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GENETIC TRANSFORMATION OF BRASSICA NAPUS
VIA AGROBACTERIUM SPP. AND THE
DEVELOPMENT OF HERBICIDE RESISTANCE

BY

PIERRE J. CHAREST, B.Sc.A., M.Sc., P.Ag.

A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGY

CARLETON UNIVERSITY

OTTAWA, ONTARIO

DECEMBER 19, 1988

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and the development of herbicide resistance.

submitted by Pierre J. Charest, B.Sc.A., M.Sc., P.Ag.
in partial fulfilment of the degree of
Doctor of Philosophy.

Chairman, Department of Biology.

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December 14, 1988.

ABSTRACT

This thesis describes the genetic transformation of Brassica napus using Agrobacterium sp. and the application of this technology to achieve sulfonylurea and imidazolinone herbicide resistance. In planta tumourigenesis induced by Agrobacterium was studied to identify strains that were efficient in the transformation of B. napus. The efficiency of A. tumefaciens nopaline Ti plasmids in contrast with the relative inefficiency of octopine Ti plasmids was demonstrated. The disarmed Ti plasmid pGV3850 derived from the nopaline pTiC58 was used to recover transgenic B. napus plants from stem explants at a frequency of c.a. 2%. Intermediate vectors containing chimeric plant genes coding for kanamycin and chloramphenicol resistances as selectable markers were employed. The nopaline synthase gene was also used as a screenable marker for genetic transformation. The chloramphenicol acetyltransferase gene was not a practical marker for Brassica genetic transformation. The transgenic B. napus plants were morphologically normal and fertile. The introduced genes were transmitted to the progeny. The mutant gene csr1-1 from A. thaliana was used to recover transgenic N. tabacum plants which were resistant to chlorsulfuron and also to other short residual sulfonylurea herbicides. When used with chlorsulfuron as a selective agent, this gene was not as efficient as the neomycin phosphotransferase gene in both N. tabacum and B. napus. The overexpression of the ALS gene fused to a 35S promoter yielded tolerance to both sulfonylurea and imidazolinone herbicides in transgenic N. tabacum. In B. napus, analysis of transgenic callus and plant obtained with the mutant csr1-1 gene indicated the presence of ALS enzyme resistant to chlorsulfuron.

RESUME

Cette thèse décrit la transformation génétique de Brassica napus avec Agrobacterium spp. et l'application de cette technologie pour le transfert de la résistance aux herbicides de types sulfonylurée et imidazolinone. La tumorigénèse in planta induite par Agrobacterium a été étudiée afin d'identifier les souches les plus efficaces pour la transformation de B. napus. L'efficacité des plasmides Ti de type nopaline en contraste avec l'inefficacité des plasmides de type octopine a été démontrée. Le plasmide désarmé pGV3850 dérivé du plasmide C58 de type nopaline a été utilisé pour régénérer des plantes transgéniques de B. napus à partir d'explants de tiges avec une fréquence de c.a. 2%. Des vecteurs intermédiaires contenant des gènes chimériques codant pour la résistance au chloramphenicol et à la kanamycine ont été utilisés comme marqueurs de sélection. Le gène de la nopaline synthase a aussi été utilisé comme marqueur de criblage pour la transformation génétique. Le gène d'acetyltransferase du chloramphenicol n'était pas un marqueur utile pour la transformation génétique des Brassica. Les plantes transgéniques de B. napus avaient une morphologie et une fertilité normales et les gènes introduits étaient transmis à la descendance. Le gène mutant csr1-1 d'A. thaliana a été utilisé pour régénérer des plants transgéniques de N. tabacum qui étaient résistants au chlorsulfuron et aussi à d'autres herbicides sulfonylurées à courte demi-vie. Lorsque utilisé avec le chlorsulfuron comme agent de sélection, ce gène n'a pas été aussi efficace que le gène de la neomycine phosphotransferase chez N. tabacum et B. napus. La sur-expression du gène de l'acetolactate synthase, fusionné au promoteur 35S, a donné une tolérance aux herbicides de types

sulfonylurée et imidazolinone chez des plants transgéniques de N. tabacum. Chez B. napus, l'analyse de cals et d'une plante transgéniques obtenus avec le gène mutant csrl-1 a révélé la présence d' ALS enzymes résistantes au chlorsulfuron.

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DEDICATION

This thesis is dedicated to my wife Danielle. Without her support, this work would have never been completed.

It is also dedicated to my parents, Rita and Arthur Charest. In spite of their modest education, they have always believed in the benefits of higher education.

Cette thèse est dédiée à mon épouse Danielle. Sans son support, ce travail n'aurait jamais été complété.

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LIST OF ABBREVIATIONS

a	alpha
a.a.	amino acid
AGS	agropine synthase
ALS	acetolactate synthase
a.i.	active ingredient
Ap	ampicillin
ATP	adenosine triphosphate
b	beta
BAA	6-benzylaminopurine
bp	base pair
CAMV	cauliflower mosaic virus
CAT	chloramphenicol acetyl transferase
Cb	carbenicillin
Cm	chloramphenicol
CTAB	cetyltrimethyl ammonium bromide
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
EDTA	ethylene diaminetetra acetic acid
EPSP	5-enol pyruvylshikimate 3-phosphate
FAD	flavine adenine dinucleotide

g	gram or centrifugal force
Gen	gentamycin
GUS	β -glucoronidase
H.A.	hormone autotrophy
HPT	hygromycin phosphotransferase
hr	hour
kb	kilobase
kg	kilogram
Km	kanamycin
L	litre
LB	left border
m	milli
M	molar
mg	milligram
mL	millilitre
mM	millimolar
μ E	microEinstein
μ g	microgram
μ L	microlitre
min	minute
Min	minimum
N	normal
NAA	1-naphtaleneacetic acid
NaI	nalidix acid
nM	nanomolar
NOS	nopaline synthase
N-P-K	nitrogen-phosphate-potassium
NPT II	neomycin phosphotransferase
OCS	octopine synthase

ONPG	o-nitro phenol-b-D-galactoside
P	promoter
PA	polyadenylation signal
pTi	tumour inducing plasmid
RB	right border
Rif	rifampicin
SDS	sodium dodecyl sulfate
sec	second
Spc	spectinomycin
Str	streptomycin
Tc	tetracycline
T-DNA	transferred DNA
UPS	upstream elements
UV	ultraviolet
VIR	virulence
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-indolyl-b-D-galactoside
Z	b-galactosidase gene
2,4-D	2,4-dichlorophenoxy acetic acid

Specific abbreviations not commonly used in this thesis are explained in the text

INTRODUCTION

Plants can regenerate in culture from single isolated cells into complete organisms identical to the parent. This unique experimental capacity makes them very attractive for molecular biology research. Plant cells can be genetically transformed with exogenous DNA and the regenerated transgenic plants with the foreign DNA integrated in their genome can be used for studying gene expression and gene regulation.

The production of transgenic plants is relatively new (section I.A.1) and most of them were obtained with model plant systems such Nicotiana tabacum (tobacco) and Petunia hybrida (petunia) which are both members of the Solanaceae family. Transgenic plants from crop species are more difficult to produce due to inefficiencies in culture systems (section I.C). The most advanced technology for genetic transformation of plants employs the phytopathogenic bacteria Agrobacterium tumefaciens (section I.C) which induces the crown gall disease. The molecular basis of this disease is the transfer of plasmid DNA (T-DNA or transferred DNA) to the plant cell genome and its expression which induces the abnormal proliferation of the transformed cell (section I.B.3). The application of this technology to crops is limited by the host range of the bacteria. Alternative methods of transformation have

been developed that are complementary to Agrobacterium transformation (section I.B.2).

The genetic transformation of plants has been achieved with selectable marker genes such as antibiotic resistance genes (section I.C.2) and recently with genes coding for agronomic traits. Characters such as resistance to viral diseases (section I.D.1), insect pests (section I.D.2), heavy metal contamination (I.D.3), herbicides (I.D.4) and fungal infection (I.D.5) have been transferred to model plant systems such as N. tabacum, P. hybrida, L. esculentum (tomato) and A. thaliana.

This thesis has as a main objective the genetic transformation of Brassica napus (rapeseed) via the Ti plasmid of Agrobacterium tumefaciens and the recovery of transgenic plants which are otherwise normal in all other aspects.

The Brassica genus is scientifically interesting for studying the principles of gene regulation and gene expression. It includes numerous important oilseed and vegetable crop species (Fig. 0.1) which are valued for products made in highly differentiated cell types. These are subdivided into subspecies that are morphologically distinct. For instance, B. napus includes subspecies of rapeseed, rutabaga, forage rape and rapekale (Prakash and Hinata 1980). Moreover, some of the Brassica species are phylogenetically linked. Three diploid species (B. nigra, B. oleracea and B. campestris) evolved by combining their genomes and this resulted in amphidiploid descendants such as B. carinata, B. juncea and B. napus (Fig. 0.1, U 1935, Downey et al. 1980, Prakash and Hinata 1980).

B. napus was chosen as the subject of this thesis for two major

Figure 0.1. Genetic relationships between the different Brassica species. The haploid number of chromosomes is indicated ($n=x$). The capital letters denote the genome composition. If it is a diploid species, the letters are in a pair. If it is an amphidiploid species originating from 2 diploid species, pairs of letters are indicated, each pair coming from each of the ancestral species.

reasons; first, good tissue culture technologies (section IV.A.2) have been developed and second, it is economically important in Canada. B. napus was valued at approximately half a billion dollars in 1984 and more than 3 million hectares are planted annually (Pearen and Beversdorf 1982, Anonymous 1985, Adolphe 1982). The cultivar Westar of B. napus has been chosen for its relative importance. It represents 55% of all Canola grown in Canada (K. Downey, Agriculture Canada, Saskatoon, Canada). Moreover, tissue culture systems that permit efficient regeneration of plants have been developed with this cultivar (Klimaszewska and Keller 1985, 1987).

