al., 1980; Zambryski et al., 1983).

The integration of T-DNA into plant DNA has been studied by analysis of T-DNA border sequences. For nopaline T-DNA, a 25 base-pair direct repeat is present at each end of the T-DNA but it is not always found in the transferred DNA (Zambryski et al., 1983). T-DNA is transcribed in the plant cell (Drummond et al., 1977) and the functions of the transcripts have been recently reviewed (Bevan and Chilton, 1982; Caplan et al., 1983). Site-directed transposon mutagenesis and deletion mutants have been

used to probe the functions of the T-DNA. Nopaline and octopine synthesis are coded for by single genes near the right borders of the nopaline T-DNA and octopine TL-DNA respectively (Garfinkel et al., 1981; Bevan et al., 1983; DeGreve et al., 1983; Depicker et al., 1983).

A part of the TL-DNA, the 9 kb "common" DNA, is highly conserved between octopine and nopaline strains (Depicker et al., 1978; Chilton et al., 1978). The conserved region codes for tumour morphology functions (Ooms et al., 1981; Garfinkel et al., 1981; Leemans et al., 1982). The organization, size and function of transcripts of the nopaline common T-DNA is very similar to that of the octopine TL-DNA (Willmitzer et al., 1983; Joos et al., 1983). Mutations in one part of the common T-DNA lead to shoot formation on certain plant hosts and mutants in an adjacent region give rise to root formation instead of the normal undifferentiated tumour. These mutants are called either shooter and rooter or shoot inhibition and root inhibition mutants. Tumour tissues incited by such mutants contain altered levels of plant growth substances (Akiyoshi et al., 1983; Caplan et al., 1983). The shoot inhibition region includes genes 1 and 2, which are adjacent on the T-DNA, and root inhibition region (gene 4) is located immediately to the right of gene 1. Strains with mutations in genes 1 and 2 are attenuated on several plant hosts and induce shoots on tobacco and Kalanchoe. Auxin restores virulence to such mutant strains when inoculated on carrot and potato discs (Leemans et al., 1982; Joos et al., 1983). Gene 5, mapping to the left of the shoot inhibition region, interacts with genes 1 and 2, but mutations in this gene are not of the shoot inhibition type (Joos et al., 1983). Gene 4 mutants give rise to tumours with roots on many plant hosts without decrease in virulence. Genes 6a and 6b lie to the right of gene 4 and are also involved in tumour morphology though the nature of their action

remains obscure (Joos et al., 1983).

Double insertion mutants and deletion mutants affecting both the shoot- and root-inhibition regions result in avirulence, but there is evidence that the T-DNA is still transferred to the plant cell (Ream et al., 1983; Leemans et al., 1982; Hille et al., 1983). Virulence studies on strains with larger T-DNA deletions show that no T-DNA genes are essential for transfer of the T-DNA to the plant DNA. The only requirement for transfer is an intact right T-DNA border (Caplan et al., 1983).

The TR-DNA of octopine strains codes for the biosynthesis of agropine (Leemans et al., 1982; Velten et al., 1983). The absence or loss of TR-DNA from tumours in culture therefore results in loss of agropine-synthesizing ability (Dahl et al., 1983).

The T-DNA can function in agrobacteria as well as in the transformed plant cell. Schroeder et al. (1983, 1984) demonstrated the transcription of several genes of the common DNA, including genes 1, 2 and 4, in Escherichia coli and in Agrobacterium. A newly found small transcript, located between genes 1 and 4, is expressed only in the bacteria.

Foreign DNA can be transferred to plant DNA using genetically engineered T-DNA in a Ti plasmid vector (Herrera-Estrella et al., 1983; Bevan et al., 1983b; Horsch et al., 1984) and can be expressed in the host plant (Murai et al., 1983).

Hairy root disease and the Ri plasmid

Hairy root, like crown gall, is a plasmid-borne disease. This was demonstrated by Moore et al. (1979) using plasmid transfer studies, though Albinger and Beiderbeck (1977) had earlier made the same suggestion without conclusive proof.

There are many similarities at the molecular genetic level between

hairy root and crown gall diseases. Firstly, opines are present in hairy root tissue. Axenic tissues of pRi strain A4 contain agropine (Tepfer and Tempé, 1981). The other silver nitrate positive opines of the agropine family, dMan1Glu, dMan1Gln and agropinic acid, are also present (Petit et al., 1983). The hairy root strains inducing roots containing known opines can be divided into two types: the agropine type and the dMan1Glu/dMan1Gln type (Petit et al., 1983). The existence of transferred T-DNA in hairy root tissue (Chilton et al., 1982; Willmitzer et al., 1982; White et al., 1982) again indicates the close relationship between hairy root and crown gall diseases. Earlier work on DNA sequence homology between the plasmids of hairy root and crown gall strains showed homology in the Vir region (White and Nester, 1980b). Huffman et al. (1984) have shown in detail the regions of homology between the Ri plasmid of A4 and the Ti plasmids and T-DNAs of pTi strains A6 (octopine) and T37 (nopaline). In addition to the Vir region homology, there is homology to the Ti plasmid T-DNA shoot inhibition region (Willmitzer et al., 1982) but this is located some distance to the right of the known pRiA4b T-DNA (Huffman et al., 1984). The total extent of homology to the Ti plasmids examined amounts to approximately 60% of the Ri plasmid. However, the T-DNA of the Ri plasmid pRiA4b contains large sequences which are not homologous to crown gall T-DNA. It is interesting that part of the Ri plasmid of agropine strains shows homology to the DNA of some plant species (Spano et al., 1982; White et al., 1983).

The plasmids of some hairy root strains exist as cointegrates of approx. 260 Md which can break into an Ri plasmid of approx. 150 Md and a smaller non-rhizogenic plasmid of approx. 110 Md (White and Nester, 1980a, 1980b). Thus plasmids of three sizes can normally be extracted from these strains. Only the cointegrate plasmid and the Ri plasmid are conjugative.

The smaller non-rhizogenic plasmid codes for the utilization of the opines dMan1Glu, dMan1Gln and agropinic acid (Petit et al., 1983).

The Ri plasmids can be considered a different type of Ti plasmid with many similarities (opines, T-DNA, Vir region homology) yet many differences (large areas with no homology to the Ti plasmid). The Ti and Ri plasmids belong to different incompatibility groups and can therefore coexist in the same cell (Costantino et al., 1980; White and Nester, 1980b).

Plant growth substances and crown gall

Plant growth substances were implicated in crown gall tumour development by Braun and Laskaris (1942) in studies on the attenuated strain A66, a derivative of virulent strain A6. Strain A66 has an auxin requirement for tumour formation. It is in fact a T-DNA gene 2 insertion mutant (Binns et al., 1982) which explains both its attenuated nature and the restoration of virulence using auxin.

Agrobacteria can produce plant growth substances, such as auxins and trans-ribosyl zeatin, in culture (Liu and Kado, 1979; Weiler and Spanier, 1981; Regier and Morris, 1982) and these may play a part in pathogenicity. The expression of TL-DNA genes by octopine bacteria (Schroeder et al., 1983, 1984) lends support to this view.

The T-DNA itself is active in altering the balance of plant growth substances in tumour tissues. This is evident from studies on the levels of auxins and cytokinins in tumours induced by strains with insertion mutations in the shoot inhibition and root inhibition regions (Akiyoshi et al., 1983).

The virulence of shoot inhibition mutants is stimulated by auxin (Leemans et al., 1982; Joos et al., 1983). This supports the notion that

genes 1 and 2 are involved in auxin metabolism. Schroeder et al. (1984) have shown that gene 2 codes for an aminohydrolase enzyme which is able to hydrolyse indole-3-acetamide to indole-3-acetic acid (IAA). Gene 1 may also be involved in the same biosynthetic pathway. The function of gene 4, which has cytokinin-like activity (Ooms et al., 1981; Leemans et al., 1982), is not known at present.

Biological control of crown gall disease

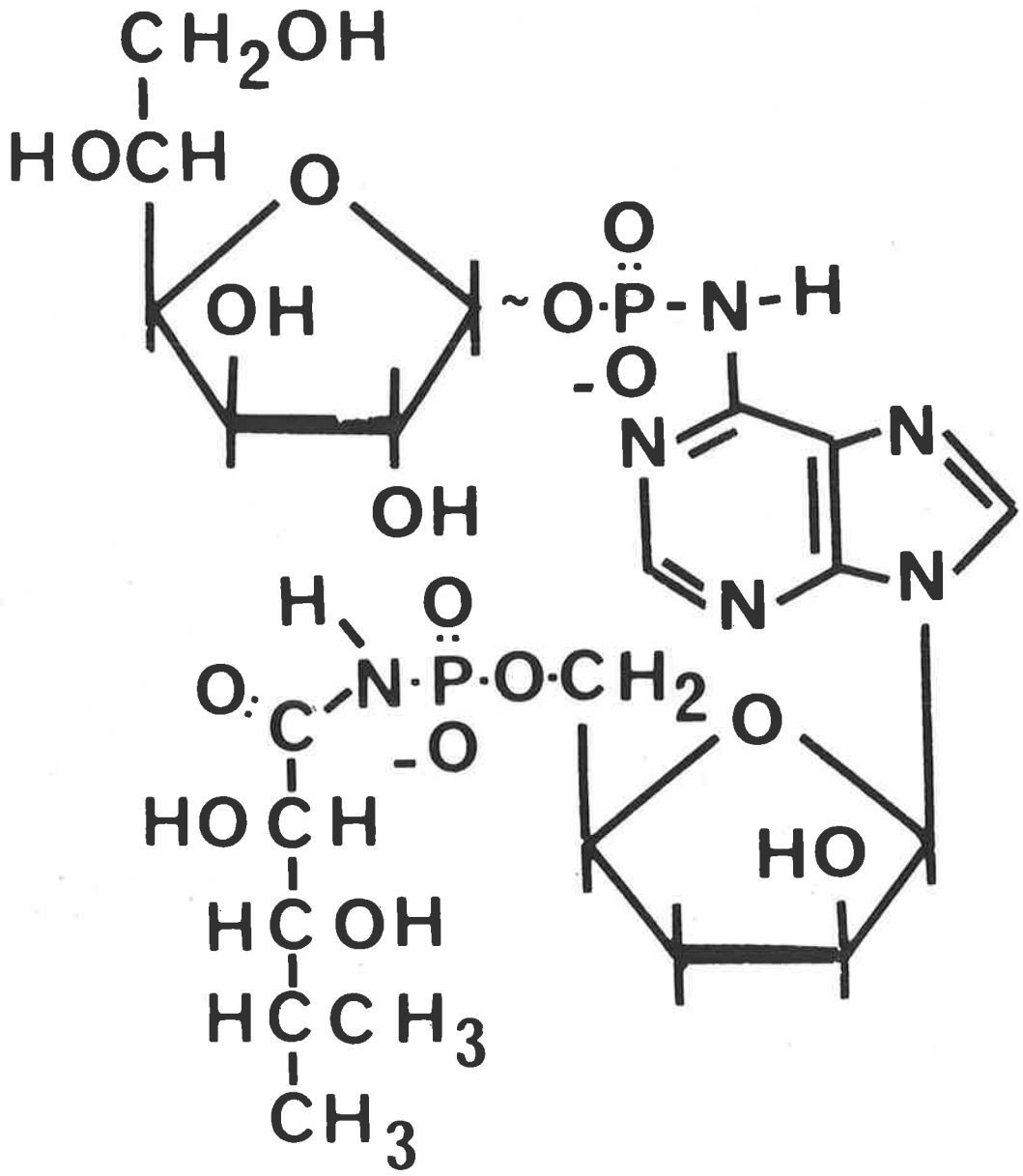
Biological control of nopaline strains of crown gall has been achieved (Kerr and Htay, 1974) and is commercially applied to a wide range of plants in different parts of the world (Moore and Warren, 1979; Kerr, 1980). Control of the disease depends upon inoculation of planting material to prevent infection. A suspension of strain K84 (A.rhizogenes, formerly biotype 2) is used for this purpose. This strain produces an antibiotic called agrocin 84 (Fig. 1-2; Tate et al., 1979) which specifically antagonizes nopaline strains. Production of agrocin 84 by K84 bacteria is coded for by a 30 Md plasmid, pAgK84 (Ellis et al., 1979; Slota and Farrand, 1982). Agrocin 84 is a fraudulent adenine nucleotide and is taken up via a permease (Murphy and Roberts, 1979) into the susceptible cells where it acts as a DNA replication terminator owing to its 3'-deoxy-arabinofuranosyl adenine nucleoside moiety (Murphy, 1981). The agrocinopines were discovered in an attempt to find a non-toxic substrate for the "agrocin 84 permease" (Ellis and Murphy, 1981).

Scope of the study

Much of the work presented in this thesis is concerned with opines, compounds which have been called the "raison d'etre" of crown gall (Lippincott, 1977).

FIGURE 1-2

The chemical structure of agrocin 84 (from Tate et al., 1979).


CH2OH
HOCH
O
OH
OH
~O·P-N-H
O
-O
N
N
N
N
H
O
O
N·P·O·CH2
C
-O
HOCH
HCOH
HCCH3
CH3
O
HO

AGROCIN 84

A major aim was to elucidate the structures of agrocinopines A and B, which had just been discovered in this laboratory (Ellis and Murphy, 1981). The isolation and structure elucidation are described in Chapter 3, showing that agrocinopines A and B are phosphodiesters of L-arabinose linked to sucrose and fructose respectively.

The biosynthesis of agropine is coded for by the TR-DNA of octopine strains (Leemans et al., 1982; Velten et al., 1983). Known compounds in the agropine family at the time the current work was commenced were agropine, dMan1Gln and dMan1Glu. When genetic studies indicated the possibility of a three-step pathway, a search was made for further related intermediates. Chapter 4 describes the isolation and characterization of two new key intermediates in agropine biosynthesis. In addition, a new opine related to agropine, and which could also be an agropine precursor, was found in IIBV7 tumours.

Because a major part of this study was related to agrocinopines, and the hairy root strains were at the time becoming subjects for study in other laboratories, it was decided to find out whether or not they are agrocinopine strains. Evidence to show that they are agrocinopine strains is reported in Chapter 5. Further studies (Chapter 6) on hairy root strains revealed a hitherto unsuspected common origin of some strains. The plasmid restriction patterns of these strains proved to be identical except for a deletion in one strain.

During an investigation of the virulence properties of agrobacteria inoculated on carrot root discs, it was found that the roots induced by cucumber hairy root strains did not contain any known opines. A search was made for their opine(s), culminating in the detection and partial characterization of a new opine for which the trivial name cucumopine is proposed. The T-DNA of these strains was detected by DNA hybridization

studies and the results are described in Chapter 7.

Another major focus of the thesis (Chapter 8) was an investigation of the transformation process using strains of Agrobacterium which were found to induce hairy root or tumours only on the apical surface of transversely cut carrot discs. The reason for the avirulence or attenuation of these strains on the basal surface was sought. The virulence properties of TL-DNA mutants in genes 1, 2 and 4 proved to be closely related to the problem. Auxins were found to be involved in tumour growth and to have their effect after the transformation step in tumour induction by strains with attenuated virulence on the basal surface.

A general discussion (Chapter 9) concludes the thesis.

CHAPTER 2

MATERIALS AND METHODS

Materials and methods used in general throughout the work are described in this chapter. Methods pertaining to particular aspects of the thesis are described in the Materials and Methods section of the relevant chapter.

Materials

Sources of materials, including chemicals and enzymes, are given in the Materials and Methods section of the appropriate chapter.

Bacterial strains, mutants and transconjugants

Bacterial strains, mutants and transconjugants used in the work are listed in the Materials and Methods section of the relevant chapter.

Growth media

Recipes for the media used are found in Appendix A.

Agrocin 84 sensitivity and the agrocinopine bioassay

Sensitivity of bacterial strains to agrocin 84 was tested by the method of Kerr and Htay (1974).

Biological activity of agrocinopines was detected by the method of Ellis and Murphy (1981). A.tumefaciens strain K84, which produces a diffusible nucleotide bacteriocin, agrocin 84 (Tate et al., 1979), was used as the producer strain. K84 bacteria were inoculated by placing a loopful of a fresh culture in the centre of a Stonier's agar plate. After a 30h incubation, the producer bacteria were killed with chloroform. The samples

to be tested for agrocinopine activity were dried on squares of Whatman No. 1 paper and placed at the expected edge of the agrocin 84 inhibition zone. The plate was overlaid with buffered soft agar containing the agrocin 84-sensitive nopaline strain A208 (Sciaky et al., 1978) of Agrobacterium as indicator strain, and incubated overnight. Agrocinopine activity appeared as an extension of the agrocin 84 inhibition zone. Control plates without agrocin 84 were prepared to ensure that the compounds isolated were not toxic in themselves.

Inoculation of plant material

Carrots and parsnips were either purchased locally or specified varieties ('Western Queen', 'Western Red' and 'Yates Topweight 556' carrots) obtained from a market garden. The carrots or parsnips were washed, peeled, dipped in ethanol and flamed. After discarding the basal 2cm of the root, discs 5 to 10mm thick were cut by transverse section, and the outer edges of each disc were trimmed with a scalpel. Discs were placed in sterile specimen containers or Linbro tissue culture trays containing 1 or 2% (w/v) agar to prevent drying. Care was taken to ensure that discs were correctly placed with either the apical or basal surface facing up, as required.

Inoculation: Bacteria were grown for 2 days at 28°C on yeast-mannitol agar. Suspensions of approximately 2×10^9 bacteria/ml were made in sterile distilled water. A small volume (10-50µl) of this suspension was used to inoculate the cambial area of the apical or basal surface of the carrot or parsnip disc. The inoculated surface faced up except where otherwise indicated. Usually 6 carrots or parsnips were used as replicates in each experiment. Discs were incubated at 25°C under low intensity fluorescent light (4.2 $\mu Em^{-2}s^{-1}$) and observed regularly between 2-6 weeks after

inoculation.

Glasshouse-grown plants used in this study were tomato (Lycopersicon esculentum, Mill., var. Early Dwarf Red), sunflower (Helianthus annuus, L., var. Hysun 10), Kalanchoe daigremontiana and Nicotiana species. Plants were inoculated on the stem of the younger internodes after wounding with a sterile needle or scalpel blade.

Axenic plant tissue

To obtain tumour tissue free from bacteria, 1 to 2 mm thick tumour slices were placed on Monnier's medium (Monnier, 1976) with Morel's vitamins (Morel and Wetmore, 1952) in 0.8% agar containing 1 mg/ml carbenicillin for 4 weeks. Tissues free of bacteria were maintained on medium without carbenicillin.

High voltage paper electrophoresis

The apparatus of Tate (1968) was used for paper electrophoresis with the following buffers and reference markers with arbitrarily assigned relative mobilities ($M_{O.G}$ and M_R) for Orange G and ribose respectively: 0.75 M formic acid/1 M acetic acid, pH 1.7 (fructose 0.0, Orange G 1.0); 0.2 M NH_4HCO_3/0.1 M NH_4OH, pH 9.2 (fructose 0.0, Orange G 1.0); 0.2 M H_3BO_3/0.2 M NH_4HCO_3/0.2 M NH_4OH, pH 9.2 (2'-deoxyadenosine 0.0, Orange G 1.0); 0.2 M Ca acetate/0.2 M acetic acid, pH 4.7 (1-O-methyl-α-D-gluco-pyranoside 0.0, Orange G 1.0); 0.1 M NaOH (glycerol 0.0, ribose 1.0).

Detection reagents for electrophoresis and chromatography

The following detection reagents were used:

(a) p-anisidine reagent of Hough et al. (1950),

(b) phosphomolybdate reagent (Harrap et al., 1960),

(c) alkaline silver nitrate dip (Trevelyan et al., 1950),

(d) the vicinal glycol reagent of Weiss and Smith (1967),

(e) a slightly modified urea-phosphoric acid dip reagent for ketoses (Avela et al., 1977) containing urea (5g) dissolved in 80% phosphoric acid (22.5 ml) diluted to 250 ml with ethanol. The air-dried paper was heated at 110°C for 3 min to yield blue spots with compounds containing ketose sugars.

(f) triphenyltetrazolium chloride dip (Trevelyan et al., 1950). Electrophoretograms were dried, dipped in a 0.25% solution of 2,3,5 triphenyltetrazolium chloride in chloroform, dried again and dipped in ethanolic NaOH (10 ml of 5 M NaOH + 90 ml ethanol). The dried electrophoretograms were steamed to reveal reducing sugars and amino acid-reducing sugar conjugates as red-pink spots. After washing in water >1h, the electrophoretograms were dried and stored.

(g) the organic acid spray reagent of Smith and Spriesterbach (1954). The electrophoretogram was sprayed with 50% ethanol containing 5% (w/v) p-anisidine and 5% (w/v) D-xylose, dried and then heated 5 min at 110°C. Mono- or di-carboxylic acids appear as brown spots.

(h) the Pauly reagent for imidazoles (Ames and Mitchell, 1952).

CHAPTER 3

THE STRUCTURE OF AGROCINOPINE A

INTRODUCTION

The agrocinopines were discovered as a result of their interaction with agrocin 84 toxicity in the agrocin bioassay (Ellis and Murphy, 1981). Two groups of agrocinopines were found; agrocinopines A and B form one group, and the second group comprises agrocinopines C and D. Nopaline tumours usually contain agrocinopines A and B and agropine tumours usually contain agrocinopines C and D. The agrocinopines act as carbon sources for agrobacteria with Ti plasmids carrying the corresponding gene(s) for opine degradation. Agrocinopines are also biologically important in that they induce conjugal transfer of Ti plasmids to non-pathogenic recipient agrobacteria (Ellis et al., 1982).

Agrocinopine A was shown by Ellis and Murphy (1981) to contain sucrose, phosphate and either D- or L- arabinose but the nature of the linkages was not established. In the present detailed study, analytically pure agrocinopine A has been shown to be a non-nitrogenous phosphodiester in which the 4' carbon of the fructosyl portion is linked to the 2 carbon of L-arabinose. The linkage position to arabinose was confirmed by a ^{13}C n.m.r. study of synthetic L-arabinose-2-phosphate. Agrocinopine B is related to agrocinopine A by loss of the glucose moiety and is the corresponding phosphodiester of D-fructose and L-arabinose.

MATERIALS AND METHODS

Materials

Snake venom phosphodiesterase, bovine spleen phosphodiesterase, alkaline phosphatase (from E.coli), D-glucose oxidase, peroxidase, ribose-5-phosphate, 1-O-methyl-α-D-glucopyranoside and 2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulphonate (ABTS) were purchased from Sigma, St. Louis, Mo., USA; α-glucosidase was from Boehringer, Mannheim, invertase from B.D.H., Poole, U.K.; 2,4-dinitrobenzenesulphonate from Eastman Kodak, Rochester, N.Y.; and 3-(trimethylsilyl)propionic acid-d_4, sodium salt (TSP) from Merck, Sharp and Dohme, Canada.

Instrumental procedures

^{13}C n.m.r. spectra were recorded at 22.49 MHz on a JEOL FX90Q spectrometer with samples dissolved in D_2O and using TSP as an internal or external reference. ^{1}H-n.m.r. spectra were recorded on the same instrument with samples in $CDCl_3$ and chemical shifts (δ) relative to p-dioxane as internal reference. Gas chromatography/mass spectrometry was carried out with a Hewlett Packard model 5992B instrument, and similarity indices for mass spectra were obtained as described by Tate et al. (1982). I.r. spectra were recorded using KCl discs (0.5% w/w samples) on a Perkin-Elmer model 983 spectrophotometer. Optical rotations were measured in a 1 dm cell on a Perkin-Elmer model 141 polarimeter. Melting points were determined using a Kofler hot stage apparatus. T.l.c. was carried out on silica gel and alumina (Eastman Kodak 13181 and 13252 with fluorescent indicator). Elemental analyses were performed by the Australian Microanalytical Service, Melbourne.

High voltage paper electrophoresis

The methods for HVPE and detection reagents used are described in Chapter 2.

pH-relative mobility profiles

The method of Tate (1981) was used to measure electrophoretic mobilities between pH 1.5 and pH 10 and to calculate pKa values from the profiles obtained. Reference markers were glycerol (0.00) and 2,4-dinitrobenzenesulphonate (1.00).

Paper chromatography

Descending paper chromatography was carried out on Whatman No. 1 paper using solvent A: ethyl acetate/pyridine/water 8:2:1 (v/v/v).

D-glucose assay

D-glucose was measured using the D-glucose oxidase/peroxidase method (Bergmeyer and Bernt, 1973) with ABTS as chromogen.

Bioassay for agrocinopines

The method is described in Chapter 2.

Tumour tissue

A.tumefaciens strain A208 (Ellis and Murphy, 1981), harbouring the T37 nopaline Ti plasmid, was used to inoculate 4 to 6 week old sunflower or tomato plants. Tumours were harvested 4 to 6 weeks after inoculation.

Extraction and purification of agrocinopines

Tomato tumour tissue (50 g f.wt.) was homogenized in 70% ethanol (2 ml/g.f.wt.) using a Polytron tissue grinder and the homogenate was centrifuged at 4000xg 10 min with two washes of the pellet. Anionic species in the combined extract were adsorbed to Dowex1-X2 (acetate) by adding resin and strirring (3h) until inorganic phosphate (Bartlett, 1959) was no longer detected in the supernatant. Approximately 0.3 ml bed vol. resin/g f.wt. tumour tissue was required. The sample resin (10 ml) was loaded on top of a column (diameter 15 mm, bed volume 40 ml) of Dowex 1-X2 (acetate) and eluted (1 ml/min) with 0.2 M pyridine/0.1 M acetic acid pH 5.4 (Liebster et al., 1961). Agrocinopines, eluting at 3 to 5 bed volumes, were detected by a phloroglucinol assay for pentoses essentially as described by Dische and Borenfreund (1957) (see Appendix C) and appropriate fractions were pooled , reduced in volume and lyophilized. The mixture (15 mg) of agrocinopines A and B was further separated by DEAE-Sephadex A25 borate chromatography (column of 100 ml bed volume, 20 mm diameter, with a linear gradient of 0 (220 ml) - 0.4 M (220 ml) triethylamine/0.8 M boric acid pH 9.4 (1 ml/min) as elution buffer (Lefebvre et al., 1964). Fractions containing agrocinopines A or B were pooled, diluted to 50 mM boric acid and adjusted to pH 6 with acetic acid. Agrocinopines were again adsorbed to 30 ml bed volume Dowex 1-X2 (acetate), boric acid was eluted with water (5 bed volumes) and the agrocinopine A or B was recovered by elution with 0.2 M pyridine/0.1 M acetic acid, pH 5.4. Finally, each agrocinopine was converted to its sodium salt using Dowex 50W-X8 (Na^+) (2 ml bed volume, 5 mm diameter column) to yield 9.9 mg (0.02 g/100 g f.wt.) Na^+ agrocinopine A and 1.4 mg (0.003 g/100 g f.wt.) Na^+ agrocinopine B. Higher yields of agrocinopine A were obtained from sunflower tumours (see Results).

Synthesis of L-arabinose-2-phosphate

The synthetic path is illustrated in Fig. 3-1 and the Roman numerals refer to the structural formulae in that figure.

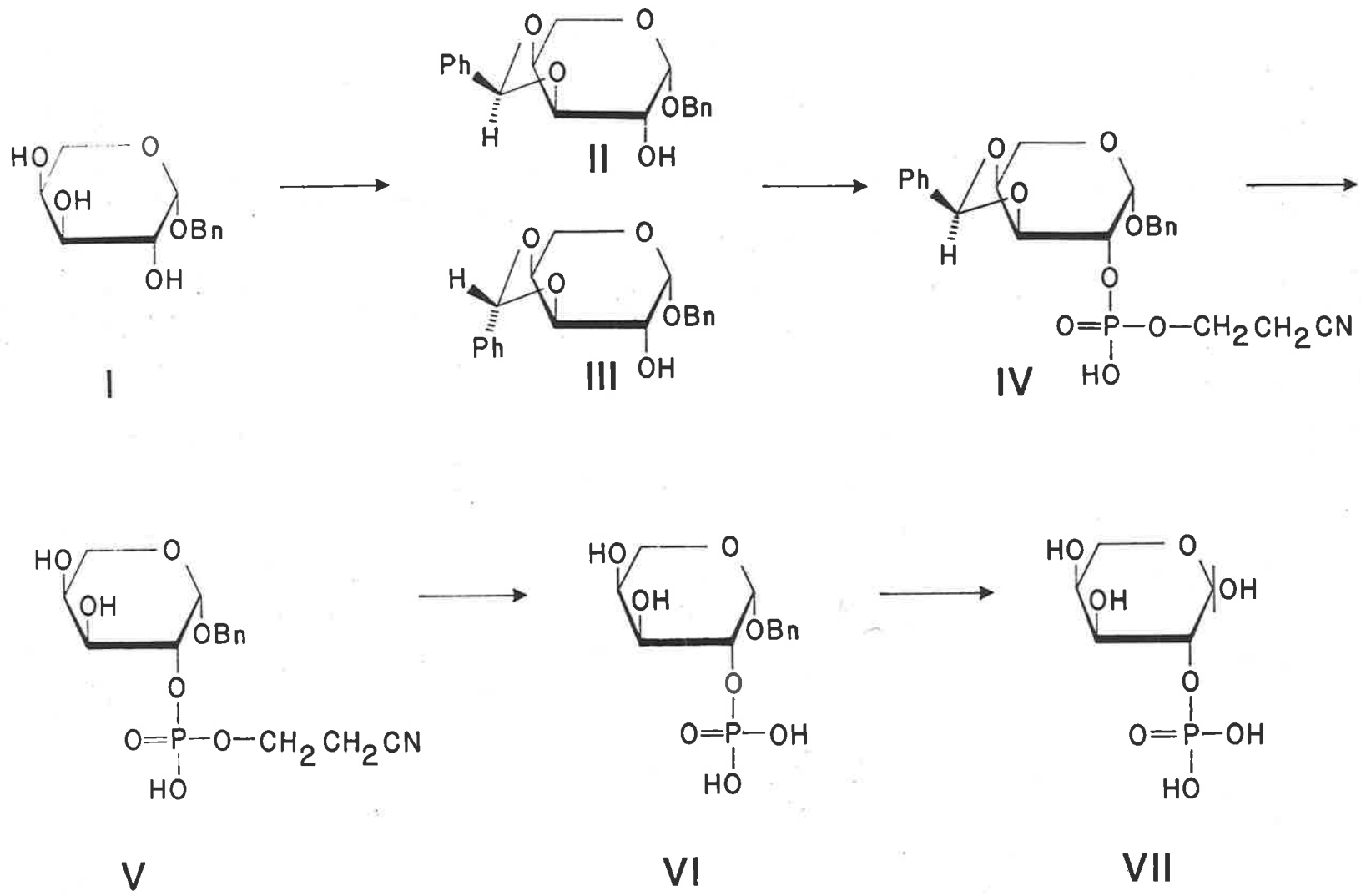
(a) 1-O-Benzyl-α-L-arabinopyranoside(I).-- Synthesis was essentially as described by Ballou (1957). L-arabinose (10g, dry) was suspended in benzyl alcohol (70 ml) in a 250 ml flask at 0°C. The flask was saturated with dry HCl (generated ex NH_4Cl, 100g, with conc. H_2SO_4, 100 ml, added dropwise) and maintained with stirring at 0°C until the arabinose dissolved (3h). After addition of ether (225 ml) at 0°C, the product separated as an oil. The oil was extracted repeatedly (5x) with ether (100 ml) and the solidified product was recrystallized 3x from n-propanol yielding I (4.6g, 29%), m.p. 167-172°C.

(b) 1-O-Benzyl-3,4-O-benzylidene-α-L-arabinopyranoside (II, III). -- A solution of I (2.43g, 10.1mmol) in α,α-dimethoxytoluene (Evans, 1972) (20g) with toluene-p-sulphonic acid (125mg) was stirred for 40h and progress of the reaction was followed by t.l.c. on silica gel (n-hexane/$CHCl_3$, 1:1 v/v). Toluene (40ml) was added, followed by 10% $KHCO_3$ (10ml, aq.) at 0°C. The toluene phase was passed through 1PS phase separator paper and the solvents removed in vacuo leaving an oily product (5.6g). Cyclohexane (50ml) was added and crystallization occurred at 4°C, three recrystallizations from cyclohexane yielding a solid (II, 0.21g, 6.3%) with m.p. 139-140°, $[\alpha]_{589}$ +165.3°, $[\alpha]_{546}$ +195.0°, $[\alpha]_{436}$ +326.2°, $[\alpha]_{365}$ +503.6° (c 0.5, ethanol)

Anal. Calculated for $C_{19}H_{20}O_5$: C 69.50; H 6.14. Found: C 69.43; H 6.02.

FIGURE 3-1

Scheme for the synthesis of L-arabinose-2-phosphate.


HO
OH
OBn
OH
I
Ph
H
II
OBn
OH
H
Ph
III
OBn
OH
Ph
H
IV
OBn
O=P–O–CH2CH2CN
HO
HO
OH
OBn
O=P–O–CH2CH2CN
HO
V
HO
OH
OBn
O=P–OH
HO
VI
HO
OH
OH
O=P–OH
HO
VII

The mother liquors were passed through alumina (20g in hexane) and eluted with diethyl ether. The second isomer (III) crystallized from the ether eluate and three recrystallizations from cyclohexane gave needles (0.25g, 7.5%) with m.p. 95.5-96.5°, $[\alpha]_{589}$ +203.8°, $[\alpha]_{546}$ +241.0°, $[\alpha]_{436}$ +409.1°, $[\alpha]_{365}$ +641.6° (c 0.5, ethanol).

Anal. Calculated for $C_{19}H_{20}O_5$: C 69.50; H 6.14. Found: C 69.40; H 5.95.

Upon ^{1}H-n.m.r., the high m.p.isomer (II) showed a benzyl proton signal at δ 4.60 ppm which corresponds to the isomer with an endo- benzyl proton (Baggett et al., 1964, 1965) whereas the low m.p. isomer (III) gave a benzyl proton signal at 4.28 ppm, corresponding to the isomer with an exo- benzyl proton. II and III were separable (R_f=0.52 and 0.58 respectively) by t.l.c. on alumina with $CHCl_3$.

(c) 1-O-Benzyl-2-O-phosphoryl-α-L-arabinopyranoside (VI). -- The procedure was based on a method (Sarfati et al., 1977) for synthesis of the D-enantiomorph. To a solution of II (164mg, 0.5mmol) in anhydrous pyridine (5ml), pyridinium 2-cyanoethylphosphate (1mmol in 1ml pyridine) and NN'-dicyclohexylcarbodiimide (1.2g in 10ml anhydrous pyridine) were added and the mixture was stirred for 48h. Water (70µl) was added, the mixture was dried in vacuo and the product was recovered by repeated (6x) aqueous extraction. The acidic conditions of the aqueous extract resulted in hydrolysis of the benzylidene group, giving V. The cyanoethyl group was cleaved by adjusting to pH 13 with NaOH (30 min) and subsequent addition of Dowex 50W(H^+) brought the pH to 7. The product (VI) was adsorbed to Dowex 1-X2(acetate), eluted with 0.4M pyridine/ 0.2M acetic acid pH 5.4, converted to the monosodium salt (63mg, 37%) and then to the disodium and

biscyclohexylammonium salts. The biscyclohexylammonium salt (m.p. 180-181°) was crystallized from acetone/water, 1:1 (v/v).

(d) L-arabinose-2-phosphate (VII).-- The disodium salt of VI (52mg) was hydrogenated 1h with PdO (Angyal and Odier, 1982) in aqueous solution, pH 9.8, yielding L-arabinose-2-phosphate, disodium salt (VII, 40mg, 98%) which was subsequently converted to the calcium salt, $[\alpha]_{589}$ +43.8°, $[\alpha]_{578}$ +44.8°, $[\alpha]_{546}$ +51.0°, $[\alpha]_{436}$ +85.7° (c 0.2 in H_2O) using Dowex 50W-X8(Ca^{++}). The literature value reported (Sarfati et al., 1977) for the D-enantiomer, Ca salt, was $[\alpha]_D^{20}$ -44.6°.

Anal. Calculated for $C_5H_9O_8PCa.H_2O$: C 22.5; H 3.0; P 11.6. Found: C 21.0; H 3.9; P 10.8.

RESULTS

1. Isolation and purification of agrocinopines

Fig. 3-2A shows the bioassay (Ellis and Murphy, 1981) used to detect agrocinopines during the fractionation procedure. Agrocinopines A and B extend the agrocin 84 inhibition zone. Analytically pure agrocinopine A was detected down to a level of 0.2µg by this method.

Agrocinopines were isolated from plant tumours by extraction with 70% ethanol followed by anion exchange separation. Fig. 3-3A shows the coincidence of biological activity, phosphorus and pentose in the eluate of a morpholine acetate gradient (0 - 0.5 M) of a Dowex 1-X2 (acetate) fractionation. Similar separations were later carried out with the volatile pyridine acetate buffer at pH 5.4 (Liebster et al., 1964). Separation of agrocinopines A and B was carried out on a borate anion exchange column (Fig. 3-3B). The DEAE-Sephadex column was eluted with a

FIGURE 3-2A

Bioassay of 0.2 to 5 µg of analytically pure agrocinopine A using the method of Ellis and Murphy (1981).
The circular inhibition zone, due to the toxicity of the fraudulent adenine nucleotide agrocin 84, is extended in the presence of agrocinopine A.

FIGURE 3-2B

Bioassay of 4 to 100 µg of synthetic L-arabinose-2-phosphate in the agrocinopine bioassay, using the method of Ellis and Murphy (1981).

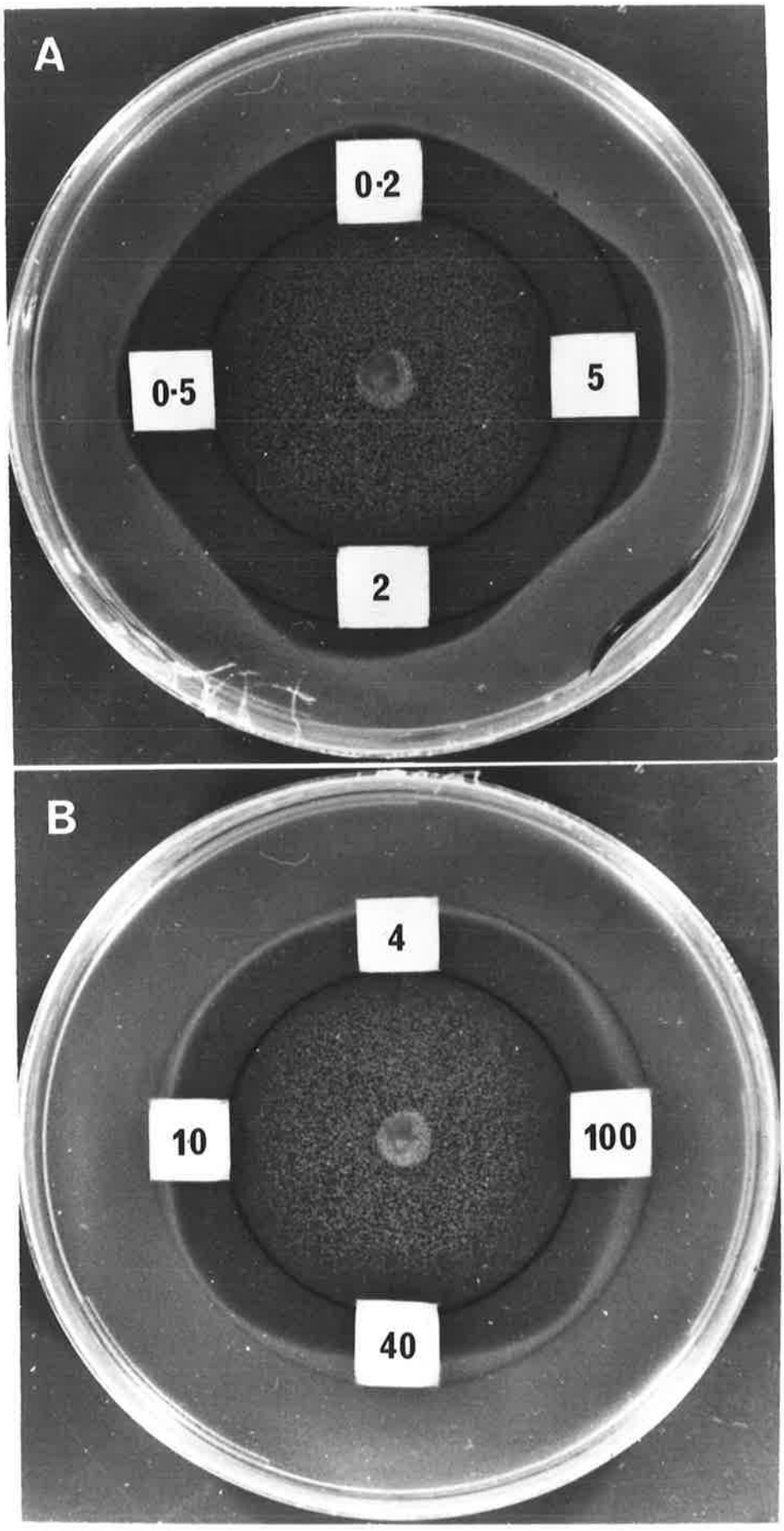

A
0·2
0·5
5
2
B
4
10
100
40

FIGURE 3-3A

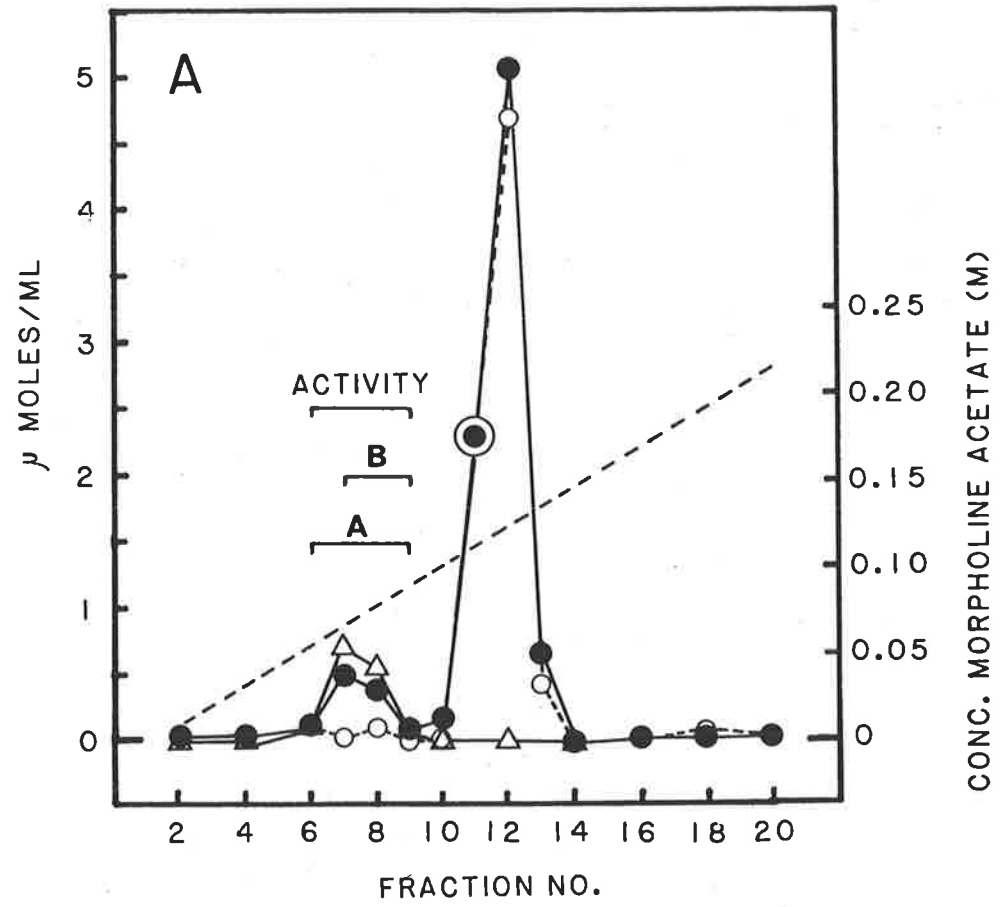
Anion exchange separation of agrocinopines and inorganic phosphate on Dowex 1-X2 (acetate) (10 ml bed vol.) using a gradient of morpholine acetate, pH 4.6 (10 ml fractions).

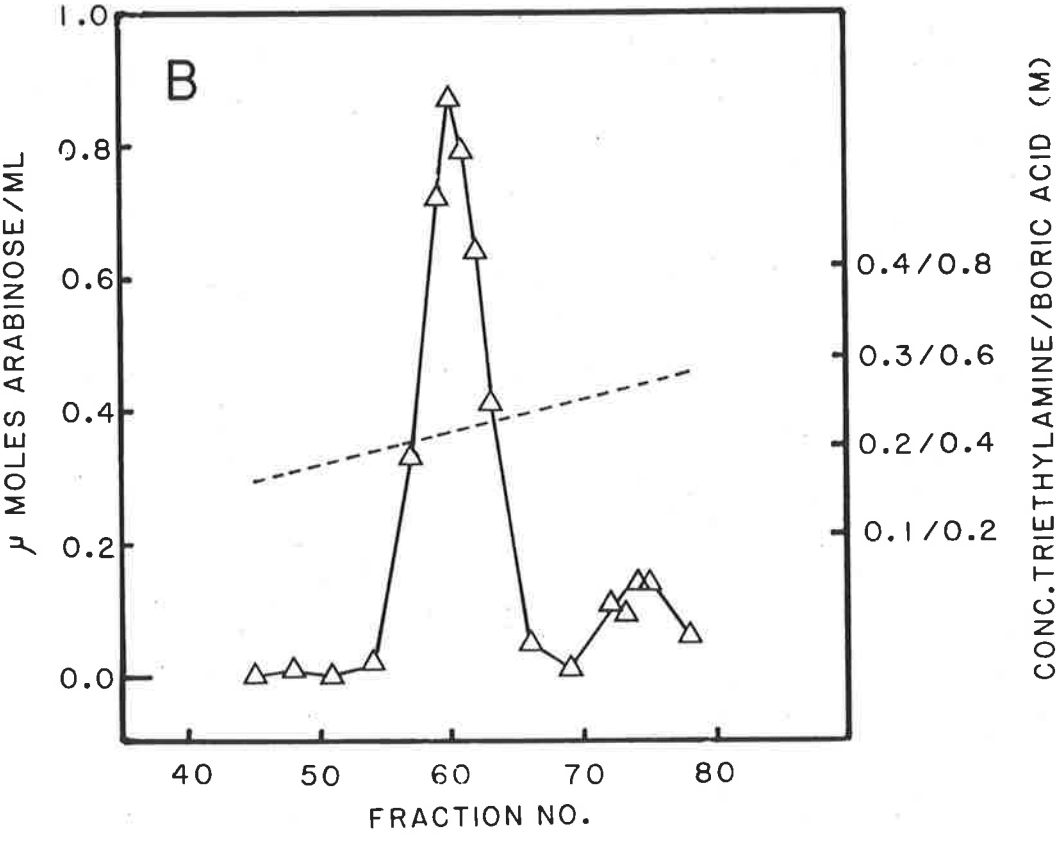
△——△ arabinose; o--o inorganic phosphate; ●——● total phosphate; - - - buffer gradient. Biological activity was found in fractions 6-9, with agrocinopine A detected in fractions 6-9 and agrocinopine B in fractions 7-9.

FIGURE 3-3B

Borate anion exchange chromatography (DEAE-Sephadex A25, 190 ml bed vol.) , of agrocinopines A and B using a triethylammonium borate pH 9.4 gradient (5 ml fractions).

Fractions 54-66 = agrocinopine A; fractions 70-78 = agrocinopine B; arabinose; - - - buffer gradient. The purified agrocinopines A and B were both active in the agrocinopine bioassay.


A
ACTIVITY
B
A
μ MOLES/ML
CONC. MORPHOLINE ACETATE (M)
FRACTION NO.
0
1
2
3
4
5
0.05
0.10
0.15
0.20
0.25
2 4 6 8 10 12 14 16 18 20


B
μ MOLES ARABINOSE/ML
CONC.TRIETHYLAMINE/BORIC ACID (M)
FRACTION NO.
0.0
0.2
0.4
0.6
0.8
1.0
0.4/0.8
0.3/0.6
0.2/0.4
0.1/0.2
40 50 60 70 80

triethylamine borate (pH 9.2) gradient (0 - 0.4 M). The borate complexing buffer was removed by rechromatography at pH 5.4 in the pyridine acetate system. Cation exchange chromatography (Dowex 50W-X8 Na^+) of the agrocinopine A pyridinium salt yielded an electrophoretically homogeneous, analytically pure sodium salt.

The overall yields of agrocinopine A, uncorrected for losses, in two extractions from sunflower tumours were 0.052 and 0.057 g/100g f.wt. Agrocinopine B was not detectable in these extractions and is therefore considered to be a breakdown product of agrocinopine A. Elemental analysis of the agrocinopine A sodium salt, $C_{17}H_{30}O_{18}PNa.3H_2O$ required C 32.4 ; H 5.8 ; N 0.0 ; P 4.9 , found: C 32.2 ; H 6.1 ; N <0.3 ; P 4.6%. A small sample of agrocinopine B (1.4 mg) was isolated from tomato tumours. The sample was electrophoretically pure, biologically active and had the properties reported by Ellis and Murphy (1981).

In one nopaline tumour extraction, a compound was found which had biological activity in the agrocinopine bioassay but could be clearly distinguished from oth agrocinopines A and B by its electrophoretic properties ($M_{O.G}$ 0.0 at pH 1.7; $M_{O.G}$ 0.55 at pH 9.2; $M_{O.G}$ 0.70 in ammonium borate, pH 9.2). This compound stained positively with the ketose and phosphate reagents but was not detectable by UV absorption. The compound was stable in solution at pH 7.8 but labile under weakly acidic and under more alkaline conditions, giving rise to agrocinopine A as a degradation product. It therefore appeared to be a molecule larger than agrocinopine A. Its lack of charge at pH 1.7 could be explained by the presence of N-containing group(s) to counteract the negatively charged phosphate group. This compound may be worthy of further investigation.

2. Physical and chemical properties

The measured optical rotations of agrocinopine A at various wavelengths were: $[\alpha]_{589}$ +47.9° ; $[\alpha]_{546}$ +56.5° ; $[\alpha]_{436}$ +93.1° ; $[\alpha]_{365}$ +139.3° (c=1, H_2O, 1=1). Infrared spectroscopy showed no carbonyl absorption and definite similarities to sucrose in the fingerprint region. The following peaks (>2% transmission) were detected: 3375, 2927, 1597, 1224, 1056, 998, 929, 883 cm^{-1}. Table 3-1 lists ^{13}C n.m.r. data for agrocinopine A, sucrose, L-arabinose-2-phosphate and L-arabinose, and the data for the corresponding reduced compounds are presented in Table 3-2.

The electrophoretic mobility profiles of agrocinopines A and B (Fig. 3-4) revealed no change in relative mobility between pH 1.5 and pH 10, demonstrating the presence of a single ionizable group with a strongly acid pK (below pH 1.5) for both molecules. This behaviour is only consistent with that of a phosphodiester and is similar to the data reported by Tate (1981) for the reference phosphodiester bis-(p-nitrophenylphosphate). By contrast, the phosphate monoesters D-ribose-5-phosphate and synthetic L-arabinose-2-phosphate (Fig. 3-4) showed the expected sharp rise in mobility between pH 6 and 7 corresponding to the second ionization of their phosphate monoester group.

3. Degradative studies on agrocinopine A

(a) Weak acid hydrolysis

Brief acid hydrolysis (1.5 M acetic acid, 110°C, 15 min) produced glucose ($M_{O.G}$, borate, 0.86), a small amount of fructose ($M_{O.G}$ 0.76), arabinose ($M_{O.G}$ 0.74 and a ketose-positive component ($M_{O.G}$ 0.95) which was indistinguishable from agrocinopine B in mobility and biological activity.

Longer acid hydrolysis (1.5 M acetic acid, 110°C, 16h) and analysis by paper chromatography (solvent A) showed the presence of arabinose

Table 3-1

^{13}C NMR spectra of agrocinopine A, sucrose, L-arabinose 2-phosphate, L-arabinose[a]

Agrocinopine A	Assgt.	Sucrose[b]	L-arabinose-2-phosphate	Arabinopyranose[c]	
105.1	F2	104.6			
96.4 $d^3J = 6.1$	A1α		97.6 $d^3J = 6.1$	97.7	1α
93.4	G1	93.1			
92.55 $d^3J = 4.9$	A1β		93.15 $d^3J = 4.9$	93.5	1β
85.2[n]					
82.0 $d^3J = 4.9$	F5	82.3			
79.6 $d^2J = 4.9$	F4	74.9			
79.4[n]					
77.95 $d^2J = 4.9$	A2α		75.8 $d^2J = 4.9$	72.9	2α
77.2 $d^3J = 4.9$	F3	77.3			
74.65 $d^2J = 4.9$	A2β		72.3 $d^2J = 4.9$	69.4	2β
73.7	G3	73.3			
73.4	G5	73.1			
72.9 $d^3J = 2.3$	A3α		73.65 $d^3J = 2.4$	73.4	3α

72.2	G2	72.0			
70.4	G4	70.1			
69.4	A4β		69.8	69.7	4β
68.8 $d^3J = 3.7$	A3β		69.85 $d^3J = 3.6$	69.7	3β
68.8	A4α		69.6	69.4	4α
66.2	A5α		67.0	67.3	5α
63.6[n]					
63.4	F6, A5β	63.3	63.3	63.5	5β
62.6	F1	62.3			
61.4	G6	61.0			

[a] Chemical shifts (ppm) calculated downfield from Me_4 Si, via an internal TSP standard. (Me_4Si = TSP - 1.8 ppm). [Abbreviations: Assgt. = assignment, G = glucose, F = fructose, A = arabinose, A1α (example) = arabinose, carbon 1, α pyranose form, d = doublet with coupling constant (Hz)]

[b] Assignments from Bax *et al.* (1981)

[c] Assignments from Gorin & Mazurek (1975)

[n] Assignment not made

Table 3-2

^{13}C NMR spectra of reduced agrocinopine A, sucrose, L-arabinitol 2-phosphate and L-arabinitol[a]

Reduced agrocinopine A	Assgt.	Sucrose[b]	L-arabinitol-2-phosphate	L-arabinitol[c]
104.9	F2	104.6		
93.2	G1	93.1		
82.0 $d^3J = 4.9$	F5	82.3		
79.45 $d^2J = 6.1$	F4	74.9		
76.85 $d^3J = 6.1$	F3	77.3		
76.25 $d^2J = 6.1$	A2		74.2 $d^2J = 4.9$	71.3
73.5	G3	73.3		
73.3	G5	73.1		
72.0	G2	72.0		
71.5	A4		72.2 72.0	
71.0 $d^3J = 4.9$	A3		71.6 $d^3J = 3.7$	71.6

70.2	G4	70.1	
63.7	A5		64.1 64.0
63.2	F6, A1	63.3	63.5 64.1
62.2	F1	62.3	
61.0	G6	61.0	

---

[a] Chemical shifts (ppm) calculated downfield from Me_4Si via an internal TSP standard. (Me_4Si = TSP - 1.8 ppm). [Abbreviations: Assgt. = assignment, G = glucose, F = fructose, A = arabinitol, F2 (example) = fructose carbon 2, d = doublet with coupling constant (Hz)]

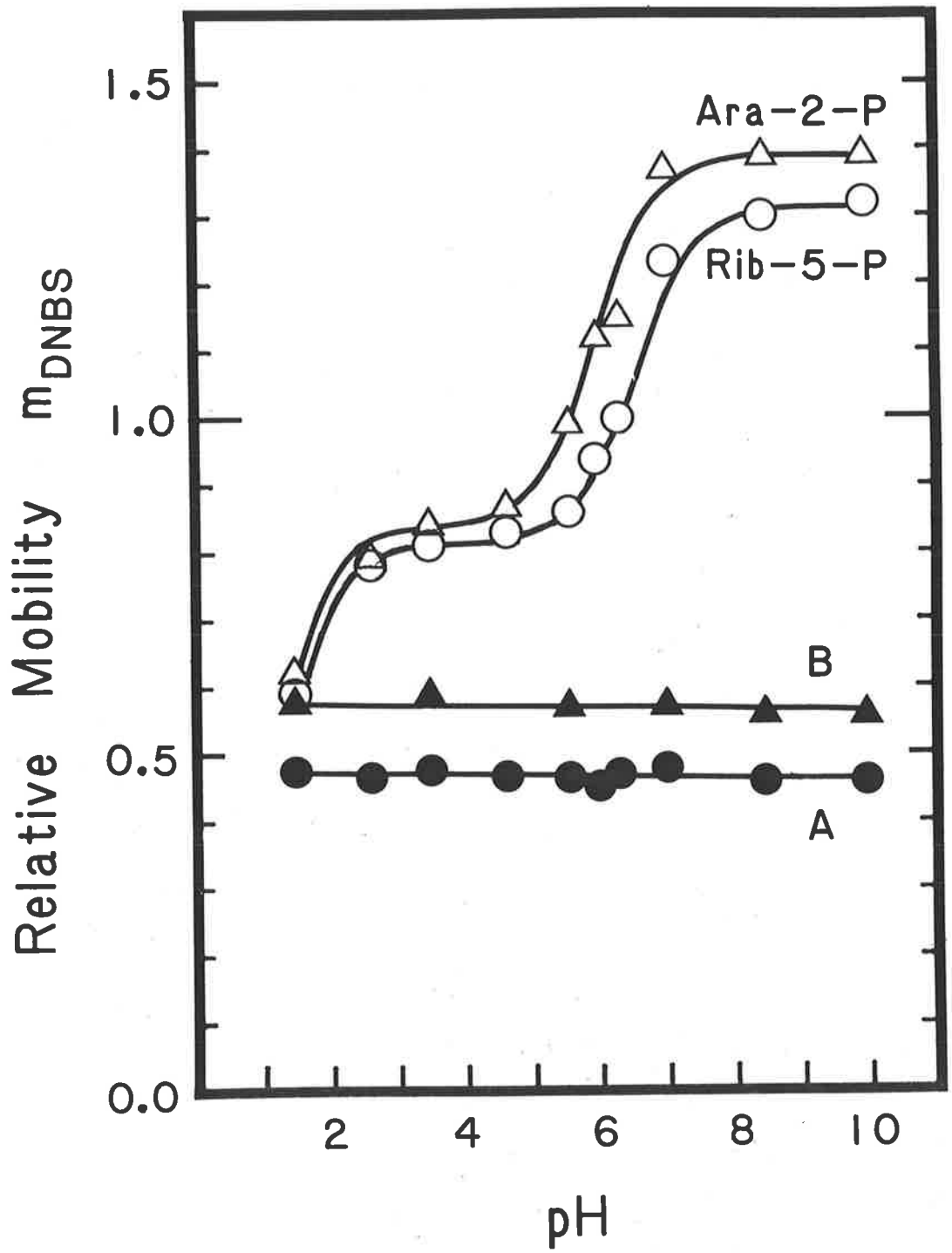
[b] Assignments from Bax *et al.* (1981)

[c] Assignments from Angyal and Le Fur (1980)

FIGURE 3-4

Electrophoretic mobility profiles for agrocinopine A (●), agrocinopine B (▲), L-arabinose-2-phosphate (△) and D-ribose-5-phosphate (○).

Mobilities are expressed relative to 2,4-dinitrobenzenesulphonate. Curves for the phosphate monoesters and pKa values were recalculated as described by Tate (1981). Note the characteristic phosphate monoester primary ionizations (L-arabinose-2-phosphate pKa_1 = 1.0, D-ribose-5-phosphate pKa_1 = 1.1) as well as their secondary ionizations (L-arabinose-2-phosphate pKa_2 = 6.0, D-ribose-5-phosphate pKa_2 = 6.5).


Relative Mobility m_{DNBS}
1.5
1.0
0.5
0.0
2
4
6
8
10
pH
Ara-2-P
Rib-5-P
B
A

(R_f 0.32) and glucose (R_f 0.26). Ketose sugars are decomposed under these hydrolysis conditions and were not detected. The identities of arabinose and glucose were confirmed by electrophoresis in 0.1 M NaOH (arabinose M_R 0.86 and glucose M_R 0.77; M_R values are mobilities recorded relative to ribose) and in ammonium borate (arabinose $M_{O.G}$ 0.76 and glucose $M_{O.G}$ 0.86).The glucose was identified as D-glucose by a positive glucose oxidase test.

(b) Weak base hydrolysis

The known and presumed products of this degradation procedure are shown in pathway (iii) in Fig. 3-5. Preparative electrophoresis (pH 1.7) of the products of a sealed tube ammonolysis (1 M NH_4OH, 110°C, 2h) of agrocinopine A revealed the presence of four silver nitrate-positive components (E,F,G,H; Fig.3-6) with $M_{O.G}$ 0.0 (E), 0.51 (F), 0.67 (G) and 0.80 (H). Their staining properties are shown in Table 3-3A. Each component was eluted and separated by paper electrophoresis in a suitable buffer.

The neutral fraction (E) separated in ammonium borate (pH 9.2) into a major ketose-positive component ($M_{O.G}$ 0.06) identical to sucrose and a minor pentose-positive component ($M_{O.G}$ 0.71) identical in its properties to arabinose.

The anionic ammonolysis products contained phosphate and each component from pH 1.7 electrophoresis was separated by electrophoresis in ammonium bicarbonate buffer pH 9.2. At this pH any phosphate monoesters would be completely ionized. The results are presented in Table 3-3B.

Component F consisted mostly of a ketose-positive compound with $M_{O.G}$ 0.91. This compound was identified as a sucrose phosphate by release of sucrose ($M_{O.G}$ 0.07 in ammonium borate) after alkaline phosphatase (EC 3.1.3.1) treatment and subsequent hydrolysis of the sucrose by invertase

FIGURE 3-5

Acid, alkaline and enzymatic degradation pathways for agrocinopine A. Bracketed components were not examined in detail. (i) α-glucosidase (pH 6.6/25°/8h); (ii) 1.5 M acetic acid/110°/15 min); (iii) 1.5 M NH_4OH/110°/2h.

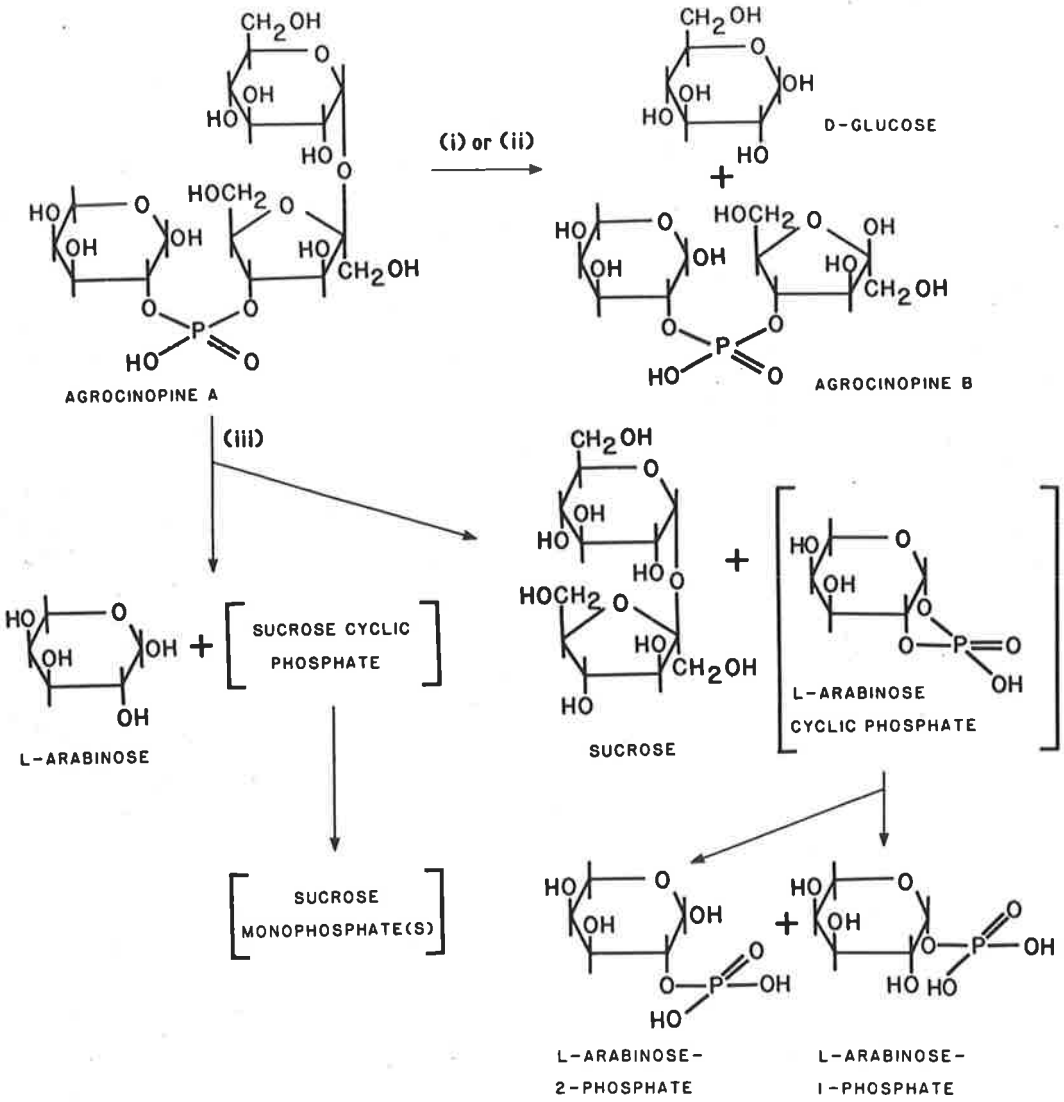

AGROCINOPINE A
(i) or (ii)
D-GLUCOSE
AGROCINOPINE B
(iii)
L-ARABINOSE
SUCROSE CYCLIC PHOSPHATE
SUCROSE
L-ARABINOSE CYCLIC PHOSPHATE
SUCROSE MONOPHOSPHATE(S)
L-ARABINOSE-2-PHOSPHATE
L-ARABINOSE-1-PHOSPHATE

FIGURE 3-6

Ammonolysis products of agrocinopine A separated by HVPE at pH 1.7 revealing four components (E, F, G and H).

The electrophoretogram was stained with (from left to right): alkaline silver nitrate, p-anisidine, ketose reagent and phosphate reagent. Product E remained at the origin; the other products were anionic.

H
G
F
E
0.80
0.67
0.51
yellow!

TABLE 3-3A

Paper electrophoresis at pH 1.7 of agrocinopine A ammonolysis products[a].

Component	$M_{0.G}$	$AgNO_3$	p-anisidine	ketose reagent
E	0.00	++	yellow/pink	++(blue)
F	0.51	+	yellow	+(blue)
G	0.67	++	pink	-
H	0.80	+	pink	-

[a] The products of ammonolysis (1 M NH_4OH, 110°C, 2h) were separated by HVPE at pH 1.7. The electrophoretic mobilities and staining properties of the four components (E,F,G and H) are recorded here. $AgNO_3$ = alkaline silver nitrate stain; ++ = strong stain reaction; + = weak stain reaction; - = did not stain.

TABLE 3-3B

Paper electrophoresis at pH 9.2 of agrocinopine A ammonolysis products[a].

Component	$M_{0.G}$	$AgNO_3$	Additional staining properties
F	0.48	+	
	0.60	+	
	0.91	+++	++ (ketose reagent)
	1.32	(+)	
G	1.24	++	++ (p-anis. pink)
	1.32	+++	+++ (p-anis. red-brown)
	1.44	+	
H	1.32	++	++ (p-anis. red-brown)
	1.44	+	n.d.

[a] Components F, G and H (see Table 3-3A) were eluted after HVPE at pH 1.7 and each was then separated by HVPE at pH 9.2 in ammonium bicarbonate buffer. The electrophoretic relative mobilities and staining properties of the compounds separated in this way are recorded. $AgNO_3$ = alkaline silver nitrate dip reagent; p-anis. = p-anisidine reagent; (+), +, ++, +++ = increasing intensity of stain reaction; n.d. = not detectable.

(EC 3.1.2.26) yielding glucose and fructose ($M_{0.G}$ 0.81 and 0.76 respectively in ammonium borate pH 9.2). Three minor components ($M_{0.G}$ 0.48, 0.60 and 1.32) were not further investigated.

Component G was resolved into 3 components at pH 9.2. Two compounds with $M_{0.G}$ 1.24 and $M_{0.G}$ 1.32 were the major products. The compound with $M_{0.G}$ 1.24 was identified as arabinose-1-phosphate by its staining properties (pink with p-anisidine) and resistance to reduction by sodium borohydride. The second major compound , $M_{0.G}$ 1.32 was reducible by sodium borohydride, stained red-brown with p-anisidine and was indistinguishable from synthetic L-arabinose-2-phosphate. These two compounds were also cleaved by alkaline phosphatase to yield arabinose (borate $M_{0.G}$ 0.74) and inorganic phosphate (borate $M_{0.G}$ 1.58). The minor product ($M_{0.G}$ 1.44) was present in insufficient quantity for further characterization.

Component H was a minor product and was also found as a degradation product in a control reaction with synthetic L-arabinose-2-phosphate. When treated with alkaline phosphatase, this degradation product released not arabinose but a small amount of ribose. No ribose was detected in acid hydrolysates of agrocinopine A, so this alkaline epimerization product was not further examined.

(c) Enzymic hydrolysis

All attempts to cleave agrocinopine A or B with snake venom phosphodiesterase (EC 3.1.4.1) and with bovine spleen phosphodiesterase (EC 3.1.4.18) have been unsuccessful. Agrocinopine A was also resistant to invertase (EC 3.2.1.26). Yeast α-glucosidase was able to slowly hydrolyse agrocinopine A at pH 6.6, 25°, yielding a mixture of agrocinopine B ($M_{0.G}$ 0.55, pH 1.7) and glucose. The glucose released was measured by the D-glucose oxidase/peroxidase method.

(d) Reduced agrocinopine A and its degradation products

Treatment of agrocinopine A (19.3mg, 0.03 mmoles) with excess sodium borohydride (0.15 mmoles) at room temperature, 1h, resulted in loss of the pentose reaction in the phloroglucinol assay and loss of biological activity in the agrocinopine bioassay (at 5µg). The reduced compound was not separable from agrocinopine A by HVPE at pH 9.2 ($M_{O.G}$ 0.44) but could be distinguished by its yellow colour reaction with p-anisidine.

Ammonolysis (1.5 M NH_4OH, 110°C, 2h) of reduced agrocinopine A (12.4 mg, 0.020 mmoles) yielded sucrose (4.9 mg, 0.014 mmoles) and several anionic products.

The identity of the sucrose was verified by infrared spectroscopy and HVPE in ammonium borate ($M_{O.G}$ 0.07).

The major anionic (phosphorylated) ammonolysis product ($M_{O.G}$ 0.58, pH 1.7) was hydrolysed in sodium acetate buffer at pH 5.4 (8h, 110°C) to yield inorganic phosphate and a polyol. Upon HVPE in 0.2 M calcium acetate, pH 4.7, (Angyal and Mills, 1979) the polyol showed only a single silver nitrate-positive component which was resolved from ribitol and xylitol, but was indistinguishable from arabinitol ($M_{O.G}$ 0.12). Acetylation of the polyol (acetic anhydride-pyridine, 1:1, v/v, 65°C, 1h) followed by sublimation (70°, 9h, 0.01mm Hg) and recrystallization from cyclohexane gave plates with mp 73.5-74.5°C. The melting point was unchanged when mixed with authentic L-arabinitol pentaacetate. By contrast, the mixed mp with authentic D-arabinitol pentaacetate was raised to 94.5-95.5°C as expected of and observed for a racemic mixture of arabinitol pentaacetates.

The specific rotation $[\alpha]_{589}$ -44.2° (c = 0.12, EtOH, 1=1) was also in reasonable agreement with the measured specific rotation $[\alpha]_{589}$ -39.7° (c = 0.12, EtOH, 1=1) for authentic L-arabinitol pentaacetate. Identical

retention times (27.1 min) upon gas chromatography (3% OV 101, Gaschrom Q, 2mm packed column, 110-170°, 16°/min) and a mass spectral similarity index of 0.995 compared to authentic L-arabinitol pentaacetate for the 10 most significant ions and their abundances 289 (3), 217 (11), 187 (15), 158 (6), 145 (18), 127 (10), 116 (80), 115 (28), 103 (14), 43 (100) added to the identity criteria. The infrared spectra of the acetylated polyol from reduced agrocinopine A and L-arabinitol pentaacetate were identical. These data clearly establish that the arabinose moiety of agrocinopine A must have the L- configuration.

4. Summary of degradation studies

(a) The analysis of agrocinopine A was consistent with a 1:1:1 ratio for sucrose, phosphate and arabinose.

(b) Since agrocinopine A can be degraded to biologically active agrocinopine B by loss of glucose, and both agrocinopines A and B are phosphodiesters, the phosphodiester linkage to sucrose must be to the fructosyl moiety.

(c) Amongst the ammonolysis products of agrocinopine A was a mixture of arabinose phosphates. One of these was resistant to sodium borohydride reduction and was therefore arabinose-1-phosphate. The other was reducible by sodium borohydride and was indistinguishable in its electrophoretic and staining properties from synthetic L-arabinose-2-phosphate. This is consistent with the degradation of agrocinopine A proceeding through the formation of a cyclic arabinose-1,2-phosphate intermediate (Fig. 3-5). Other ammonolysis products included sucrose, a small amount of arabinose, and a sucrose phosphate.

(d) Agrocinopine A was reducible by sodium borohydride to the arabinitol phosphodiester. This, taken together with the presumed cyclic 1,2 phosphate mentioned in (c) above, is consistent with a phosphodiester linkage to the 2-carbon atom of arabinose.

(e) L-arabinitol was isolated from the ammonolysis of borohydride-reduced agrocinopine A. The arabinose in agrocinopine A must therefore have the L- configuration.

5. Synthesis of L-arabinose-2-phosphate

L-arabinose-2-phosphate was synthesized in order to confirm the position of the phosphodiester linkage to arabinose in agrocinopine A. The synthetic product had optical rotation $[\alpha]_D$ $+43.8^o$, similar in magnitude but opposite in sign to that reported for the D- enantiomorph, $[\alpha]_D$ -44.6^o, synthesized by Sarfati et al. (1977) by a different route. The L-arabinose-2-phosphate was biologically active and inhibited the toxic effect of agrocin 84 in the agrocin bioassay (Fig. 3-2B) at a level of 10µg.

6. The position of the phosphodiester linkage

The best evidence for the position of the phosphodiester linkage comes from ^{13}C n.m.r. studies. The results are shown in Fig. 3-7 and Table 3-1. The results for the corresponding borohydride-reduced compounds are presented in Fig. 3-8 and Table 3-2.

Data already published for sucrose, L-arabinose and L-arabinitol (Pfeffer et al, 1979; Gorin and Mazurek, 1975; Angyal and LeFur, 1981) were recorded again and were in good agreement with the published data, apart from very small (0.1-0.2 ppm) upfield shifts.

The assignments of the arabinose signals in agrocinopine A are shown

FIGURE 3-7

^{13}C n.m.r. spectra of (a) sucrose, (b) agrocinopine A, sodium salt, (c) L-arabinose-2-phosphate, disodium salt and (d) L-arabinose.