The genetic transformation method exploited the A. tumefaciens encoded gene transfer system. This seemed to be a logical choice because B. napus is an excellent host for this plant pathogen (section III.A.1). Normal transgenic plants unaltered in morphology or agronomic performance were sought.

The application of genetic transformation technology to B. napus varietal development may be an important long term objective; however, the completion of less ambitious goals such as the development of genetic technologies was hoped for in this thesis. Herbicide resistance was a realistic agronomic trait that could be achieved. It would also be of great value since at least 12% of the rapeseed crop is lost to weeds. This represents around 100 million dollars (Chandler et al. 1984). Furthermore, the recent development of 2 new classes of herbicides (sulfonylurea and imidazolinone, section V.A.2) coincided with the discovery of mutant genes encoding enzymes resistant to these chemicals (Haughn and Somerville 1987). The use of these genes in the genetic transformation of B. napus provided one approach for developing herbicide resistant lines.

To be successful, a logical progression of experiments had to be executed:

- The study of wild type genetic transformation by Agrobacterium which was needed to determine the Ti or Ri plasmid best suited for B. napus.

- The development of an ex planta transformation procedure using Agrobacterium was needed for the regeneration of normal transgenic plants.

- The development of selection protocols was needed for the efficient recovery of genetically transformed tissues.

- The adaptation of a vectoring strategy for efficient gene transfer from A. tumefaciens to B. napus.

- The assessment of herbicide resistance as a potential marker for genetic transformation as well as its agronomic value for B. napus.

The overall objectives of this work in term of the scientific contribution to the field are to increase the knowledge of the genetic transformation of B. napus using Agrobacterium and to evaluate the potential applications of herbicide resistance in the genetic engineering of B. napus.

This thesis begins by reviewing literature relevant to the development of transformation technology, the recovery of transgenic plant species and the use of agronomically relevant genes in transformation experiments. This is followed by a detailed description of the materials and methods employed. The Chapters III, IV and V present the core of the data generated. Chapter III describes the genetic transformation of B. napus by oncogenic Agrobacterium strains.

Chapter IV describes the genetic engineering of transgenic B. napus plants. Chapter V evaluates the application of herbicide resistance in the recovery of transgenic B. napus. The thesis is concluded by a general discussion which links these areas of research together. The significance of this work and questions arising from it are assessed.

CHAPTER 1 - LITERATURE REVIEW

I.A.1 Terminology

A characteristic of a rapidly evolving area is very often the confusion in the terminology employed. To avoid this, the most general terms will be defined in this section. The first expression with the broadest meaning is genetic manipulation which includes any manipulation or modification that can be performed on the genome of an organism. Genetic manipulation covers plant breeding, somatic hybridization and genetic transformation. Genetic transformation is more restricted and refers to the transfer and integration of exogenous DNA to the genome of an organism. This is done with or without gene isolation or modification and includes chromosome transformation, Agrobacterium-induced tumourigenesis and genetic engineering. The latter expression will be used in this thesis to refer to the introduction of an engineered (or modified) piece of DNA into the genome of a living organism to deliberately produce a specific characteristic. The organism may or may not be regenerated into its original form.

I.A.2 Milestones in plant molecular biology and plant genetic engineering

The field of plant genetic engineering is no more than seven years old. The first evidence for this type of genetic manipulation was published in 1981 by Wullems and co-workers. In this study, N. tabacum teratomas (differentiated tumourous tissues produced by A. tumefaciens transformation) were grafted on the stem of normal N. tabacum plants to obtain fertile flowers and seeds. The transferred genes were not modified and the plants regenerated were essentially chimeras. The transferred DNA was transmitted to the progeny (Wullems et al. 1981). This work was preceded by the regeneration of transgenic N. tabacum obtained from Agrobacterium rhizogenes transformed hairy roots (Ackermann 1977) but it went almost unnoticed because the nature of A. rhizogenes transformation was not understood at that time. It was only in 1983 that the first critical evidence of plant genetic engineering was published by Barton and co-workers. A modified gene located on the T-DNA (transferred DNA) of the A. tumefaciens Ti plasmid (tumour inducing) was introduced into N. tabacum. Normal transgenic plants were regenerated and the introduced DNA was transmitted to the progeny.

Scientific milestones headed earlier opened the way for these breakthroughs in technology. Table I.1 gives a list of what the author believes are some of the most important steps in the evolution of plant molecular biology and plant genetic engineering. Basic research on Agrobacterium generated the knowledge required for the recovery of transgenic plants using this natural gene transfer mechanism. Other basic tools for modifying crop plants have evolved and a fair understanding of plant gene structure has been acquired. However,

TABLE I.1. Scientific Milestones in Plant Genetic Engineering

Year	Event
1907	-Discovery of the causative agent of the crown gall disease; <u>Agrobacterium tumefaciens</u> (Smith and Townsend 1907)
1930	-Discovery of the causative agent of the hairy root disease; <u>Agrobacterium rhizogenes</u> (Riker 1930)
1974	-Discovery of large plasmids in <u>A. tumefaciens</u> (Zaenen et al. 1974)
1975	-Identification of Ti plasmids as responsible for tumour induction in plants (Watson et al. 1975, Van Larebeke et al. 1975)
1977	-Regeneration of <u>N. tabacum</u> plants from <u>A. rhizogenes</u> transformed roots (Ackermann 1977)
	-First isolation of a plant gene (large subunit of ribulose biphosphate carboxylase from chloroplast genome) from <u>Zea mays</u> (Coen et al. 1977)
	-Evidence of genetic transformation by the T-DNA of <u>A. tumefaciens</u> Ti plasmids in plant tissues (Chilton et al. 1977)
1981	-Recovery of a genetically transformed plant (Wullems et al. 1981)
1983	-Plant genetic engineering with a modified T-DNA (Barton et al. 1983)
	-Plant genetic engineering with functional bacterial chimeric genes (Herrera-Estrella et al. 1983a, 1983b, Fraley et al. 1983)
1985	-Plant chloroplast genetic transformation (De Block et al. 1985)
	-Transfer of herbicide tolerance in transgenic <u>N. tabacum</u> (Comai et al. 1985)
	-Presence of gene silencers and enhancers in plants (Timko et al. 1985)
1987	-Site directed gene transfer in plant (Wirtz et al. 1987)
1988	-Regeneration of a transgenic monocot (<u>Zea mays</u>) (Rhodes et al. 1988)

little is known about plant biochemistry, its relation with gene expression and how these principles determine crop quality or productivity.

1.B Genetic transformation methods

1.B.1 Introduction

Great progress has been made in plant genetic engineering and numerous transformation methods other than A. tumefaciens-mediated transformation have been developed. Stable integration of the introduced DNA has been achieved with isolated DNA fragments; total genomic DNA, chromosomes, and even isolated nuclei. This contrasts with transient gene transfer where the DNA is not integrated into the plant cell genome but is expressed for a relatively short period of time and subsequently lost. This includes virus-mediated gene transfer (CAMV, gemini virus) although genes are maintained by extrachromosomal replication (Davies et al. 1987, Gronenborn 1987). The advantage of transient gene expression is the rapid assessment of gene expression by introducing very high copy numbers into the host cells. This type of gene transfer will not be reviewed.

A novel type of genetic transformation has been established by combining Agrobacterium-mediated gene transfer and virus infection. The genomes of some viruses have been cloned in the T-DNA of A. tumefaciens Ti plasmid vectors and transferred to plant cells by either viral or bacterial inoculation methods. It is only necessary for a few cells to be genetically transformed to achieve virus infection of the entire plants (Grimsley and Bisaro 1987). This can be considered as an amplification of the transformed cell phenotype but the plants are not transgenic organisms.

There are two kinds of gene transformation approaches. Direct gene transfer can be accomplished using methods which deliver DNA directly to the plant cell without any intermediates. Indirect gene transfer employs an intermediate organism such as the plant pathogens A. tumefaciens and A. rhizogenes to transmit DNA to the plant genome. It must first be introduced to the intermediate organism (Agrobacterium) which in turn delivers the DNA to the plant genome via the T-DNA (transferred DNA).

I.B.2 Direct gene transformation methods

Direct transformation methods overcome host range limitations inherent to the use of Agrobacterium species. For monocotyledon transformation, in particular the Graminae, this is very important because these species fall outside the host range of A. tumefaciens. Most of these methods rely on protoplasts from which plant regeneration can be achieved. Except for extremely efficient methods such as microinjection, selection for the introduced DNA with the help of a marker gene such as antibiotic resistance is needed to recover transformed cells.

I.B.2.a Polyethylene glycol or polyvinyl alcohol delivery

Polyethylene glycol or polyvinyl alcohol are used to permeabilize the plant protoplast membrane in order to stimulate the passive uptake of naked DNA or precipitated DNA (with calcium ions) into the plant cell (Freeman et al. 1984, Chen et al. 1987, Junker et al. 1982, Negrutiu et al. 1987). Factors such as concentration of polyethylene glycol or polyvinyl alcohol and concentration of vector and carrier DNA

influence the rates of transformation (Shillito et al. 1985). Stable transformation was obtained with N. tabacum (Kohler et al. 1987a), Saccharum spp. (Chen et al. 1987), Triticum monococcum (Lorz et al. 1985), Vigna aconitifolia (Kohler et al. 1987a, 1987b), Oryza sativa (Uchimiya et al. 1986), P. hybrida (Kruger-Lebus and Potrykus 1987), Lolium multiflorum (Lorz et al. 1985), B. campestris (Paszkowski et al. 1986) and Nicotiana plumbaginifolia (Negrutiu et al. 1987). Frequencies of transformation are relatively low in the order of 10^{-6} to 10^{-5} per protoplast; however, frequencies of 10^{-3} to 10^{-4} have been obtained after optimization of factors with P. hybrida (Kruger-Lebus and Potrykus 1987). Transient expression was obtained with Zea mays, Hordeum vulgare, O. sativa, Secale cereale (Junker et al. 1987) and T. monococcum (Weer and Lorz 1986). Transgenic plants have been obtained with P. hybrida (Kruger-Lebus and Potrykus 1987).