Chemical shifts (ppm) are calculated relative to Me_4Si via an internal TSP reference (Me_4Si = TSP-1.8 ppm). The broken lines show provisional assignments of agrocinopine A and L-arabinose-2-phosphate signals from the published data for sucrose (Bax et al., 1981; and L-arabinose (Gorin and Mazurek, 1975).

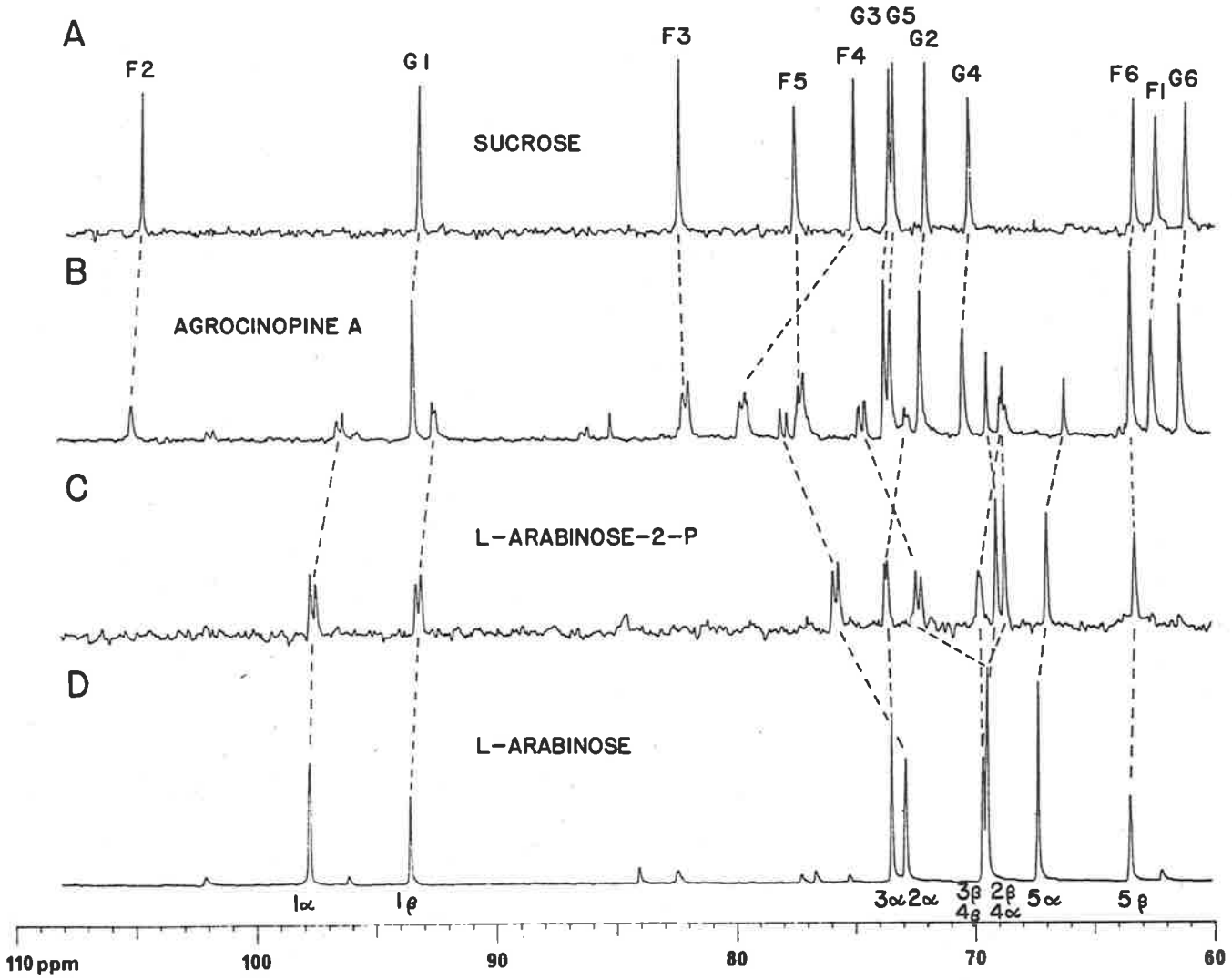
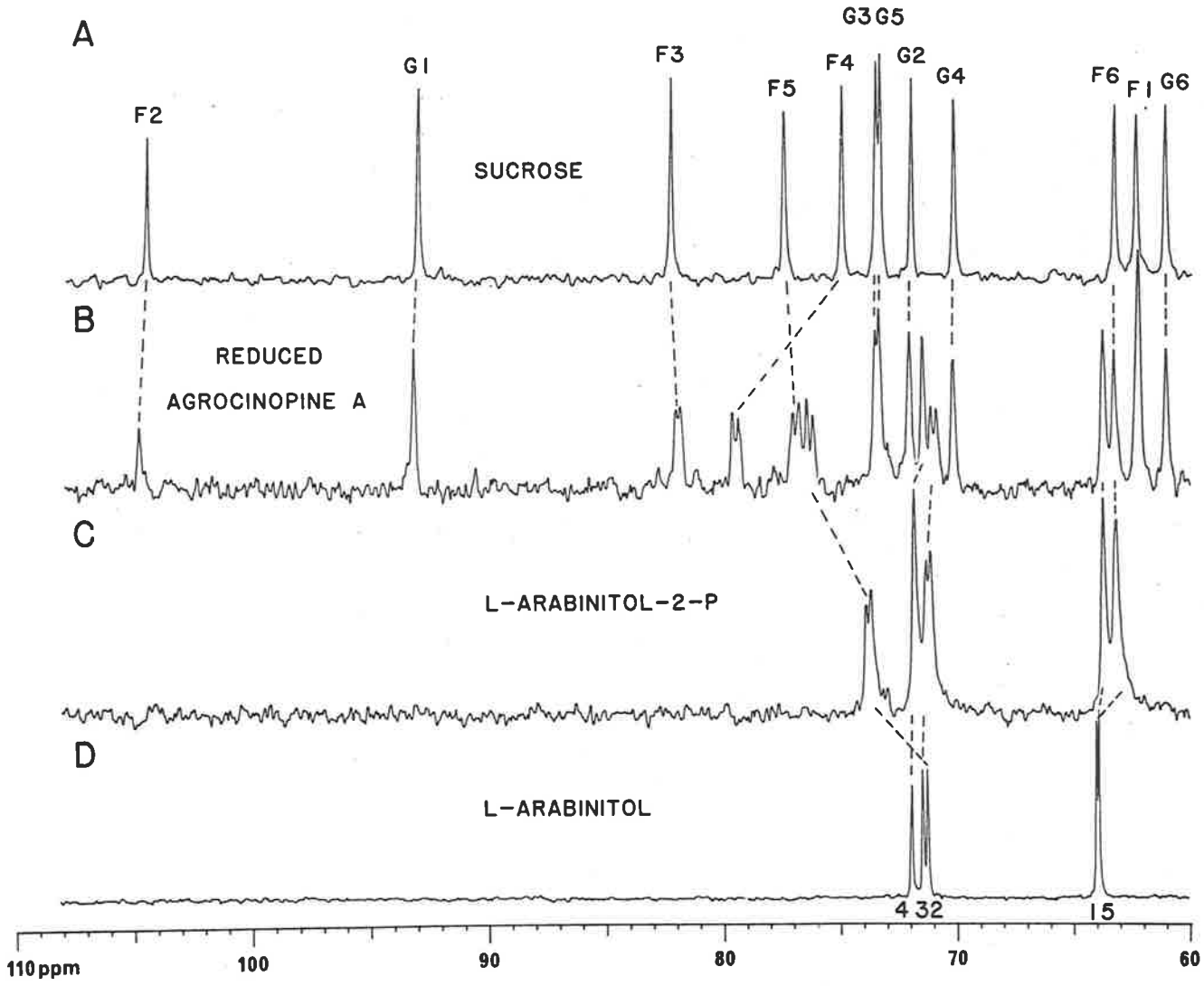

A
F2
G1
F3
F5
F4
G3 G5
G2
G4
F6
F1
G6
SUCROSE
B
AGROCINOPINE A
C
L-ARABINOSE-2-P
D
L-ARABINOSE
1α
1β
3α 2α
3β
4β
2β
4α
5α
5β
110 ppm
100
90
80
70
60

FIGURE 3-8

^{13}C n.m.r. spectra of (a) sucrose, (b) borohydride-reduced agrocinopine A, sodium salt, (c) L-arabinitol-2-phosphate, disodium salt and (d) L-arabinitol.

Chemical shifts (ppm) are calculated relative to Me_4Si via an internal TSP reference (Me_4Si = TSP-1.8 ppm). The broken lines show provisional assignments of reduced agrocinopine A and L-arabinitol-2-phosphate signals from the published data for sucrose (Bax et al., 1981) and L-arabinitol (Angyal and LeFur, 1980).


A
F2
G1
F3
F5
F4
G3 G5
G2
G4
F6
F1
G6
SUCROSE
B
REDUCED
AGROCINOPINE A
C
L-ARABINITOL-2-P
D
L-ARABINITOL
4 3 2
1 5
110 ppm
100
90
80
70
60

in Fig. 3-7 and Table 3-1. Comparison of the spectra for L-arabinose and L-arabinose-2-phosphate revealed that carbon-2 had undergone a large (2.9 ppm) downfield displacement for both α and β anomers upon substitution. Almost all other arabinose signals were displaced slightly upfield. The 2-bond coupling for carbon-2 was 4.9 Hz. The anomeric carbon signals were doublets with 3-bond couplings of 6.1 and 4.9 Hz and the 3-bond couplings for the C-3 signals were 2.4 and 3.6 Hz for the α and β anomers respectively.

The split anomeric signals were clearly visible in the agrocinopine A spectrum; they were slightly further upfield but had the same relative positions and exhibited the same coupling constants as in synthetic L-arabinose-2-phosphate. The carbon-2 signals were displaced even further downfield (2.2 ppm) than in L-arabinose-2-phosphate. All other arabinose signals except one were located and tentatively assigned in the agrocinopine A spectrum. The carbon-5 signal for the β anomer (A-5β) was presumed to be obscured by one of the sucrose primary alcohol signals, probably the F-6 signal because this was much larger in agrocinopine A than in sucrose.

Comparison of the spectra for agrocinopine A and reduced agrocinopine A showed that the arabinose anomeric signals disappeared upon reduction. Thus although the signals were doublets in the agrocinopine A spectrum, the phosphate group cannot be bonded directly to the anomeric carbon.

Signals in the agrocinopine A spectrum originating from sucrose were identified by comparison with authentic sucrose and using the assignments of Bax et al. (1981). The following sucrose peaks were essentially unchanged in agrocinopine A: the anomeric carbon atoms F-2 and G-1, the primary alcohol groups not on the sugar rings (F-6, F-1 and G-6) and the members of the glucose ring (G-2, -3, -4 and -5). None of the signals

originating from the glucose moiety were split. The only sucrose signals to be split by the proximity of the phosphate group were F-3, F-4 and F-5. The one possible interpretation is that the phosphate group is attached to carbon-4 of the fructosyl moiety in agrocinopine A. The identity of the F-3, -4 and -5 signals was confirmed by the fact that they remained unchanged (apart from a 0.2 ppm upfield shift) after sodium borohydride reduction (Fig. 3-8).

The coupling constants for F-3, -4 and -5 were 4.9 Hz in each case and thus no distinction between 2- and 3-bond couplings was possible. Relative to the corresponding sucrose signals, peaks F-3 and F-5 showed a slight upfield displacement (0.1 and 0.3 ppm respectively) and F-4 was shifted downfield 4.9 ppm. This large downfield displacement is characteristic of a phosphate-substituted carbon (Gorin, 1981; Mantsch and Smith, 1972; Gronenborn et al., 1983). At higher resolution, the F-4 peak showed more complex splitting than the other split signals. This is presumed to be caused by bond angle changes of the phosphodiester group (Gorenstein and Kar, 1975) due to alternate conformations of the agrocinopine A molecule, or to the presence of detectable amounts of arabinofuranose anomers as shown below.

Comparison of the L-arabinose-2-phosphate and agrocinopine A data with the α and β arabinopyranose data of Gorin and Mazurek (1975) reveals such similarities that the arabinose in the former two compounds must be present mostly in the pyranose form, with only a small proportion in the furanose form, as discussed below.

The agrocinopine A spectrum (Fig. 3-7) showed two small doublets (101.8 ppm, d^3J = 6.1 Hz and 95.8 ppm d^3J = 2.4 Hz) in the anomeric region with similar chemical shifts to two small anomeric signals (102.1 and 96.1 ppm) in the arabinose spectrum. These can reasonably be assigned to the α-

and β- furanosyl anomers respectively, from the data for methyl α- and β-arabinofuranosides (C-1 = 109.3 and 103.2 ppm) reported by Gorin and Mazurek (1975). The corresponding signals for L-arabinose-2-phosphate were not observed with certainty due to the lower signal to noise ratio. This may be interpreted as evidence for the presence of α and β furanose forms in the agrocinopine A anomeric equilibrium mixture. Since reduced agrocinopine A is inactive in the agrocinopine bioassay, it is likely that an anomeric ring structure is necessary for recognition by the agrocinopine permease.

Further confirmation of agrocinopine A signal assignments was possible by comparison (Table 3-2) of the spectrum for borohydride-reduced agrocinopine A with arabinitol and using the ^{13}C n.m.r. assignments reported for arabinitol by Angyal and LeFur (1980). Because of the absence of anomeric equilibria in reduced agrocinopine A, the arabinitol signals were altered in chemical shift, fewer in number and of markedly enhanced intensity compared to the arabinose signals of agrocinopine A. In contrast, signals originating from sucrose in the reduced agrocinopine A remained essentially unaltered relative to agrocinopine A. The P-O-C-C coupling of carbon 1 in both arabinitol-2-phosphate and reduced agrocinopine A broadened the signal but was too small to resolve the signal into a doublet.

DISCUSSION

The data presented provide good evidence that the non-nitrogenous opines agrocinopines A and B are the sucrose and fructose phosphodiesters of L-arabinose-2-phosphate shown in Fig. 3-5. As is the case with all previously described opines, agrocinopines A and B are formed from two common low molecular weight plant metabolites linked in an unusual way.

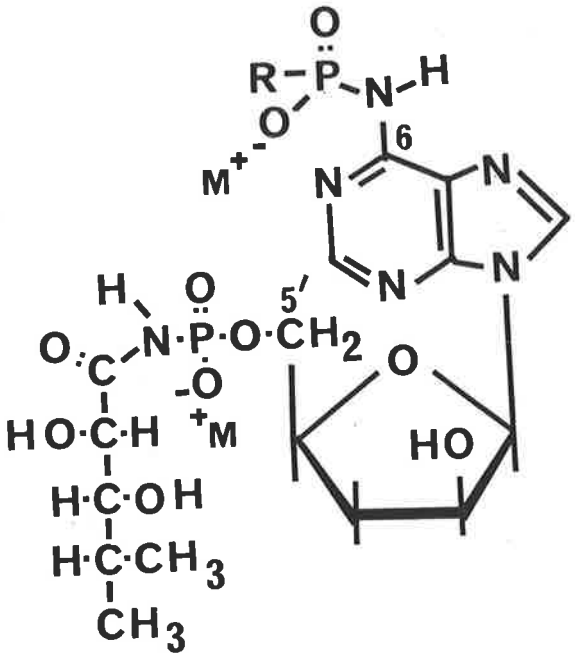
Agrocinopine B was not found in some nopaline tumour extracts and may be obtained from agrocinopine A by mild acid hydrolysis or α-glucosidase action. It is considered to be a degradation product of agrocinopine A.

Agrocinopines A and B were discovered in a bioassay involving an interaction between agrocinopines and agrocin 84 toxicity (Ellis and Murphy, 1981). The L-arabinose moiety is required for biological activity because reduced agrocinopine A with its L-arabinitol moiety was inactive in this bioassay. The action of agrocinopine A in the bioassay is to fully induce a permease for its uptake which is normally only partly expressed (Ellis and Murphy, 1981). This permease induction makes cells of the indicator strain sensitive to a lower concentration of agrocin 84, resulting in an extension of the inhibition zone. It appears that the arabinose portion of agrocinopine A is required for induction of the permease, but whether it is also required for uptake, i.e. recognition of agrocinopine A by the permease, is not known. L-arabinose-2-phosphate (4 to 100 µg) did not extend the agrocin 84 inhibition zone and at 100 µg counteracted the toxicity of agrocin 84. Agrocinopine B is thus the smallest molecule so far reported active in extending the inhibition zone in the agrocin 84 bioassay.

Agrocin 84 is toxic to only a limited range of agrobacteria and its specificity lies in its D-glucofuranosyl substituent (Tate et al., 1979). This substituent may mimic the arabinose moiety of agrocinopine A in the process of uptake into the bacterial cell but the agrocinopine A molecule differs somewhat from agrocin 84 in this region. The arabinose moiety in agrocinopine A is in the pyranose form, whereas the glucosyl moiety of agrocin 84 is in the D-furanose form. The possibility that the phosphoramidate linkage to the D-glucosyl moiety of agrocin 84 may be to carbon 2 has not been excluded (Kerr and Tate, 1984; Fig. 3-9). Fig. 3-9

FIGURE 3-9

The structural formula of agrocin 84, from Kerr and Tate (1984); R = glucofuranosyl moiety. This figure shows that the position of the phosphoramidate linkage to the D-glucosyl moiety has not been unequivocally determined (c.f. Fig. 1-2).



AGROCIN 84

shows an amended structure of agrocin 84 in which the position of the phosphoramidate linkage to glucose is not defined. Agrocinopine A is very similar to agrocinopine C which has a glucopyranosyl moiety linked with a phosphodiester bridge via the 2-carbon, also to sucrose (Savage, 1983). Thus the configuration around the 1-, 2- and 3-carbon atoms of L-arabinose in agrocinopines A and B and around the reducible D-glucose moiety in agrocinopines C and D are the same, and this configuration may play an important role in biological activity. In contrast to the similarities between the arabinose and glucose moieties, the remaining portions of the agrocin and agrocinopine molecules differ markedly in structure. The agrocin 84 molecule contains a fraudulent nucleotide which is responsible for its toxic effect on sensitive agrobacteria (Murphy, 1981), whereas agrocinopine A simply contains sucrose phosphate which acts as a bacterial nutrient source. The range of nutrients available to agrobacteria in the form of opines can now be extended to include phosphorus.

The agrocinopines are unusual conjugates of two common plant metabolites. In the case of agrocinopine A, the common plant transport sugar, sucrose, and the pentose sugar L-arabinose are linked to form a phosphodiester. Agrocinopines C and D are phosphodiesters similar to agrocinopines A and B but differing in some respects. In agrocinopine C, sucrose is linked via its glucosyl moiety (6-position) to the 2-position of D-glucose (Savage, 1983). These relatively simple molecules have not been reported in normal plant tissues.

The biosynthesis of agrocinopine A is coded by a T-DNA gene in nopaline strains. Joos et al. (1983) have located this gene immediately to the left of the "common DNA", the region of the T-DNA common to both nopaline and octopine Ti-plasmids. The enzyme(s) responsible and the substrates involved have not yet been identified. The only sucrose

phosphate so far described from normal plant tissue is sucrose-6'-phosphate (Preiss, 1982). The substrate for the formation of agrocinopine A, which is phosphorylated in the 4'- position, may either be present in normal plant tissue or specifically synthesized in crown gall tissue at the direction of the T-DNA. In view of the involvement of nucleotide sugars in the biosynthesis of sucrose and starch (Preiss, 1982), such intermediates may also be important in agrocinopine biosynthesis. An additional argument for the involvement of nucleotides is the well-documented role of cytosine nucleotides in phosphodiester formation in the synthesis of phosphoglycerides (Kennedy, 1962).

One of the synthetic intermediate in arabinose-2-phosphate synthesis, 1-O-benzyl-3,4-O-benzylidene- -L-arabinopyranoside, could be used in the chemical synthesis of agrocinopine A. However, a method for obtaining sucrose protected in all positions except fructose carbon 4 is still required for synthesis of the complete opine. Partial benzylation of sucrose yielding a heptabenzyl sucrose is known (Tate, personal communication) but it is not known in this case whether the F-4 position remains free, as would be required for a synthesis of agrocinopine A.

CHAPTER 4

AGROPINE BIOSYNTHESIS

INTRODUCTION

Trivial names have been commonly used for opines but, for the agropine family, which includes a number of closely related compounds, a more meaningful system of nomenclature is now essential. The abbreviations used here have been derived by converting the commonly accepted sugar abbreviation to a polyol abbreviation by adding the letter "1" and coupling this to the appropriate amino acid abbreviation. Thus the opine N^2-(1' deoxy-D-mannitol-1'-yl)-L-glutamine (structure VIII, Fig. 4-1), also known as mannopine, is referred to as dMan1Gln. Agropinic acid (XIV), a lactam of dMan1Gln, is designated dMan1-5-oxo-Pro, but no abbreviation is used for agropine (XII).

Agropine (XII) was first found in octopine tumours of A.tumefaciens (Firmin and Fenwick, 1978) and later in "null type" crown gall tumours (Guyon et al., 1980). It has also been found in hairy root tissue (Tepfer and Tempé, 1981; Petit et al., 1983). The chemically related compounds N^2-(1'-deoxymannityl)-L-glutamate (dMan1Glu, VII) and N^2-(1'-deoxy-mannityl)-L-glutamine (dMan1Gln, VIII), called mannopinic acid and mannopine respectively by Tempé and coworkers, are present in tumours and hairy roots containing agropine (Dahl et al., 1983; Petit et al., 1983). Hairy root tissues induced by some strains contain dMan1Gln and dMan1Glu but lack agropine (Petit et al., 1983) and a distinction can therefore be made between agropine and dMan1Gln (also known as mannopine) hairy root strains.

Agropine can be prepared chemically by lactonization of dMan1Gln (Tate et al., 1982; Petit et al., 1983) and dMan1Gln was for this reason thought

to be a good candidate for the precursor of agropine in the biosynthetic pathway. It has been suggested that agropine biosynthesis might involve at least two steps (Tate et al., 1982; Dahl et al., 1983; Petit et al., 1983). The two steps suggested were Schiff base formation between L-glutamine and D-mannose to form dMan1Gln and then lactonization to agropine. This is in contrast to the simpler biosyntheses of octopine and nopaline, which involve only a single step, coded for by a single gene on the T-DNA (Depicker et al., 1983; DeGreve et al., 1982). Agropine biosynthesis in octopine crown gall tumours is coded by the TR-DNA (Leemans et al., 1982; Velten et al., 1983). Velten and coworkers found five TR-DNA transcripts and provided evidence that at least two and possibly three of these transcripts were involved in the agropine biosynthetic pathway, but did not ascribe particular functions to the transcripts.

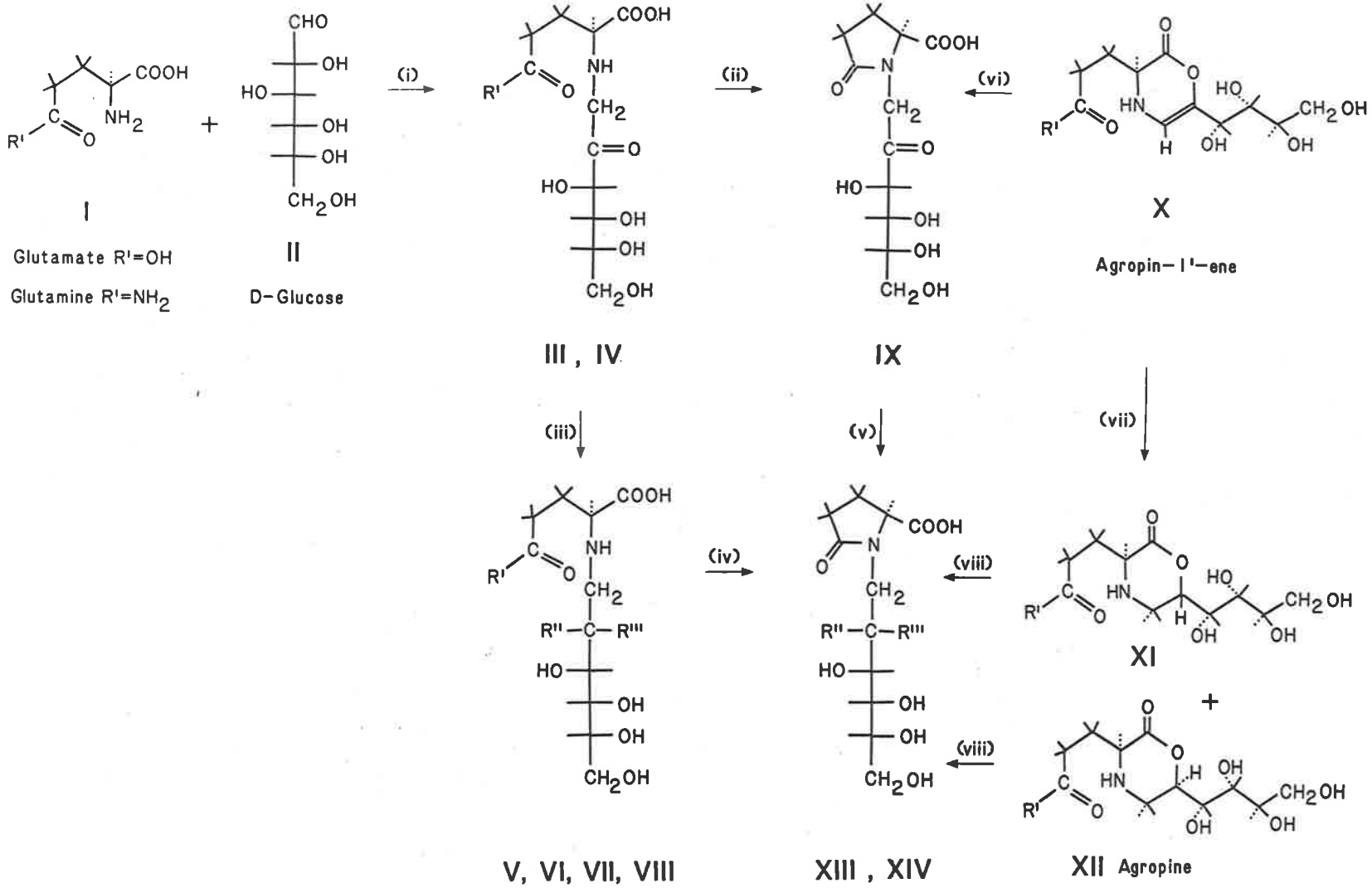
This study, in conjunction with the work of Dr. J. Ellis on the genetics of agropine biosynthesis, demonstrates that 3 steps are involved in the agropine biosynthetic pathway and that the fructosyl amino acids deoxyfructosylglutamate (dFruGlu, III) and deoxyfructosylglutamine (dFruGln, IV) are the precursors of dMan1Glu and dMan1Gln. The chemical identity of these fructosyl amino acids, their close relationship to dMan1Glu and dMan1Gln and evidence for their opine nature are demonstrated. The number of opines in the agropine group is thus extended. An unusual combination of opines in tumours of strain IIBV7 is reported, as well as the discovery of a new agropine-related opine, agropin-1'-ene (X), in tumours of this strain.

FIGURE 4-1

Synthesis of the agropine family of opines.

I, L-glutamate (R'=OH), L-glutamine (R'=NH_2); II, D-glucose; III, dFruGlu (R'=OH); IV, dFruGln (R'=NH_2); V, dGlc1Glu (R'=OH, R"=H, R'"=OH); VI, dGlc1Gln (R'=NH_2, R"=H, R'"=OH); VII, dMan1Glu, (R'=OH, R"=OH, R'"=H); VIII, dMan1Gln (R'=NH_2, R"=OH, R'"=H); IX, dFru-5-oxo-Pro; X, agropin-1'-ene (R'=NH_2); XI (VI 1',2' lactone, R'=NH_2); XII, agropine (VIII 1',2' lactone, R'=NH_2); XIII, dGlc1-5-oxo-Pro (R"=H, R'"=OH); XIV, dMan1-5-oxo-Pro (agropinic acid, R"=OH, R'"=H).

Reaction conditions (i) D-glucose (100 mmoles), Na-L-glutamate (100 mmoles), $Na_2S_2O_5$ (200 mmoles), H_2O (5 ml), 100°C, 45 min; D-glucose (56 mmoles), 17.5 M acetic acid (150 ml), L-glutamine (25 mmoles), 100°C, 30 min (<u>in vacuo</u>). (ii) neutral pH, 110°C, 30 min. (iii) Excess (5x) $NaBH_4$, pH 9.2, 30 min. (iv) 3 M NH_4OH, 110°C, 2 h). (v) Excess (5x) $NaBH_4$, pH 9.2, 30 min. (vi) neutral pH, 110°C, 30 min. (vii) Pt/H_2, 4 h, 17.5 M acetic acid. (viii) 3 M NH_4OH, 110°C, 2 h.


I
Glutamate R'=OH
Glutamine R'=NH2
II
D-Glucose
(i)
III , IV
(ii)
IX
(vi)
X
Agropin-1'-ene
(iii)
V, VI, VII, VIII
(iv)
(v)
XIII , XIV
(viii)
(viii)
(vii)
XI
XII Agropine

MATERIALS AND METHODS

Materials

Synthetic agropine, dMan1Glu, dMan1Gln, dGlc1Glu, dFruGlu and dFruGln were obtained from Dr.M.E.Tate. The fructosylamino acids were prepared by the method of Anet and Reynolds (1957).

Tumour tissue

Tumour tissue was obtained from carrot discs or *Kalanchoe daigremontiana* plants inoculated with suspensions of bacteria in sterile distilled water. Carrot discs were prepared for inoculation as described in Chapter 2. *Kalanchoe* plants were inoculated by wounding the upper stem internodes with a sterile scalpel blade and applying the bacterial suspension. Axenic tumour tissue was obtained as described in Chapter 2.

Extraction and purification of opines

Tumour tissue was extracted with 70% ethanol by maceration with a glass rod in a 1.5 ml centrifuge tube. The extracts were centrifuged, and the supernatants used for preparative electrophoresis on Whatman No.1 paper.

High Voltage Paper Electrophoresis (HVPE)

HVPE was carried out as outlined in Chapter 2. Buffers used were: 0.75 M formic acid/1 M acetic acid, pH 1.7; 0.2 M sodium formate, pH 2.8; 0.2 M NH_4CO_3/0.1 M NH_4OH, pH 9.2; 0.2 M NH_4CO_3/0.2 M boric acid/0.2 M NH_4OH, pH 9.2.

Detection reagents used are described in Chapter 2.

Paper chromatography

Descending paper chromatography on Whatman No.1 paper was carried out using solvent A: ethyl acetate/ pyridine/ water (8:2:1, v/v/v) for 18h.

Infrared spectra

I.r. spectra were recorded on a Perkin-Elmer model 983 spectrophotometer using KCl discs (0.5% w/w samples).

Biological utilization experiments

1. Compounds extracted from tumours: Utilization of compounds purified by preparative HVPE was tested in liquid culture. The filter-sterilized compound to be tested was dissolved in either Petit's salts (see Appendix A) without added N or C or in Bergersen's salts (Appendix A) with 0.5 mg/ml mannitol in a sterile 1.5 ml tube. A suspension of bacteria (1/10 volume) was added and the tubes were incubated on a rotary shaker at 25°C. Samples were taken at 0, 24 and 72 h and analysed by HVPE and staining with alkaline silver nitrate reagent to monitor disappearance of compounds from the culture medium. HVPE buffers used were (a) ammonium bicarbonate, pH 9.2, for dFruGlu and dFruGln and their reduction products and (b) ammonium borate, pH 9.2, for agropine, agropin-1'-ene and its reduction product.

2. Synthetic compounds: dFruGlu (III) and dFruGln (IV) were prepared by Dr. M.E.Tate from D-glucose and L-glutamate, and D-glucose and L-glutamine respectively by the method of Anet and Reynolds (1957). The ability of strains to utilize dFruGlu as sole C source was tested by growth on Petit's salts in purified agar containing 0.2% dFruGlu.

RESULTS

1. Occurrence and identification of deoxyfructosylglutamate (dFruGlu) and deoxyfructosylglutamine (dFruGln).

The ketose-positive compounds deoxyfructosylglutamate (dFruGlu, III) and deoxyfructosylglutamine (dFruGln, IV) were first found in tumours of strain TR104 (= NCPPB5), a slow-growing octopine strain, and later in tumour tissue of other agropine strains (A.tumefaciens A6, B6 and A281). They are also present in hairy roots of strain 8196 which contain no agropine but do contain both dManlGlu (VII) and dManlGln (VIII). These fructosylamino acids were not detectable in tumours incited by nopaline strain C58.

Upon paper electrophoresis at pH 1.7, dFruGlu and dFruGln had the same mobility as dManlGlu and dManlGln, but all four compounds separated clearly after elution of the dManlGlu band and electrophoresis in NH_4CO_3, pH 9.2. The former two compounds were also clearly distinguished from dManlGlu and dManlGln by their positive stain with both p-anisidine (yellow) and triphenyltetrazolium chloride reagent (Table 4-1). Fig. 4-2 shows the separation by electrophoresis at pH 9.2 (NH_4CO_3) of dFruGlu, dManlGlu, dFruGln and dManlGln isolated from tumours incited by different agropine strains. Identical results were obtained with extracts from tumours on carrot discs and on Kalanchoe stems; the presence of these compounds thus depended on the inciting strain of bacteria and not on the host plant. Axenic hairy roots induced by pRi strain 8196 also contained dFruGlu and dFruGln. Extracts of normal plant tissue contained no detectable dFruGlu or dFruGln, although there is evidence for the presence of other fructosyl amino acids in some untransformed plant tissues (see Results, section 5). Cultured axenic tumour tissue of TR104 contained

TABLE 4-1

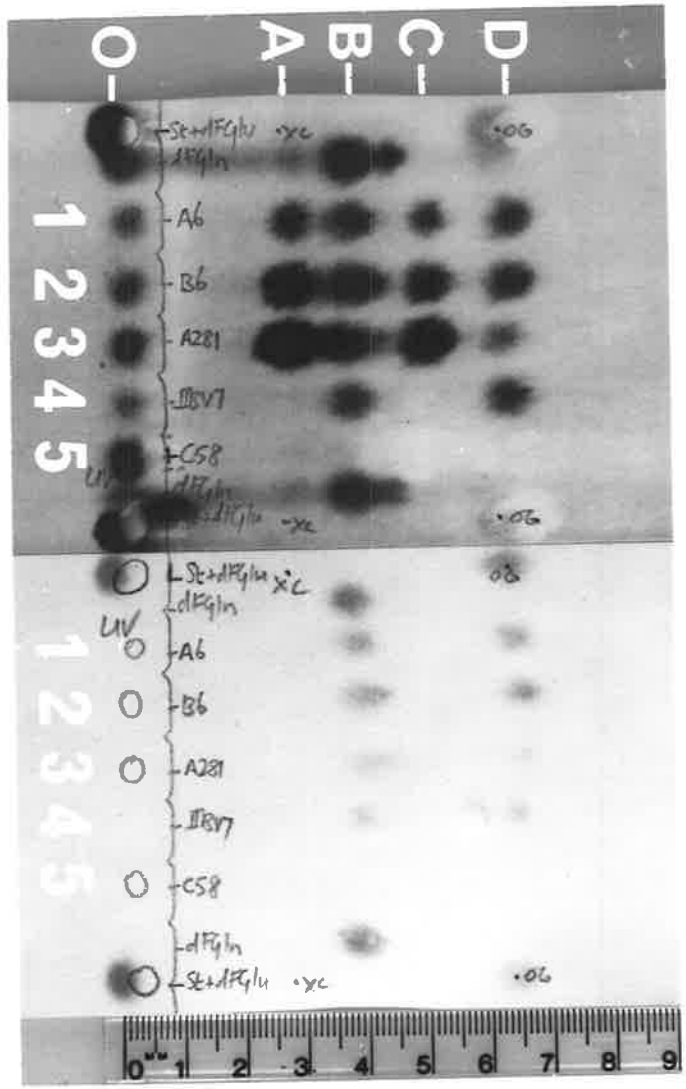
Staining properties of compounds extracted from tumours incited by A.tumefaciens strain TR104 on carrot discs. These compounds all had $M_{O.G}$ -0.28 at pH 1.7.

	dFruGlu	dFruGln	dManlGlu	dManlGln
Alkaline silver nitrate	+	+	+	+
Ninhydrin	+	+	+	+
p-Anisidine	+(yellow)	+(yellow)	-	-
Urea-phosphoric acid ketose reagent	+(blue)	+(blue)	-	-
Triphenyltetrazolium reagent	+(red)	+(red)	-	-

FIGURE 4-2

Electrophoretic separation of agropine family opines at pH 9.2.

Tumour extracts were electrophoresed at pH 1.7 and compounds having $M_{O.G}$ -0.28 were eluted. These compounds were then electrophoresed in ammonium bicarbonate, pH 9.2 and the electrophoretogram was stained with alkaline silver nitrate (left hand side), and triphenyltetrazolium chloride (right hand side). Tumours were incited by the following strains: Lane 1, A6; Lane 2, B6; Lane 3, A281; Lane 4, IIBV7; Lane 5, C58. Compound A = dMan1Gln; B = dFruGln; C = dMan1Glu; D = dFruGlu; O = origin.


O
A
B
C
D
1 2 3 4 5
St+dFGlu
dFGln
A6
B6
A281
IIBV7
C58
dFGln
St+dFGlu
UV
1 2 3 4 5
St+dFGlu
dFGln
A6
B6
A281
IIBV7
C58
dFGln
St+dFGlu
0 1 2 3 4 5 6 7 8 9

dFruGlu and dFruGln, demonstrating that these compounds are neither bacterial products nor a result of bacterial metabolic activity. In these respects, dFruGlu and dFruGln conform to the operational definition of an opine (Schell et al., 1979). The bacterial utilization of these compounds is presented in Results section 2.