1.B.2.b Electroporation

Electroporation is used to increase the frequency of transformation of the PEG or PVA method (Shillito et al. 1985, Potrykus et al. 1987). Subjecting protoplasts to a high-voltage electric pulse in the presence of DNA and PEG enhances the passage of DNA into plant protoplasts and changes the permeability of the cell membrane (Potrykus et al. 1987, Shillito et al. 1985). Both stable integration (Langridge et al. 1985, Hauptmann et al. 1988, Bradshaw et al. 1987, Rhodes et al. 1988, Fromm et al. 1986) and transient expression have been achieved with this method (Guerche et al. 1987a, Bradshaw et al. 1987, Ou-Lee et al. 1986, Thornburg et al. 1987). The frequencies of transformation are in the order of 10^{-3} to 10^{-4} per protoplast.

The use of protoplasts is a prerequisite. This limits the number of plant species that are now amenable for transformation by electroporation. Regeneration of transgenic plants from electroporated protoplasts has been done with Zea mays (Rhodes et al. 1988) and N. tabacum (Shillito et al. 1985).

I.B.2.c Liposome-mediated delivery

In this approach, DNA is encapsulated in a phospholipid vesicle, the liposome which is fused with plant protoplasts by polyethylene glycol or cationic treatments (Deshayes et al. 1985, Rosenberg et al. 1988, Fraley 1983, Caboche and Deshayes 1984). The protection of nucleic acids from degradation by nucleases in the medium or the cytoplasm is the main advantage of liposomes. Frequencies of transformation of 10^{-5} to 10^{-6} per protoplast have been obtained (Deshayes et al. 1985, Caboche and Deshayes 1984, Fraley 1983). This method has been used successfully with N. tabacum protoplasts and transgenic plants were regenerated. Genetic analyses indicated that marker genes were transmitted to the progeny (Deshayes et al. 1985, Caboche and Deshayes 1984).

I.B.2.d Spheroplast-mediated delivery

Fusion of plant protoplasts with spheroplasts prepared from Escherichia coli or A. tumefaciens was accomplished with polyvinyl alcohol or polyethylene glycol. The DNA inside E. coli or A. tumefaciens was integrated into the protoplast genome. This method has been used to transform Vinca rosea (Hasezawa et al. 1981, 1983) with E. coli and A. tumefaciens (Matsui et al. 1983), B. napus with E. coli

(Tanaka et al. 1984), N. tabacum with A. tumefaciens and E. coli (Hain et al. 1984) and O. sativa with A. tumefaciens spheroplasts (Baba et al. 1986). Frequencies of transformation of 10^{-4} to 10^{-3} per protoplast have been obtained (Hain et al. 1984, Baba et al. 1986) but no transgenic plants have been regenerated. A drawback of this method is the introduction of more DNA than desired which cannot be avoided. This could lead to mutagenesis and recombination events in genes that could be detrimental for the organism as shown in bacterial transformation (Grist and Butler 1983).

I.B.2.e Microinjection

Microinjection permits the direct and precise delivery of DNA into the plant cell or its nucleus. The DNA can be introduced into the nucleus of isolated protoplasts (Reich et al. 1986a, 1986b, 1986c, Miki et al. 1987, Crossway et al. 1986) or into plant cells (Crossway et al. 1986, Neuhaus et al. 1987) with a microsyringe under microscopic examination. For injection in the nucleus, dyes in combination with fluorescence microscopy (Reich et al. 1986a) or bright field microscopy (Crossway et al. 1986) can be used to visualize the nucleus. Protoplasts may be held with a holding pipette or embedded in a gelling agent. The precision of DNA delivery and the extremely high transformation frequencies are the main advantages of this method. Transformation frequencies of 15 to 26% with Medicago sativa, 66% with B. napus and 14% with N. tabacum were obtained by intranuclear microinjection (Reich et al. 1986b, Crossway et al. 1986, Miki et al. 1987) of protoplasts. With cytoplasmic microinjection of N. tabacum protoplasts, 6% transformation was obtained (Crossway et al. 1986).

Recently, the microinjection of B. napus microspore derived embryos gave transformation frequencies of 10 to 30% (Neuhaus et al. 1987). Transgenic plants were obtained with N. tabacum (Neuhaus et al. 1987, Crossway et al. 1986) and B. napus (Neuhaus et al. 1987). Microinjection is however a tedious procedure and the equipment needed is expensive. Moreover, a limited number of cells can be microinjected in one experiment.

I.B.2.f Microprojection or biolistic

Biolistic is a new technique in which microprojectiles made by coating small tungsten (Klein et al. 1987, 1988) or gold (Christou et al. 1988, McCabe et al. 1988) particles with DNA are accelerated with a particle gun to velocities that permit the penetration of intact cells. Factors affecting transformation are the volume and size of the microprojectile, the amount of DNA, the presence of CaCl_2 and spermidine and the vacuum strength (Klein et al. 1988). Expression of transferred DNA has been shown in Z. mays (Klein et al. 1988), Allium cepa (Klein et al. 1987) and Glycine max (Christou et al. 1988, McCabe et al. 1988). Transgenic G. max plants were recovered following meristem microprojection (McCabe et al. 1988). The use of intact cells and tissues with microprojection is a major advantage because it bypasses tissue culture steps. Chloroplast (Boynton et al. 1988) and mitochondrial (Johnston et al. 1988) transformation with biolistic have been accomplished in Chlamydomonas reinhardtii and Saccharomyces cerevisiae respectively.

I.B.2.g Macroinjection

Macroinjection is the injection of DNA with a syringe into the floral parts of a plant. Successful transformation has been reported only once (DeLa Pena et al. 1987). Transgenic Secale cereale plants were obtained by injecting DNA (plasmid with NPT II gene) into young floral tillers which are sensitive to colchicine or caffeine treatment suggesting that they were also permeable to other molecules such as DNA (Puertas et al. 1984). Transformation of seedlings from the macroinjected plants were verified by detection of the NPT II gene and resistance to kanamycin. The frequency of transformation was relatively low (0.3%). The advantage of macroinjection, if proven repeatable, is that it bypasses limitations of the tissue culture procedures for the recovery of transgenic cereal plants.

I.B.2.h Laser microbeam delivery

This method involves the puncture of plant cells by a UV laser microbeam which allows the entry of DNA into the cell (Weber et al. 1988). No direct proof of transient expression or stable transformation has been reported. Consequently, no transgenic plants have been regenerated. The advantage of such a method would be the use of intact cells.

I.B.3 Indirect gene transfer

Agrobacterium is qualified as a natural genetic engineer because of its ability to transfer and integrate DNA into plant genomes through a unique interkingdom gene transfer mechanism. This property has been exploited for genetic engineering of plants with A. tumefaciens and A. rhizogenes. Both are phytopathogenic bacteria and members of the

Rhizobiaceae family. The species tumefaciens is the causative agent of the crown gall disease and rhizogenes is responsible for the hairy root disease (Lippincott et al. 1981). Both species induce diseases in a wide range of gymnosperms and dicotyledonous angiosperms (DeCleene and DeLey 1976,1981). Monocotyledonous angiosperms are naturally resistant to Agrobacterium infection (DeCleene and DeLey 1985).

I.B.3.a Natural genetic transformation by Agrobacterium

The diseases are due to large plasmids (150-250 Kb) present in Agrobacterium that are called tumour inducing plasmids (Ti plasmid or pTi) for A. tumefaciens and root inducing plasmid (Ri plasmid or pRi) for A. rhizogenes (Watson et al. 1975, Van Larebeke et al. 1975, White and Nester 1980a,1980b). These plasmids code for the disease characters and when they are transferred from one Agrobacterium to another or even to Rhizobium, the ability to induce a specific disease is also transferred (Watson et al. 1975). The Ti plasmids are classified according to 4 categories; nopaline, octopine, agropine and succinamopine plasmids (Petit et al. 1983, Nester et al. 1984, Eckes 1987) and Ri plasmids according to 3 categories; mannopine, agropine and cucumopine plasmids (Biot et al. 1987) (Table 1.2). These compounds or opines are amino acid or sugar derivatives specifically produced by plant tumour tissues and can be used by Agrobacterium as a sole source of carbon and nitrogen (Petit et al. 1983, Nester et al. 1984, Eckes et al. 1987).

I.B.3.a.(1) The role of the T-DNA

The crown gall and hairy root diseases are caused by the

TABLE I.2. Classes of Ti and Ri Plasmids of Agrobacterium

Class of plasmids	Opines synthesized	example	Incompatibility group
A. tumefaciens			
Octopine	octopine family +agropine family	pTiA6 pTiB6S3 pTiAch5 pTiC58 pTiT37 pTiB0542	Rh-1
Nopaline	nopaline family +agrocinnopines A and B		Rh-1
Agropine	agropine family +agrocinnopines C and D		Rh-2
Succinamopine	succinamopine family	pTiAT181	?
A. rhizogenes			
Agropine	agropine family +agrocinnopines A and B	pRiA4	Rh-3
Mannopine	agropine family (except agropine) + agrocinnopines C + D	pRi8196	Rh-3
Cucumopine	cucumopine	NCPB2659	?
The octopine family includes octopine, octopinic acid, lysopine and histopine, the nopaline family includes nopaline and nopalinic acid, the succinamopine family includes succinamopine, succinamopine lactam and succinopine lactam and the agropine family includes agropine, mannopine, mannopinic acid and agropinic acid. ?= not determined			

expression of hormone synthesis genes (oncogenicity or onc genes) located on a portion of the Ti or Ri plasmids that is transferred and integrated into the plant genome (reviewed by Weiler and Schroder 1987, Spena et al. 1987, Birot et al. 1987) and called T-DNA or transferred DNA (Chilton et al. 1977, 1982). With the hormone genes on the T-DNA, are also located opine synthase genes which will be expressed in transformed tissues and produce the specific opines corresponding to the Ti plasmid class (Nester et al. 1984, Eckes et al. 1987). A schematic representation of various T-DNA is given in Figure I.1.

The genes inside the T-DNA can be deleted without effect on its processing, transfer and integration. Two 25 bp repeats at the borders of all T-DNAs are however needed for their efficient transfer. The right border has been found to be essential whereas the left border can be deleted and T-DNA transfer will still occur (Wang et al. 1987a, Peralta and Ream 1985a, 1985b). In octopine plasmids such as pTiA6NC, a 23 bp sequence next to the right border (the overdrive) was found to stimulate T-DNA transmission (Pelranta et al. 1986). Furthermore, T-DNA transfer in nopaline pTiC58 has been found to be affected positively by sequences close to the right border and negatively by sequences close to the left border (Wang et al. 1987a). Both studies favor the hypothesis of polar transfer of T-DNA to plant cells.

The most probable mechanism for T-DNA transfer involves single-stranded site specific nicking of the borders and the production of a single stranded T-DNA (T-strand) transfer intermediate by displacement (Christie et al. 1988, Yanofsky et al. 1986, Stachel et al. 1987, Veluthambi et al. 1988, Wang et al. 1987b). An alternative

Figure I.1. Structure of various T-DNA from Ti and Ri plasmids.

T_L -DNA= Left T-DNA, T_R -DNA= right T-DNA. LB= left border, RB= right border. Hormone synthesis genes; aux= auxine synthesis, cyt= cytokinin synthesis. Opine synthesis genes; ags= agropine synthesis, nos= nopaline synthesis, ocs= octopine synthesis. cT-DNA= DNA hybridizing with plant DNA. Adapted from Nester et al. (1984).

view is the double stranded site specific nicking of the right border and the generation of a circular or linear double stranded T-DNA (Koukolikova-Nicola et al. 1985, Veluthambi et al. 1987, Alt-Moerbe et al. 1986, Yamamoto et al. 1987, Jayaswal et al. 1987). However, it is possible that such molecules are generated by the experimental procedures and may not be involved in T-DNA transfer to the plant cell. Moreover, the frequency of such an event is relatively low (Koukolikova-Nicola et al. 1985).

Some of the hormones genes on the T-DNA have also been found important in the control of the host range of A. tumefaciens (Yanofsky et al. 1985a, 1985b). Their actions are at the tumour formation level and not at the transformation stage.

It is important to notice that the T-DNA genes have evolved to be expressed in the plant cell and not in the bacteria. The hormone imbalance caused by the T-DNA genes in the plant tissues will induce the disease; tumour formation in the case of Ti plasmids and hairy roots in the case of Ri plasmids. The opines produced in the tumour or the root will be used by Agrobacteria and allow the colonization of the plant tissues. The opines will also induce Agrobacterium mating and transfer of Ti or Ri plasmids.

I.B.3.a.(2) The role of the VIR region

Another region, called virulence or VIR region, of about 35 kb on the Ti or Ri plasmids is essential for the transfer of the T-DNA to the

plant genome. It acts in trans to the T-DNA (Hoekema et al. 1983). The genes of the VIR region of the Ti or Ri plasmids are responsible for the processing of the T-DNA and its transfer to the plant cell. They are also involved in the host specificity of the Ti or Ri plasmids (Knauf et al. 1982, Thomashow et al. 1980, Loper and Kado 1979). Six VIR operons (VIR A,B,C,D,E,G) have been identified in both octopine (Stachel and Nester 1986) and nopaline (Rogowsky et al. 1987) Ti plasmids. Homology between VIR regions of octopine, nopaline Ti plasmids and Ri plasmids has been found (Janssens et al. 1986, Rogowsky et al. 1987, Knauf et al. 1984, Risuelo et al. 1982, Jouanin 1984). In octopine Ti plasmids, a 7th operon, VIR F, has been identified which does not have any counterparts in other Ti plasmid types (Stachel and Nester 1986). In nopaline Ti plasmids, the VIR region contains the locus *tzs* which encodes an enzyme for the synthesis of transzeatin in Agrobacteria (Janssens et al. 1986). The VIR F and *tzs* loci are induced by plant exudates.

The VIR A,B,D,G operons are essential for tumour induction. The VIR C,E, and F are necessary only with certain plant species (Stachel and Nester 1986, Rogowsky et al. 1987). The VIR A and G operons are expressed constitutively in Agrobacterium but the VIR G operon can be induced under specific conditions. The VIR B,C,D, E and F operons are induced by cocultivation of Agrobacterium with plant cells or by incubation with plant phenolics such as acetosyringone or hydroxy-acetosyringone (Stachel and Nester 1986, Stachel et al. 1985, 1986a, Rogowsky et al. 1987).

The roles of the VIR genes have not been completely elucidated but strong experimental evidences suggest possible functions for most of them. The VIR A operon which contains one open reading frame (ORF) (Melchers et al. 1987) is thought to code for an inner membrane sensory protein for plant signal molecules (Stachel and Nester 1986, Rogowsky et al. 1987, Melchers et al. 1987). The VIR A protein would in turn activate directly or indirectly the VIR G protein product. This last operon comprises one ORF which codes for a positive regulatory protein which activates the other VIR genes (Stachel and Nester 1986, Rogowsky et al. 1987, Winans et al. 1986). VIR A and VIR G affect host range specificity (Jin et al. 1987, Leroux et al. 1987).

The other VIR operons are more complex and only a few proteins have been assigned potential roles. The VIR D operon possesses five ORFs (Porter et al. 1987). VIR D1 and VIR D2 proteins are thought to be part of a site specific endonuclease that cleaves the bottom strand of the T-DNA at the borders (Porter et al. 1987, Yanofsky et al. 1986, Stachel et al. 1987, 1986b, Jayaswal et al. 1987, Alt-Moerbe et al. 1986) generating the T-strand molecule in Agrobacterium by displacement. The VIR E operon includes 2 ORFs in pTiA6 and at least 5 in pTiC58 (McBride and Knauf 1988, Hirooka et al. 1987). The VIR E2 gene product has been found to bind to single stranded DNA and is located in the cytoplasm, the inner and outer membranes and in the periplasmic fractions of acetosyringone induced A. tumefaciens (Christie et al. 1988). The VIR E2 protein is thought to be involved in the processing of the T-DNA and with the T-strand protection (Christie et al. 1988). The VIR E operon has a role in the host range specificity (McBride and Knauf 1988, Hirooka and Kado 1986). However,

VIR E is not strictly required for tumour formation and can be complemented by co-infection with an A. tumefaciens strain containing a non-defective VIR E operon (Gardner and Knauf 1986).

The VIR B operon includes 11 ORFs (Ward et al. 1988). There is some evidence of membrane association of the VIR B gene products suggesting that it would be involved with the formation of a trans-membrane complex for the delivery of the T-DNA to the plant cell. The VIR B1 to VIR B9 are localized in the cell envelope (Thompson et al. 1988). VIR B4 and VIR B11 contain also sequences needed for binding ATP which support the idea of a delivery complex for T-DNA (Thompson et al. 1988). The VIR B operon influences the host range specificity of A. tumefaciens (Jin et al. 1987).

The VIR C locus contains two ORFs (Yanofsky and Nester 1986) and it affects host range. It also elicits an hypersensitive response on Vitis spp. and renders the plant resistant to tumour induction by A. tumefaciens (Yanofsky and Nester 1986).

The Table 1.3 presents a summary of the VIR gene functions.

I.B.3.a.(3) The role of Agrobacterium chromosomal genes

Tumour induction on plants by Agrobacterium is dependant on multiple factors. The host plant has to be susceptible to Agrobacterium which in turn has to be virulent on the host. The host range of Agrobacterium is dependant upon the expression of the T-DNA hormone gene in the transformed cell (see section I.B.3.a.(2)), the virulence region of the Ti or Ri plasmids (see section I.B.3.a.(2)) and the chromosomal genes of Agrobacterium. The steps that are under the control of the bacterial chromosome (Matthysse 1983, Matthysse et al.

TABLE I.3. Summary of Possible Functions for Chromosomal and

<u>Virulence Genes Involved in Agrobacterium Transformation</u>	
Chromosomal genes	Functions
<u>chv a</u> , <u>chv b</u> , <u>att</u> , <u>exo C</u>	Attachment to plant cell
<u>ros</u>	VIR gene regulation
"chemotaxis"	<u>Agrobacterium</u> chemotaxis
<u>Virulence genes</u>	
Functions	
VIR A	chemoreceptor, activator of VIR G
B	transmembrane complex
C	host range specificity
D	site specific endonuclease
E	T-DNA processing and protection
F	host range specificity
G	positive regulator of VIR B,C,D,E,F and tzs

1981) are the loose and reversible site specific attachment of Agrobacterium to the plant host cell which is followed by the synthesis of cellulose fibrils by Agrobacterium to cover the plant cell surface. This last step is irreversible and Agrobacterium is tightly bound to the host cell. Finally, clusters of Agrobacterium are entrapped into the cellulose fibril web.

The chromosomal genes involved in these steps have been identified and mapped by transposon mutagenesis. The genes chvA and chvB (Douglas *et al.* 1987) are involved in the synthesis of cellulose fibrils and mutants in these loci are defective in tight plant attachment. The chvB locus encodes the synthesis of β -1,2-glucan that is found in periplasmic and extracellular spaces (Zorreguieta *et al.* 1988). The motility of Agrobacterium and the ability to transfer the pTi by conjugation is affected by these genes. Two other genes are also involved in Agrobacterium attachment to the plant. The gene Att is associated with synthesis of polypeptides involved in Agrobacterium spheroplast formation (Matthysse 1987, Robertson *et al.* 1988). Mutants of this gene fail to attach to plant cells. The last gene is exoC (or pscA) and it encodes the synthesis of succinoglycan needed for the synthesis of cellulose fibrils (Tomashow *et al.* 1987, Cangelosi *et al.* 1987).