The chemical identity of dFruGlu and dFruGln was confirmed by analysis of their reduction products, by analysis of the products of degradation by heating, and by comparison with synthetic dFruGlu and dFruGln. The identity criteria are listed in Table 4-2 for dFruGlu and Table 4-3 for dFruGln.

Natural dFruGlu from TR104 tumours was purified by preparative electrophoresis at pH 9.2 ($M_{O.G}$ 1.04). The purified compound was smoothly reduced by sodium borohydride (5x excess, pH 9.2, 30 min), giving 2 products, in equal amounts, which were indistinguishable from dManlGlu and N^2-(1'-deoxyglucityl)-L-glutamate (dGlclGlu, V) in both their staining properties and in their electrophoretic mobility at pH 1.7 and pH 9.2. Similarly, natural dFruGln was isolated by preparative electrophoresis at pH 9.2 ($M_{O.G}$ 0.65) and it too was smoothly reduced by sodium borohydride (5x excess, pH 9.2, 30 min) giving two products which were indistinguishable from dManlGln and N^2-(1'-deoxyglucityl)-L-glutamine (dGlclGln, VI) using the same criteria.

Synthetic dManlGlu and dManlGln undergo a characteristic cyclization reaction to form dManl-5-oxo-Pro (agropinic acid, XIV) upon heating in 3 M ammonia at 110°C. Similarly, dGlclGlu and dGlclGln form dGlcl-5-oxo-Pro (glucoagropinic acid, XIII) when treated in the same way, and the two isomers XIII and XIV are separable by electrophoresis at pH 2.8. Therefore, to check the identity of the compounds obtained after sodium borohydride reduction of natural dFruGlu and dFruGln, the reduction

TABLE 4-2

Chemical and biological identity criteria for dFruGlu[a].

	dFruGlu synthetic	dFruGlu ex TR104	Reduction products dFruGlu ex TR104	dManlGlu synthetic	dGlclGlu ex TR104
$M_{O.G}$ pH 1.7	-0.28	-0.28	-0.28	-0.29	-0.27
$M_{O.G}$ pH 9.2	1.04	1.04	0.82 & 0.91	0.84	0.92
$M_{O.G}$ pH 9.2 Borate	1.28	1.27	1.07	1.06	1.07
$R_{Glucose}$	0.13	0.14	n.d.	0.11	n.d.
Biological utilization					
by strain C58C1	+	+	- and -	-	-
TR104	+	+	+ and +	+	+
B6	+	+	+ and +	+	+

[a] Electrophoretic mobilities are expressed relative to Orange G ($M_{O.G}$). Buffer systems used for electrophoresis were: 0.75 M formic acid/ 1 M acetic acid, pH 1.7; 0.2 M NH_4HCO_3/0.1M NH_4OH, pH 9.2; 0.2 M NH_4HCO_3/0.2 M boric acid/ 0.2 M NH_4OH, pH 9.2. Paper chromatography was carried out using ethyl acetate/pyridine/water 8:2:1 (v/v/v) and migration expressed relative to glucose ($R_{Glucose}$). n.d.= not determined.

TABLE 4-3

Chemical and biological identity criteria for dFruGln[a]

	dFruGln synthetic	dFruGln ex TR104	Reduction products dFruGln ex TR104	dManlGln synthetic	dGlclGln ex TR104
$M_{O.G}$ pH1.7	-0.26	-0.28	-0.28	-0.28	-0.27
$M_{O.G}$ pH9.2	0.65	0.63	0.44 & 0.52	0.46	0.54
$M_{O.G}$ pH9.2 Borate	0.93	0.93	0.73 & 0.76	0.76	0.80
$R_{Glucose}$	0.23	0.22	n.d.	0.19	n.d.
Biological utilization					
by strain C58C1	+		- and -	-	
TR104	+		+ and +	+	
B6	+		+ and +	+	

[a] Conditions and symbols as for Table 4-2.

products were heated (110°C, 3h, 3 M NH_4OH) and the products analysed by electrophoresis in sodium formate, pH 2.8. The resultant products had the same electrophoretic mobilities as XIII ($M_{O.G}$ 0.46) and XIV ($M_{O.G}$ 0.43).

It was thus possible to reduce dFruGlu (III) and dFruGln (IV) to compounds with the same properties as synthetic V, VI VII and VIII and these reduction products were then converted by ammonolysis to compounds with the same properties as synthetic XIII and XIV. The reactions are illustrated in Fig. 4-1 (step iii followed by step iv).

On heating at neutral pH (110°, 30 min), natural and synthetic dFruGlu and dFruGln underwent a cyclization reaction (Fig. 4-1, step ii) to form a product resembling agropinic acid. The product (IX) had the same electrophoretic mobility ($M_{O.G}$ = 0.05) as agropinic acid at pH 1.7 but could be distinguished from agropinic acid by its positive stain with the triphenyltetrazolium reagent.

The identity criteria (Tables 4-2 and 4-3) show that natural dFruGlu and dFruGln had the same properties as their synthetic counterparts upon electrophoresis in several buffer systems and paper chromatography. The reduction products were indistinguishable from dMan1Glu and dGlc1Glu in the case of dFruGlu and from dMan1Gln and dGlc1Gln in the case of dFruGln.

2. Biological utilization of dFruGlu, dFruGln and their reduction products.

As a further test of identity, natural dFruGlu and dFruGln from TR104 tumours and also their reduction products were used in a biological utilization experiment. The results are presented in Tables 4-2 and 4-3.

The utilization of dFruGlu and dFruGln was not specific; they were utilized by Ti-plasmidless control strain C58C1. After dFruGlu and dFruGln

were chemically reduced, the utilization of the products was specifically Ti- and Ri-plasmid-coded. The reduced compounds were utilized by TR104 and B6 but no longer utilized by C58C1. Thus dFruGlu and dFruGln utilization was not restricted to Ti plasmid-harbouring strains, but the utilization of their reduction products was specifically Ti/Ri plasmid-coded. This is further evidence for the close relationship of dFruGlu and dFruGln to the reduced opines dManlGlu and dManlGln.

A minor but surprising point was that hairy root strain C58C1(pRi8196) utilized both isomers (dManlGlu and dGlclGlu) of dFruGlu reduction but only one isomer (dManlGln) of dFruGln reduction.

3. The opines of strain IIBV7

Analysis of tumour tissue of strain IIBV7 from carrot discs and Kalanchoe revealed an unusual picture which has relevance to agropine biosynthesis.

Apart from nopaline ($M_{O.G}$ -0.42 at pH 1.7, $M_{O.G}$ 0.75 at pH 9.2, guanidine positive) and agrocinopine C ($M_{O.G}$ 0.44 at pH 1.7, $M_{O.G}$ 0.41 at pH 9.2, $M_{O.G}$ 0.54 in ammonium borate, pH 9.2, yellow staining with p-anisidine), the tumours contained dFruGlu and dFruGln, but not the reduced relatives dManlGlu and dManlGln (see Fig. 4-2). The identities of dFruGlu and dFruGln were confirmed by:

(1) staining properties: triphenyltetrazolium-positive and yellow with p-anisidine

(2) reduction with sodium borohydride, giving two products behaving like dManlGlu/dGlclGlu and dManlGln/dGlclGln respectively

(3) heating (neutral pH, 110°C, 30 min.) which gave a triphenyltetrazolium-positive product with $M_{O.G}$ 0.04 at pH 1.7.

In all these properties the natural compounds from IIBV7 tumours were indistinguishable from synthetic dFruGlu and dFruGln.

An agropine-like compound which stained positively with the triphenyltetrazolium reagent was also found in IIBV7 tumours. This compound, designated agropin-1'-ene, is described in the next section (Results, section 4).

Axenic IIBV7 tumour tissue was found to contain all these opines.

4. Characterization and occurrence of agropin-1'-ene

Agropin-1'-ene was first identified in IIBV7 tumours, where it is present in large amounts, and later in B6 tumours where it is difficult to detect owing to the low levels present. This new compound was similar but not identical to agropine in its electrophoretic mobilities and, in contrast to agropine, stained positively with the triphenyltetrazolium reagent. For these reasons it appeared that it might have a similar relationship to agropine as dFruGlu and dFruGln to dMan1Glu and dMan1Gln respectively.

Agropin-1'-ene was purified to electrophoretic homogeneity by preparative HVPE at pH 1.7 and then at pH 9.2 in ammonium bicarbonate buffer.

Some properties of agropine and agropin-1'-ene are compared in Tables 4-4A and 4-4B. Agropin-1'-ene was separable from agropine at pH 1.7 and pH 9.2 and was most easily separable in ammonium borate, pH 9.2 (Table 4-4A). Agropin-1'-ene was reducible by Pt/H2 (17.5 M acetic acid, 4h) to a compound which no longer stained with the triphenyltetrazolium reagent (Table 4-4A) and which was indistinguishable from agropine in its electrophoretic mobilities (Table 4-4A) and in biological utilization studies using strains of Agrobacterium (Table 4-4B). Agropin-1'-ene was

TABLE 4-4A

Electrophoretic and staining properties of (a) agropin-1'-ene from IIBV7 tumours, (b) its major reduction product, and (c) synthetic agropine[a].

	Agropin-1'-ene	Reduced agropin-1'-ene (major product)	Agropine
$M_{O.G}$ pH 1.7	-0.49 (Ag,T)	-0.53 (Ag)	-0.53 (Ag)
$M_{O.G}$ pH 9.2	0.04 (Ag,T)	n.d.	0.00 (Ag)
$M_{O.G}$ pH 9.2 Ammonium borate	0.69 (Ag,T)	0.62 (Ag)	0.61 (Ag)

[a] Electrophoretic relative mobilities are expressed relative to Orange G ($M_{O.G}$). Ag = silver nitrate-positive; T = triphenyltetrazolium-positive; n.d. = not determined.

TABLE 4-4B

Bacterial degradation of (a) agropin-1'-ene from IIBV7 tumours, (b) its major reduction product, and (c) synthetic agropine[a].

Bacterial strain	Agropin-1'-ene	Reduced agropin-1'-ene (major product)	Agropine
C58C1	-	-	-
IIBV7	+	-	-
B6	-	+	+

[a] + = degraded; - = not degraded

resistant to reduction by sodium borohydride.

Agropin-1'-ene was specifically utilized by bacteria of strain IIBV7 but not by C58C1 or B6 (Table 4-4B). Agropine, on the contrary, was utilized by B6 but not by IIBV7 or C58C1. Upon Pt/H2 reduction, agropin-1'-ene was converted from a compound utilized by IIBV7 to a compound which was indistinguishable from agropine by HVPE, was utilized by B6 and no longer utilized by IIBV7.

If agropin-1'-ene were structurally related to dFruGlu and dFruGln, heating at neutral pH may be expected to form a similar cyclized lactam product (IX) to that formed by heating of the latter two compounds (see Results, section 1). Agropin-1'-ene, dFruGlu and dFruGln were heated (neutral pH, 110^{o}C, 30 min.) and the products analysed by HVPE. The results are shown in Table 4-5. The product formed by heating agropin-1'-ene (X) was indistinguishable from the slower moving product formed by heating dFruGlu (III) and dFruGln (IV) by HVPE and paper chromatography. This again indicates that compounds III, IV and X are related. Agropinic acid (XIV), which is a structurally related reduced lactam, had similar properties. Heating dFruGlu and dFruGln unexpectedly seemed to produce two products which were separable by HVPE at pH 9.2 in ammonium bicarbonate buffer and chromatography in solvent A but not by HVPE at pH 1.7 or in ammonium borate buffer, pH 9.2. The agropin-1'-ene degradation product appeared to correspond to the slower moving of the two degradation products of dFruGlu and dFruGln.

The infrared spectra of agropin-1'-ene and agropine show similar properties (Fig. 4-3). The following peaks were detected at >2% transmission for (a) agropine, synthetic: 3368, 2919, 2849, 2357, 2326, 1691, 1607, 1397, 1298, 1077 cm^{-1} and (b) agropin-1'-ene: 3383, 2327, 1597, 1402, 1350, 1084, 932 cm^{-1}.

TABLE 4-5

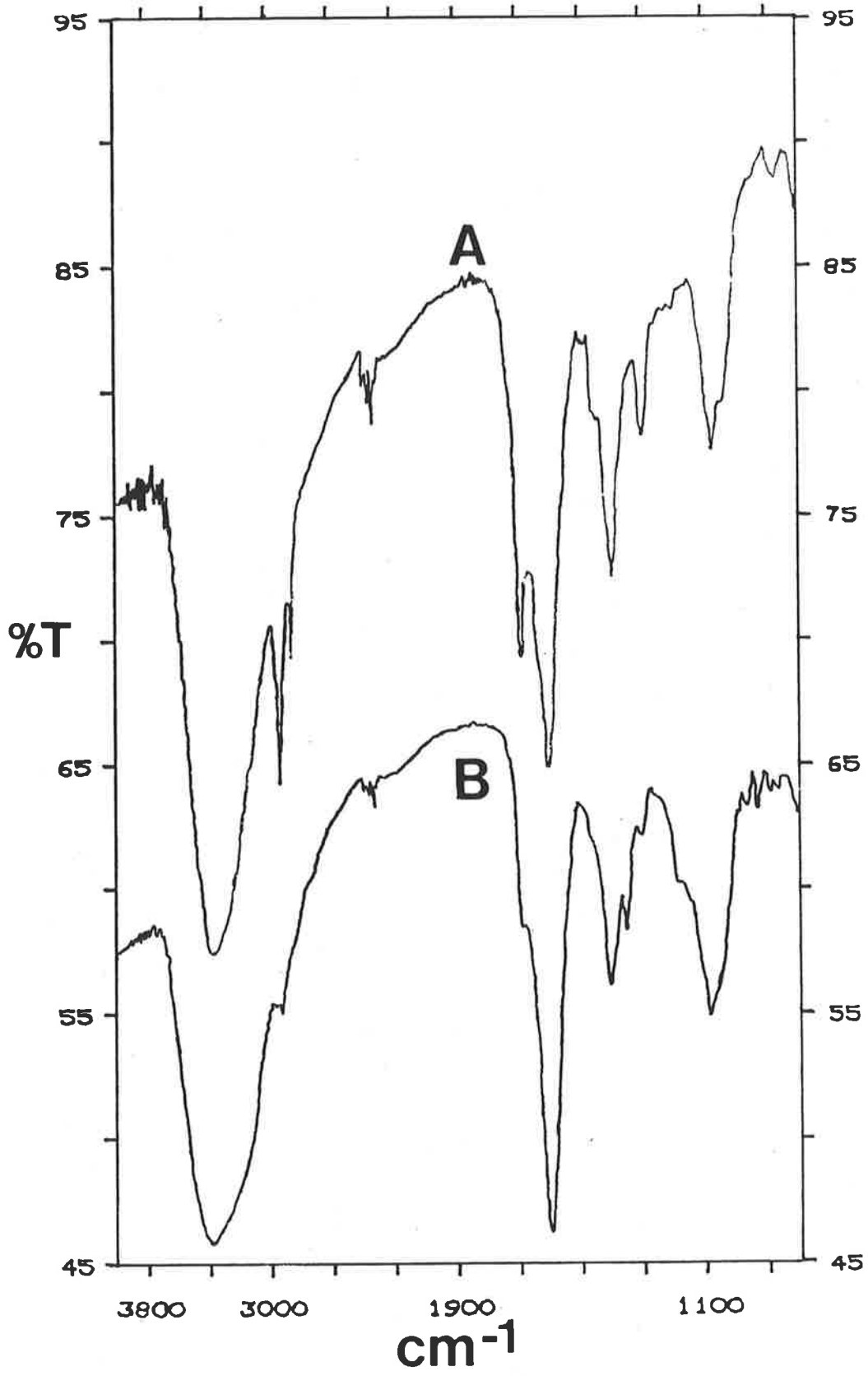
Electrophoretic and chromatographic properties of products obtained by heating dFruGlu, dFruGln and agropin-1'-ene[a].

	Agropinic acid	dFruGlu product	dFruGln product	Agropin-1'-ene product
$M_{O.G}$ pH 1.7	0.05	0.04	0.04	0.04
$M_{O.G}$ pH 9.2	0.66	0.67,0.72	0.66,0.74	0.68
$M_{O.G}$ pH 9.2 ammonium borate	n.d.	1.06	1.06	1.06
$R_{Glucose}$ (Solvent A)	0.26	0.30,0.34	0.30,0.34	0.31

[a] The compounds were heated at 110°C for 30 min at neutral pH. The properties of agropinic acid are included for comparison.

FIGURE 4-3

Infrared spectra of synthetic agropine (A) and natural agropin-1'-ene (B).


95
85
75
65
55
45
%T
A
B
3800
3000
1900
1100
cm-1

A search was made for agropin-1'-ene in B6 tumours, despite the fact that B6 bacteria did not utilize agropin-1'-ene isolated from IIBV7 tumours. The agropine region ($M_{O.G}$ -0.53) from preparative electrophoresis at pH 1.7 was eluted, concentrated, and again subjected to HVPE as a band, this time at pH 9.2 in ammonium bicarbonate buffer, in order to separate agropin-1'-ene from dFruGlu and dFruGln. The origin ($M_{O.G}$ 0.08) from this electrophoretogram was eluted and the eluate dried under nitrogen. This eluate contained a small amount of triphenyltetrazolium-positive material which had the same mobility as agropin-1'-ene in ammonium borate pH 9.2 and which cyclized in the same way as agropin-1'-ene (neutral pH, 110°C, 30min.) to form a triphenyltetrazolium-positive compound with $M_{O.G}$ 0.04 at pH 1.7.

5. Opines of the agropine family

The structures of the compounds of the agropine family of opines, now extended in number are presented in Fig. 4-1 and their chemical interrrelationships are also indicated in this Figure. The chemical interconversions not yet described will now be outlined.

Synthetic dFruGlu (III) was converted to a mixture of agropinic acid (XIV) and glucoagropinic acid (XIII) by the two routes indicated in Fig. 4-1. The first route, via steps (ii) and (v), involved heating at 110°C at neutral pH to give dFru-5-oxo-Pro (IX) followed by sodium borohydride reduction to give two isomeric products (XIII and XIV) separable by HVPE in sodium formate, pH 2.8. In the alternative route, proceeding via steps (iii) and (iv), III was first reduced by sodium borohydride to give dGlc1Glu (V) and dMan1Glu (VII) which were then subjected to ammonolysis at 110°C for 2h. Again, two isomers (XIII and XIV) were formed and these were separable by HVPE at pH 2.8.

Careful analysis of the products of Pt/H2 reduction of agropin-1'-ene (X) revealed that not one but two isomers of agropine were formed. This was demonstrated as follows. After chemical reduction of X, the products were subjected to ammonolysis (3 M NH_4OH, 110°C, 2h) and two isomers of agropinic acid were subsequently detected upon separation of the ammonolysis products by HVPE in sodium formate buffer, pH 2.8. As judged by intensity of silver nitrate staining, approximately 95% was agropinic acid (dMan1-5-oxo-Pro, XIV) and 5% was glucoagropinic acid (dGlc1-5-oxo-Pro, XIII). From this it can be inferred that the original reduction with Pt/H2 produced 95% agropine and 5% "glucoagropine" (XI). The reduction must have had a preferred stereochemical orientation giving almost all one isomer.

Opines, according to the operational definition of Schell et al. (1979), should be specifically synthesized by crown gall tumour tissues and should not be detectable in normal plant tissue. Normal callus tissue from carrot discs was extracted with 70% ethanol in an attempt to find deoxyfructosylamino acids. The ethanol extract was electrophoresed at pH 1.7 and the dFruGlu region ($M_{O.G}$ -0.28) was eluted. A faint triphenyl-tetrazolium-positive band was visible in this region. The eluate was concentrated and electrophoresed again at pH 1.7. In contrast to authentic and natural dFruGlu and dFruGln which did not break down under the same conditions, the triphenyltetrazolium-positive material now appeared at the origin, probably as a result of acid hydrolysis. Thus, although normal callus may contain other deoxyfructosyl amino acids, they are clearly distinguishable from dFruGlu and dFruGln on the basis of acid-lability.

6. Intermediates of agropine biosynthesis in tumours incited by TR-DNA mutants

Tumours incited on carrot discs or Kalanchoe stems by strains with TR-DNA mutations were analysed for opine content by HVPE. Ethanol extracts of the tumours were electrophoresed at pH 1.7, followed by elution and HVPE at pH 9.2 in ammonium bicarbonate buffer. In some cases, extracts of tumours from Kalanchoe stems were obtained from Dr.J.Ellis.

The positions of the mutations and transcripts on the TR-DNA are shown in Fig. 4-4 and the results are presented in Table 4-6. Tumours incited by strains mutated in transcript 2' contained no opines of the agropine family. Mutations in transcript 1' eliminated agropine and dMan1Glu/dMan1Gln synthesis but allowed synthesis of dFruGlu and dFruGln. Tumours induced by strains with mutations in transcript 0' lacked only agropine. Wild type tumours, as expected, contained all compounds of the agropine biosynthetic pathway.

DISCUSSION

1. The agropine biosynthetic pathway

The TR arm of the octopine T-DNA has been shown to code for agropine biosynthesis (Leemans et al., 1982; Velten et al., 1983). Genetic analysis by site-directed mutagenesis of the TR-DNA of strain B6 has shown that agropine biosynthesis is a three-step pathway (Velten et al., 1983; Ellis et al., 1984; Fig. 4-5). The three TR genes involved are 0', 1' and 2'. Tumours incited by strains with mutations in these TR-DNA genes were analysed for the presence of dFruGlu, dFruGln, dMan1Glu, dMan1Gln and agropine. The results revealed a pattern consistent with a three-step pathway, the in vivo precursors of dMan1Glu and dMan1Gln being dFruGlu and

FIGURE 4-4

TR-DNA map of restriction fragments EcoRI 12 and 20, showing the location of deletion and insertion mutations.

□ indicates a transposon insertion mutant; ▼ indicates an *in vitro* resistance gene insertion at a restriction site; Δ indicates a deletion. The locations of the TR-DNA transcripts, taken from Velten *et al.* (1983), are shown above the restriction map. Restriction sites: E = EcoRI; H = HindIII; P = PstI; S = SalI.

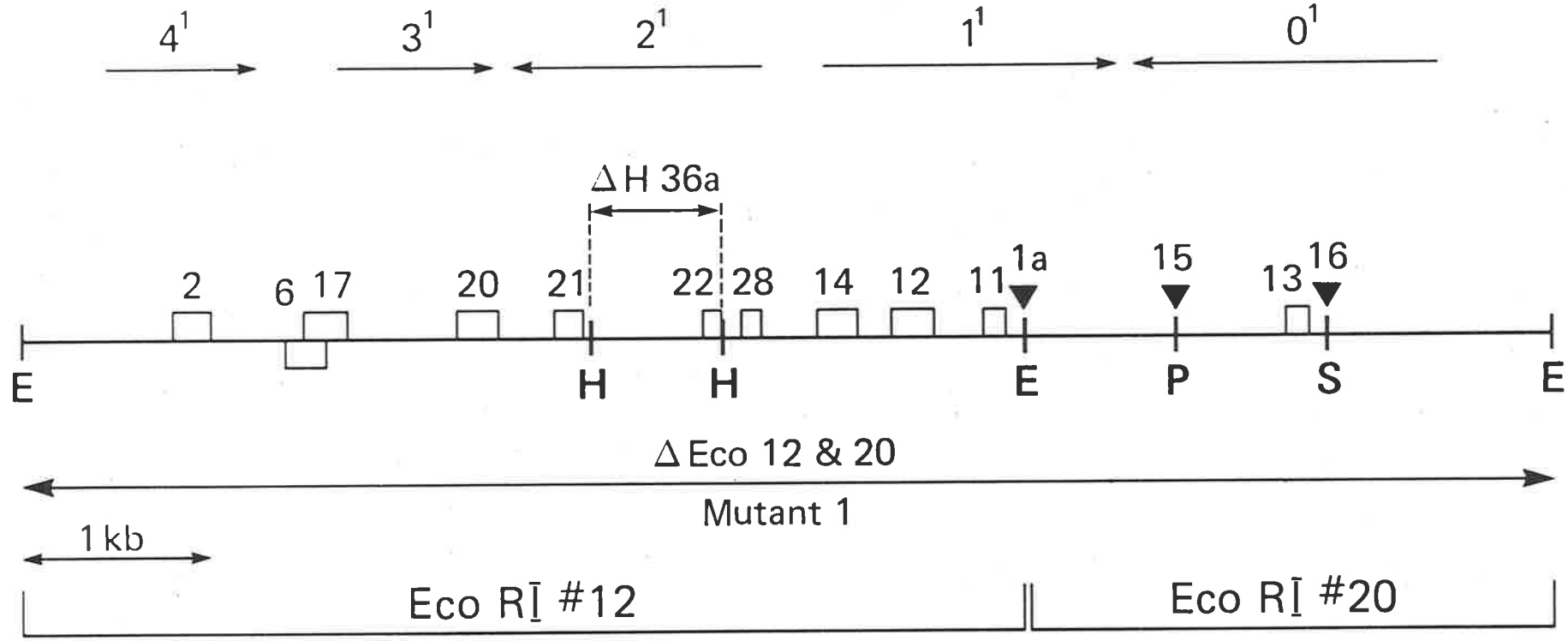

4¹
3¹
2¹
1¹
0¹
ΔH 36a
2
6
17
20
21
22
28
14
12
11
1a
15
13
16
E
H
H
E
P
S
E
ΔEco 12 & 20
Mutant 1
1 kb
Eco RI #12
Eco RI #20

TABLE 4-6

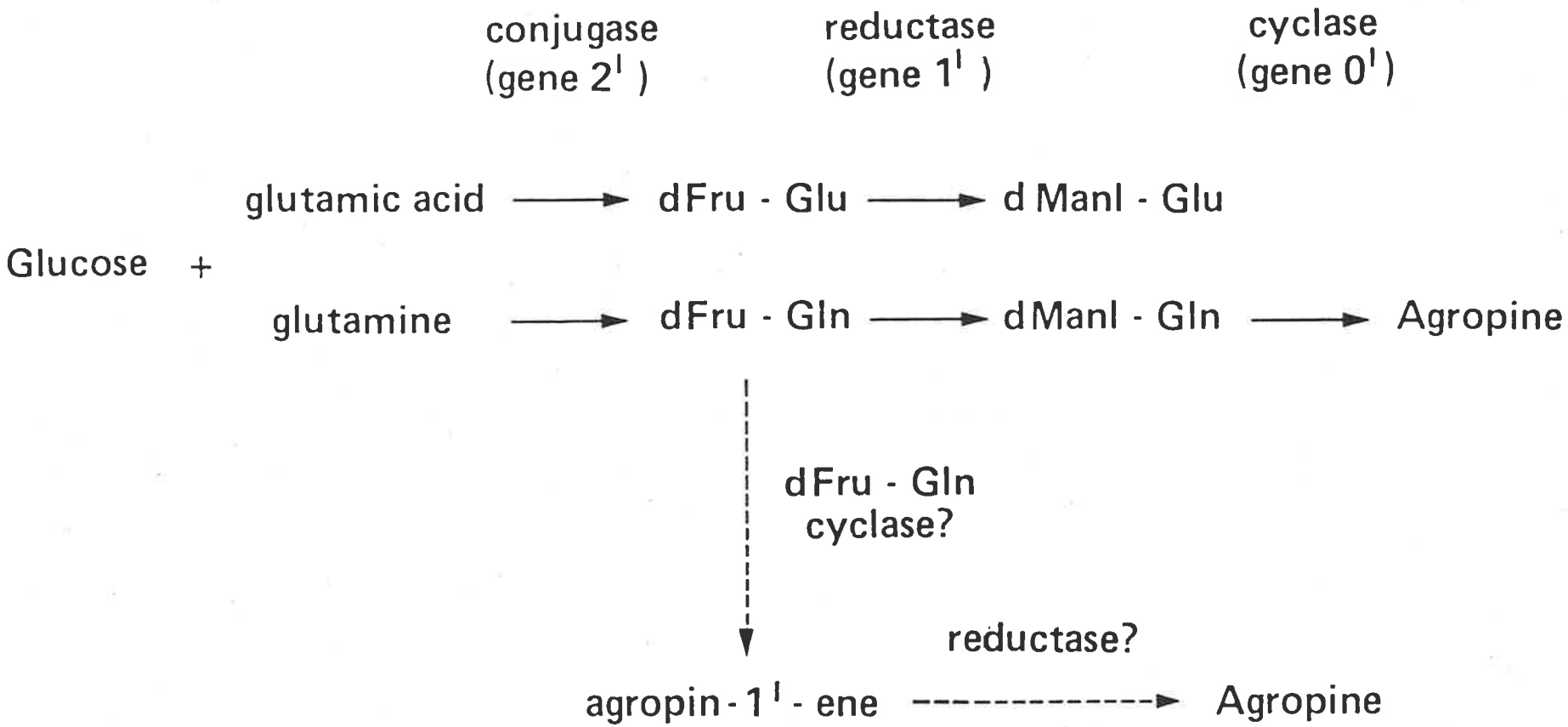
Agropine family opines in tumours incited by strains with TR-DNA mutations[a].

Mutant strain	OPINES IN TUMOUR		
	dFruGlu/dFruGln	dManlGlu/dManlGln	agropine
22	-	-	-
1a	+	-	-
11	+	-	-
12	+	-	-
14	+	-	-
13	+	+	-
15	+	+	-
16	+	+	-
TR104 (wild type)	+	+	+
B6 (wild type)	+	+	+

[a] Ethanolic tumour extracts were electrophoresed at pH 1.7. The band comprising the region $M_{O.G}$ -0.28 was eluted and electrophoresed at pH 9.2 to separate dFruGlu, dFruGln, dManlGlu and dManlGln. The fructosyl amino acids were distinguished by their positive stain with triphenyltetrazolium chloride.

FIGURE 4-5

Biosynthetic pathways to agropine.


conjugase
(gene 2')
reductase
(gene 1')
cyclase
(gene 0')
glutamic acid
dFru - Glu
d Manl - Glu
Glucose +
glutamine
dFru - Gln
dManl - Gln
Agropine
dFru - Gln
cyclase?
reductase?
agropin - 1' - ene
Agropine

dFruGln respectively.

The substrates proposed for the first step of the pathway are D-glucose and L-glutamate or L-glutamine, by analogy to the chemical synthesis of dFruGlu and dFruGln. Prior to the discovery of the fructosylamino acids in agropine tumours, D-mannose was suggested as a possible reducing sugar substrate for agropine biosynthesis (Tate et al., 1982; Dahl et al., 1983) but this is now considered unlikely. The two substrates for the first step must be linked by a "conjugase", coded for by gene 2', that enzymically catalyses the acid-catalysed condensation and Amadori rearrangement used for the chemical synthesis. Some support for D-glucose as a substrate for the first step of the pathway comes from the observation (Firmin and Fenwick, 1978) that the ^{14}C label of glucose fed to tumours incited by A.tumefaciens strains A66 and B6 appeared in agropine.

In the second step, the fructosyl amino acids are stereospecifically reduced by a reductase enzyme, coded by gene 1', to dMan1Glu and dMan1Gln. By contrast, a smooth non-stereospecific chemical reduction of dFruGlu with sodium borohydride produces both the deoxymannityl- and deoxyglucityl- isomers.

The final step in the biosynthesis could be either via lactonization of dMan1Gln or reduction of agropin-1'-ene. Agropin-1'-ene would in this case have to be formed by lactonization of an enolic form of dFruGln. At present there is no proof of the correctness of either alternative but indications from (a) the very low levels of agropin-1'-ene in tumours incited by strains with TR-DNA mutations and (b) the fact that it is not utilized by B6 bacteria, are that agropin-1'-ene is unimportant in agropine biosynthesis by tumours of this strain. Agropine biosynthesis is therefore more likely to proceed via lactonization of dMan1Gln. The

enzymes of the agropine biosynthetic pathway are yet to be isolated and characterized.

2. Deoxyfructosylamino acids

Whether the agropine precursors dFruGlu and dFruGln conform fully to the operational definition of an opine of Schell et al. (1979) remains to be seen. Although their synthesis is T-DNA-coded, and they are utilized by bacteria with the corresponding Ti plasmid, it has not been demonstrated that this utilization is Ti plasmid-coded for any dManlGln or agropine strain. A Ti-plasmidless derivative of octopine strain B6 is able to utilize dFruGlu as sole C source (A.Kerr, personal communication), but whether utilization is also coded for by the Ti plasmid of B6 remains to be tested. Simultaneous transfer, to a non-utilizing recipient, of both the Ti plasmid and the ability to utilize dFruGlu and dFruGln is required to prove that utilization is encoded by the B6 Ti plasmid.

The ability to utilize deoxyfructosyl amino acids may have spread from agropine or dManlGln strains to other strains of Agrobacterium by plasmid transfer and recombination. Alternatively, this ability could have evolved separately in the different strains. Experiments by A. Kerr and J.Tempé have shown that the ability of C58C1 to utilize deoxyfructosyl amino acids as sole C source is coded for by the large cryptic plasmid. Thus strain C58pTi$^-$pAt$^-$, lacking the cryptic plasmid, was unable to utilize deoxyfructosylamino acids and is a suitable recipient for plasmid transfer studies. Deoxyfructosylglutamate was a poorer carbon source than dManlGlu for bacterial strains such as B6 (A.Kerr, personal communication).

It was expected that dFruGlu and dFruGln might be found in transformed tissues of all crown gall and hairy root agropine or dManlGln strains if the biosynthetic pathway were the same in all cases. Tumours of the

octopine/agropine strains A6 and B6 and the agropine/agrocinopine C strain Bo542 all contained dFruGlu and dFruGln. Hairy root tissue of 8196, a dManlGln strain (Petit et al., 1983), also contained dFruGlu and dFruGln. This suggests that the pathway in the dManlGln hairy root strains also proceeds via the first two steps of the agropine pathway described above. In this study, no silver nitrate-positive opines were detected in axenic roots induced by hairy root strain TR105 (also known as strain 1855), even though it is known to be an agropine strain (Petit et al., 1983). These results do not allow any conclusions to be drawn about the pathway in that strain. The loss of the ability of hairy root tissue to synthesize agropine family opines has been reported by Petit et al. (1983) who found that some axenic hairy root cultures continued synthesizing agropine whereas others lost this capacity. This situation may be similar to the octopine pTi strains (Thomashow et al., 1980) where the TR-DNA is sometimes absent from axenic tumour cultures, leading to loss of agropine synthesis.

There is evidence, then, that part of the agropine biosynthetic pathway operates in the same way in pRi strain 8196 as in octopine pTi strain B6, since 8196 roots contain dFruGlu/dFruGln and dManlGlu/dManlGln. The cyclization step to agropine is absent in 8196 but at least two pRi8196 genes may code for the same reactions as in the pTi strains. Homology has indeed been found between the T-DNAs of pRi strain 15834 and the octopine pTi strain Ach5 (Willmitzer et al., 1982). The left portion of EcoRI fragment 20 (Fig. 4-4) of the Ach5 TR-DNA hybridized with a part of the 15834 T-DNA. This may well be that part of the T-DNA coding for agropine synthesis in 15834 and would appear to correspond to gene 1' in B6 if it is assumed that TR-DNAs are similar in octopine strains B6 and Ach5.

Fructosyl amino acids, including dFruGlu and dFruGln are commonly found in plant extracts as a result of "non-enzymic browning" (Anet and Reynolds,1957). In plant extracts such as fruit purees, conditions are favourable for the simple chemical condensation of glucose with amino acids to form a variety of fructosylamino acid conjugates. Anet and Reynolds (1957) worked mainly on deoxyfructosylaspartate and deoxy-fructosylasparagine, but glutamate and glutamine react in the same way as aspartate and asparagine as they are closely related amino acids. Commercial yeast extract also contains a number of different fructosylamino acids, including dFruGlu and dFruGln. These latter two compounds from yeast extract were identified by their electrophoretic mobility, staining properties and by the products of their chemical reduction (this work, data not shown). The fructosylamino acids are presumably formed under the conditions used for the industrial preparation of the yeast extract, as the presence of such compounds in processed foods is well known (American Chemical Society Symposium Series, vol. 215 (1983) "The Maillard reaction in foods and nutrition").

Deoxyfructosylglutamate and dFruGln were not detected in in untransformed carrot callus, but a very careful search should be made for these compounds in untransformed plant tissue considering the wider occurrence of deoxyfructosyl amino acids in plant extracts. The possibility arises that deoxyfructosyl amino acids may be formed in wounded plant tissues if conditions are conducive to chemical conjugation of glucose and amino acids in the "wound juice". Wounding is a requirement for transformation and any deoxyfructosyl amino acids formed non-enzymically could act as a stimulus or signal to the bacteria for the presence of wounded plant tissue. It may even allow speculation about the origin of opine synthase genes. If the ability to utilize deoxyfructosyl

amino acids from wound tissue evolved in agrobacteria as an initial step, the genes for utilization could later have been transferred to the T-DNA and there operated in reverse, with appropriate energy input, to direct the production of large amounts of these substances in tumours.

Dahl et al. (1983) suggested that biosynthesis of dMan1Glu might be either T-DNA-coded or that it might arise from "spontaneous chemical or enzymatic hydrolysis" of dMan1Gln. Deoxyfructosyl glutamate could be formed by similar alternative routes: either T-DNA-coded or from breakdown of dFruGln. If dFruGlu biosynthesis were T-DNA-coded, then the same enzyme must be able to accept both glutamate and glutamine as substrates, since only one gene (gene 2') is involved in the biosynthetic reaction in which both dFruGlu and dFruGln appear as products. Such a lack of substrate specificity would parallel that of lysopine dehydrogenase (LpDH) which accepts various amino acids in the synthesis of the octopine family of opines (Hack and Kemp, 1977; Otten, 1979). LpDH is able to accept lysine, ornithine or histidine as well arginine in the reductive condensation with pyruvic acid (Goldmann, 1977). The evidence presented here, obtained from analysis of tumours incited by strains with TR-DNA mutations, is compatible with both of the above explanations for dFruGlu biosynthesis.