Other chromosomal genes are involved in functions other than Agrobacterium attachment to the plant cell. The gene ros regulates the expression of the VIR genes (Close *et al.* 1985). This locus acts as a negative regulator of VIR genes and mutants have VIR expression levels that are elevated 300 fold. The Agrobacterium colony morphology is also affected. Yet other chromosomal genes are responsible for

Agrobacterium chemotaxis toward the plant. These genes have not been mapped or characterized (Parke et al. 1987, Loake et al. 1988, Ashby et al. 1987). The chemotaxis system is based on attraction of bacteria to chemicals such as sugars, amino acids (Loake et al. 1988) and phenolics which are released from the plant (Parke et al. 1987). Phenolics such as acetosyringone and hydroxyacetosyringone also act as chemoattractants involved in the mechanism encoded by the Ti plasmid and apparently by the VIR A and G genes (Shaw et al. 1988, Ashby et al. 1988)

The Ti and Ri plasmids also contain bacterial genes that are involved in opine catabolism, conjugal transfer of the plasmids, replication functions, agrocline sensitivity and phage exclusion (Nester and Kosuge 1981).

A. tumefaciens genetic transformation process via the Ti plasmid is illustrated in Figure 1.2.

I.B.3.b Genetic engineering of plants with Agrobacterium Ti or Ri plasmids

Ti and Ri plasmids have been used as vectors for plant genetic engineering. The T-DNA of Ti plasmids have been disarmed by replacing the oncogenic functions (or hormone genes) with other DNA fragments (Zambryski et al. 1983). Table I.4 gives a list of disarmed Ti plasmids. No disarmed Ri plasmid is available. The first type of disarmed Ti plasmids was engineered by exchanging the oncogenic genes with pBR322 sequences via homologous gene recombination techniques (Zambryski et al. 1983, Van Haute et al. 1983). Both T-DNA borders were retained in this vector and the DNA to be transferred to the plant

Figure I.2. Schematic representation of molecular events involved in T-DNA transfer.

The chromosomal genes involved in genetic transformation are chvA,B, att, exo C, Ros and Chem. The VIR genes are A,B,G,C,D,E and are induced by chemical such as acetosyringone through the chemoreceptor (VIR A gene products) which in turn activates the VIR G gene products. Then, these activates the other VIR genes. The VIR D gene products are involved in T-DNA processing (L= left border, R= right border) and the VIR E gene products in the transfer process. VIR B gene products are involved in a transmembrane complex for the transfer of the linear T-DNA. Once the T-DNA is integrated in the plant genome, the hormone genes (aux, cyt) and opine synthase genes (ops) are expressed in the plant cell.

opine catabolism

T-DNA

UVR region

TABLE I.4. List of Engineered Disarmed Ti Plasmids and their Origin

Disarmed Ti plasmid	Original Ti plasmid	Type	Reference
PGV3850	pTiC58	cointegrate	Zambryski <u>et al.</u> 1983
pMP90	pTiC58	binary	Koncz and Schell 1986
pAL4404	pTiAch5	binary	Hoekema <u>et al.</u> 1983
pEHA1001	pTiBo542	binary	Hood <u>et al.</u> 1986
pTiB6S3-SE	pTiB6S3	cointegrate	Fraley <u>et al.</u> 1985
pTiB6S3-NE	pTiB6S3	binary	Rogers and Klee 1987
pTiT37-SE	pTiT37	cointegrate	Rogers and Klee 1987

was introduced between them through homologous recombination with pBR322 sequences. The genes are cloned and manipulated in E. coli and transferred by mating to A. tumefaciens. For this purpose, intermediate vectors with a narrow host range replicon are used because they cannot replicate autonomously in A. tumefaciens. To be maintained, cointegration with the Ti plasmid is needed and antibiotic resistance selection pressure on the intermediate plasmid ensures stability of the integration. In most cases, a third plasmid is needed to mobilize the intermediate plasmid from E. coli to Agrobacterium. This plasmid is called helper plasmid and is normally present in a second E. coli strain. Triparental mating is performed by mixing two E. coli strains and the Agrobacterium strain together and selection is applied to rescue strains in which the cointegration of the intermediate plasmid with the Ti plasmid vector has occurred (Van Haute et al. 1983).

A second type of disarmed Ti plasmids has been constructed without the T-DNA borders (Hoekema et al. 1983, Koncz and Schell 1986, Hood et al. 1986). They can only be used in trans to another vector containing the T-DNA borders. These have been constructed with a wide host range replicon which allows their autonomous replication in Agrobacterium. The T-DNA borders permit their processing by VIR functions carried in trans either by disarmed or wild type Ti plasmids or Ri plasmids. This type of plasmid is called a binary vector since two different plasmids are needed in Agrobacterium for T-DNA transfer (Bevan 1984, Hoekema et al. 1983). The binary vector is manipulated in E. coli and transferred to Agrobacterium by mating as described before. Antibiotic selection pressure is also needed to maintain the plasmid in Agrobacterium (Bevan

1984, Van Haute et al. 1983). The process of triparental mating is illustrated in Figure I.3.

I.C Recovery of transgenic plants

Using the methods of direct and indirect gene transfer described previously, transgenic plants have been recovered. In order to evaluate the status of this technology, a survey of plant species that have been genetically engineered was compiled from the published literature. This work was divided into species for which transgenic plants have been regenerated (Table I.5) and could not be regenerated from transformed tissues (Table I.6). This survey illustrates the severe constraints imposed by tissue culture systems on the development of technologies for producing transgenic plants.

I.C.1 Survey of genetically transformed plant species

A review of the literature reveals that transgenic plants have been obtained with 33 plant species including dicots, monocots and trees (Table I.7). About 1/4 of these species (8) belong to the Solanaceae. In the Cruciferae family, transgenic B. napus has been the object of many transformation reports. This is partly explained by the fact that the Solanaceae have the best tissue culture aptitudes followed by the Brassicaceae in particular B. napus. Most of the work published outside these two groups are single reports indicating that the application of transformation technologies is still in the early stages of development. Monocots have been extremely difficult species to engineer due to the lack of regenerable tissue culture systems and their recalcitrance to Agrobacterium transformation. Monocot

Figure 1.3. Process of triparental mating between E. coli and A. tumefaciens.

The binary (IVB) or cointegrating (IVC) vectors in E. coli (E. coli I) are mobilized with the help of a helper plasmid (pHE) in another E. coli (E. coli II) strain which possesses the transfer (TRA) and mobilization (MOB) genes. Their gene products act in trans on the bacterial origin of mobilization (BOM) sites of the intermediate vectors. These are transferred to A. tumefaciens. The intermediate vector of cointegrating type has a narrow host range origin of replication (NORI) and must be integrated in the Ti plasmid for stable maintenance (A. tumefaciens A). The intermediate vector of binary vector has a wide host range origin of replication (WOR1) and can be autonomously maintained in A. tumefaciens (B).

TABLE I.5. Survey of Plant Species where Transgenic Plants have been Recovered

Species	Common name	Methods	Marker	References
-Dicotyledons				
-Solanaceae				
<u>Lycopersicon esculentum</u>	tomato	A. tumefaciens	NPT II	Koornneef et al. 1986, McCormick et al. 1986, Bino et al. 1987
<u>Lycopersicon peruvianum</u>		A. rhizogenes	NPT II	Shahin et al. 1986b
<u>Nicotiana glauca</u>		A. rhizogenes	H.R.	Morgan et al. 1987
<u>Nicotiana plumbaginifolia</u>		A. rhizogenes	NPT II	Morgan et al. 1987
		A. tumefaciens	Bleo	Hille et al. 1986
<u>Nicotiana tabacum</u>	tobacco	A. tumefaciens	NPT II	Trinh et al. 1987
		A. tumefaciens	NPT II	Horsch et al. 1985
		" "	SPT	Jones et al. 1987
		" "	HPT	Waldron et al. 1985
		" "	CAT	De Block et al. 1984
<u>Petunia hybrida</u>	petunia	electroporation	NPT II	Paszkowski et al. 1984
		A. tumefaciens	NPT II	Walroth et al. 1986, Meyer et al. 1987,
		" "		Sanders et al. 1987
		" "	DHFR	Eichholtz et al. 1987
<u>Solanum nigrum</u>	black nightshade	A. rhizogenes	H.R.	Wei et al. 1986
<u>Solanum tuberosum</u>	potato	A. tumefaciens	NPT II	Shahin et al. 1986, Ooms et al. 1987,
-Leguminosae				Eckes et al. 1986, Twell and Ooms 1987
<u>Glycine max</u>	soybean	A. tumefaciens	NPT II	Hinchee et al. 1988
		microprojection	NPT II	McCabe et al. 1988
<u>Lotus corniculatus</u>	trefoil	A. rhizogenes	H.R., CAT	Stougaard-Jensen et al. 1986
<u>Medicago sativa</u>	alfalfa	A. tumefaciens	NPT II	Shahin et al. 1986a
<u>Medicago varia</u>	alfalfa	A. tumefaciens	NPT II	Deak et al. 1986
<u>Stylosanthes spp.</u>	Townsville stylo	A. tumefaciens	NPT II	Manvers 1988
<u>Trifolium repens</u>	white clover	A. tumefaciens	NPT II	White and Greenwood 1987
-Cruciferae				
<u>Arabidopsis thaliana</u>		A. tumefaciens	HPT	Lloyd et al. 1986
		" "	NPT II	Feldmann and Marks 1987

TABLE 1.5.(continued) Survey of Plant Species where Transgenic Plants have been Recovered

Species	Common name	Methods	Marker	References
<u>Brassica napus</u>	rapeseed	<u>A. tumefaciens</u> " " <u>A. tumefaciens</u> electroporation microinjection <u>A. rhizogenes</u> <u>A. tumefaciens</u>	NPT II CAT DHFR NPT II NPT II NPT II H.R. NPT II	Charest <u>et al.</u> 1988 Pua <u>et al.</u> 1987 Fry <u>et al.</u> 1987 Guerche <u>et al.</u> 1987a Neuhaus <u>et al.</u> 1987 David and Tempe 1988 Basiran <u>et al.</u> 1988, Jordan and McHughen 1988
<u>Brassica oleracea</u>	cauliflower			
<u>Linum usitatissimum</u>	flax			
-Other dicotyledons				
<u>Apium graveolens</u>	celery	<u>A. tumefaciens</u>	NPT II	Catlin <u>et al.</u> 1988
<u>Armoracia lapathifolia</u>	horseradish	<u>A. rhizogenes</u>	H.R.	Noda <u>et al.</u> 1987
<u>Atropa belladonna</u>		<u>A. rhizogenes</u>	H.R.	Kamada <u>et al.</u> 1986
<u>Convolvulus arvensis</u>	morning glory	<u>A. rhizogenes</u>	H.R.	Slightom <u>et al.</u> 1985
<u>Cucumis sativus</u>	cucumber	<u>A. rhizogenes</u>	NPT II	Trulson <u>et al.</u> 1986
<u>Daucus carota</u>	carrot	<u>A. rhizogenes</u>	H.R.	David <u>et al.</u> 1984
<u>Gossypium hirsutum</u>	cotton	<u>A. tumefaciens</u>	NPT II	CAT Firoozabady <u>et al.</u> 1987, Umbeck <u>et al.</u> 1987
<u>Helianthus annuus</u>	sunflower	<u>A. tumefaciens</u>	NPT II	Everett <u>et al.</u> 1987
<u>Lactuca sativa</u>	lettuce	<u>A. tumefaciens</u>	NPT II	Michelmores <u>et al.</u> 1987
-Monocotyledons				
<u>Asparagus officinalis</u>	asparagus	<u>A. tumefaciens</u>	NPT II	Bytebier <u>et al.</u> 1987
<u>Oryza sativa</u>	rice	electroporation	NPT II	Toryama <u>et al.</u> 1988
<u>Secale cereale</u>	rye	Macroinjection	NPT II	De La Pena <u>et al.</u> 1987
<u>Zea mays</u>	corn	electroporation	NPT II	Rhodes <u>et al.</u> 1988
-Trees				
<u>Juglans regia</u>	walnut	<u>A. tumefaciens</u>	NPT II	McGranahan <u>et al.</u> 1988
<u>Populus spp.</u>	poplar	<u>A. tumefaciens</u>	NPT II	Fillatti <u>et al.</u> 1987b
			EPSP	

This list includes all the transgenic plant species that have been reported in the literature. In plant species where normal transgenic plants were obtained, transgenic plants obtained through A. rhizogenes are omitted. NPT II= neomycin phosphotransferase II, H.R.= hairy root, H.A.= hormone autotrophy, Bleo= bleomycin resistance, SPT= spectinomycin phosphotransferase, HPT= hygromycin phosphotransferase, CAT= chloramphenicol acetyltransferase, DHFR= dihydrofolate reductase, EPSP= enolphosphopyruvate synthase.

TABLE 1.6. Plant Species where Transgenic Plants have not been Regenerated from Transformed Tissues

Plant species recovered	Common name	Methods	Marker	References
-Dicotyledons				
<u>Beta vulgaris</u>	sugarbeet	<u>A. tumefaciens</u>	OCS	Harpster et al. 1988
<u>Crepis capillaris</u>		<u>A. rhizogenes</u>	chitinase	
<u>Hyoscyamus muticus</u>		<u>A. tumefaciens</u>	H.R.	Ambros et al. 1986
<u>Stylosanthes humilis</u>		<u>A. tumefaciens</u>	H.A.	Hanold 1983
<u>hamata</u>		<u>A. tumefaciens</u>	NPT II	Manners 1987
<u>guianensis</u>		<u>A. tumefaciens</u>	NPT II	
<u>scabra</u>		<u>A. tumefaciens</u>	NPT II	
<u>Vigna unguiculata</u>	cowpea	<u>A. tumefaciens</u>	NPT II	Garcia et al. 1986
<u>Vigna aconitifolia</u>	moth bean	<u>A. tumefaciens</u>	NPT II	Kohler et al. 1987
<u>Vinca rosea</u>		electroporation	NPT II	Hasezawa et al. 1981, 1983
-Monocotyledons		spheroplasts	NPT II	
<u>Chlorophytum capense</u>	narcissus	<u>A. tumefaciens</u>	Nos	Lorz et al. 1985
<u>Dioscera bulbifera</u>	yam	<u>A. tumefaciens</u>	H.A.	Schafer et al. 1987
<u>Gladiolus spp.</u>	italian ryegrass	<u>A. tumefaciens</u>	H.A.	Graves and Goldman 1987
<u>Lolium multiflorum</u>		PEG	NPT II	Lorz et al. 1985
<u>Panicum maximum</u>		electroporation	DHFR	Hauptman et al. 1988
<u>Saccharum spp.</u>	sugarcane	PEG	NPT II	Chen et al. 1987
<u>Triticum monococcum</u>	einkorn wheat	electroporation	HPT	Lorz et al. 1985

* The list of plant species does not include all the attempts that have been reported.

OCS= octopine synthase, H.R.= hairy roots, NPT II= neomycin phosphotransferase II, H.A.= hormone autotrophy, Nos= nopaline synthase, HPT= hygromycin phosphotransferase.

Table I.7. Total Number of Species from which Plants and Transformed Calli

have been Recovered			
		Transgenic plants recovered	No regeneration
		Normal phenotype	hairy root phenotype calli
-DICOTYLEDONS			
<u>Solanaceae</u>	5	3	
<u>Leguminosae</u>	5	1	7
<u>Cruciferae</u>	3	1	
others	5	4	3
-MONOCOTYLEDONS			
	4		7
-TREE	2		1
Total			
	24	9	18

transformation has been the focus of major research efforts worldwide but only 4 successes have been reported.

Agrobacterium-mediated transformation has been the most extensively developed method for plant genetic engineering of dicots. The neomycin phosphotransferase gene (NPT II) has been a very useful marker for recovering transgenic tissues in monocots, dicots and trees. For monocots methods other than Agrobacterium-mediated gene transfer have been used successfully.

I.C.2 Genetic transformation markers and gene construction

Two general strategies can be followed to recover transgenic plants after protoplast, cell, tissue or explant transformations. The first approach is to screen all the plants that have been regenerated from a transformation experiment for the expression of a screenable (or reporter) gene. The second way is to select the transformed protoplasts, cells, tissues or explants for resistance to a selective agent which is conferred by a selectable gene marker. Both types of marker genes are chimeric constructions that contain all the signals needed for expression in the plant but may not be of plant origin (see next paragraph for a discussion on the gene construction).

The Table I.8 gives a list of marker genes used in plant genetic engineering experiments and their provenance.

Reporter gene markers have a gene product or enzyme activity that can be easily detected. Very sensitive screening procedures have been developed for the nopaline synthase gene (Holbrook et al. 1986), the chloramphenicol acetyltransferase gene (De Block et al. 1984), b-glucuronidase gene (Jefferson et al. 1987) and luciferase gene

TABLE I.8. Marker Genes for Plant Genetic Engineering

Screenable marker	Source	Substrate	Reference
-nopaline synthase	Ti plasmid	¹⁴ C-arginine	Holbrook <u>et al.</u> 1986
-octopine synthase	Ti plasmid	¹⁴ C-arginine	Holbrook <u>et al.</u> 1986
-chloramphenicol acetyltransferase	Tn9	¹⁴ C-chloramphenicol	De Block <u>et al.</u> 1984
-neomycin phosphotransferase	Tn5	g- ³² P- ATP	Herrera-Estrella <u>et al.</u> 1983a
-luciferase	<u>Photenus pyralis</u>	luciferine	Ow <u>et al.</u> 1986
-luciferase	<u>Vibrio harveyi</u>	decanal	Koncz <u>et al.</u> 1987
-b-glucuronidase	<u>E. coli</u>	X-glu, MUG, .PNPG	Jefferson <u>et al.</u> 1987
-b-galactosidase	<u>E. coli</u>	X-gal, ONPG	Helmer <u>et al.</u> 1984
Selectable marker	Source	Resistance	Reference
-chloramphenicol acetyltransferase	Tn9	chloramphenicol	De Block <u>et al.</u> 1984
-neomycin phosphotransferase I	Tn601	kanamycin	Fraley <u>et al.</u> 1983
-neomycin phosphotransferase II	Tn5	kanamycin	Fraley <u>et al.</u> 1983
		geneticin	Herrera-Estrella <u>et al.</u> 1983a
-hygromycin phosphotransferase	<u>E. coli</u>	paromomycin	Guerche <u>et al.</u> 1987b
-dihydrofolate reductase	PR67	hygromycin	Maldrón <u>et al.</u> 1985
	Mouse	methotrexate	Herrera-Estrella <u>et al.</u> 1983a
-streptomycin phosphotransferase	Tn5	methotrexate	Elchholtz <u>et al.</u> 1987
-bleomycin resistance	Tn5	streptomycin	Jones <u>et al.</u> 1987
		bleomycin	Hille <u>et al.</u> 1986

X-glu= 5-bromo-4-chloro-3-indolyl glucuronide, MUG= 4-methyl umbelliferyl glucuronide, PNPG= p-nitrophenol glucuronide, X-gal= 5-bromo-4-chloro-3-indolyl galactoside, ONPG= 0-nitrophynyl-b-D-galactopyranoside.