3. Opines of strain IIBV7

The opines of IIBV7 are relevant to agropine biosynthesis but they are also an interesting case in their own right, owing to the unusual combination of opines found in this strain. The tumours contain large amounts of (a) dFruGlu, (b) dFruGln and (c) agropin-1'-ene. These compounds can be chemically reduced to (a) dMan1Glu and dGlc1Glu, (b) dMan1Gln and dGlc1Gln and (c) agropine respectively. The reduced compounds are not found in the tumours, so IIBV7 T-DNA must lack the

reductase(s) coded for by TR-DNA transcript 1' in strains such as A6, B6 and 8196 which do contain the related reduced compounds dMan1Gln and dMan1Glu. One would still expect some homology between the T-DNA of IIBV7 and B6 in the region coding for the presumed "conjugase" (gene 2') linking D-glucose with L-glutamate or L-glutamine. Hybridization studies using the T-DNAs of these strains would indicate whether any homology exists in this region. A comparison of the T-DNAs of strains B6 and IIBV7 could also offer clues about the relationship between agropine and agropin-1'-ene biosynthesis, as it is not certain at present whether agropin-1'-ene in B6 tumours is a precursor of agropine.

Agropin-1'-ene behaved as an opine for strain IIBV7; it was specifically utilized by IIBV7 bacteria, although plasmid transfer studies are required to show whether or not this function is Ti plasmid-coded. The proposed structure (X) of agropin-1'-ene is primarily based on its smooth catalytic hydrogenation (Fig. 4-1, step vii) to agropine (the major isomer) and glucoagropine, both of which were shown by lactamization studies to produce the corresponding deoxymannityl- (XIV) and deoxyglucityl- (XIII) agropinic acids (Fig. 4-1, step viii). The resistance of agropin-1'-ene to sodium borohydride reduction and its staining properties (triphenyltetrazolium positive, weak ketose reaction) are consistent with the tentative structure (X). Agropin-1'-ene could conceivably be synthesized from dFruGln by a cyclization reaction. If agropine is synthesized by lactonization of dMan1Gln, then agropin-1'-ene may be synthesized from an enolic form of dFruGln by a similar type of enzyme.

The other opines of IIBV7, nopaline and agrocinopine C, have not been found together in the same strain before. Nopaline tumours usually contain agrocinopine A, and agrocinopine C is normally found in agropine tumours.

Kerr and Roberts (1976) reported that IIBV7 was resistant to agrocin 84, unlike other nopaline strains. This was confirmed in the present study, in which it was also shown that sensitivity to agrocin 84 was induced by agrocinopine C. Induction of agrocin 84-sensitivity by agrocinopine C has previously been observed with other agrocinopine C strains (Ellis and Murphy, 1981). The inability of Ellis et al. (1982) to induce conjugal transfer of the IIBV7 plasmid with agrocinopine A, the usual conjugative opine of nopaline strains, can now be explained, as IIBV7 is not an agrocinopine A strain. The conjugative opine of IIBV7 has not yet been identified, but agrocinopine C, the conjugative opine in other agrocinopine C strains (Ellis et al., 1982), may be expected to induce conjugal transfer of the IIBV7 Ti plasmid.

How the particular combination of opines found in IIBV7 tumours came to be coded for by the IIBV7 T-DNA can only be guessed at. The situation may have arisen by recombination between the T-DNAs of a nopaline strain and an agropine strain, with loss of the "reductase" catalyzing dMan1Glu and dMan1Gln synthesis. The presence of opines of three families in tumours incited by a single wild type strain has only been recorded previously for strain Bo542. Tumours of this latter strain contain agropine (Guyon et al., 1980), agrocinopine C (Ellis and Murphy, 1981) and leucinopine (Chang et al., 1983). Certainly the particular combination of opine families found in IIBV7 tumours (nopaline, agrocinopine and agropine) is unique amongst known strains of Agrobacterium.

CHAPTER 5

AGROCINOPINES AND HAIRY ROOT STRAINS

INTRODUCTION

Agrocin 84, a fraudulent adenine nucleotide antibiotic, is taken up by sensitive pTi strains of Agrobacterium via a specific permease (Murphy and Roberts, 1979). The agrocinopines were discovered after it was reasoned that the agrocin 84 molecule might be using the same permease as a hypothetical non-toxic opine from the plant tumours. The agrocinopines were then found to fulfil the role of non-toxic substrates which compete with agrocin 84 for uptake into the bacteria. These opines are readily detected by their effects on the inhibition zones in the agrocin 84 bioassay (Ellis and Murphy, 1981; this thesis, Chapter 3).

Hairy root strains were reported to be agrocin 84-sensitive by Albinger and Beiderbeck (1977). Sensitivity to agrocin 84 and pathogenicity were simultaneously transferable to a resistant non-pathogenic recipient, indicating that they were both likely to be plasmid-borne traits. White and Nester (1980a) demonstrated the presence of three plasmids, designated pAr15834a (107 Md), pAr15834b (154 Md) and pAr15834c (258 Md), in hairy root strain 15834 and that the 154 Md plasmid was the root-inducing (Ri) plasmid. They reported also that agrocin 84 sensitivity was coded for by the small non-rhizogenic plasmid (pAr15834a).

Owing to the agrocin 84 sensitivity of the hairy root strains, experiments were done to determine whether or not hairy root strains were also of the agrocinopine type. Since this work was begun, Petit et al. (1983) have independently presented evidence that agropine strain 15834 is of the agrocinopine A type and dMan1Gln (also known as mannopine) strain

8196 is of the agrocinopine C type. This has been corroborated in the present investigation in which agrocin sensitivity and agrocinopine utilization were studied.

MATERIALS AND METHODS

Agrocin 84-sensitivity

The methods used are described in Chapter 2.

Agrocinopines

Agrocinopine A was purified as described in Chapter 3. For utilization studies, agrocinopine C was partly purified from A281 tumour extracts by anion exchange chromatography. For studies on the interaction with agrocin 84 toxicity, agrocinopine C was further purified by preparative HVPE at pH 9.2 in ammonium bicarbonate buffer.

Agrocinopine utilization

Agrocinopines were filter sterilized before use and made to a final concentration of 0.2% in Bergersen's salts with 1 mg/ml ammonium sulphate. Bacteria from fresh yeast-mannitol slopes were added (0.01ml suspension to 0.09ml medium) and incubated on a rotary shaker at 25°C. Samples were taken at 0, 24 and 72 hours and analysed for disappearance of opines by HVPE at pH 9.2. Agrocinopines were detected with the alkaline silver nitrate reagent.

Bacterial strains and transconjugants

The bacterial strains used and their sources were: C58C1 from J.Schell; A208 and A281 from M-D.Chilton; A4 and NT1(pArA4a, pRiA4b,

pArA4c) from L.Moore; TR101 and TR7 from ICPB; 8196 and C58C1(pRi8196b) from A.Petit; C58C1(pArA4a), C58C1(pRiA4b) and C58C1(pRiTR105) from A.Kerr; 2655, 2657 and 2659 from NCPPB.

RESULTS

Agrocin 84 sensitivity of hairy root strains.

Table 5-1 shows the agrocin 84 sensitivity of hairy root strains. All strains tested, except the negative control (Ti-plasmidless C58C1) and the cucumber hairy root strains NCPPB 2655 and 2659, were to some degree sensitive to agrocin 84. Owing to variation in the degree of sensitivity of the hairy root strains to agrocin 84, experiments were carried out three times. The fact that agrocin sensitivity was observed implies that these strains are of the agrocinopine type.

In order to determine whether strains were of the agrocinopine A or C type, the effects of agrocinopines on agrocin 84 sensitivity were determined and the results are listed in Table 5-1. With dMan1Glu strains TR101, TR7 and 8196, agrocinopine C caused extension of the agrocin 84 inhibition zone, but agrocinopine A had no effect.

The transconjugant of agropine strain A4 harbouring all three plasmids, pArA4a (107 Md), pRiA4b (154 Md) and pArA4c (259 Md), showed an extension of the inhibition zone with both agrocinopines A and C.

The transconjugant C58C1(pRiA4b), containing only the A4 Ri plasmid, and the TR105 transconjugant both behaved in the same way; the inhibition zone was extended by agrocinopine A but agrocinopine C had no effect. The transconjugant containing only the small non-rhizogenic plasmid pRiA4a was agrocin-sensitive but it is curious that this sensitivity was strongly counteracted by both agrocinopines A and C.

TABLE 5-1

AGROCIN 84 SENSITIVITY OF HAIRY ROOT STRAINS[a]

STRAIN AND PLASMID CONTENT	AGROCIN 84 SENSITIVITY	AGROCIN 84 INHIBITION ZONE EXTENDED BY: AGROCINOPINE A	AGROCINOPINE C
C58C1	-	-	-
A208 (harbours pTiT37)	+	+	-[b]
NT1(pRiA4b, pArA4a, pArA4c)	+	+	+
C58C1(pArA4a)	+	-[b]	-[b]
C58C1(pRiA4b)	+[c]	+	-
C58C1(pRiTR105)	+[c]	+	-
TR101	+	-	+
TR7	+	-	+
8196	+	-	+
NCPPB 2655	-	-	-
NCPPB 2659	-	-	-

[a] overall sensitivity varied markedly between experiments.

[b] counteracted agrocin 84 toxicity.

[c] weakly sensitive

The agrocin sensitivity of C58C1(pArA4a) was intermediate between the weak sensitivity of C58C1(pRiA4b) and the marked sensitivity of the transconjugant containing all three plasmids.

The cucumber hairy root strains NCPPB 2655, 2657 and 2659 were all resistant to agrocin 84 and sensitivity was not inducible by agrocinopines A or C.

Utilization of agrocinopines by hairy root strains.

The results of agrocinopine utilization experiments are set out in Table 5-2. Ti plasmidless C58C1 was the negative control and tumorigenic strains A208 and A281 were used as positive controls for agrocinopines A and C respectively.

The dMan1Gln strain 8196 utilized agrocinopine C but not agrocinopine A whereas agropine strain A4 utilized both agrocinopines A and C. The transconjugant harbouring pRiA4b also utilized both agrocinopines. Surprisingly, the transconjugant with the non-rhizogenic plasmid (pArA4a) utilized agrocinopine C but not agrocinopine A.

Neither of the agrocinopines was utilized by the cucumber hairy root strains.

DISCUSSION

Results obtained for the dMan1Gln hairy root strains TR7, 8196 and TR101 provide straightforward evidence that they are agrocinopine C strains. Their ability to utilize agrocinopine C but not agrocinopine A and the interaction of agrocin 84 toxicity with agrocinopine C but not with agrocinopine A support the conclusion of Petit et al. (1983) that these strains are of the agrocinopine C type. Agrocinopine C utilization

TABLE 5-2

UTILIZATION OF AGROCINOPINES BY HAIRY ROOT STRAINS[a]

STRAIN	UTILIZATION	
	AGROCINOPINE A	AGROCINOPINE C
C58C1	-	-
A208 (harbours pTiT37)	+	n.t.
A281 (harbours pTiBo542)	n.t.	+
A4	+	+
C58C1(pArA4a)	-	+
C58C1(pRiA4b)	+	+
8196	-	+
C58C1(pRi8196)	n.t.	+
NCPPB 2655	-	-
NCPPB 2657	-	-
NCPPB 2659	-	-

[a] n.t.= not tested

must be coded for by the Ri plasmid pRi8196, as this opine was utilized by the Ri plasmid transconjugant. Direct chemical evidence for the presence of agrocinopine C in hairy root extracts is still required in order to confirm these results.

Results obtained for agropine hairy root strains TR105 and A4 support the conclusions of Petit et al. (1983) that they are agrocinopine A strains. Agrocinopine A utilization is coded for by the Ri plasmid pRiA4b but not by the small plasmid pArA4a which unexpectedly appears to code for agrocinopine C utilization. The relationship between agrocinopine C and the agropine/agrocinopine A hairy root strains remains unclear. For example, it is not known why the transconjugant containing pRiA4a has the same reaction to both agrocinopines A and C in the agrocin 84 bioassay (Table 5-1) but utilizes only agrocinopine C and not agrocinopine A.

White and Nester (1980b) reported that the plasmids of A4 and 15834 are very similar in size and restriction patterns and (1980a) that pAr15834a codes for agrocin 84 sensitivity. The present study reports that pArA4a also codes for agrocin 84 sensitivity. In addition, it is demonstrated here that pRiA4a codes for the utilization of agrocinopine C. The Ri plasmid pRiA4b (equivalent to pRi15834b), however, also codes for agrocin 84 sensitivity, though this sensitivity is difficult to detect, and in addition codes for the utilization of both agrocinopines A and C. It is not clear at this stage why agrocin sensitivity and agrocinopine C catabolism are encoded by two different plasmids in the same strain. The results do show that these functions on the two plasmids either operate differently or are regulated in a different manner.

Strain A4, which harbours three plasmids, was much more sensitive to agrocin 84 than were the transconjugants C58C1(pRiA4b) or C58C1(pArA4a). The greater sensitivity of A4 could be due to cooperation for permease

induction between the two smaller plasmids when both are together in the same strain. More detailed studies on the uptake of agrocin 84 are required to clarify the situation.

On the basis of the results presented, the cucumber hairy root strains NCPPB2655 and 2659 appear not to be of the agrocinopine type. This is discussed further in Chapter 7.

The variation in sensitivity between agrocin 84 bioassay experiments with the hairy root strains was problematic. The use of Hendson's medium (Hendson et al., 1983) rather than Stonier's medium has been beneficial in this respect (A.Kerr, personal communication).

CHAPTER 6

THE ORIGINAL SOURCES OF HAIRY ROOT ISOLATES AND THEIR IMPLICATIONS FOR COMPARATIVE STUDIES

INTRODUCTION

A number of bacterial strains isolated in early studies on crown gall and hairy root diseases are still used in present-day research on *Agrobacterium*. Use of the single-cell cultures originally isolated by Wright *et al.* (1930) has been particularly widespread. Since these single-cell cultures are kept in several bacterial collections and many laboratories, some are now known by more than one designation. It is thus possible to inadvertantly do comparative work on cultures which came from the same parent cell. Evidence is presented that this is the case for *Agrobacterium rhizogenes* ICPB TR7 and NCIB 8196 which are both descended from single-cell strain C-1 isolated in the late 1920's by Wright and his coworkers in A.J.Riker's laboratory. The evidence was compiled from a literature search and from information given by culture collections.

The plasmids and their restriction patterns for four dMan1Gln hairy root strains including TR7 and 8196 were compared, revealing identical patterns except for a 3.0 Md deletion in the smallest TR7 plasmid.

MATERIALS AND METHODS

Plasmid isolation

The plasmid isolation and restriction analysis in this part of the work were performed by J.-S.Shim. The method used was modified from that of Birnboim and Doly (1979) by Dr.S.K.Farrand (personal communication). The solutions used are described in detail in Appendix B.

Cells were grown to late log phase in 1 ml L-broth, pelleted (2 min) in a microcentrifuge tube and resuspended in 1 ml TE buffer. After adding 5 M NaCl (0.1 ml) and 10% Na sarkosyl (0.01 ml) and mixing, the suspension was again pelleted. The pellet was resuspended in Solution 1 (0.1 ml) and kept on ice 5 min. Solution 2 (0.2 ml) was quickly added, the tube was inverted to mix, and allowed to stand 15 min at room temperature and 2 M Tris-HCl pH 7 (0.05 ml) was then added. After 30 min (room temperature), 5 M NaCl (0.05 ml) was added with gentle mixing.

The mixture was extracted with phenol saturated with 3% NaCl (0.4 ml of a solution stored at 4°C before use) approximately 5 min with gentle agitation. After centrifugation (10 min at 4°C), the upper (aqueous) phase was transferred to a new centrifuge tube and 2 volumes of absolute ethanol (-20°C) were added. The solution was kept at -20°C for >2h. The DNA was collected by centrifugation (10 min at 4°C), the ethanol was decanted and the pellet air-dried.

For agarose gel electrophoresis, the DNA was redissolved in LTE buffer (0.05 ml), mixed with tracking dye (0.02 ml DNA solution + 0.01 ml dye) and 0.01 ml of this solution was loaded per well. Gel electrophoresis was carried out in 0.7% agarose (Seakem) at 150 Volts (approx. 35 mA) for 4.5 h.

Restriction analysis

The DNA pellet from the plasmid isolation procedure was dissolved in LTE buffer (0.1 ml) and Solution 3 (0.15 ml) was added with gentle inversion to mix. Sodium acetate (3 M, pH 5.2, 0.1 ml) was added with gentle inversion and the mixture was incubated on ice 30 min. After centrifugation (15 min at 4°C), the liquid was decanted to a 1.5 ml centrifuge tube on ice and absolute ethanol (0.7 ml, kept at -20°C) was

added to precipitate the DNA. The DNA was collected by centrifugation (10 min, 4°C), the ethanol was decanted, and the pellet was allowed to drain well.

The dry pellet was redissolved in TE buffer containing RNase (0.1 ml stock solution), transferred to a 0.5 ml centrifuge tube and was incubated at 37°C for 30 min. Potassium acetate (5 M, pH 5, 0.01 ml) was added and the solution was mixed well. Absolute ethanol (0.2 ml, kept at -20°C) was added and the DNA collected as described above. The ethanol was decanted and the pellet was rinsed with 70% ethanol (kept at -20°C) followed by centrifugation (4°C, 10 min). The 70% ethanol was decanted and the tube was allowed to drain thoroughly before the pellet was redissolved in LTE buffer (0.06 ml). Aliquots (0.015 ml) were taken for restriction enzyme digestion using EcoRI and BamHI (New England Biolabs). The digests were carried out at 37°C for 2 h. The reaction was stopped by the addition of tracking dye (0.02 ml; 5% glycerol, 2.5% Ficoll, 0.025% Bromophenol blue) and 0.04 ml was loaded for gel electrophoresis. The gel (0.7% agarose in Tris borate, pH 8.0, 20 x 25 cm) was run for 22 h at 35 Volts (30 mA), stained with ethidium bromide (2 µg/ml) and was photographed on Polaroid type 665 positive/negative film under UV (302 nm) illumination.

RESULTS AND DISCUSSION

Literature survey

Riker et al. (1930) made many isolations of Phytomonas (now Agrobacterium) from crown gall on various plant hosts and from hairy root on roots of apple trees. A few of the apple isolates appeared to them to be mixed crown gall and hairy root cultures even after four successive

purifications by the dilution plate method. To be certain of obtaining pure cultures, several single-cell isolations were made by mechanically isolating single bacteria from the cultures already four times purified (Wright et al., 1930). Single-cell isolations were made from one tumour-inducing culture, two hairy root cultures and one apparently mixed culture. Five single-cell isolations, designated C-1, C-10, C-11, C-12, and C-13 were made from one of the hairy root parent cultures, culture T-32, from hairy root of apple. Daughter cultures originating from C-1 and C-10 have been used in many studies on Agrobacterium and hairy root disease.

Cultures descended from C-1 have been sent to culture collections and laboratories by the following routes:

(1) Strain NCIB 8196 was obtained from the U.S. Department of Agriculture's Northern Regional Research Laboratory (NRRL, now NRRC) as NRRL B-193. Strain NRRL B-193 was in turn received from A.J.Riker in 1941 as C-1.

(2) M.P.Starr's ICPB TR7 was obtained from A.W.Hofer in 1943 as C-1 (Starr, 1946). Hofer had previously received C-1 as one of the single-cell strains from the University of Wisconsin, where Riker worked. According to the American Type Culture Collection catalogue (1974 edition), Hofer's C-1 was sent to him by E.M.Hildebrand. A survey of the literature showed that Hildebrand worked closely with Riker and others at the University of Wisconsin (Riker and Hildebrand, 1934; Riker et al., 1932, 1934) and in his own work (Hildebrand, 1934) used hairy root culture C-1 from Riker's laboratory. He states in this work that he compared the pathogenicity of C-1 regularly with its sister single-cell strains C-10, C-11, C-12 and C-13. It was undoubtedly Hildebrand who sent A. rhizogenes C-1 to Hofer from the University of Wisconsin.

Strains ICPB TR7 and NCIB 8196 are therefore descended from the same bacterial cell. A. rhizogenes C-1 was first used under the designation TR7 by Starr (1946). The daughter culture NCIB 8196 was apparently first used in a published work by DeLey et al. (1966) who included this strain, as well as TR7, in a taxonomic study. In view of the common origin of these strains, it is not surprising that the same DNA melting temperature (95.1°C) and DNA base composition (62.8% GC) were reported for both.

TR7 and 8196 have both been included in the same study on several other occasions e.g. Lippincott and Lippincott (1969), DeCleene and DeLey (1981) and Costantino et al. (1981). Minor differences between the strains were noted in the latter two papers and these differences are described below.

DeCleene and DeLey (1981), in Table III of their paper on the host range of hairy root disease, reported the same result for pathogenicity of 8196 and TR7 on four out of five hosts tested but a difference was reported for the virulence of these two strains on Vicia faba. When inoculated on this host, DeCleene and DeLey observed "neoplasms without external roots or shoots" with 8196 but no reaction with TR7.

Costantino et al. (1981) isolated plasmids from several hairy root strains and compared their restriction endonuclease digestion patterns. The plasmids of 8196 had molecular weights of $(98 \pm 1) \times 10^6$ and $(137 \pm 2) \times 10^6$ by contour length measurements from electron micrographs. These results agreed well with those reported for TR7 by Currier and Nester (1976): $(98 \pm 3.6) \times 10^6$ and $(140 \pm 4.7) \times 10^6$. Although the plasmids of the two strains were identical in size, the restriction patterns published by Costantino et al. (1981) did show a difference. The EcoRI cleavage patterns (their Fig. 4) were identical except in the case of the second-largest restriction fragment. Their interpretation was that

there was either a single deletion in TR7 or an insertion in 8196. This is a noteworthy result, considering the common origin of these plasmids. There is a precedent for this type of change in plasmid size upon subculturing. Sciaky et al. (1978) have demonstrated strain divergence in the Ti plasmid of A. tumefaciens B6, with SmaI fragment 2 of pTiB6-T being 1.1Md larger than the same fragment in pTiB6-806.

Comparison of plasmids of dMan1Gln hairy root strains

In the present study, the plasmids of all four dMan1Gln (also known as mannopine) pRi strains were investigated. These four strains show attenuated virulence on the basal surface of carrot root discs (see Chapter 8). Fig. 6-1 shows that each strain harbours three plasmids and that there is no detectable difference in plasmid size between strains.

The plasmid restriction patterns of TR7, 8196, ATCC 11325 and TR101 as well as C58C1(pRi8196) are shown in Fig. 6-2. There was a single difference between TR7 and 8196 in the second-largest EcoRI fragment, which confirms the result of Costantino et al. (1981). This second-largest fragment was 3.0 Md smaller in TR7 than in 8196 but by comparison with the 8196 transconjugant the difference did not appear to be on the Ri plasmid and must therefore be on the smaller non-rhizogenic plasmid.

Strain 11325 was classed as a nopaline strain by White and Nester (1980b) and Petit et al. (1983) who both obtained it from J.A.Lippincott. However, I found that a culture of 11325 obtained from ATCC in 1982 was rhizogenic on carrot root discs and closely resembled TR7 and 8196 as described above. Strain 11325 has been designated the type strain for hairy root by Skerman et al. (1980) and as the culture held by ATCC is a rhizogenic dMan1Gln strain, this culture collection should be used as a future source of the strain.

FIGURE 6-1

Agarose gel electrophoresis of plasmids from dMan1Gln type hairy root strains.

Lane A: C58C1(pRi 8196b); B: TR101; C: TR7; D: 8196; E: 11325.

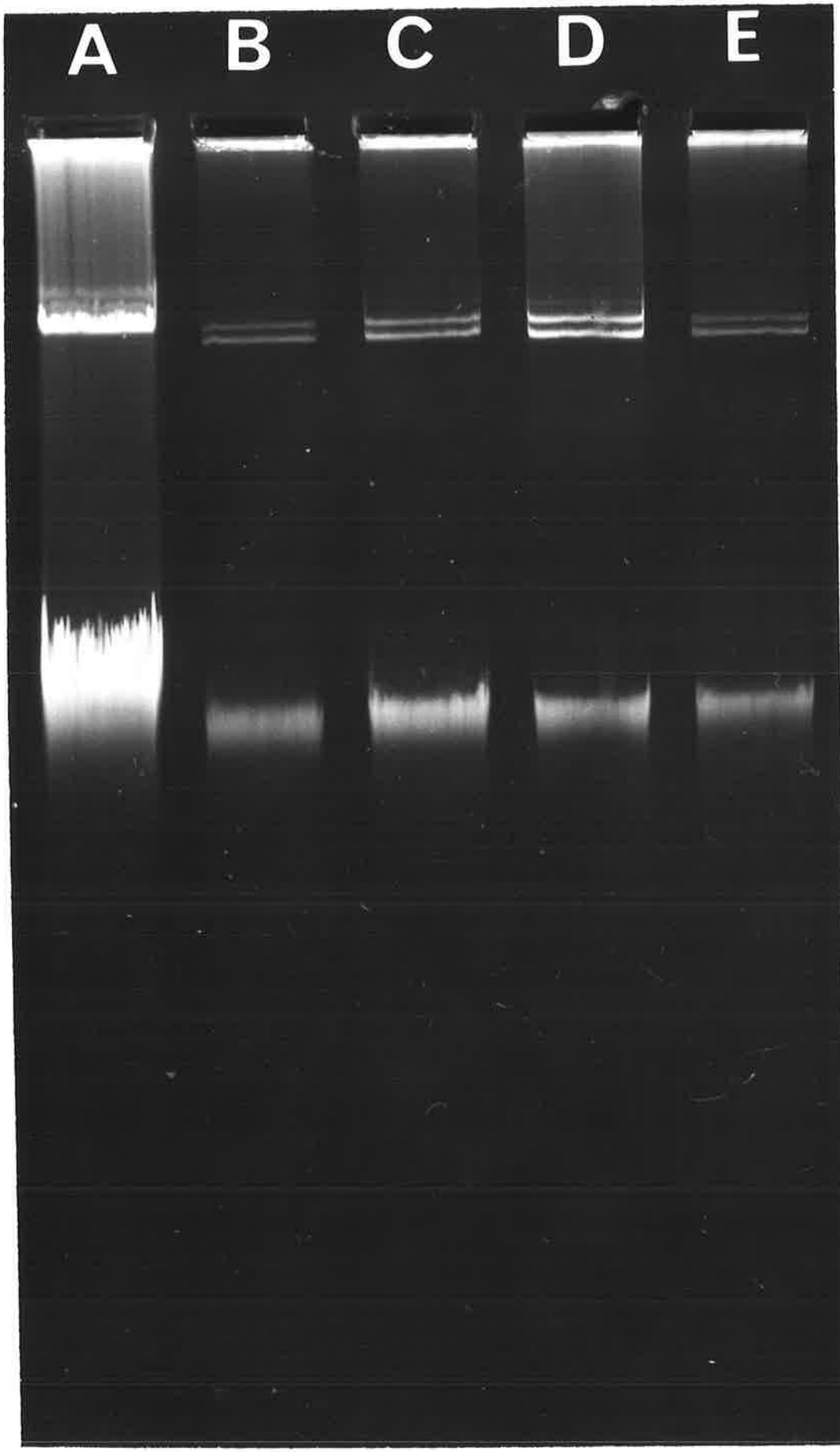
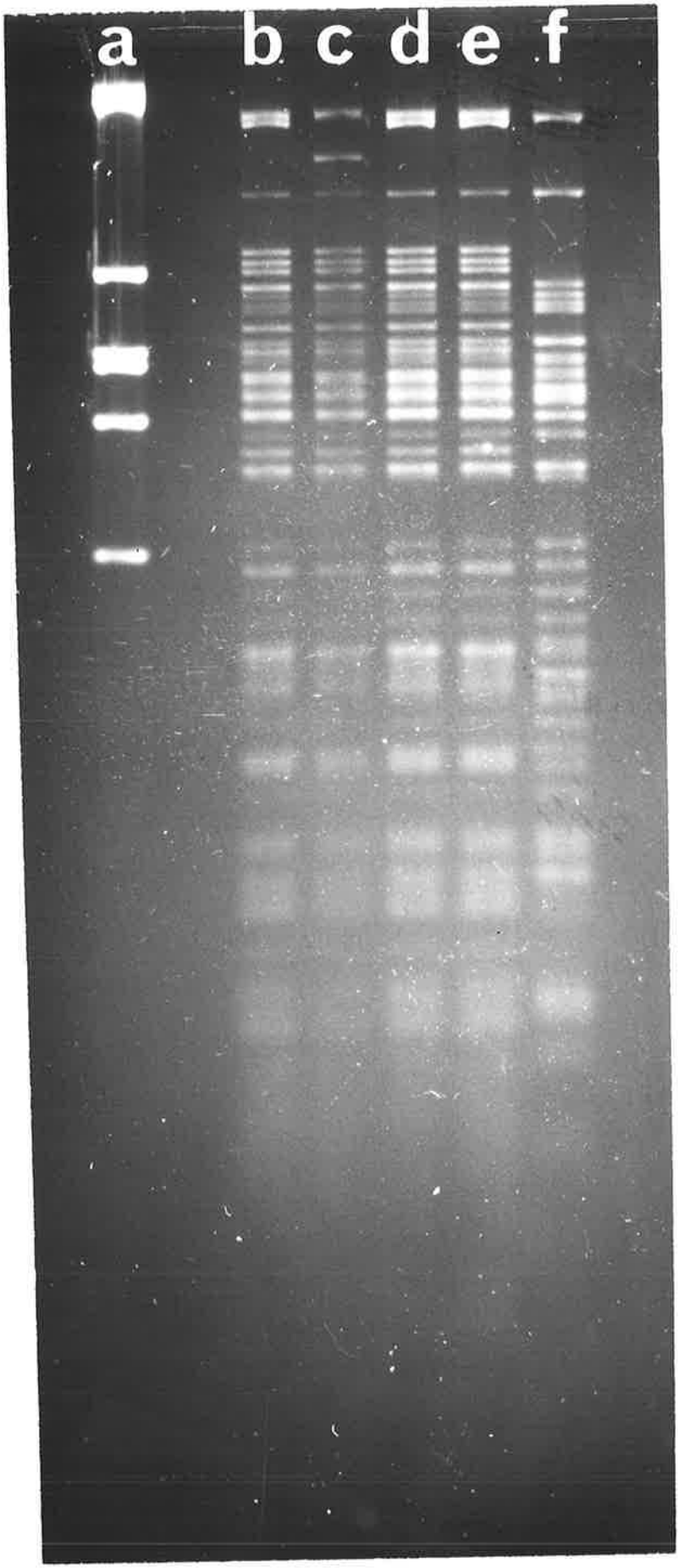

A
B
C
D
E

FIGURE 6-2

Restriction endonuclease analysis of plasmids of dMan1Gln type hairy root strains.

The plasmids were digested with EcoRI.

Lane A: λ-DNA (EcoRI digest); B: TR101; C: TR7; D: 8196; E 11325; F: C58C1(pRi 8196b).


a
b
c
d
e
f

It is remarkable that strains TR101 and 11325 had identical plasmid restriction patterns to strain 8196 using both EcoRI (Fig. 6-2) and BamHI (data not shown). These strains are all of the same opine type, are all A.rhizogenes (formerly biotype 2), and also show the same attenuated virulence on the basal surface of carrot root discs (see Chapter 8). The origins of strains TR101 and 11325 are obscure. TR101 was originally isolated in the USA but no further information could be obtained anywhere in the literature about its origin. ATCC 11325 was deposited in the American Type Culture Collection by A.J.Riker in August 1952 without any further information on its origin. In view of the similarities outlined above, and the fact that 11325 came from Riker, it is possible, if not probable, that these two strains both came from the same origin as TR7 and 8196 and at the very least they ought not to be treated as independent isolates. If they are all derived from the same isolate, then the plasmid difference between TR7 and 8196 must be due to a 3.0 Md deletion in the smallest TR7 plasmid and not to an insertion in the corresponding 8196 plasmid, since the restriction patterns for all strains except TR7 were identical.

CHAPTER 7

THE OPINES AND T-DNA OF CUCUMBER HAIRY ROOT STRAINS

INTRODUCTION

In terms of opines and T-DNA, the A.tumefaciens (formerly biotype 1) hairy root strains, originally isolated from cucumber in the U.K., are a little-known group. Costantino et al. (1981) found that these strains (NCPPB 2655, 2657 and 2659) contained plasmids which showed "substantial" sequence homology with the Ri plasmid of agropine hairy root strain TR105. They concluded from homology studies between plasmids of the cucumber strains that the single plasmid in strain 2659 was also present in the other cucumber strains. These other strains harboured additional plasmids showing no homology with the 2659 plasmid. Risuleo et al. (1982) found that the 2659 plasmid showed similarities to the C58 (nopaline) Ti-plasmid in the Vir region but not in the T-DNA region. Despite the large amount of work done on the cucumber strain plasmids, it has not been proven that the disease is plasmid-coded in these strains. It was simply inferred that the common plasmid was an Ri plasmid by analogy to other hairy root strains (Risuleo et al., 1982).

My interest in the cucumber hairy root strains lay in the fact that they show attenuated virulence on the basal surface of carrot root discs and in this respect closely resembled the 8196 group of dMan1Gln hairy root strains (see Chapter 8). An investigation was made of the opines and T-DNA of these strains, to find out whether they were related to the dMan1Gln group.

MATERIALS AND METHODS

Bacterial strains

A.tumefaciens (formerly biotype 1) strains 2655, 2657 and 2659 were obtained from NCPPB, Harpenden, Herts. U.K. Strain C58C1 was obtained from J.Schell, C58pAt$^-$ was from A.Petit, and A6 and IIBV7 were from G.Morel.

Opine utilization

1. Octopine, nopaline and deoxymannitylglutamate (dManlGlu)

The filter-sterilized opines were made to a final concentration of 0.2% in Petit's medium (see Appendix A) solidified with 2% agar. Octopine and nopaline were tested as sole carbon and nitrogen source; dManlGlu was tested as sole C source, with 0.1% $(NH_4)_2SO_4$ being added as N source.

Freshly grown bacteria (2 day yeast-mannitol slopes) were suspended in sterile distilled water, streaked on to the agar and incubated several days at 28^{o}C before growth was recorded.

2. Agropine

Utilization of agropine (0.2% in Petit's medium + 0.1% $(NH_4)_2SO_4$) was tested in liquid culture. The medium, (15µl) was dispensed into sterile microcentrifuge tubes and bacteria from 2-day yeast-mannitol slopes were added with a sterile needle. The tubes were incubated at 28^{o}C. Positive and negative control strains were included.

Samples were taken at 0h and 24h, and the disappearance of agropine was monitored by HVPE in formic acid/acetic acid pH 1.7, followed by staining with alkaline silver nitrate.

3. Agrocinopines A and C

Agrocinopines were used at a concentration of 0.2% in Bergersen's salts (see Appendix A) + 0.1% $(NH_4)_2SO_4$. The bacterial suspension (0.01 ml) was added to the medium (0.09ml) in a sterile culture tube. The tubes were incubated at 25°C on a rotary shaker and samples (5µl) were taken for analysis by HVPE in NH_4HCO_3 pH 9.2 (20 min at 1500V) to follow the disappearance of agrocinopines. Alkaline silver nitrate was used for detection.

4. Cucumopine

The opine was biologically purified by growing strain C58C1 on the hairy root extract which was obtained as described below under "Extraction of opines from hairy root tissue". The compound was further purified by ion exchange chromatography on Dowex 50W-X8(H^+) using 1.5 M ammonia for elution, following the method of Guyon et al. (1980). The fractions containing cucumopine were reduced in volume on a rotary evaporator and the partly purified compound was filter sterilized. For opine degradation studies, 1/5 volume of 5x Bergersen's salts with 1 mg/ml ammonium sulphate was added, followed by 1/10 vol of bacterial suspension. The total volume (0.1 ml) was incubated in Falcon 5 ml sterile tubes on a rotary shaker at 25°C. The disappearance of cucumopine was monitored by sampling at 0, 24 and 72 h and analysis by HVPE (pH 1.7), using alkaline silver nitrate as detection reagent.

Agrocin 84 sensitivity

Agrocin 84 sensitivity of bacteria and the effects of agrocinopines A and C were determined by the method of Ellis and Murphy (1981) (see Chapter 2).

High voltage paper electrophoresis

Methods for HVPE and detection reagents are described in Chapter 2. Additional buffers used were: 0.1 M formic acid/0.05 M NaOH, pH 3.25; 0.05 M citric acid/NaOH to pH 5.0; 0.1 M KH_2PO_4/KOH to pH 7.4; 0.1 M $NaHCO_3$/0.05M NaOH, pH 10.3; 0.1 M $KHCO_3$/KOH to pH 11.2; 0.1 M KH_2PO_4, pH 12.

Carrot inoculations

Carrots were inoculated as described in Chapter 2.

Extraction of opines from hairy root tissue

Hairy root tissue (approx. 10g) was harvested from carrot discs 3 months after inoculation or from axenic tissue of 2659 roots grown on Monnier's salts (Monnier, 1976) plus Morel's vitamins (Morel and Wetmore, 1952) and sucrose (see Appendix A). The roots were homogenized in 70% ethanol (5 ml/g f.wt.) using a Polytron tissue grinder. The homogenate was centrifuged (4,000xg 10min), the supernatant was decanted and the pellet was rinsed with 70% ethanol. The combined ethanol extract was dried *in vacuo* at 35°C and was subsequently used in biological purification and utilization studies.