(Ow et al. 1986, Koncz et al. 1987). All these genes can be detected by their enzymatic activities. The b-glucuronidase and luciferase genes can be visualized in situ by their end products (Jefferson et al. 1987, Ow et al. 1986, Koncz et al. 1987). The expression of the b-galactosidase and luciferase genes can be detected by incubating the transformed plant tissues with their respective substrates without destroying the tissues tested (Helmer et al. 1984, Ow et al. 1986, Koncz et al. 1987).

Selectable gene markers are used to discriminate transformed cells from non transformed cells. Generally, the selectable markers are genes that will detoxify an antibiotic or an antimetabolite added to the culture medium. An exception to this rule is the dihydrofolate reductase which is a mutant form of a similar enzyme present in plants resistant to the drug methotrexate (Eichholtz et al. 1987). The advantage of selection is to reduce the amount of non-transformed material to be handled. In certain plant tissue culture systems, where transformants are very infrequent, the selective agent is ineffective in the recovery of the transformed cells because they may be sensitive to the metabolites released by the surrounding dying non-transformed cells. Neomycin phosphotransferase II has been the most widely used selectable marker. This is probably due to the availability of numerous gene constructions, the relative low cost of kanamycin and the possible use of kanamycin analogues such as geneticin, neomycin and paromomycin (Herrera-Estrella et al. 1983a, Fraley et al. 1983, Guerche et al. 1987b).

To express these marker genes in the plants, the coding regions have been fused to plant promoter, enhancers and termination sequences.

These sequences are often taken from the nopaline synthase (Bevan et al. 1983, Depicker et al. 1983) octopine synthase (De Greve et al. 1983), mannopine synthase (DiRita and Gelvin 1987) genes of the Ti plasmids, and from the 19S and 35S gene transcripts of the cauliflower mosaic virus (Lawton et al. 1987, Odell et al. 1985). The promoter and enhancer sequences of these genes were thought to give constitutive expression of the chimeric genes in transgenic plant tissues (Odell et al. 1985, An 1986, Shaw et al. 1984, Koziel et al. 1984, DiRita and Gelvin 1987); however, tissue or cell specificity was reported for the 35S promoter (Nagata et al. 1987). The 35S promoter promotes a higher level of transcription in transgenic N. tabacum than the nopaline synthase promoter (Harpster et al. 1988, Sanders et al. 1987).

I.D Genetic engineering of plants with genes of agronomic value

The genetic engineering of plants with genes of agronomic value has been limited to single gene traits. Virus disease, insect, herbicide and heavy metal resistance have been engineered in model plant systems such as N. tabacum, P. hybrida, L. esculentum and A. thaliana. Engineering of fungal disease resistance has been attempted but with no conclusive results. The last part of this literature review will deal with the accomplishment in this area of plant genetic engineering.

I.D.1 Viral disease resistance

Viral disease resistance in transgenic plants have been obtained through three different mechanisms (Table I.9). The first mimics the

TABLE I.9. Virus Protection in Transgenic Plants

<u>Virus</u>	<u>Plant</u>	<u>Mechanism</u>	<u>Reference</u>
cucumber mosaic virus (CMV)	<u>N. tabacum</u>	Satellite RNA	Baulcombe <u>et al.</u> 1986
	<u>N. tabacum</u>	coat protein	Harrison <u>et al.</u> 1987
	<u>N. tabacum</u>	antisense to CP	Cuozzo <u>et al.</u> 1988
tobacco mosaic virus (TMV)	<u>L. esculentum</u>	coat protein	Nelson <u>et al.</u> 1988
tomato mosaic virus (ToMV)	<u>N. tabacum</u>	coat protein	Powell-Abell <u>et al.</u> 1988
alfalfa mosaic virus (AIMV)	<u>N. tabacum</u>	coat protein	Loesh-Fries <u>et al.</u> 1987
	<u>N. tabacum</u>	coat protein	Tumer <u>et al.</u> 1987
	<u>L. esculentum</u>		
potato virus X (PVX)	<u>N. tabacum</u>	coat protein	Hemenway <u>et al.</u> 1988
		antisense	
tobacco ringspot virus	<u>N. tabacum</u>	satellite RNA	Gerlach <u>et al.</u> 1987

phenomenon of virus cross protection in which the infection of a plant with an avirulent or mild strain of virus prevents the development of severe symptoms when the same plant is challenged with a severe strain of the same virus (Fulton, 1986). This type of virus cross protection has been achieved in transgenic plants by cloning the virus coat protein gene cDNA and integrating it into the plant. Transgenic plants which were expressing the virus coat protein gene have been recovered for the cucumber mosaic virus (CMV) in N. tabacum (Cuozzo et al. 1988), tobacco mosaic virus (TMV) in L. esculentum and N. tabacum (Powell-Abel et al. 1986, Nelson et al. 1988), alfalfa mosaic virus (AIMV) in L. esculentum and N. tabacum (Loesch-Fries et al. 1987, Tumer et al. 1987) and potato virus X (PVX) in N. tabacum (Hemenway et al. 1988). In all cases, a delay in systemic spread of the virus, a decrease in the number of lesions and a diminution of plants with symptoms are observed in transgenic plants. This kind of protection can be partially overcome by using an RNA inoculum instead of virions (Tumer et al. 1987). Potential explanations for this induced resistance are the blockage of initial virion uptake into the cell and the prevention of uncoating once the virion is inside the cell (Hemenway et al. 1988, Tumer et al. 1987, Cuozzo et al. 1988). It has also been postulated that the coat protein may inhibit viral RNA transcription (Loesch-Fries et al. 1987) and the viral spread from cell to cell (Cuozzo et al. 1988). In the case of PVX protection, the RNA inoculum did not overcome the protection as seen with the other virus (Hemenway et al. 1988). The PVX coat protein or its RNA are thought to inhibit early events during RNA infection. Field tests with transgenic L. esculentum plants expressing a TMV coat protein gene have shown that

resistance to TMV and ToMV (tomato mosaic virus) can be achieved (Nelson et al. 1988).

The second mechanism of protection used in transgenic plants is the introduction of an antisense gene to the virus coat protein gene. The antisense RNA could hybridize either to the coat protein of the infecting virus or its RNA and prevent replication. This approach has been used with CMV in N. tabacum (Cuozzo et al. 1988) and with PVX in N. tabacum (Hemenway et al. 1988). However, the resistance obtained is functional at low level of inoculum only.

The last strategy is based on the introduction of a gene coding for the virus satellite RNA which is present in certain plant RNA viruses which suppresses in some cases the symptoms of virus infection. Cloned satellite RNA expressing their DNA with CMV (Harrison et al. 1987) and with the tobacco ringspot virus (TobRV) (Gerlach et al. 1987) in N. tabacum showed decrease in the intensity of symptoms. Interaction of the satellite RNA with the viral RNA is a possible explanation for this type of tolerance. Two major limitations of this approach are that the resistance is host specific and virus specific because the satellite RNAs are found only in some RNA viruses.

I.D.2 Insect resistance

Insect resistance in transgenic plants has been achieved using two different types of genes. In both instances, A. tumefaciens-mediated transformation was used to obtain transgenic plants. The first type comprises genes encoding insect toxins produced by Bacillus thuringiensis. During bacterial sporulation, these toxins are produced as crystalline proteinaceous inclusion bodies. They are

dissolved in the alkaline condition of the insect midgut (Aronson et al. 1986) and cleaved to yield toxic peptides. These peptides are not toxic to other organisms than insects. Different strains of B. thuringiensis exists and they are active against specific insects. The strain israelensis acts against Diptera. The strains kurstaki and berliner act against Lepidoptera. The strains tenebrionis and san diego act against Coleoptera (Aronson et al. 1986).

A truncated toxin gene of B. thuringiensis var. berliner was used to produce transgenic N. tabacum resistant to the insect larvae of Manduca sexta (tobacco hornworm) (Vaeck et al. 1987). The use of a truncated gene was necessary to obtain biologically significant levels of toxin proteins. The level of resistance was dependant on the level of expression. Similar results were obtained with the B. thuringiensis var. kurstaki toxin gene in transgenic N. tabacum (Barton et al. 1987). The plants were resistant to the tobacco hornworm. Furthermore, insect mortality was increased and plant damage by other insects (Heliothis virescens; cotton bollworm, Heliothis zea; corn earworm, Spodoptera exigua; beet armyworm) was decreased. The same gene used in transgenic L. esculentum in a truncated form reduced the damage by the larvae of the tobacco hornworm (Fischhoff et al. 1987). The transgenic L. esculentum plants were also resistant to H. virescens (cotton bollworm) and H. zea (corn ear worm).

The other class of genes that has been used in genetic engineering experiments encodes the proteinase inhibitor which is present in the leaves of various species (Gatehouse et al. 1980, Gatehouse and Boulter 1983). They are induced in some cases by insect attack and inhibit the digestive enzymes of the insects. Transgenic N. tabacum plants

obtained via A. tumefaciens transformation have been engineered that express a cowpea (Vigna unguiculata) trypsin inhibitor gene (Hilder et al. 1987). The transgenic plants were tolerant to the tobacco budworm (H. virescens). The advantage of this approach is the potential for resistance to a wide range of insects (Heliothis, Spodoptera, Diabrotica and Tribolium) in contrast to the B. thuringiensis toxin genes which are more specific.

I.D.3 Heavy metal resistance

Some soils have been contaminated with heavy metals such as Cd, Hg, Zn and Cu by fertilizers or sewage sludge (Poon 1984, Davis 1984). Plants which sequester the heavy metals in specific tissues which are not consumed by human or animals may protect the other tissues from contamination. Plants have their own Cd binding proteins (phytochelatins) (Grill et al. 1985); however, by genetic engineering the amount of metal binding proteins in transgenic plants can be increased. A chimeric mouse metallothionein gene has transferred by A. tumefaciens-mediated transformation to transgenic N. tabacum (Maiti et al. 1988); however, the transgenic metallothionein gene has not been tested for its function in metal binding. In a different study, the chinese hamster metallothionein II gene was inserted into an engineered CAMV vector and B. campestris plants infected (Lefebvre et al. 1987). The transfected plants showed Cd tolerance and they depleted Cd pools in leaf tissues. Together, these studies shows that an animal metallothionein may function in transgenic plants.