Selectivity of opine utilization

Dried ethanol extracts of hairy root tissue, prepared as described in the previous section, were dissolved in distilled water (50 mg dry wt. extract/ml) and were then filter sterilized. The extracts were diluted 1:5 into Bergersen's salts including thiamine (1 mg/ml), biotin (0.25 mg/ml) and $(NH_4)_2SO_4$ (0.1%). *A.tumefaciens* control strains C58C1 and C58pAt$^-$, as

well as the inciting hairy root strain, were grown on separate samples of the extracts (0.02 ml bacterial suspension in 1 ml medium). The cultures were incubated on a rotary shaker at 25°C. Samples (0.2 ml) were taken at 4, 30 and 72h, blown dry under nitrogen, dissolved in 25µl water and 5µl samples were used for HVPE in formic acid/acetic acid, pH 1.7.

Hairy root DNA extraction

DNA extraction was essentially as described by Chilton et al. (1982) except that the final dialysis step was omitted. Axenic hairy roots (37g f.wt.) of strain NCPPB 2659, grown on Monnier's salts and Morel's vitamins (see Appendix A) at 25°C in the dark, were used for extraction. The roots were frozen in liquid nitrogen, mixed with dry ice and were then ground, while frozen, in a coffee grinder. An emulsion (40 ml), containing: (A) phenol equilibrated with 0.02 M Tris buffer pH 8.0, 10 mM Tris, 50 mM EDTA, 50 mM NaCl, 1 mM $CaCl_2$, 0.4 mg/ml ethidium bromide (40 ml) and (B) 25% SDS (2.4 ml), was added. The emulsion was allowed to melt, stirred slowly for 5 min on a magnetic stirrer at room temperature and was then centrifuged in Corex tubes (20 min at 6000 rpm in an SW50 rotor). The supernatant (DNA fraction) was collected, reextracted with equilibrated phenol and was decanted into a measuring cylinder. To precipitate the DNA, 3 M Na acetate (1/10 vol) and isopropanol (0.75 vol) were added. The solution was mixed and was allowed to stand at -15°C overnight. The DNA was collected by centrifugation (6000 rpm, SW 50, 20 min) and the tubes were inverted to drain. The white precipitate was redissolved in 10 mM Tris/5 mM EDTA, pH 8.0 (8 ml) and was centrifuged to equilibrium in a CsCl gradient. Caesium chloride (7.83 g) was dissolved directly in the DNA solution, ethidium bromide was added to a final concentration of 0.4 mg/ml and the solutions were centrifuged 72 h at 33,000 rpm in an SW50.1 rotor.

The DNA was visualized with UV light and was removed from the gradient with an 18-gauge syringe. The ethidium bromide was then extracted using isopropanol equilibrated with 20x SSC (an equal volume of isopropanol was added and shaken, and the isopropanol phase was removed; the extraction was repeated until the isopropanol was clear and then repeated twice more). The aqueous phase was diluted with an equal volume of water, and 1/10 vol. of Na acetate was added, followed by 0.75 vol. isopropanol. The DNA was spooled on to the tip of a Pasteur pipette, rinsed by dipping in 70% ethanol, allowed to dry and was then redissolved in 0.3 ml of 10 mM Tris/1 mM EDTA, pH 8.0. The DNA concentration was approximately 1 mg/ml as judged by agarose gel electrophoresis of samples compared with known amounts of lambda DNA.

T-DNA analysis by Southern blot hybridization

Southern transfer and hybridization were carried out as described by Maniatis et al. (1982).

Plant DNA (5µg/well) was digested with BamHI, HindIII, EcoRI and KpnI (3h, 37°C, 100U enzyme from New England Biolabs) and then electrophoresed in horizontal 0.8% agarose gels (8mm thick, in Tris borate buffer) for 15h (75V, 68mA).

The probe used for hybridization was a mixture of clones 118 and 119 (from M.-D. Chilton; see Koplow et al., 1984) which together span the T-DNA of pRi 8196. The probe (300ng, clones 118 and 119 in approximate ratio 1:3 plus 5ng lambda DNA) was labelled by nick translation (Amersham kit) to a specific activity of 4.3×10^8 dpm/µg DNA using [γ-^{32}P]-dCTP (from BRESA, Adelaide)

Hybridization was carried out in 6x SSC and 5x Denhardt's solution at 68°C for 23h. Blots were washed at 58°C in 0.1xSSC to remove excess probe

and then dried. Hybridized blots were autoradiographed using Fuji X-ray film at -80°C for up to 2 weeks.

RESULTS

Opines of cucumber hairy root strains

Table 7-1 and Fig. 7-1 show the utilization of opines by the cucumber hairy root strains. Octopine and nopaline were not utilized as sole C and N sources, nor were dMan1Glu, agropine or agrocinopines A and C used as sole sources of carbon.

Strains 2655 and 2659 were resistant to agrocin 84 and agrocinopines A and C did not induce sensitivity at the levels tested (10µg). Both these results are evidence that strains 2655 and 2657 are not of the agrocinopine A or C type.

Ethanol extracts of hairy root tissues incited by strains 2655, 2657 and 2659 were analysed for opines by HVPE. Upon electrophoresis at pH 1.7, followed by elution of the dMan1Glu region ($M_{O.G}$ approx. -0.25) and then HVPE in NH_4HCO_3 pH 9.2, no silver nitrate-positive opines of the agropine family were detected. After elecrophoresis at pH 1.7, no octopine or nopaline was detected with the guanidine stain, nor were agrocinopines A or C detected by the p-anisidine or alkaline silver nitrate reagents. Compounds running in the agrocinopine region at pH 1.7 were further concentrated by eluting that region and drying the eluate, followed by HVPE in NH_4HCO_3 pH 9.2. Agrocinopines were not detectable even after this concentration step.

A spot staining negatively (white) with alkaline silver nitrate and which was absent from normal carrot callus was noted in hairy root extracts of all three strains tested (2655, 2657 and 2659). This compound

TABLE 7-1

UTILIZATION OF OPINES BY CUCUMBER HAIRY ROOT STRAINS[a]

Utilization of octopine, nopaline and dManlGlu was assessed by growth of bacterial strains on solid media. The degradation of cucumopine extracted from 2659 hairy root tissue was assessed by disappearance of the compound from liquid culture medium. The positive control for octopine and dManlGlu was A.tumefaciens A6, and A.tumefaciens IIBV7 was the positive control for nopaline.

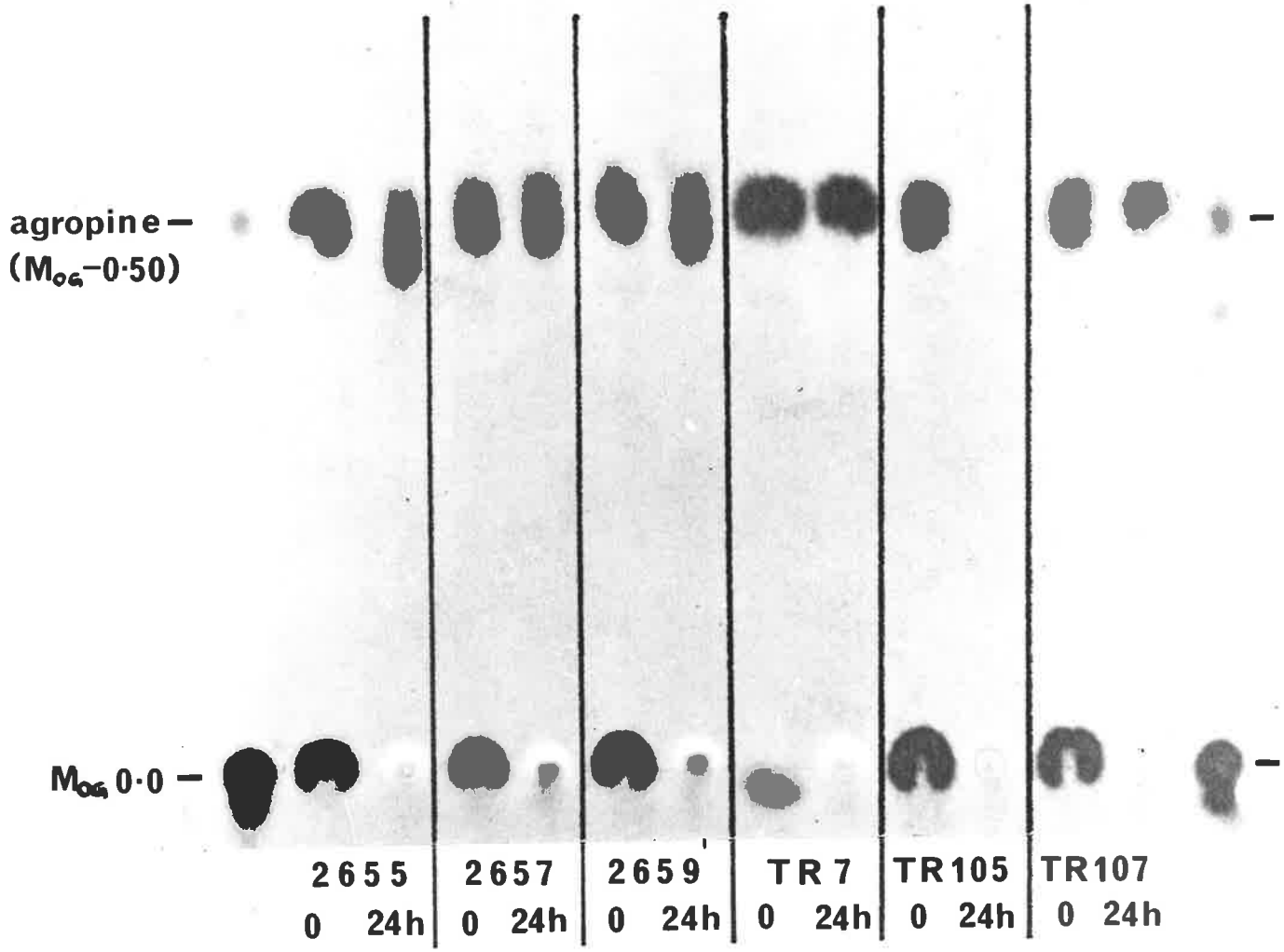
STRAIN	CUCUMOPINE	OCTOPINE sole C & N	NOPALINE sole C & N	dMANLGLU sole N
2655	+	-	-	-
2657	+	-	-	-
2659	+	-	-	-
A6	-	+	-	+
IIBV7	-	-	+	-

[a] + = utilized; - = not utilized.

FIGURE 7-1

Degradation of agropine by hairy root strains.

Samples were taken at 0 and 24 h and analysed by HVPE at pH 1.7. Agropine was detected with the alkaline silver nitrate reagent.


agropine –
(M_{oc}–0·50)
M_{oc} 0·0 –
2655
0 24h
2657
0 24h
2659
0 24h
TR 7
0 24h
TR105
0 24h
TR107
0 24h

stained positively with a detection reagent for organic acids and stained yellow-red with the Pauly reagent for imidazoles but reacted only weakly with ninhydrin. To test whether it was an opine, biological utilization experiments were performed. Extracts of hairy root tissue incited by strains 2655, 2657 and 2659 as well as extracts of 2659-induced axenic root tissue were incubated with bacteria of strains C58C1, C58pAt$^-$ and the respective inciting strain. Fig. 7-2 shows the result of one such experiment, using strain 2659 and an extract of 2659 axenic roots. The silver nitrate-negative compound ($M_{O.G}$ -0.21) was selectively utilized by strain 2659, showing that it indeed behaved like an opine. Identical results were obtained with strains 2655 and 2657 when grown on extracts of hairy roots induced by strains 2655 and 2657 respectively. In a separate experiment, the compound ($M_{O.G}$ -0.21, pH 1.7) present in 2659 roots was degraded by all the cucumber strains tested (2655, 2657 and 2659) but not by pTi strains A6 or IIBV7 or by the negative control C58C1 (Fig. 7-3).

The electrophoretic relative mobilities of octopine, nopaline and the silver nitrate-negative compound over a range of pH values are listed in Table 7-2. The mobility of the 2659 cucumopine increased much more than that of octopine or nopaline above pH 5, and the 2659 opine was more similar to nopaline than to octopine in its pH mobility profile.

T-DNA of cucumber hairy root strain 2659

A search was made for the presumed T-DNA of cucumber hairy root strain 2659. The T-DNA of pRi8196 was used as a probe, on the assumption that there would be at least some homology despite differences in opine type between strains 2659 and 8196. DNA was extracted from axenic 2659-induced roots and was digested, apparently to completion, by EcoRI and HindIII. Digests with BamHI and KpnI appeared to be incomplete as many high

FIGURE 7-2

Biological utilization of cationic (pH 1.7) compounds extracted from hairy roots induced by strain NCPPB 2659.

The inciting strain (2659) and two control strains (C58C1 and C58pAt$^-$) were tested as utilizers. Samples were taken at 4, 30 and 72 h and analysed by HVPE at pH 1.7. Cucumopine, staining white with alkaline silver nitrate, moved just behind the dMan1Glu reference. Fructose = zero relative mobility marker; 2'dA = 2'-deoxyadenosine ($M_{O.G}$ = -0.82).

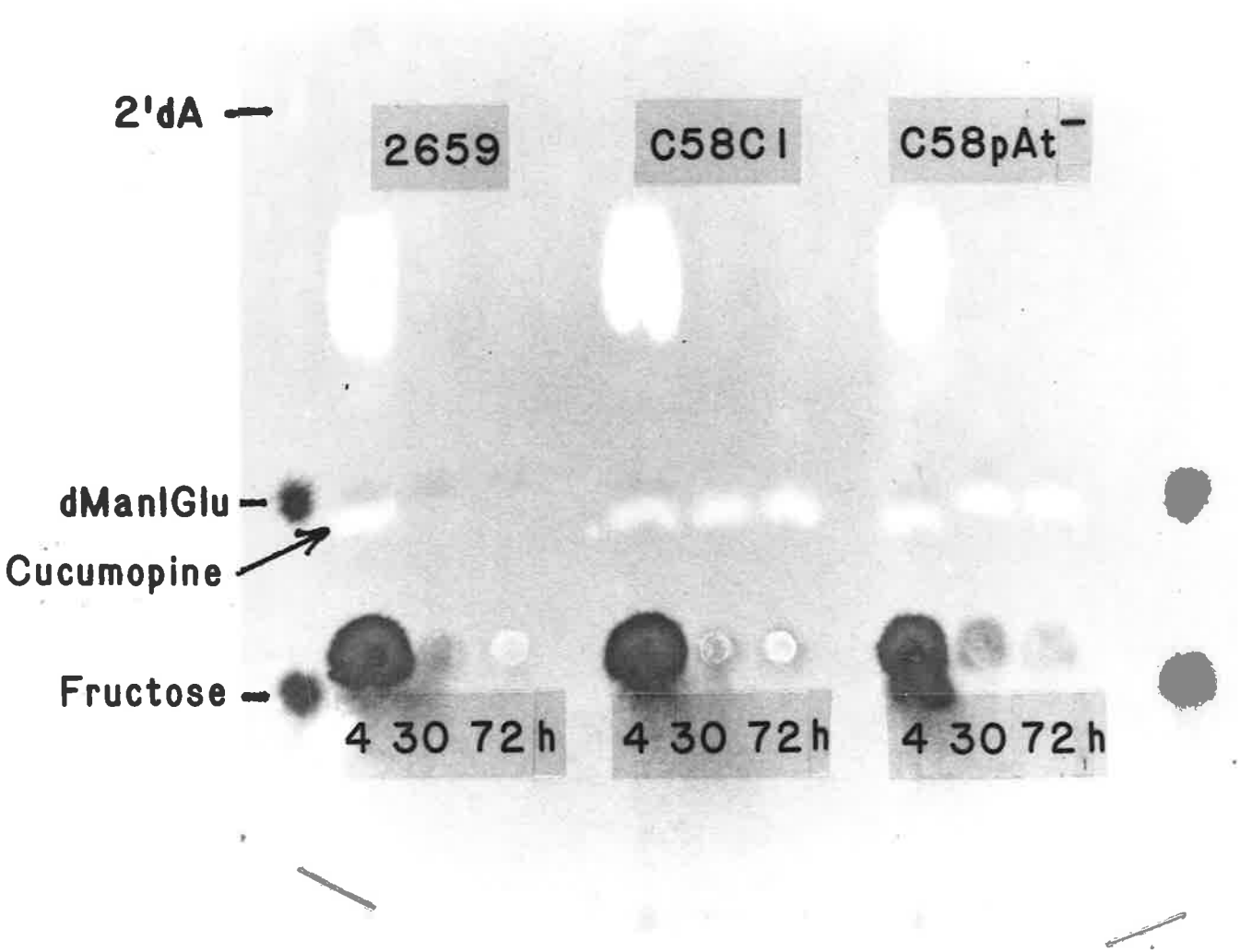

2'dA
2659
C58C1
C58pAt
dManIGlu
Cucumopine
Fructose
4 30 72 h
4 30 72 h
4 30 72 h

FIGURE 7-3

Degradation, by strains of Agrobacterium, of cucumopine isolated from hairy roots incited by strain 2659.

Samples were taken at 0 and 24 h and were analysed by HVPE at pH 1.7. Cucumopine (C) was detected by its negative (white) stain with alkaline silver nitrate. F = fructose, $M_{O.G}$ 0.0; M = dMan1Glu, $M_{O.G}$ -0.28.

Bacterial strains used were: 1, 2655; 2, 2657; 3, 2659; 4, IIBV7; 5, A6; 6, C58C1.
The results after 72h (data not shown) were the same those shown here for the 24h samples.

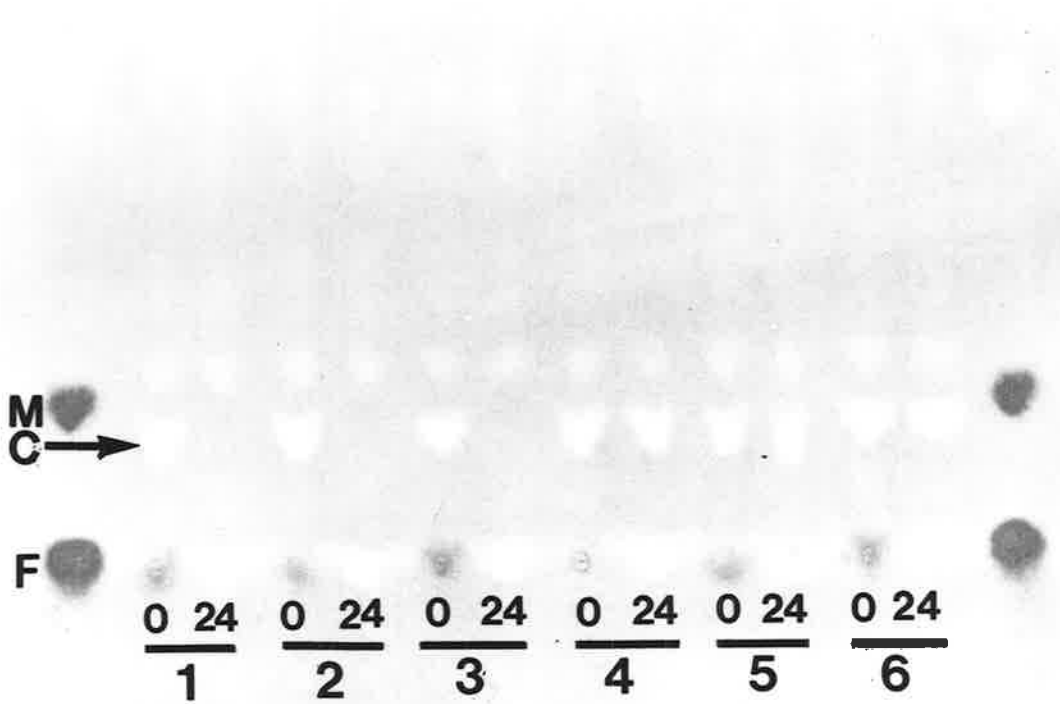

MC
F
0 24
1
0 24
2
0 24
3
0 24
4
0 24
5
0 24
6

TABLE 7-2

Electrophoretic relative mobilities ($M_{O.G}$), over a range of pH values, of octopine, nopaline, histopine and of cucumopine extracted from hairy root tissue of strain 2659. Fructose was used as the non-migrating marker.

pH	$M_{O.G}$			
	OCTOPINE	NOPALINE	HISTOPINE	CUCUMOPINE
1.7	-0.58	-0.46	-0.47	-0.21
3.25	-0.03	0.16	0.00	0.27
5.0	0.08	0.69	0.18	0.96
7.4	0.25	0.86	1.00	1.70
9.2	0.33	0.86	1.07	1.56
10.3	0.59	1.01	1.28	1.66
11.2	0.69	1.21	1.48	1.94
12.0	0.69	1.18	1.33	1.92

molecular weight fragments were present. Fig. 7-4 shows the hybridization of pRi 8196 T-DNA to uncut plant DNA (lane 2), the BamHI digest (lane 3) and the HindIII digest (lane 4). Hybridization was observed in all three lanes. Four bands were visible in the BamHI digest and six bands with the HindIII digest (the second HindIII band from the top is a doublet), indicating the presence of T-DNA in 2659 hairy roots and also that there is a reasonable amount of homology between 2659 and 8196 T-DNAs. Table 7-3 lists the sizes of the 2659-transformed root DNA fragments which hybridized to the 8196 T-DNA probe.

DISCUSSION

Three cucumber hairy root strains, NCPPB 2655, 2657 and 2659 were investigated, with 2659 chosen for a more detailed analysis since it harbours only one plasmid whereas the other strains have two (Costantino et al., 1981). None of the well known families of opines were utilized by the bacteria, nor were they detected in hairy root tissue, indicating that these strains form a separate group based on opine type.

Electrophoresis of hairy root extracts of all three strains revealed a characteristic silver nitrate-negative (white-staining) compound which was cationic at pH 1.7 ($M_{O.G}$ -0.21). This compound was present appeared in axenic 2659 roots and was not detected in normal carrot callus tissue. The silver nitrate-negative compound was selectively utilized by the inciting bacteria and in these respects behaved like an opine. The trivial name suggested for this compound is "cucumopine", based on Cucumis, the generic name for cucumber, from which the strains were originally isolated. Larger amounts of the cucumopines are needed before studies on their utilization as sole C and/or N sources can be undertaken.

FIGURE 7-4

Southern blot hybridization of pRi 8196 T-DNA to DNA extracted from hairy root tissue incited by strain 2659.

The probe contained lambda DNA and clones 118 and 119 (from M.-D.Chilton) of pRi 8196. These clones span the T-DNA of strain 8196.

A = lambda DNA cut with HindIII

B = uncut 2659 hairy root DNA

C = 2659 hairy root DNA cut with BamHI

D = 2659 hairy root DNA cut with HindIII

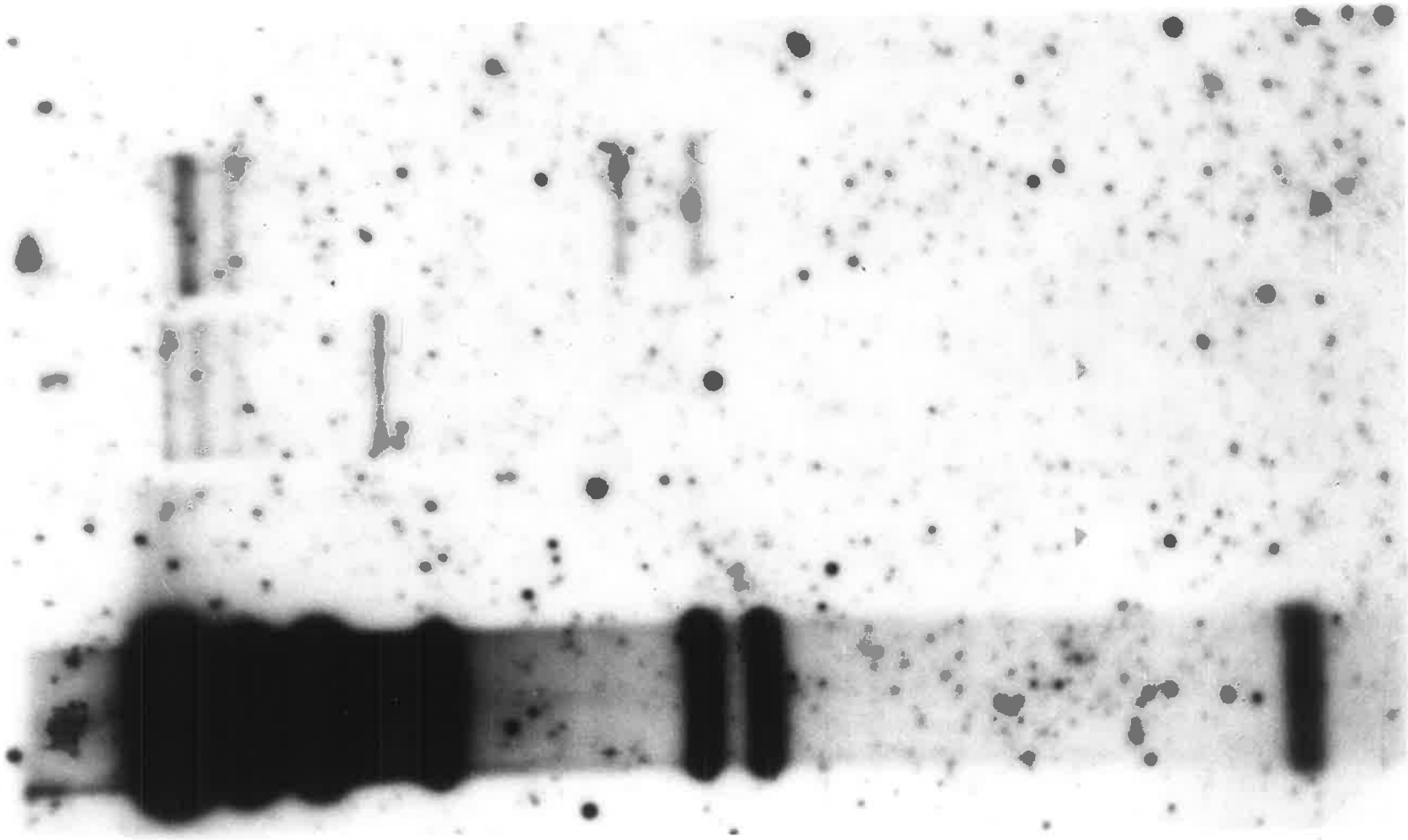


TABLE 7-3

Restriction endonuclease digestion of axenic 2659 hairy root DNA: sizes of root DNA fragments which hybridized to T-DNA of pRi8196.

DNA extracted from hairy roots induced by strain 2659 was cut with BamHI and HindIII. The distance of the bands from the origin, and their sizes, are recorded here. The marker fragments used were lambda DNA cut with HindIII.

BamHI			HindIII		
Band	distance(mm)	size(kbp)	Band	distance(mm)	size(kbp)
1	4	20.2	1	5	19.1
2	13.7	11.7	2	11	13.3
3	22	8.7	3	12.6	12.4
4	52	4.7	4	20.8	9.0
			5	102.7	2.4
			6	118	2.2

The negative staining of cucumopine with silver nitrate is indicative of a non-reducing insoluble silver salt and the weak ninhydrin reaction is suggestive of an imino acid. The presence of a mono- or di-carboxylic acid is indicated by its positive reaction with the organic acid stain. The positive reaction with the Pauly reagent for imidazoles could be taken as evidence for the presence of an imidazole group but cucumopine stained a yellow-red colour, whereas reference compounds with an imidazole group (e.g. histidine) stain bright red. It is therefore possible that the molecule contains a modified imidazole group. The change in electrophoretic mobility of cucumopine over a range of pH was similar to that observed for nopaline but cucumopine was more anionic than nopaline above pH 5. The observed difference in mobility suggests that cucumopine contains one more ionizable group than nopaline. This extra ionization could be due to the presence of one more carboxyl group than in nopaline, which has three such groups, or to the presence of an imidazole group as well as three carboxyl groups. If cucumopine is indeed an imino acid, further information about its structure may be gained by cleavage of the molecule with permanganate, followed by identification of the amino acid cleavage products. Cucumopine is unlikely to be identical to leucinopine (Chang et al., 1983), asparaginopine or glutaminopine (Chang and Chen, 1983) owing to both its apparent highly acidic nature and its positive stain reaction with the Pauly reagent.

Cucumopines from hairy root tissue of all three cucumber strains (2655, 2657 and 2659) had the same electrophoretic mobility, and biological utilization experiments demonstrated that bacteria of strains 2655 and 2657 were able to degrade the 2659 cucumopine. The cucumopines of all three strains may therefore be identical. Even if they are not identical, the results do indicate that the catabolic enzymes for

cucumopine are very similar in all three strains of bacteria. Costantino et al. (1981) suggested that the plasmid in 2659 is present in all the cucumber strains and that it is an Ri plasmid. This is additional evidence that the cucumopines may be the same in all three strains.

The finding, in this work, that DNA in axenic 2659 root tissue hybridizes to the T-DNA of hairy root strain 8196 strongly implies that the 2659 plasmid is an Ri plasmid. Costantino et al. (1981) have already demonstrated substantial sequence homology between the biotype 1 (which includes strain 2659) and biotype 2 (including strain 8196) hairy root plasmids. The present work demonstrates the presence of T-DNA in the 2659 root tissue and that there is homology with 8196 T-DNA, despite differences in opine type between the two strains. At least some of the homology detected between 8196 and 2659 plasmids by Costantino et al. (1981) must therefore be in the T-DNA region.

The more heavily labelled bands in Fig. 7-4 are good candidates for internal T-DNA fragments of strain 2659, and the fainter bands are likely to be either T-DNA border fragments or internal fragments showing less homology to 8196 T-DNA. Further work, including the analysis of plasmid DNA from strain 2659, is necessary for the unequivocal identification of the border and internal fragments of the 2659 T-DNA. In the present study, no control hybridization with normal carrot DNA was run at the same time, and it could be argued that the T-DNA of dMan1Gln strain 8196 may have hybridized to sequences common to normal carrot DNA. This possibility seems remote, however, as Chilton et al. (1982) reported no homology between pRi 8196 DNA and normal carrot root DNA. Spano et al. (1982) found homology between normal carrot root DNA and areas in or near the T-DNA on the Ri plasmid of agropine strain 1855, but the T-DNA of strain 1855 contains an insert which is not present in the T-DNA of dMan1Gln strain

8196 (Spano et al., 1982; Koplow et al., 1984). The results of Chilton et al. (1982) and those of Spano et al (1982) are therefore not incompatible.

Risuleo et al. (1982) infer by analogy to the dMan1Gln and agropine hairy root strains that the ability of cucumber strains to cause hairy root is plasmid-coded. Conclusive proof of this assertion requires co-transfer of both the plasmid and virulence to a non-pathogenic receptor strain. Strain 2659 harbours a single plasmid and would therefore be the strain of choice as a donor in plasmid transfer experiments. Plasmid transfer could be achieved by transformation of a suitable recipient strain with 2659 plasmid DNA. Alternatively, the cucumopine of strain 2659 could be tested for its ability to induce bacterial conjugation and plasmid transfer.

The finding of opines in tumours or hairy roots of "null-type" strains was predicted by Tempé and Goldmann (1982), who argued that opines are of central importance in the biology of crown gall and hairy root diseases. The cucumber hairy root strains were found to be "null-type" strains in the present study, and cucumopine was subsequently discovered in hairy root tissue induced by these strains. Opines have been found in all null-type transformed tissues so far examined, and can be considered a common feature of the transformation of plant tissues by different pathogenic strains of Agrobacterium.

CHAPTER 8

VIRULENCE PROPERTIES OF STRAINS OF AGROBACTERIUM ON THE APICAL AND BASAL SURFACES OF CARROT ROOT DISCS

INTRODUCTION

Several genes in the TL-DNA of octopine Ti plasmids code for tumour morphology functions (Caplan et al., 1983). TL-DNA genes 1 and 2 have been implicated in the control of the auxin balance of transformed tissue (Ooms et al., 1981; Akiyoshi et al., 1983; Caplan et al., 1983). The normal function of these genes has been termed shoot inhibition, as mutations in these genes cause shoot formation on tobacco tumours (Leemans et al., 1982). A "cytokinin-like" function has been ascribed to an adjacent root inhibition gene which, when mutated, gives rise to root formation (Ooms et al., 1981; Leemans et al., 1982).

Mutants in the shoot inhibition region have been described as attenuated (Onc^{att}) on several hosts including carrot (Leemans et al., 1982; Joos et al., 1983). Virulence was restored to Onc^{att} shoot-inducing mutants by addition of auxin to inoculated carrot root discs (Leemans et al., 1982).

The object of the work described in this chapter was to study the crown gall and hairy root transformation process. Differences in virulence properties between wild type strains and strains with T-DNA mutations in the tumour morphology region led to the identification of T-DNA functions differentially affecting virulence on the apical and basal surfaces of carrot root discs. Differences were also found among wild type hairy root strains in their virulence properties on carrot root discs. The basis of

these differences is not known, but may be due to functions similar to those of the crown gall T-DNA shoot inhibition region.

MATERIALS AND METHODS

Plant material

Carrots (Daucus carota, L.) and parsnips (Pastinaca sativa, L.) were either purchased locally or specified varieties ('Western Queen', 'Western Red' and 'Yates Topweight 556' carrots) obtained from a market garden. The carrots or parsnips were washed, peeled, dipped in ethanol and flamed. The basal 2 cm of the root were discarded, and discs 5 to 10mm thick were cut by transverse section, proceeding towards the apex of the root. The outer edges of each disc were trimmed with a scalpel. Discs were placed in sterile specimen containers (70 ml capacity, 43 mm diameter) or Linbro 12-well tissue culture trays (well diameter 24 mm) containing 1 or 2% (w/v) water agar to prevent drying. Care was taken to ensure that discs were correctly placed with either the apical or basal surface facing up, as required. Plant growth substances or antibiotics were added to the agar where specified.

Nicotiana glutinosa and tomato (Lycopersicon esculentum, Mill., var. early Dwarf Red) were grown in a glasshouse for 8 and 4 weeks respectively before inoculation.

Inoculation

Bacteria were grown for 2 days at 28°C on yeast-mannitol agar. Suspensions of approximately 2×10^9 bacteria/ml were made in sterile distilled water. A small volume (10-50µl) of this suspension was used to inoculate the cambial area of the apical or basal surface of the carrot or

parsnip disc. The inoculated surface faced up except where otherwise indicated. Variation in response between discs taken from the same carrot was small but variation between individual carrots was large. For this reason, 6 carrots or parsnips were used as replicates in the survey of virulence of wild type strains. Discs were incubated at 25°C under fluorescent light (4.2μE m^{-2} sec^{-1}) and observed regularly between 2-6 weeks after inoculation.

For complementation experiments, bacteria of the two strains to be tested were mixed in approximately equal cell densities in sterile distilled water before inoculation, except in the case of the nitroso-guanidine mutants SA 2139 and SA 2201. In the experiment involving the latter two strains, bacteria of one strain were taken from an Oxoid nutrient agar plate with a sterile toothpick and applied to the cambium of the basal surface of the carrot disc. The second strain was immediately applied directly over the first strain.

The effect of auxin (5 x 10^6 M NAA) on the virulence of hairy root strain TR7 on the basal surface was tested using 50 replicate carrots. Five discs were cut from each carrot, and were used for the following 5 treatments: (1) uninoculated basal control, (2) uninoculated basal control + NAA, (3) TR7 basal inoculation, (4) TR7 basal inoculation + NAA, (5) TR7 apical inoculation (positive control). The inoculated surface faced downwards on water agar or water agar containing 5 x 10^6 M NAA. Root formation was assessed 56 days after inoculation. In some cases, roots which developed were tested for the presence of opines by HVPE of ethanolic extracts, as described below under "Detection of opines...".

The virulence of strains with T-DNA mutations was compared quantitatively using the following method. Fourteen discs, 5 mm thick, were cut from each of 8 carrots (8 replicates). The discs from a single

carrot were randomly assigned to seven treatments (six strains and one water control) which were each applied to the apical and to the basal surface (total 7 x 2 = 14 discs). The discs were inoculated 24 hours after cutting, and tumour growth was measured four weeks after inoculation by (a) photography, to enable the proportion of the carrot disc cambium covered by tumour growth to be measured, and (b) removing the tumour tissues with a scalpel and weighing. The experiment was carried out twice: firstly with carrots of smaller diameter using Linbro 12-well tissue culture trays for incubation of the discs, and secondly with carrots of larger diameter, incubating the discs in sterile specimen containers of 70 ml capacity.

Glasshouse-grown plants were inoculated on the younger stem internodes after wounding with a sterile needle or scalpel blade.

Axenic plant tissue

In order to obtain tumour tissue free from bacteria, tumour slices 1 to 2 mm thick were placed on Monnier's medium (Monnier, 1976) with Morel's vitamins (Morel and Wetmore, 1952) in 0.8% agar containing carbenicillin (1 mg/ml) for 4 weeks. Tissues free of bacteria were maintained on medium without carbenicillin. Tissue growth was measured non-destructively by area determination from a photograph.

Detection of opines in tumour and hairy root tissues

Opines were extracted by homogenizing plant tissue in ethanol (1 ml/g f.wt.). After centrifugation, approximately 10µl of the supernatant solution was separated by paper electrophoresis on Whatman No.1 paper using the apparatus of Tate (1968) and employing the following buffers: 0.75 M formic acid/1 M acetic acid, pH 1.7, and 0.2 M NH_4CO_3/0.1 M NH_4OH

pH 9.2. Mobilities are expressed relative to Orange G (1.0) with fructose as non-migrating marker. Agropine, dMan1Glu and dMan1Gln were detected using the alkaline silver nitrate dip reagent of Trevelyan et al. (1950).

Bacterial strains

Bacterial mutant strains are listed in Table 8-1. Strains carrying TL-DNA mutations were provided by Dr.D.Garfinkel, University of Washington, Seattle, and Prof.J.Schell, Max Planck Institut fuer Zuechtungsforschung, Cologne. Sources of wild type strains are included in Table 8-2.

Non-pathogenic isolates used in complementation studies (see Results section 7) were: K112, K130, K237, K368, K372, K408, K410, K434, K458, K505, K535 and K550.

Transconjugants: These were obtained by Prof.A.Kerr using the following methods. In all crosses, 48-hour cultures on Y-M agar of both donor and recipient were used. The recipient was a Ti-plasmid-cured strain derived from C58 and was resistant to rifampicin and streptomycin. Selective media were based on a minimal agar medium lacking a source of carbon and nitrogen and containing 0.1mg/ml rifampicin and 0.5mg/ml streptomycin to counterselect the donor.

(a) C58C1(pRiTR105): The in planta cross method of Kerr (1969) was used. The donor strain TR105 and the recipient were suspended in sterile distilled water, mixed in a ratio of 1:2 and added to hairy roots induced by strain TR105 on carrot discs. After 3 days, the inoculated roots were transferred to buffered saline, the bacteria were suspended and 5µl of the suspension was added to selective medium containing 0.2% agropine as sole source of carbon and nitrogen. Transconjugants combining the antibiotic resistance of the recipient and the agropine catabolism of pRiTR105 were

TABLE 8-1.

Bacterial strains carrying Ti plasmid mutations.

Strain and plasmid content	Reference
A136(pTiA6NCtms-328::Tn5)	Garfinkel et al. (1981)
A136(pTiA6NCtms-394::Tn3)	"
A136(pTiA6NCtmr-149::Tn5)	"
A136(pTiA6NCtmr-338::Tn5)	"
A136(pTiA6NCtml-336::Tn5)	"
A136(pTiA6NCtml-346::Tn5)	"
C58C1(pGV2206)	Leemans et al. (1982)
" (pGV2210)	"
" (pGV2216)	"
" (pGV2219)	"
" (pGV2224)	"
A1007(pTiA6NConc-3::Tn5)	Klee et al. (1982)
A1010(pTiA6NConc-5::Tn5)	"
A1023(pTiB$_6$806onc-13::Tn5)	"
A1028(pTiB$_6$806onc-16::Tn5)	"
A1040(pTiB$_6$806onc-21::Tn5)	"
A1069(pTiB$_6$806onc-25::Tn5)	"
A1074(pTiB$_6$806onc-24::Tn5)	"

isolated after 4 days and purified.

(b) C58C1(pTiA6), C58C1(pTiA66) and C58C1(pTiTR104): The drop method of Ellis et al. (1982) was used. The donors were grown for 48 hours on basal agar medium plus 0.2% octopine, and were then suspended and added to a lawn of recipient growing on selective medium plus 0.2% octopine.

Selective media

The selective media of Brisbane and Kerr (1983) were used in the isolation of agrobacteria from inoculated carrot discs.

RESULTS

1. Inoculation with wild type strains

Table 8-2 shows the classification of wild type Agrobacterium strains according to their virulence properties on the basal surface of carrot root discs. Most pathogenic strains were able to induce tumours or hairy root on both the apical surface (facing the root tip) and the basal surface (facing the shoot). These strains are referred to as Bas^{+} (Fig. 8-1A). Some wild type strains, however, were either avirulent or much attenuated on the basal surface. These strains are called Bas^{att} (Fig. 8-1B). Of 22 wild type pTi strains drawn from all 3 species of Agrobacterium, only one (A.tumefaciens TR104, a slow-growing octopine strain also known under the designation NCPPB5) was Bas^{att}. In contrast, pRi strains were more commonly Bas^{att} than Bas^{+}. The Bas^{att} phenotype was observed irrespective of whether the inoculated surface faced up or down on the agar.

On uninoculated carrot discs a callus usually formed around the cambium, but only on the apical surface (Fig. 8-1C). In the case of pRi

TABLE 8-2.

Virulence properties of *Agrobacterium* strains.

Wild type *Agrobacterium* strains are classified according to virulence properties on the basal surface of carrot root discs. Bas^{+} strains are virulent on both apical and basal surfaces; Bas^{att} strains show marked attenuation of virulence on the basal surface (facing the shoot). Sources of strains are listed below. Strains *A. tumefaciens* IIBNV6 and *A. rhizogenes* TR107 were avirulent on both apical and basal surfaces.

		Bas^{+}	Bas^{att}
A.tumefaciens (=Biotype 1)	pTi	A6, B6, C58, Ach5, IIBV7, TR108, 15955, T37, 4, 925, 1641	pTi TR104
	pRi		pRi 2655, 2657, 2659
A. rhizogenes (=Biotype 2)	pTi	27, 29, 35, 49, TT133, 108	
	pRi	TR105, A4, 15834	pRi TR7, 8196, TR101, 11325
A. rubi (=Biotype 3)	pTi	Ag84, 305, 308, 374	

Sources of strains: 15955 & 11325, American Type Culture Collection, Rockville, Maryland, U.S.A.; TR101 & TR107, J.DeLey, Ghent, Belgium; C58, R.Hamilton, Pennsylvania, U.S.A.; TR104, TR108, TT133, TR105 & TR7, International Collection of Phytopathogenic Bacteria, Davis, California, U.S.A.; 27, 29, 35, 49, 108, 305, 308 & 374, A.Kerr, Adelaide, Australia; 8196, 15834 & IIBNV6, J.Lippincott, Evanston, Illinois, U.S.A.; A6, B6, IIBV7 & T37, G.Morel, Versailles, France; A4, L.Moore, Corvallis, Oregon, U.S.A.; 4, 925, 1641, 2655, 2657 & 2659, National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.; Ag84, C.Panagopoulos, Athens, Greece; Ach5, J.Schell, Cologne, W.Germany.

FIGURE 8-1

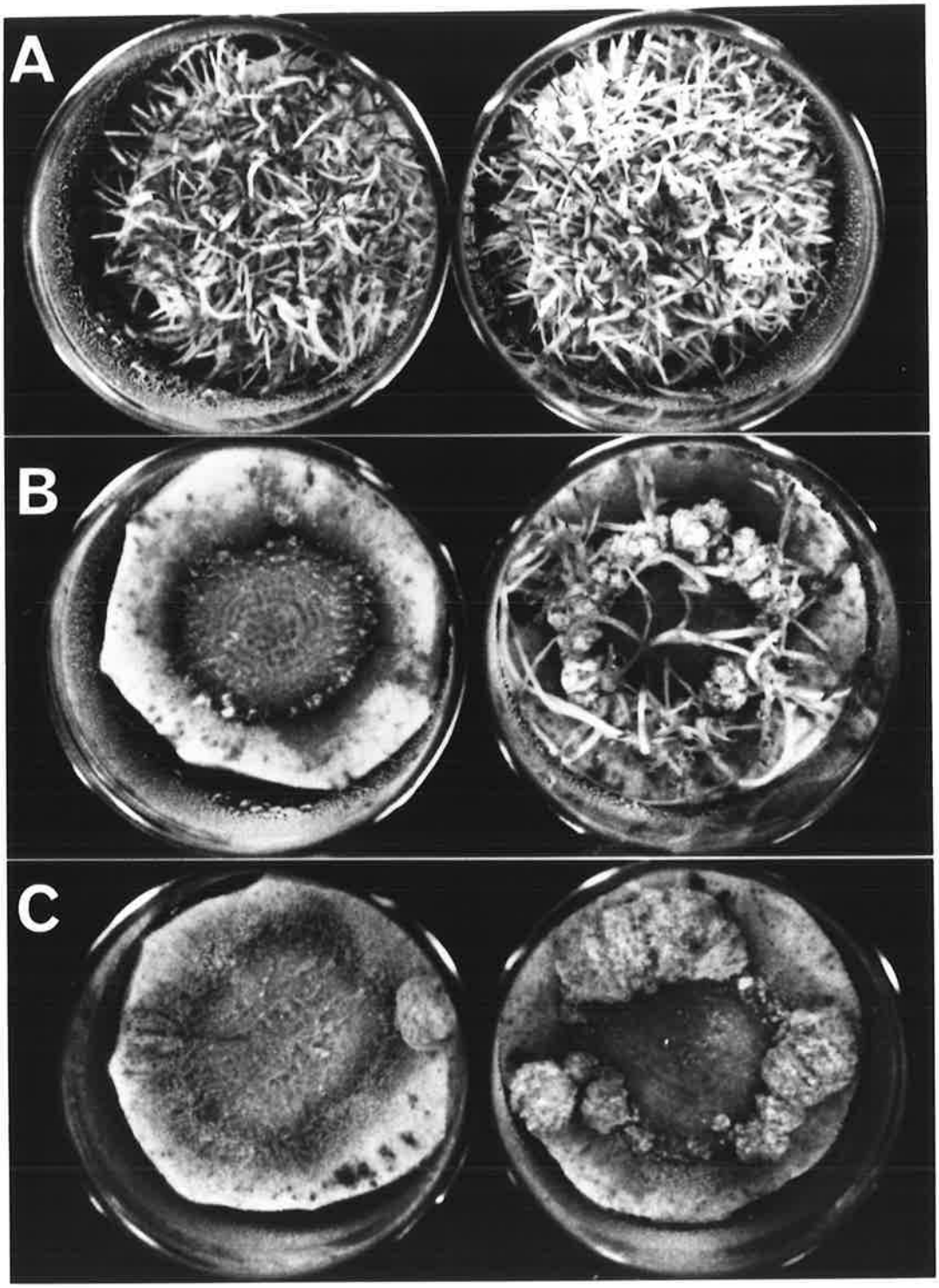
Inoculations of hairy root on parsnip discs.

A. TR105 is highly virulent on both apical and basal surfaces.

B. TR7 is avirulent on the basal surface and mildly virulent on the apical surface.

C. Uninoculated discs showing callus formation on the apical cambium but not on the basal cambium. Callus has also developed on a lateral root exposed on the phloem of the basal surface by cutting the disc.

Photographed 40 days after inoculation.


A
B
C
BASAL
APICAL

strains, root formation was used as a morphological marker to demonstrate that transformation of the apical surface had indeed occurred. For pTi strain TR104, apical transformed tissue was distinguished from untransformed callus tissue by hormone-independent growth on culture medium and by the presence of silver nitrate-positive opines, which were detected by paper electrophoresis of ethanol extracts at pH 1.7 and pH 9.2.

Callus growth was observed on the phloem of the basal surface of uninoculated discs where a lateral root was close to the basal cut surface. Tumours or hairy roots were also able to develop in such situations on discs inoculated with Bas^{att} strains.

Parsnip root discs responded in the same way as carrot discs to inoculation with Bas^{+} and Bas^{att} strains. No difference in response was found between carrot varieties tested.

The effect of varying time between slicing the carrot disc (i.e. wounding) and inoculation was tested. The Bas^{att} phenotype was observed with pRi strain TR7 whether discs were inoculated 0, 1, 2, 3, 4 or 10 days after slicing the discs. The Bas^{+} phenotype of pRi strain TR105 was also independent of time after inoculation although inoculations made 10 days after slicing the discs were less effective at inducing root formation.

The Bas^{att} phenotype of TR7 was found to be independent of the position on the carrot from which the discs were taken.

2. T-DNA tumour morphology mutants of pTi strains

Tables 8-3A and 8-3B show the virulence of octopine strains of Agrobacterium carrying different TL-DNA mutations, when inoculated on carrot root discs. Fig. 8-2 shows the location of mutations on the T-DNA of the octopine Ti plasmid. Virulence was assessed by measuring (a) tumour

TABLE 8-3A.

Virulence, on carrot root discs, of strains of Agrobacterium carrying TL-DNA deletion mutations[a].

Ti plasmid or mutant Ti plasmid	Mean tumor weight (mg/disc)				% cambial circumference covered with tumors			
	Expt.1		Expt.2		Expt.1		Expt.2	
	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
pGV3111	86	61	407	252	69	34	86	68
pGV2206	56	0.7	227	6	46	2	65	3
pGV2216	14	0.0	84	0.3	14	0	40	0
pGV2219	56	0.3	193	1.5	45	0	58	4
pGV2210	9	0.0	44	0.2	18	0	25	0
pGV2224	6	0.0	11	0.0	2	0	11	0
H_2O control	15	0.0	26	0.0				

[a] Bacteria were inoculated on the apical and basal surfaces of carrot root discs and the tumors were harvested and weighed 4 weeks after inoculation. The proportion of cambial circumference covered with tumors was measured from a photograph taken immediately before tumors were harvested. The wild type parent strain (positive control) harboured the octopine B6 Ti plasmid pGV3111 an the deletion mutants were derived from this plasmid (Leemans et al., 1982). The negative H_2O control was uninoculated. Expt.1: mean of 7 replicates; Expt.2: mean of 8 replicates.

TABLE 8-3B.

Virulence, on carrot root discs, of strains of Agrobacterium carrying TL-DNA insertion mutations[a].

Ti plasmid or mutant Ti plasmid	Mean tumor weight (mg/disc)				% cambial circumference covered with tumors			
	Expt.1		Expt.2		Expt.1		Expt.2	
	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
A6	67	22	346	177	43	34	78	66
A66	23	0.0	191	5	28	0	36	0
tms-394	47	0.0	315	21	45	0	38	7
tms-328	27	0.7	157	10	52	1	65	5
tmr-149	42	38	259	219	71	41	87	70
tmr-338	71	48	265	179	81	52	84	75
H_2O control	21	1.0	36	0				

[a] Bacteria were inoculated on the apical and basal surfaces of carrot root discs and the tumors were harvested and weighed 4 weeks after inoculation. The proportion of cambial circumference covered with tumors was measured from a photograph. A strain harbouring the A6 Ti plasmid was the positive control and the insertion mutants were derived from this plasmid (Binns et al., 1982; Garfinkel et al., 1981). All plasmids were in the same genetic background (strain C58C1). The negative H_2O control was uninoculated. Expt.1: mean of 8 replicates; Expt.2: mean of 5 replicates.

FIGURE 8-2

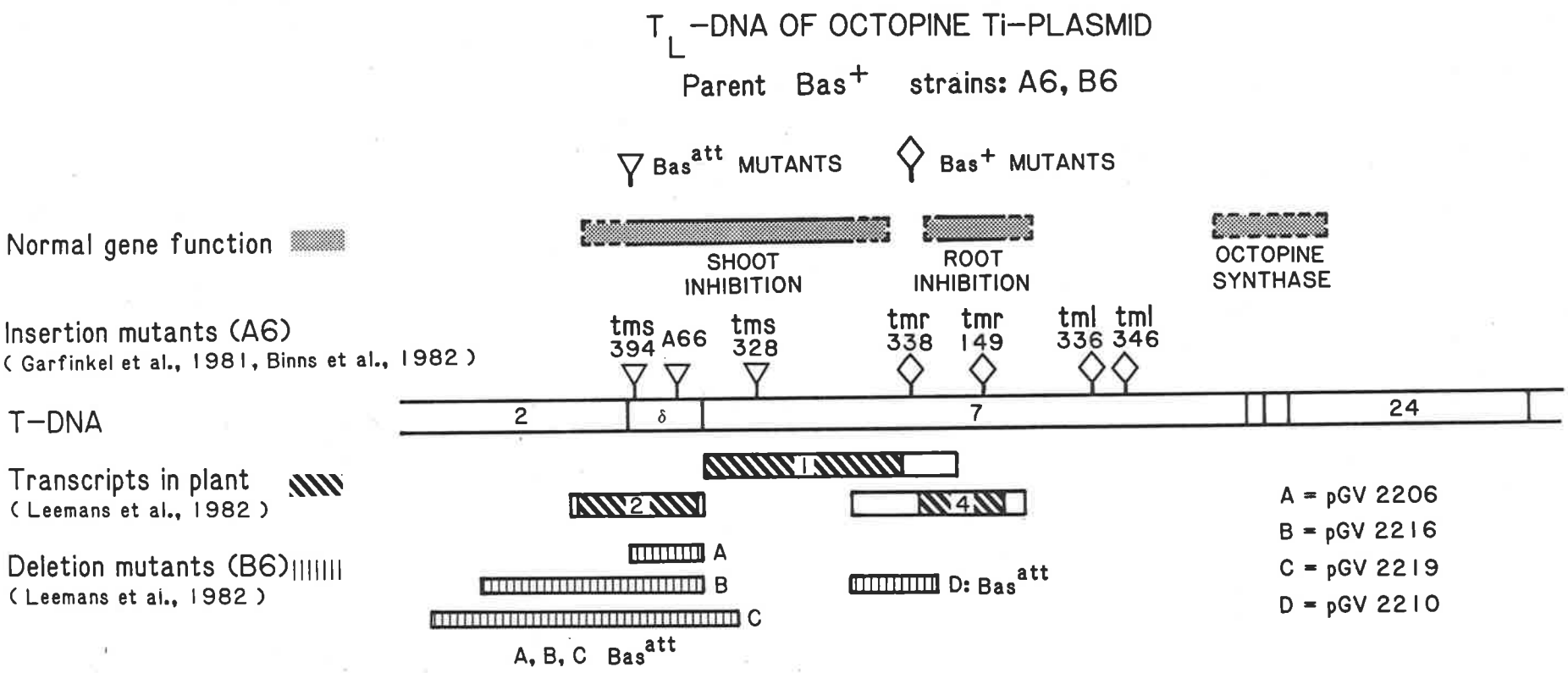
Virulence properties of T-DNA tumour morphology mutants of pTi strains A6 and B6 of Agrobacterium when inoculated on carrot root discs.

The TL-DNA of the octopine parent strains is presented as the EcoRI restriction map of Thomashow et al. (1980). The positions of the "normal gene function" regions are taken from Garfinkel et al. (1981).

Mutations causing marked attenuation of virulence on the basal surface (Bas^{att}) map in the shoot inhibition region. TL-DNA mutations referred to as (Bas^{+}) do not decrease virulence on the basal surface.

The map positions of the deletion mutations are from Leemans et al. (1982) and the designations used in this figure are:

A = pGV2206; B = pGV2216; C = pGV2219; D = pGV2210.


T L -DNA OF OCTOPINE Ti-PLASMID
Parent Bas+ strains: A6, B6
Bas att MUTANTS
Bas+ MUTANTS
Normal gene function
SHOOT INHIBITION
ROOT INHIBITION
OCTOPINE SYNTHASE
Insertion mutants (A6)
(Garfinkel et al., 1981, Binns et al., 1982)
tms 394
A66
tms 328
tmr 338
tmr 149
tml 336
tml 346
T-DNA
2
δ
7
24
Transcripts in plant
(Leemans et al., 1982)
1
2
4
Deletion mutants (B6)
(Leemans et al., 1982)
A
B
C
A, B, C Bas att
D: Bas att
A = pGV 2206
B = pGV 2216
C = pGV 2219
D = pGV 2210

weight per disc and (b) the proportion of cambial circumference covered by tumours. There was good agreement between results obtained by these two different methods. Tumour weights were higher in Experiment 2 than in Experiment 1 due to the larger diameter of the carrots used. In each experiment there was large variation between individual carrots in the amount of tumour tissue formed.

Wild type parent octopine strains A6 and B6 were virulent on both the apical and basal surfaces (Bas^{+}). Strains with mutations tmr-338 and tmr-149 in the root inhibition region (TL-DNA gene 4) were also Bas^{+}. The strains with mutations tml-336 and tml-346, mutated in genes 6a and 6b respectively, were Bas^{+} (data not shown).

Strains with shoot inhibition mutations in either gene 1 or gene 2, or both, (insertion mutants tms-328, tms-394 and A66, and deletion mutants pGV2206, pGV2216 and pGV2219) were markedly attenuated or avirulent on the basal surface (Bas^{att}) but were virulent on the apical surface. Virulence of the Bas^{att} mutant strains was decreased to a greater extent on the basal surface than on the apical surface, relative to the virulence of the wild type parents. This was more clearly seen when using the data for the proportion of cambium covered by tumours than when tumour weight was used as a measure of virulence. An exception was in Expt.1 with mutant strain pGV2216, where low virulence was recorded on both surfaces by both methods. Tumour weight measurements do not take into account variation in the size of discs and the associated difference in the amount of cambium available for tumour formation. The virulence of Bas^{att} strains on the apical surface was confirmed by the detection of opines of the agropine family in ethanol extracts of apical tissues. The agropine family of opines includes agropine, dMan1Glu and dMan1Gln, which can be used

as chemical markers for transformed tissue incited by octopine strains (Petit et al., 1983).

The virulence of the strain carrying the deletion pGV2210, in which genes 1 and 4 are inactive (Leemans et al., 1982), was low on the apical surface but opines of the agropine family were detected, indicating that transformation must have occurred.

A strain carrying the double deletion pGV2224, which combines the deletions of pGV2210 and pGV2224 and is therefore mutated in genes 1, 2 and 4, exhibited even lower virulence on the apical surface. Ethanol extracts of apical tissues from discs inoculated with pGV2224 contained no detectable agropine, dMan1Glu, dMan1Gln or octopine.

Axenic carrot tumour tissue transformed by mutant tms-328 grew rapidly when first placed on culture medium, but growth had almost ceased after 6 months in culture. Growth could be restored by addition of auxin (10^{-7}M 2,4-D) as shown by an experiment in which uniform pieces of tms-328 axenic carrot tissue were placed on Monnier's medium and on Monnier's medium + 10^{-7}M 2,4-D. The results are shown in Fig. 8-3A. After 20 days, the mean areas per tissue piece were (a) Monnier's - 2,4-D: 0.52 $\pm$0.12 cm^2 (13 pieces) and (b) Monnier's + 2,4-D: 1.46$\pm$0.48 cm^2 (11 pieces). On carrots, this mutant tumour tissue, which is known to form shoots on tobacco (Garfinkel et al., 1981) did not form shoots either as primary tumours on carrot discs or in axenic culture. In contrast, axenically cultured carrot tissue of tmr-338 and tmr-149 mutants developed roots which were able to grow without addition of plant growth substances.

3. Virulence properties of transconjugants of pTi and pRi strains

Ti- and Ri-plasmid transconjugants were obtained using recipient strains C58C1 or NT1, which are both Ti-plasmidless derivatives of A.tumefaciens C58.

FIGURE 8-3A

The effect of auxin (2,4-D) on growth of axenic carrot tumour tissue incited by shoot inhibition mutant tms-328.
The tumour tissue had been kept in axenic culture on a medium containing Monnier's salts and Morel's vitamins for 6 months prior to this experiment.
The plates were photographed 20 days after placing tumour pieces on Monnier's medium with or without addition of 2,4-D.

FIGURE 8-3B

Complementation between Bas^{att} mutants tms-328 and tms-394 on the basal surface of carrot root discs.
The discs were taken from four different carrots and were photographed 32 days after inoculation.

A


tms328 on carrot
0
10-7 M 2,4-D
20days

B


COMPLEMENTATION
tms 328
tms 328 + tms 394
tms 394

The virulence of pTi strains A6 and A66 and their transconjugants is shown in Fig. 8-4A. The Bas^{+} phenotype of A6 and the Bas^{att} phenotype of A66 were unaltered in the C58C1 genetic background. The pTi transconjugants of Bas^{+} strains Bo542 and T37 were still Bas^{+}, and the transconjugant of Bas^{att} strain TR104 was Bas^{att} (data not shown). Thus no change in virulence properties was observed upon plasmid transfer.

The virulence of pRi strains 8196, TR105 and A4 and their respective transconjugants is presented in Table 8-4. The Ri plasmid transconjugants of 8196 (Bas^{att}) and TR105 and A4 (both Bas^{+}) were obtained by transfer of the Ri plasmid from an A.rhizogenes (biotype 2) chromosomal background to an A.tumefaciens (biotype 1) background. The pRi transconjugants were all Bas^{att}. The virulence of pRi 8196 was unchanged whereas pRi TR105 and pRi A4 decreased markedly in virulence relative to the wild type parent strains, as measured by the fresh weight and number of roots induced (Table 8-3 and Fig. 8-4B).

4. Effect of auxin on virulence of hairy root strain TR7 on the basal surface.

In order to test the effect of auxin on the virulence of TR7 (Bas^{att}) on the basal surface, α-naphthalene acetic acid (NAA), pH6, was supplied at 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M in agar to carrot discs inoculated on the basal surface with TR7 bacteria. The inoculated surface faced the agar. Control inoculations of TR7 on the apical surface showed good root growth with the inoculated surface facing the agar. When the basal surface was inoculated with TR7, roots formed when NAA was present at 10^{-6} and 10^{-5}M. The roots contained dMan1Glu and dMan1Gln which are the opines characteristic of roots induced by strain TR7 (Petit et al., 1983).

In a subsequent experiment, $5x10^{-6}$M NAA was used and the results are

FIGURE 8-4A

Virulence, on the apical and basal surfaces of carrot root discs, of octopine pTi strain A6, its attenuated relative A66, and their transconjugants in the C58C1 genetic background.
Photographed 28 days after inoculation. A = apical; B = basal; TC = transconjugant.

Plan of inoculations:

H_2O A	H_2O B	TR105 A	TR105 B
A6 A	A6 B	A6TC A	A6TC B
A66 A	A66 B	A66TC A	A66TC B

FIGURE 8-4B

Virulence, on the apical and basal surfaces of carrot root discs, of hairy root strains 8196, TR105, A4 and their transconjugants in the C58C1 genetic background.
Photographed 60 days after inoculation.
A = apical; B = basal; TC = transconjugant.

Plan of inoculations:

8196 A	8196 B	8196TC A	8196TC B
TR105 A	TR105 B	TR105TC A	TR105TC B
A4 A	A4 B	A4TC A	A4TC B

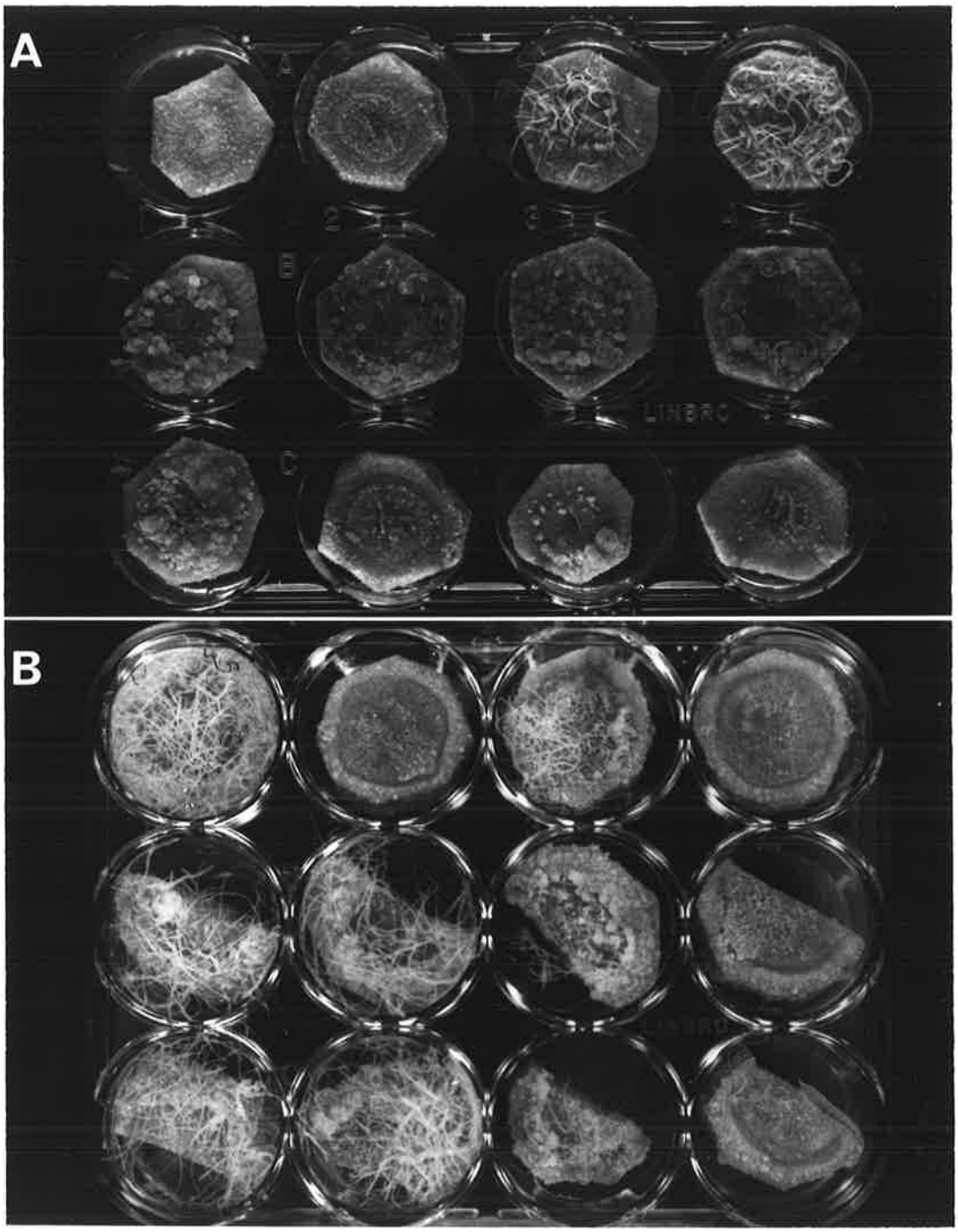

A
B

TABLE 8-4

Virulence of pRi strains and their transconjugants on the apical and basal surfaces of carrot root discs[a].

STRAIN	TUMOUR/ROOT TISSUE, FRESH WT. (mg), ON INOCULATED SURFACE	
	APICAL	BASAL
8196	118	0
C58C1(pRi8196)	31	0
TR105	226	233
C58C1(pRiTR105)	39	3
A4	166	117
NT1(pArA4a,b,c)	52	4

[a] The mean fresh weight of tumour plus root tissue on the inoculated surface was measured 82 days after inoculation. Mean of 4 replicates.

shown in Table 8-5. A χ^2 test revealed highly significant differences (χ^2=90.5, p<0.001) between the five treatments. Although it is not usual to perform repeated tests on subsets of the data, this was done for the comparison of particular interest between TR7 and TR7+NAA on the basal surface. When TR7 was inoculated on the basal surface, the TR7+NAA treatment induced the formation of roots on more discs than TR7 alone and the results for the two treatments were significantly different (χ^2 = 10.3, p<0.01). Auxin stimulated hairy root growth on 36% (=18/50) of carrots although the amount of root growth was less than on control apical inoculations in almost all cases. The presence of marker compounds dMan1Glu and dMan1Gln (M_{OG} -0.28 at pH1.7) in the roots induced on the basal surface indicated that they were transformed roots.

5. Can auxin applied to the basal surface after transformation restore virulence to Bas^{att} shoot inhibition mutants?

Experiments had shown an auxin requirement for tms-328-transformed axenic carrot tissue (see section 2). An experiment was done to determine whether or not auxin (NAA) promoted tumour growth when applied after transformation of the basal surface by strains with shoot inhibition mutations in genes 1 and 2.

Strains tms-328 (a gene 1 mutant), A66 (a gene 2 mutant) and A6 (wild type positive control) were inoculated on the basal surface of carrot discs (9 replicates). One set of discs was kept at 25^{o}C for 4 days to allow transformation to proceed and another set of discs was kept at 32^{o}C for the same period, to prevent transformation (Braun, 1947). After the 4 days allowed for transformation, the bacteria were killed by placing the discs, basal surface down, on agar containing carbenicillin (0.2 mg/ml) and vancomycin (0.1 mg/ml). The auxin treaments were supplemented with

TABLE 8-5.

Effect of auxin on hairy root induction on carrot discs inoculated with strain TR7.

Hairy root formation on carrot root discs. Fifty carrots were used as replicates and 5 discs from each used for the 5 treatments. The inoculated surface faced water agar with or without $5x10^{-6}$M NAA.

TREATMENT	INOCULATED SURFACE	NO. OF DISCS WITH ROOTS ON INOCULATED SURFACE	TOTAL DISCS
WATER	BASAL	0	50
WATER +NAA	BASAL	0	50
TR7	BASAL	5	50
TR7 +NAA	BASAL	23	50
TR7	APICAL	41	50

5 x 10^6 M NAA at the same time and all discs were incubated at 25°C from this time on.

In order to check the effectiveness of the antibiotic treatment, discs were pressed (basal surface) on to medium selective for A. tumefaciens (biotype 1) at the end of the experiment. One carrot showed the presence of agrobacteria.

The results are presented in Table 8-6. The treatments which were kept at 32°C before antibiotic treatment showed no tumour growth, indicating that transformation had been prevented. No growth was observed in the 32°C treatment supplemented with auxin, demonstrating that auxin alone did not stimulate the growth of callus tissue and also that antibiotic treatment effectively prevented the bacteria from transforming cells at the basal surface even though the temperature was brought down to 25°C 4 days after inoculation.

Tumours formed on 8 out of 9 carrot discs with strain A6, the positive control. In the treatment without added auxin, tumours formed on two carrot discs for both tms-328 and A66. Auxin stimulated tumour growth on the basal surface on 4 out of 9 discs with tms-328 and on 6 out of 9 discs with A66.

The tumours which formed on the basal surface were checked for the presence of opines by HVPE of ethanol extracts at pH 1.7 followed by staining of the electophoretograms with alkaline silver nitrate. All such tumours contained dMan1Glu, dMan1Gln and agropine, which are markers for transformed tissue of octopine strains.

It is interesting to note that on some discs which had been inoculated on the basal surface with Bas^{att} strains tms-328 and A66, tumour growth occurred on the apical (non-inoculated) surface. The transformed nature of the apical tissue was confirmed by the presence of silver nitrate-positive

TABLE 8-6

The effect of auxin applied after transformation to T-DNA shoot inhibition mutants on the basal surface of carrot root discs[a].

TREATMENT	TEMP. DURING TRANSFORMATION (°C)	NO. OF DISCS WITH BASAL TUMOURS
H_2O - AUXIN	25	0
H_2O + AUXIN	25	0
A6 - AUXIN	25	8
A6 - AUXIN	32	0
tms-328 - AUXIN	25	2
tms-328 + AUXIN	25	6
tms-328 - AUXIN	32	0
tms-328 + AUXIN	32	0
A66 - AUXIN	25	2
A66 + AUXIN	25	8
A66 - AUXIN	32	0
A66 + AUXIN	32	0

[a] Discs from 9 different carrots were used, with one disc per treatment from each carrot. Results were assessed 5 weeks after inoculation.

opines. The phenomenon of transformation of the non-inoculated surface was also observed with Bas^{att} pRi strain TR7 (see Results section 8).

6. Complementation between Bas^{att} TL-DNA mutants

Two Bas^{att} strains with TL-DNA mutations tms-394 (a gene 2 mutant) and tms-328 (a gene 1 mutant) were inoculated on the basal surface of carrot root discs at the same time and in approximately equal bacterial cell densities. This coinoculation resulted in the formation of more primary tumours, covering a larger area of the cambium, than when either mutant strain was inoculated by itself. The amount of cambium (% of circumference) covered by tumour growth was measured 40 days after inoculation. Results for the 3 treatments were as follows (mean of 4 carrots): (1) tms-328: 2.7%, (2) tms-328 + tms-394: 47.5%, (3) tms-394: 2.7% (see Fig. 8-3B). These two strains, with mutations in different genes of the shoot inhibition region, were thus able to complement each other and restore virulence on the basal surface. The results were statistically significant ($H = 6.49$, $p<0.05$) as determined by the Kruskal-Wallis one way analysis of variance by ranks.

7. Further complementation studies using Basatt hairy root strains

(a) Complementation with Bas^{+} strains.

Successful complementation leading to root formation on the basal surface was achieved by coinoculating a Bas^{att} hairy root strain with a Bas^{+} pTi strain. When Bas^{att} pRi strain TR7 was coinoculated on the basal surface of carrot discs with Bas^{+} pTi strains A6, B6 or C58, roots developed on discs where tumours had been induced by the tumorigenic strain. These roots grew from the tumours themselves or from the cambium close to the tumours. Axenic cultures of roots taken from discs inoculated

with both TR7 and A6 were analysed for opines to determine whether or not TR7 had super-transformed the A6 tumour cells. The roots contained only dMan1Glu and dMan1Gln, which are the opines characteristic of TR7 hairy root. Octopine and agropine, which are markers for tissue transformed by A6, were not detectable.

In contrast to the coinoculation studies with Bas^{+} pathogenic agrobacteria, no complementation was observed upon coinoculation of strain TR7 on the basal surface with any of the 12 non-pathogenic isolates of Agrobacterium tested.

(b) Complementation with a pair of complementing Ti plasmid mutants.

The mutant strains used were SA 2139 and SA 2201, which are both avirulent nitrosoguanidine-induced mutants of the 15955 Ti plasmid. These mutant strains are able to complement each other, resulting in tumour formation on carrot discs (S.K.Farrand, personal communication).

In the present study, complementation between SA 2139 and SA 2201 was not at all efficient with respect to the induction of tumours on the basal surface. When either SA2139 or SA2201 were coinoculated with the Bas^{att} pRi strain 8196, complementation was observed in both cases but again the process was not efficient; the tumours formed were small in size and the degree of reproducibility was low. The combination 8196 + SA 2139 produced tumours from which roots or root-like structures protruded in some cases. The combination 8196 + SA 2201 produced tumours only.

(c) Complementation with Vir region mutants.

Seven different mutants in the Vir region were each coinoculated on the basal surface with Bas^{att} pRi strain TR7. The only combination to

produce any growth on the basal surface was TR7 + A1028, but A1028 alone was also weakly virulent on the basal surface.

(d) Complementation between the two groups of Bas^{att} hairy root strains.

Coinoculation of pRi strains NCPPB 2659 and TR7 did not result in hairy root formation. These two strains were thus unable to complement each other.

8. Bas^{att} strains: virulence on the apical surface of basal-inoculated discs

Inoculation of pRi strain TR7 on the basal surface occasionally resulted in root proliferation on the apical (uninoculated) surface, although no roots were formed on the basal (inoculated) surface. It was suspected that bacteria could move to the uninoculated side of the disc and there transform and induce hairy root. Indeed, the xylem vessels in carrot root discs (diameter up to 60 µm) could easily accomodate motile agrobacteria (approx. 2 µm x 1 µm in size). In order to test the hypothesis, bacteria of pRi strains TR7 and TR105 were inoculated on the uppermost surface (either apical or basal) of carrot discs placed on 2.3% Oxoid nutrient agar. After 2 days the discs were removed and growth of bacteria was observed on the nutrient agar several days later. The identity of the bacteria was checked by (a) testing for growth on culture media which are selective for the different Agrobacterium species (Brisbane and Kerr, 1983) and (b) testing for pathogenicity on carrot root discs. The bacteria were shown to be pRi strains of A. rhizogenes (biotype 2).

Virulence on the apical surface after inoculation of the basal surface was also noted for Bas^{att} strains with shoot inhibition mutations (see section 5).

9. Virulence of hairy root strains on other plant hosts

Bas^{att} and Bas^{+} hairy root strains were inoculated on three other plant hosts:

(a) Tomato. The results are shown in Figs. 8-5A and 8-5B. No visible callus was formed in the control (water) inoculations. The tumours induced by Bas^{att} strains TR7, TR101 and 8196 were barely detectable. The Bas^{+} strains TR105, A4 and 15834 were all able to induce tumours with short roots or root-like structures.

(b) Nicotiana glutinosa. Strain TR105 (Bas^{+}) induced tumour growth only and was mildly virulent. The Bas^{att} strains 8196 and NCPPB 2659 were only weakly virulent (data not shown). None of the pRi strains tested were able to induce the formation of roots on this host.

(c) Sunflower. Both the Bas^{+} and Bas^{att} groups of pRi strains were avirulent on sunflower stems.

DISCUSSION

Natural callus formation on the apical surface of uninoculated carrot discs (Gautheret, 1944) is a result of cell division in the cambium and, in its unorganized shape, the callus resembles crown gall tumour tissue. This callus does not develop on the basal surface, indicating an intrinsic polarity in the carrot disc which is unaffected by the orientation of the disc.

Klein and Tenenbaum (1955) noted that the amount of crown gall tumour

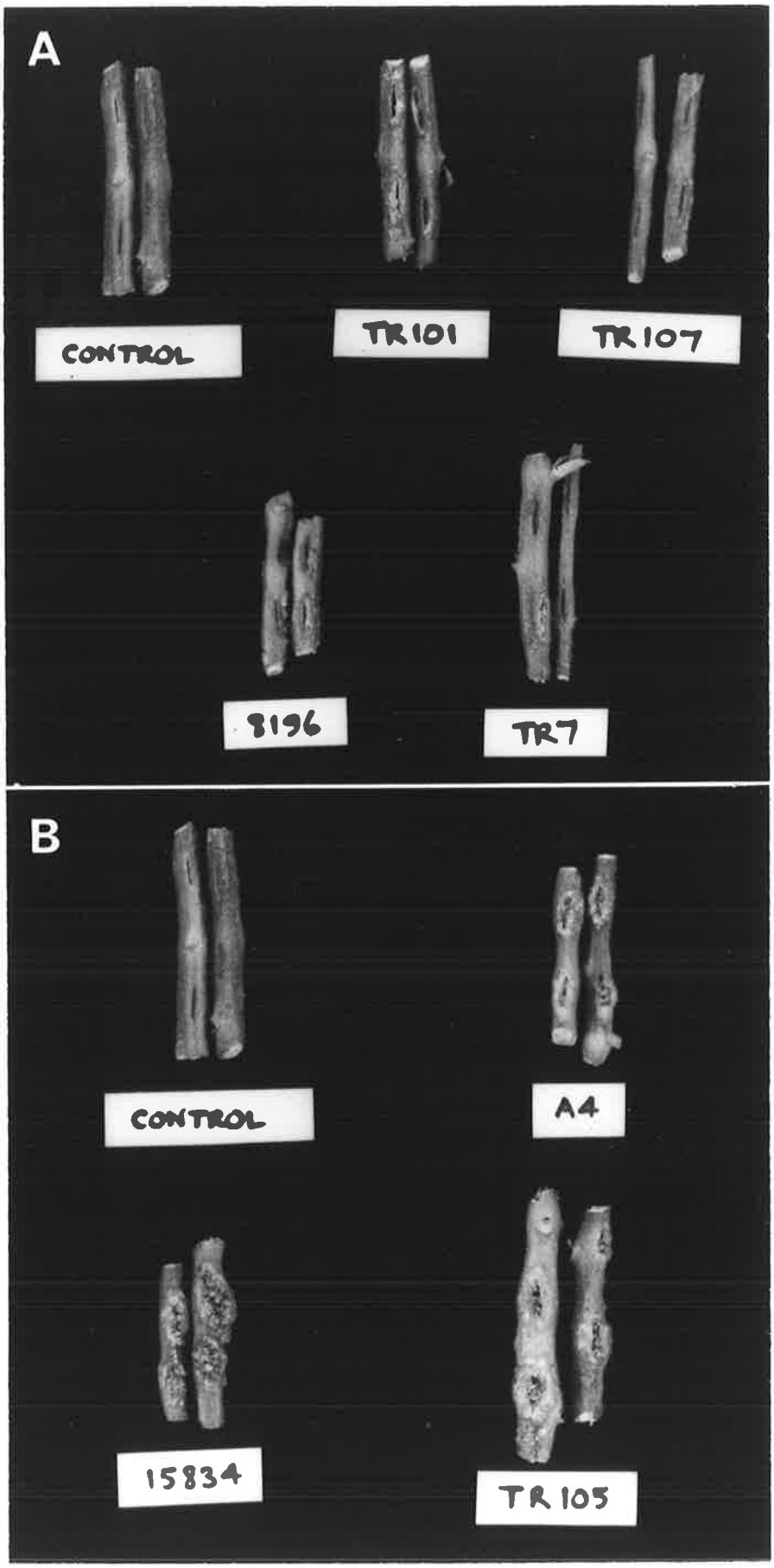
FIGURE 8-5A and 8-5B

Inoculation of hairy root strains on tomato stems.

A: Virulence of Bas^{att} strains TR101, 8196 and TR7. Control stems were inoculated with sterile water; strain TR107 is plasmidless and avirulent.

B: Virulence of Bas^{+} strains A4, 15834 and TR105. Controls were inoculated with sterile water.

Photographed 34 days after inoculation.


A
CONTROL
TR101
TR107
8196
TR7
B
CONTROL
A4
15834
TR105

tissue formed on the apical surface (facing the root tip) was twice that formed on the basal surface, whereas DeRopp (1950) reported no difference between the two surfaces in the amount of tumour tissue formed. Magnus (1918) observed either the former or the latter type of result, depending on the carrot variety used. The botanical term "polarity" was used in all this early work to describe differences in amount of tumour tissue on the two surfaces. The spectacular and morphologically unequivocal polarity with hairy root strains (Fig.8-1) has not been previously reported. I have avoided using the term "polarity" which has other connotations in the crown gall literature and prefer to use Bas^{+} and Bas^{att} to refer to tumour or hairy root formation on the basal surface, with virulence on the apical surface being implied. Wild type Bas^{att} strains, including one tumour-inducing strain and several hairy root-inducing strains, are almost avirulent on the basal surface (Fig. 8-1).

Both callus formation and virulence of Bas^{att} strains appear to be auxin-requiring and auxin level is the only factor found thus far to increase the virulence of Bas^{att} strains on the basal surface. Addition of auxin in the form of NAA to inoculations of hairy root strain TR7 on the basal surface stimulated formation of transformed roots, although not in all replicates. NAA was effective at concentrations between 10^{-6} and 10^{-5}M. Leemans *et al.* (1982) and Joos *et al.*(1983) reported the restoration of virulence to Bas^{att} T-DNA mutants on carrot and potato discs by addition of auxin, but no quantitative data were presented. The T-DNA regions involved in attenuated virulence on the basal surface are genes 1 and 2, which appear to control the auxin balance of tumour tissue (Caplan *et al.*, 1983). A lack of auxin at the basal surface could provide an explanation for the decreased ability of Bas^{att} strains to form tumours at that surface.

It is a widely held view that auxin transport in plants is unidirectional (polar) from shoot tip to root tip (Goldsmith 1977). Higher levels of auxin at the apical than the basal surface of carrot root discs have been measured (Pilet, 1967). Pilet provided evidence that this was due to increased auxin synthesis at the apical surface and increased catabolism at the basal surface. This is in contrast to the view of Gautheret (1944) that unidirectional auxin transport towards the apical surface was responsible for callus growth on the apical surface. It is conceivable that both mechanisms operate together to account for larger amounts of auxin at the apical than the basal surface. The results presented here are further evidence for higher levels of auxin at the apical surface than at the basal surface. There is now clear evidence (Jacobs and Gilbert, 1983) that the auxin transport carrier is present only at the lower end (i.e. facing the root tip) pea stem cells. Such a situation, if demonstrated in carrot cells, would provide a rational explanation for the Bas^{att} phenotype.

Auxin could conceivably act either before or after transformation in stimulating virulence of Bas^{att} strains. Axenic tissue induced by a strain carrying the mutant tms-328 (a TL-DNA gene 1 mutant) is auxin-dependent, and hence auxin must act after transformation in this case. The evidence that tumour growth of Bas^{att} shoot inhibition mutants is stimulated by auxin application after transformation (Results, section 5) also indicates that auxin acts after this stage. Taken together, the available evidence strongly suggests that cells on the basal surface of discs inoculated with such mutants can be transformed without subsequent tumour growth occurring. An external agent, in this case applied auxin, can apparently stimulate the growth of previously transformed plant cells and this effect does not require the presence of living bacteria of the inciting strain.

Klein and Link (1952) and Braun and Laskaris (1942), working with the attenuated strain A66 (which is now known to be a TL-DNA gene 2 mutant) also favoured a role for auxin after transformation.

There is evidence that even avirulent mutant strains with large TL-DNA deletions can transfer their T-DNA to the plant cell without subsequent tumour formation. Evidence for this is the detection of octopine synthase in wound tissue of carrot discs inoculated with avirulent strain carrying the large TL-DNA double deletion pGV2224 (Leemans et al., 1982). In the present work, no opines were found in the tissue which was inoculated pGV2224 but the octopine synthase assay used by Leemans et al. (1982) is a more sensitive test for transformation. The inability to detect agropine in this tissue could be due to loss of TR-DNA, a not infrequent occurrence (Thomashow et al., 1980). Transfer of T-DNA to the plant cell by avirulent strains suggests that Bas^{att} strains transform cells at the basal surface and that auxin stimulates their subsequent growth. The possibility of a role for auxin before or at transformation is not excluded, however. The expression of TL-DNA genes by agrobacteria (Schroeder et al., 1983) raises the interesting possibility that the bacteria may play an active role in inducing plant cell division as part of the transformation process. Whether the stimulus for tumour growth in the wild type (Bas^{+}) situation comes solely from the T-DNA in the transformed cell, or whether there is also some contribution from the bacteria, is not known at present.

Mutations in T-DNA genes 1 or 2 decreased the virulence of agrobacteria on carrot root discs to a greater extent on the basal than on the apical surface. Strains mutated in gene 4 (root inhibition) alone did not show decreased virulence on either surface but inactivation of both genes 1 and 4, as in the deletion mutant pGV2210, led to a marked decrease in virulence on both the apical and basal surfaces. This suggests that the

small TL-DNA transcript found between genes 1 and 4 and which is expressed only in the bacteria (Schroeder et al., 1983) may play a role in virulence on the apical surface.

The wild type Bas^{att} pRi strains and the Bas^{att} T-DNA shoot inhibition mutants are similar in their response to auxin, but the particular property of the Ri plasmid of pRi Bas^{att} strains causing the attenuation of basal virulence has not been identified. On the basis of the results obtained with the Ri plasmid transconjugants, chromosomally coded functions appear to be important in the virulence of Bas^{+} wild type hairy root strains. However, until the results obtained with the transconjugants are confirmed, a role of the Ri plasmid cannot be excluded. In this respect it is noteworthy that the T-DNAs of TR105 (Bas^{+}) and 8196 (Bas^{att}) are very similar, except that an insert in the central region of TR105 T-DNA is absent from 8196 T-DNA (Spano et al., 1982). It is also interesting that Huffman et al. (1984) provide evidence that there are two T-DNAs on the Ri plasmid of Bas^{+} pRi strains such as A4. The newly discovered T-DNA in this pRi strain shows homology with the shoot inhibition region of octopine and nopaline T-DNA. If this T-DNA were missing from the Ri plasmids of the Bas^{att}, this could provide an explanation for the difference in virulence properties between the two groups of strains. An analysis of the TR7 or 8196 Ri plasmid is required to test this possibility. In addition, other experiments could be done to test whether the Bas^{+} and Bas^{att} phenomena in hairy root strains are plasmid- or chromosomally-coded. One possibility would be to cure strains TR105 (Bas^{+}) and 8196 (Bas^{att}) and then to transform these cured recipients in order to obtain strains with the Ri plasmid of 8196 in the TR105 background and vice versa. These transformants would then be tested for pathogenicity on the apical and basal surfaces.

There are basic differences between pRi and pTi strains which are as yet unexplained in molecular terms. For example, Bas^{att} pRi strains are root-inducing yet the pTi Bas^{att} T-DNA mutants are shoot-inducing on tobacco (Garfinkel et al., 1981; Binns et al. 1982) despite the similar auxin responses mentioned above.

The ability of agrobacteria to move through carrot root discs and transform the apical surface after basal inoculation caused difficulties in work designed to test the effects of chemicals or other factors on the virulence of Bas^{att} strains at the basal surface. If the presence of opines at the basal surface is used as a test for transformation, it must be borne in mind that opines can be produced by tumours at the apical surface and these opines can diffuse from the site of production to the basal surface. By contrast, an enzyme assay for an opine synthase is a more reliable test for transformation of the basal surface, because its diffusion would be strictly limited. In the case of octopine, the enzyme assay for lysopine dehydrogenase (Otten and Schilperoort, 1978) can be used, but no such test is yet available for dMan1Glu and dMan1Gln which are the opines of strain TR7.

The complementation observed between strains with mutations tms-328 and tms-394 in the shoot inhibition region differs from previously reported complementation (Ooms et al., 1982) between strains with mutations in the T-DNA morphology region in two respects. Firstly, tms-328 and tms-394 are mutations in different genes (genes 1 and 2) of the same (shoot inhibition) region of the T-DNA, and secondly the complementation reported here resulted in the restoration of virulence in a situation where each mutated strain by itself was markedly attenuated. The complementation observed by Ooms et al. (1982), on the other hand, involved the formation of tumours with wild type morphology on tobacco

stems after coinoculation with a root inhibition and a shoot inhibition mutant. The complementation observed here between tms-328 and tms-394 was not due to bacterial conjugation and T-DNA recombination (Donner, personal communication) and could therefore be due to either double infection of a single plant cell or cross feeding between transformed cells from separate transformation events as suggested by Ooms et al.(1982). Alternatively, evidence for production of plant growth substances by agrobacteria due to bacterial expression of TL-DNA genes (Schroeder et al., 1983) suggests that complementation between bacterial mutant strains for production of such substances may occur.

The induction of root formation by TR7 when coinoculated with Bas^{+} strains on the basal surface of carrot discs could be due to growth factors produced either by bacteria of the Bas^{+} strain, by the tumours induced by those bacteria, or both. The opine analysis of the roots showed that TR7 had transformed normal plant cells and had not super-transformed tumour cells induced by the Bas^{+} strain. The basal root induction by TR7 when coinoculated with Vir region mutant A1028 could be due to the fact that A1028 alone was not completely avirulent, with the resultant A1028-induced tumour growth being sufficient to stimulate TR7 root growth.

The list of wild type Bas^{att} strains is almost identical to the list of "receptor" strains identified by Lippincott and Lippincott (1970) in their mixed inoculation studies on Pinto bean leaves. Their "receptor" strains were 8196, TR7, TR101, 11325, IIBNV6 and TR104(=ATCC13333). If the same factor(s) were involved both in differential apical/basal virulence on carrot root discs and in Pinto bean leaf complementation, then strain IIBNV6, a "receptor" strain on Pinto bean leaves, should be virulent on the apical surface of carrot root discs. In the present study, no opine markers were detected in apical tissues inoculated with IIBNV6, nor were

such tissues able to grow in culture in the absence of plant growth substances. Strain IIBNV6 may lack more function(s) necessary for tumorigenic ability than do the Bas^{att} strains listed in Table 8-2.

The auxin-dependence of axenic tissue of tms-328 on carrot is in marked contrast to the results of Joos et al. (1983), who found no auxin-dependent lines from tumours induced by mutants in genes 1 or 2. These results were apparently obtained with tobacco, where mutants in genes 1 and 2 induce formation of tumours with shoots which would provide a source of auxin. Binns et al. (1982) reported, also for tobacco, that tumours induced by mutant strain A66 (Bas^{att}) are auxin-dependent in culture unless buds are formed. The reason for the auxin-dependence of tms-328 carrot tissue is may lie in its inability to form shoots which would be a potential supply of auxin.

Although the reason for basal hairy root attenuation is not known, the genetic analysis of the crown gall TL-DNA has shown that the Bas^{att} phenotype is caused by mutations in genes 1 and 2. The Bas^{att} phenotype may well provide a suitable screening test for T-DNA mutations in these genes. T-DNA analysis of A66 (Binns et al., 1982) revealed an insertion in gene 2, and a similar analysis of the Ti plasmid of TR104 (also Bas^{att}) is warranted.

Inoculation of Bas^{+} and Bas^{att} pRi strains on several plant species indicated that the Bas^{+} strains were more virulent than the Bas^{att} strains. The host range for hairy root compiled by DeCleene and DeLey (1981), a study in which the basal-attenuated strain TR7 was used for the majority of the inoculations, may therefore be underestimating the number of plant species susceptible to the disease.

CHAPTER 9

GENERAL DISCUSSION

Opines are considered to be of fundamental importance in the biology of crown gall (Tempé and Goldmann, 1982). Since the recent discovery of opines in hairy root tissue (Tepfer and Tempé, 1981; Petit et al., 1983), their role in hairy root disease has been considered similarly important. Opines are synthesized at the direction of the T-DNA in tumour and hairy root cells and are not present in normal plant tissue. The type of opine synthesized in the transformed cells depends on the inciting bacterial strain (Petit et al., 1970). The opines are conjugates of simple plant metabolites and act as nutrient sources for bacteria harbouring the corresponding Ti- or Ri- plasmid. The bacteria are thus ensured of a supply of C, N and P with a minimum of competition from other organisms. The bacteria virtually "farm" the plant host which provides the opines synthesized in tumour tissue. The ability to induce the synthesis of opines and the ability to utilize them, which are both Ti- or Ri-plasmid-coded traits, would confer a considerable selective advantage on the bacteria harbouring these plasmids. The spread of Ti plasmids in a population of agrobacteria is ensured because they are self-transmissible plasmids and, in addition, some opines act as inducers of plasmid transfer by bacterial conjugation (Kerr and Ellis, 1982). Opines have in fact been called the "raison d'etre" of crown gall (Lippincott,1977) and the "biological rationale for the existence of crown gall" (Guyon et al., 1980).

The structures of the phosphorylated sugar opines agrocinopines A and B have now been determined and are presented in Chapter 3 of this thesis. They are phosphodiesters of L-arabinose linked from the 2-position to the

4'-fructosyl carbon of sucrose and D-fructose respectively. These opines are thus formed by the linkage of two simple plant constituents in an uncommon manner. This is the case for all opines described so far and the simplicity of the structures is a common feature. This simplicity would also serve to minimize the number of enzymes and hence the amount of T-DNA required to code for opine synthesis. The economy of this method of diverting the plant's nutrients to a bacterial food source is striking and is unique among known parasitic relationships.

The agropine family of opines is another group of simple molecules, but differing from other opines in that not one but three enzymes are involved in agropine biosynthesis. The steps involved are the condensation of simple precursors, followed by reduction and cyclization reactions. As part of a collaborative study with Dr.J.Ellis, who investigated the genetic aspects of agropine biosynthesis, the work outlined in this thesis details the discovery of two new key intermediates in agropine biosynthesis: these are the deoxyfructosylamino acids dFruGlu and dFruGln (Chapter 4; Ellis *et al.*, 1984). These compounds are formed in the first step of the pathway, which involves condensation of glucose and the corresponding amino acid as probable substrates. Deoxyfructosylglutamate and dFruGln are subsequently reduced to dManlGlu and dManlGln respectively. These new intermediates are synthesized at the direction of the T-DNA, as demonstrated by T-DNA mutagenesis studies, and in this respect conform to the definition (Schell *et al.*, 1979) of an opine. According to this definition, the utilization of the compound must also be Ti plasmid-coded but this has not been demonstrated for dFruGlu and dFruGln. To answer this question it will be necessary to perform plasmid transfer studies using a recipient strain (e.g. C58pAt$^-$pTi$^-$) which is unable to utilize these compounds. The ability to utilize the

fructosylamino acids as sole C source is certainly more widespread than simply on the Ti plasmid because both strain C58C1 and a pTi-cured derivative of strain B6 are utilizers of these Amadori rearrangement products.

The operational definition of an opine given by Schell et al. (1979), which applies specifically to crown gall (and now, by extension, to hairy root disease), is very useful in practical terms as it is clear and precise. According to this definition, opine biosynthesis should occur specifically in crown gall tumour cells and should be directed by the T-DNA; utilization should be encoded by Ti- or Ri- plasmid-borne genes. The use of this definition enables a series of simple experiments to be carried out to determine whether or not a compound has the properties required of an opine. The somewhat broader definition of Petit et al. (1978) is much more subject to interpretation, with ill-defined concepts such as the idea that an opine creates "favourable environmental conditions" for the pathogen. This definition casts the net widely and does not preclude the existence of compounds which could be classed as opines in other plant-microbe interactions, or indeed any host-parasite or symbiotic interaction. As an example, opine-like compounds have been found in Rhizobium-induced nodules (Tempé et al., 1982). Since the term opine has such strong connections with crown gall and hairy root diseases, it would be preferable to coin new names for compounds with similar biological roles found in other interactions between organisms, for example in the Rhizobium-plant association.

The opine concept, as it applies to crown gall and hairy root, has now been extended to the "null-type" cucumber hairy root strains with the discovery and partial characterization of cucumopine, as described in this thesis. These cucumber hairy root strains were found, in this work, not to

induce the synthesis of any of the previously described opines and a successful search for a new opine revealed the presence of a compound which is selectively degraded by the inciting strains and has chemical properties distinct from any of the currently known opines. The structure of cucumopine is not known at present, but the charge characteristics and its weak ninhydrin reaction are consistent with the presence of yet another imino acid and its staining properties suggest the presence of an imidazole or modified imidazole group.

The prediction has been made that opines should be found in null-type tumours or hairy roots on the basis of their biological importance in the Agrobacterium-plant interaction (Guyon et al., 1980). The prediction has been fulfilled in every case so far examined including agropine pTi strains (Guyon et al., 1980), agropine pRi strains (Tepfer and Tempé, 1981), dMan1Gln pRi strains (Petit et al., 1983), succinamopine (= asparaginopine) and glutaminopine strains EU6 and 181 (Chang and Chen, 1983; Chilton et al., 1984). It could now be argued that the opine concept applies to pathogenic agrobacteria and their Ti- or Ri- plasmids in general, and any investigation of remaining known or undiscovered null type strains is only likely to further strengthen this argument. Testing recently-found opines such as cucumopine, leucinopine and succinamopine for their ability to induce conjugal plasmid transfer may reveal more conjugative opines.

Most types of transformed crown gall or hairy root tissues contain opines belonging to two different families (Ellis, 1981; Table 1-1). On the basis of the amounts of opines found in tumour tissues, Ellis (1981) introduced the idea that each strain had a "major" nutritional opine and a "minor" conjugative opine. The discovery of more opine types and strains with three types of opines (these are strain IIBV7, Chapter 4 and

strain Bo542, see Chapter 1) requires that this idea be modified or extended. The upper limit on the number of opine types found in any tumour or hairy root tissue would undoubtedly be low, owing to the limits imposed by T-DNA size. The size of the T-DNA in the "three-opine family" strains Bo542 and IIBV7 is not known at present. The presence of nopaline, agrocinopine C, dFruGlu, dFruGln and agropin-1'-ene in IIBV7 tumours presents an interesting case in terms of Ti plasmid and T-DNA evolution. The apparently hybrid nature of the IIBV7 plasmid, as seen in its combination of opines, could have arisen either by recombination of previously existing T-DNAs or by independent evolution. The former explanation seems more likely, as the three opine types are an unusual combination. This combination could have evolved after cointegrate formation between a nopaline and an agropine Ti plasmid with subsequent loss of the reductase in the agropine biosynthetic pathway.

The origin of the genes for opine biosynthesis and catabolism has not generally been considered in the literature. Conceptual difficulties arise in considering the evolution a new metabolic function, such as the biosynthesis of nopaline and octopine, which are not found in normal plant tissues. In the case of the fructosylamino acids dFruGlu and dFruGln, steps in the evolutionary process can be envisaged as discussed in Chapter 4. The idea rests on the assumption that fructosylamino acids are likely to be present in "wound juice". More work on this topic is required to test the validity of this notion.

The acquisition of the ability to utilize novel growth substrates by bacteria is an apparent "evolutionary jump" in the organism's ability to interact with its environment. This phenomenon has been studied by experimental evolutionists, and is reviewed by Hall (1983). The results demonstrate that such apparent jumps are in fact due to regulatory or

structural mutations in previously existing genes and that these genes are involved in metabolic pathways for compounds closely related to the novel substrate. Thus the observed phenotypic jump is not necessarily a result of the sudden evolution of a new gene but can be explained by more subtle changes at the molecular genetic level. The review by Hall (1983) outlines approaches which, if applied to agrobacteria, may help in understanding the evolution of the genes for the synthesis and catabolism of opines.

The aim of the latter part of the thesis (Chapter 8) was to gain an insight into the crown gall transformation process. The avirulence of some hairy root strains and the attenuated virulence of strain A66, when inoculated on the basal surface of carrot root discs, was the starting point for the work. Experiments indicated that of the factors tested, auxins were the only compounds to have any effect in stimulating virulence of these strains on the basal surface. But the auxin effect was by no means quantitative, and it is therefore possible that other factor(s) are also involved in the attenuation of basal virulence. Publications which appeared during the course of this study (Leemans et al., 1982; Joos et al., 1983) reported the basal attenuation of strains with mutations in the T-DNA shoot inhibition region (TL-DNA genes 1 and 2) and the effect of auxin in promoting virulence of these strains. In addition, Binns et al. (1982) demonstrated that A66 is a natural gene 2 insertion mutant. TL-DNA genes 1 and 2 have thus been described as having auxin-like effects. Several lines of evidence point to the involvement of auxin in attenuated virulence on the basal surface of carrot root discs:

1. There is evidence (Goldsmith, 1977) that auxin transport in plants is polar, and that it moves towards the apex of the root. The strains whose virulence on the basal surface is promoted by auxin are naturally virulent on the apical surface.

2. Axenic carrot tumour tissue incited by the gene 1 mutant tms-328 requires auxin for growth.

3. Evidence is increasing that genes 1 and 2 are directly involved in auxin metabolism (Schroeder et al., 1984), both in the tumour and in the bacteria.

The evidence presented in Chapter 8 suggests that the auxin supplement has its tumour growth-stimulating effect after the transformation step, i.e. after T-DNA transfer to the plant DNA. This is also supported by evidence that disarmed Ti plasmids, lacking functional tumorigenicity genes ("common" T-DNA genes), can transfer their DNA to the plant cell (Leemans et al., 1982; Hille et al., 1983; Ream et al., 1983). Transformation without subsequent tumour growth is therefore possible. The role of auxin after transformation would seem to be the stimulation of cell division at the onset of tumour growth. The Bas^{+} strains seem adapted to be virulent in situations where there is little or no cell division in the host, such as the carrot disc basal surface. Bas^{att} strains , on the other hand, appear to require host cell division or auxin, or both, for virulence. This is related to the plant wound response, a process which is not well understood at present (Kahl, 1982). Wounding stimulates a large variety of responses culminating in most cases in cell division. However there is no cell division after wounding on both the carrot disc basal surface and the decapitated pea hypocotyl. It is in such situations that the difference between Bas^{+} and Bas^{att} strains becomes apparent. It is noteworthy that Van Slogteren (1983) observed avirulence of strains with shoot inhibition mutations when inoculated on decapitated pea hypocotyls.

The transformation process, whereby the T-DNA is transferred from the bacterium to the plant cell, remains largely a mystery. For example, how the DNA is moved into the plant cell and how much of the Ti plasmid enters

the plant cell are not known. The idea of a circular free replicon of T-DNA as an intermediate excised from the Ti plasmid was suggested by Chilton (1982) but as yet there is no experimental data to support it.

Significant advances in our understanding of transformation to the tumorous state are likely to come from further investigation of the functions of T-DNA genes. The study of the translation products of genes 1 and 2 and their involvement in auxin metabolism has already proved fruitful (Schroeder et al., 1984). The function of gene 4, the "cytokinin-like" locus is unknown but the function of this single gene certainly has a profound effect on the transformed plant cell. Deletion of both the shoot inhibition and root inhibition regions, comprising only 3 or 4 genes, removes the tumorous phenotype while still allowing T-DNA transfer (Leemans et al., 1982; Hille et al., 1983; Ream et al., 1983). This is an important result, firstly for the use of the Ti plasmid in plant genetic engineering and also because it again indicates that the T-DNA encodes the minimum number of genes required for functions to be expressed in the plant cell. In addition to transcripts with known functions, there remain more identified T-DNA transcripts whose functions are yet to be determined. These occur, for example, on the left hand end of the nopaline T-DNA (Joos et al., 1983; Willmitzer et al., 1983).

The Ri plasmids and their T-DNAs are less well understood than the Ti plasmids at this stage. Despite similarities between the two types of strains, as shown by DNA homology studies (White and Nester, 1980b; Willmitzer et al., 1982; Huffman et al., 1984) there are fundamental differences. This is clear from the fact that they have been distinguished as two different diseases. The basis for these differences may well be found in the T-DNA regions which are not common to both the Ri and Ti plasmids. The Ri plasmid T-DNA regions which are not homologous to the Ti

plasmid T-DNA may code for hitherto unrecognized phytohormone biosynthesis. The possible existence of two T-DNAs in the Ri plasmid of strain A4 (Huffman et al., 1984) is intriguing and, if confirmed by subsequent studies, could offer insight into the mechanism whereby the Ri plasmid and its T-DNA are transferred to the plant cell. The detection of homology between part of the Ri plasmid T-DNA and normal plant DNA of carrot and Nicotiana glauca (Spano et al., 1982; White et al., 1983) also offers new opportunities for an increase in our understanding of the Agrobacterium-plant interaction.

APPENDIX A

CULTURE MEDIA

1. Yeast mannitol agar

K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.2g
$CaCl_2$	0.2g
$FeCl_3$	0.01g
yeast extract	1.0g
mannitol	10.0g
Davis agar	15g
dist. water	to 1 litre

2. Petit's salts (Petit and Tempé, 1978)

K_2HPO_4	10.5g
KH_2PO_4	4.5g
$MgSO_4.7H_2O$	0.2g
$CaCl_2$	0.01g
$FeSO_4$	5.0mg
$MnCl_2$	2.0mg
biotin	0.2mg
purified agar (separately sterilized)	20g
dist. water	to 1 litre

Minimal medium may be supplemented with 2 g/1 $(NH_4)_2SO_4$ and 1 g/1 mannitol.

3. Stonier's medium (Stonier, 1960)

K citrate	10.0g
NH_4NO_3	2.7g
Na glutamate	2.0g
K_2HPO_4	0.88g
NaH_2PO_4	0.3g
$MgSO_4.7H_2O$	0.2g
NaCl	0.2g
$CaSO_4$	0.1g
$Fe(NO_3)_3$	5.0mg
$MnCl_2$	0.1mg
$ZnCl_2$	0.5mg
biotin	0.2g
agar	15g
dist. water	to 1 litre
	(pH to 7.0)

4. Modified Bergersen's salts (Bergersen, 1961; modified by J. Tempé).

$Na_2HPO_4.12H_2O$	0.45g
$MgSO_4.7H_2O$	0.1g
$CaCl_2$	0.04g
$FeCl_3$	0.02g
thiamine	1.0mg
biotin	0.25mg
dist. water	to 1 litre
	(pH to 7.0)

Bergersen's medium may be supplemented with 2 g/l mannitol and 1 g/l $(NH_4)_2SO_4$. The salt solutions are stored as 5x concentrates. Yeast extract

(50 - 100 mg/1) may be used in place of thiamine and biotin. The medium can be used at half strength.

5. Monnier's salts with Morel's vitamins (Monnier, 1976; Morel and Wetmore, 1952).

KNO_3	1.9g	
$CaCl_2.2H_2O$	0.88g	
NH_4NO_3	0.825g	Use 100 ml/1 of a 10x
$MgSO_4.7H_2O$	0.37g	stock solution.
KCl	0.35g	
KH_2PO_4	0.17g	
Na_2EDTA	14.9mg	Use 2 ml/1 of a 500x
$FeSO_4.7H_2O$	11.1mg	stock solution.
H_3BO_3	14.4mg	
$MnSO_4.H_2O$	33.6mg	
$ZnSO_4.7H_2O$	21.0mg	
KI	1.66mg	Use 2 ml/1 of a 500x
$Na_2MoO_4.2H_2O$	0.5mg	stock solution
$CuSO_4.5H_2O$	0.05mg	
$CoCl_2.6H_2O$	0.05mg	

Ca pantothenate	1.0mg	
myo-inositol	100.0mg	
biotin	0.01mg	Use 2 ml/1 of a 500x
nicotinic acid	1.0mg	stock solution.
pyridoxine (Vit. B6)	1.0mg	(Filter-sterilize
thiamine (Vit. B1)	1.0mg	stock separately.)

sucrose	30g
agar	7g
dist. water	to 1 litre
	(pH to 6 with NaOH)

6. Selective media for (a) A. tumefaciens (biotype 1) and (b) A. rhizogenes (biotype 2) (Brisbane and Kerr, 1983).

(a) A. tumefaciens (biotype 1)

L-arabinitol	3.04g
NH_4NO_3	0.16g
KH_2PO_4	0.54g
K_2HPO_4	1.04g
$MgSO_4.7H_2O$	0.25g
Na taurocholate	0.29g
crystal violet (1% aq.)	2.0ml
Davis agar	15.0g
dist. H_2O	to 1 litre

After autoclaving, add, per 100 ml, 2% aq. actidione (1.0 ml) and 1% aq. $Na_2SeO_3.5H_2O$ (1.0 ml).

(b) A. rhizogenes (biotype 2)

erythritol	3.05g
NH_4NO_3	0.16g
KH_2PO_4	0.54g
K_2HPO_4	1.04g
$MgSO_4.7H_2O$	0.25g
Na taurocholate	0.29g
yeast extract (1.0% aq.)	1.0ml
malachite green (0.1% aq.)	5.0ml
Davis agar	15g
dist. H_2O	to 1 litre

After autoclaving, add, per 100 ml, 2% aq. actidione (1.0 ml) and 1% aq. $Na_2SeO_3.5H_2O$ (1.0 ml).

APPENDIX B

SOLUTIONS FOR PLASMID ISOLATION AND RESTRICTION ANALYSIS

1. TE buffer: 50 mM Tris-HCl, 20 mM EDTA, pH 8.0
2. LTE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
3. TES buffer: 30 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, pH 8.0
4. KOAc (5 M, pH 5): To 60 ml KOAc (5 M) add glacial acetic acid (11.5 ml) and H_2O (28.5 ml).
5. Solution 1: 50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0, made to 2 mg/ml lysozyme just prior to use.
6. Solution 2: 0.2 N NaOH, 1.0% SDS, made fresh each day from 2 N NaOH and 10% SDS.
7. Solution 3: 50 mM Tris, 20 mM EDTA, 1% SDS, pH 12.35
8. RNase stock solution: RNase was dissolved in 10 mM Tris, 15 mM NaCl, 5% glycerol, pH 7.4 at 2.5 mg/ml. The solution was heated (100°C, 15 min), allowed to cool slowly to room temperature and stored as 0.02 ml aliquots at -20°C.
9. Electrophoresis buffer (0.089 M Tris/0.089 M borate, pH 8.0): Sigma 7-9 (10.78g), boric acid (5.5g) and disodium EDTA (0.93g) were made to 1 l with distilled water. This can also be made as a 5x stock solution.
10. Agarose gel (0.7%): Agarose (0.875g Seakem) was melted in electrophoresis buffer (125 ml), cooled to 60°C and poured.

APPENDIX C

COLORIMETRIC PENTOSE DETERMINATION

(slightly modified from Dische and Borenfreund, 1957).

1. 0.2 ml sample (5 to 50 µg pentose).
2. Add 2.5 ml reagent* and mix.
3. Boil 15 min in a vigorously boiling water bath.
4. Cool tubes in tapwater, measure A_{552} (λ_{max}) and subtract A_{510}.

* Reagent (prepared freshly each time):

110 ml glacial acetic acid, redistilled¶.

2 ml conc. HCl

4.5 ml of 5% phloroglucinol in glacial acetic acid

1 ml of 0.8% glucose

¶ Glacial acetic acid was redistilled under vacuum after refluxing (2h) with chromium trioxide (1 to 2% w/w) to remove acetaldehyde and other oxidizable compounds (Wiberg, 1960). N.B. leave 1/3 volume when redistilling: risk of explosion.

APPENDIX D

PUBLICATIONS

M.H.Ryder, M.E.Tate and G.P.Jones (1984)

Agrocinopine A, a Ti plasmid-coded enzyme product, is a phosphodiester of sucrose and L-arabinose.

J.Biol.Chem. 259, 9704-

J.G.Ellis, M.H.Ryder and M.E.Tate (1984)

Agrobacterium tumefaciens T_R-DNA encodes a pathway for agropine biosynthesis.

Mol.Gen.Genet. 195, 466-473.

M.H.Ryder, M.E.Tate and A.Kerr

Virulence properties of strains of Agrobacterium on the apical and basal surfaces of carrot root discs.

Plant Physiol., submitted for publication

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