I.D.4 Herbicide resistance

Herbicide resistance in plants is the subject of tremendous activity due to its commercial importance. Herbicide resistance in transgenic plants can be achieved by 3 different strategies (Table I.10); the introduction of a mutant gene coding for an enzyme with a modified herbicide binding site, the introduction of a chimeric gene which overproduces the herbicide target enzyme and the introduction of a gene that codes for an enzyme that detoxifies the herbicide (Table I.10). All three approaches have been exploited.

The strategy involving the use of herbicide resistant mutant genes has been used successfully with the herbicides glyphosate (Monsanto) and chlorsulfuron (DuPont). Glyphosate (or N-phosphonomethyl glycine) is a broad spectrum herbicide acting against the 5-enol pyruvylshikimate 3-phosphate synthase enzyme (EPSP synthase) in the shikimate pathway leading to the synthesis of aromatic amino acids (Steinrucken and Amrhein 1980). When the herbicide is active, shikimate accumulates and starvation of aromatic amino acid occurs. A mutant gene from the bacteria Salmonella typhimurium has been isolated in which a substitution of a proline to a serine in the enzyme resulted in a decrease in the affinity of the herbicide for the enzyme without affecting its function (Comai et al. 1985). This gene fused to octopine synthase and mannopine synthase promoter sequences was introduced in N. tabacum and L. esculentum to give tolerance to the herbicide glyphosate. The transgenic plants were not killed by the herbicide but the yield was reduced by 30 to 70% (Comai et al. 1985, Fillatti et al. 1987b). Similar results were obtained in transformed B. campestris tumour tissues using the same approach (Thompson et al. 1987b).

TABLE I.10. Genetic Engineering of Herbicide Resistance in Plants

Herbicide	Plant	Mechanism	Gene	Reference
Chlorsulfuron	<u>N. tabacum</u>	mutation	acetolactate synthase	Haughn <u>et al.</u> 1988
	<u>L. esculentum</u>	mutation	acetolactate synthase	Mazur <u>et al.</u> 1987a,b
	<u>N. tabacum</u>	mutation	acetolactate synthase	Lee <u>et al.</u> 1988
Glyphosate	<u>N. tabacum</u>	mutation	aroA	Comai <u>et al.</u> 1985
	<u>B. campestris</u> *	mutation	aroA	Thompson <u>et al.</u> 1987b
	<u>L. esculentum</u>	mutation	aroA	Fillatti <u>et al.</u> 1987a
	<u>P. hybrida</u>	overexpression	EPSP synthase	Shah <u>et al.</u> 1986
Phosphinotricin	<u>A. thaliana</u>	overexpression	EPSP synthase	Klee <u>et al.</u> 1987
	<u>L. esculentum</u>	detoxification	phosphinotricin	
			acetyltransferase	De Block <u>et al.</u> 1987

*B. campestris tumour tissues

A gene encoding a chlorsulfuron resistant acetolactate synthase (csr1-1) isolated from the mutant A. thaliana line GH50 has been used to recover transgenic N. tabacum plants (Haughn and Sommerville 1988). This gene conferred resistance to chlorsulfuron and sulfometuron methyl in transgenic N. tabacum. These two herbicides are members of the sulfonylureas which is a new class of herbicides inhibiting acetolactate synthase (ALS) which is an essential enzyme in the pathway for branched-chain amino acid biosynthesis (Ray et al. 1986). The ALS enzyme, although coded by a nuclear gene, is localized in the chloroplast which is the site of amino acid biosynthesis (Mazur et al. 1987a, 1987b). Similar results were obtained with two N. tabacum mutant genes (C3 of the SuRA locus and Hra-S4 of the SuRB locus). Transgenic N. tabacum and L. esculentum were resistant to high levels of chlorsulfuron and sulfometuron methyl (Mazur et al. 1987a, 1987b, Lee et al. 1988). Further discussion on the sulfonylurea and imidazolinone herbicides and their mode of action on the ALS enzyme will be presented in Chapter V.

The second approach to herbicide resistance in transgenic plants is the overexpression of the target enzyme. It has been used to achieve resistance to the herbicide glyphosate. The EPSP synthase gene isolated from an overproducer P. hybrida cell line was fused to the CAMV 35S promoter and reintroduced into P. hybrida. The transgenic plants were resistant to over 4 times the glyphosate dose required to kill the plants in the field (Shah et al. 1986). A similar strategy was used with an EPSP synthase gene from A. thaliana isolated with the P. hybrida EPSP synthase gene as the probe. The A. thaliana EPSP synthase gene was fused to 35S promoter sequences and reintroduced A.

thaliana. The transgenic A. thaliana plants were resistant to glyphosate in vitro (Klee et al. 1987).

In plants, the EPSP gene is a nuclear encoded gene which is synthesized as a cytoplasmic precursor and targetted to the chloroplast (Della-Cioppa et al. 1987). This could explain why Comai et al. (1985) observed only tolerance to glyphosate with the bacterial AroA gene. Tolerance here is defined as the survival of the plants with a delay in growth whereas resistance is defined as the complete absence of symptoms of toxicity. The use of plant genes with native transit peptide sequences (Shah et al. 1986, Klee et al. 1987) may have ensured transport to the chloroplast; whereas the aroA encoded enzyme lacked a transit sequence and may have been restricted to the cytoplasm.

The third strategy is to use a detoxifying enzyme gene in transgenic plants to give herbicide resistance. This approach was used in transgenic N. tabacum, L. esculentum and S. tuberosum plants to obtain resistance to phosphinotricin and bialaphos (De Block et al. 1987). Bialaphos is an antibiotic produced by Streptomyces hygroscopicus (Imai et al. 1984, 1985). The antibiotic is phosphinotricin with two alanine residues. The phosphinotricin is chemically synthesized and is an analogue of glutamic acid (Bayer et al. 1972). These two herbicides are inhibitors of the glutamine synthetase enzyme which plays a major role in assimilation of ammonia and regulation of nitrogen metabolism in plants (Tachibana et al. 1986). When the enzyme is inhibited by one of these two herbicides, ammonia is accumulated and kills the plant cells. The detoxification gene was isolated from S. hygroscopicus which produces Bialaphos (Thompson et al. 1987a). This gene (bar) encodes a phosphinotricin

acetyltransferase enzyme which acetylates the free NH_2 group of phosphinotricin and bialaphos and prevents its action. The bar gene was fused to 35S promoter sequences and introduced into N. tabacum, L. esculentum and S. tuberosum to give resistance to phosphinotricine (Basta) and Bialaphos (Herbiace) up to a dose of 20L/ha which is 3 to 8 times the level used in the field (De Block et al. 1987).

II.D.5 Fungal pathogen resistance

Fungus resistance has been attempted by using chitinase genes isolated from Serratia marcescens (Jones et al. 1986) in A. tumefaciens mediated transformation experiments. The resistance was expected because chitinase hydrolyses chitin of fungus such as Fusarium oxysporum which is a plant pathogen (Jones et al. 1986). Transgenic plants were obtained with P. hybrida (Jones et al. 1985) and N. tabacum (Jones et al. 1988) but assessment of fungus resistance was not made.

CHAPTER II

MATERIAL AND METHODS

II.A Special chemicals used and sources

This section will list the antibiotics, solvents used in stock solutions and sources (Table II.1). The herbicides used, their molecular weights and sources are also indicated (Table II.2).

II.B Bacterial species, strains and plasmids

II.B.1 Storage conditions

The bacterial strains were grown overnight in liquid media with the appropriate antibiotics (see section II.B.2) to late log phase. The bacteria pelleted and resuspended in 5mM $MgSO_4$, 40% (v/v) glycerol (Enquist and Sternberg 1979). The suspensions were stored at $-20^{\circ}C$ for frequent use and at $-70^{\circ}C$ for long term storage. Viability at $-70^{\circ}C$ has been observed after 2 years for A. tumefaciens and E. coli.

II.B.2 Media for bacterial growth

Unless indicated, E. coli and A. tumefaciens strains were grown aseptically in LB medium (10g/L tryptone, 5g/L yeast extract, 5g/L

TABLE II.1. Antibiotics and Sources

Antibiotics	Solvent	Source
Bleomycin	water	Sigma Chemical Co.
Carbenicillin	water	Ayerst Laboratories Ltd.
Cefotaxime sodium	water	Roussel Canada Inc.
Chloramphenicol	ethanol 95% v/v	Sigma Chemical Co.
Claforan	water	Terochem Laboratories Ltd.
Clavulin 250	added directly§	Beecham Laboratories Inc.
Geneticin sulfate	water	Sigma Chemical Co.
Gentamycin sulfate	water	Sigma Chemical Co.
Hygromycin B	water	Boehringer-Manheim Canada
Kanamycin sulfate	water	Sigma Chemical Co.
Methotrexate	water (alkali)	Sigma Chemical Co.
Nalidixic acid	water (alkali)	Sigma Chemical Co.
Paromomycin sulfate	water	Warner Lambert Co.
Rifampicin	methanol	Sigma Chemical Co.
Spectinomycin	water	Sigma Chemical Co.
Streptomycin sulfate	water	Sigma Chemical Co.
Tetracycline	ethanol 95% v/v	Sigma Chemical Co.
Timentin¶	water	Beecham Laboratories Inc.
Tunicamycin	water (alkali)	Boehringer-Manheim Canada

§ Clavulin 250 was added directly to the tissue culture medium as a sterile powder before pouring, it is composed of 250mg of clavulanic acid and 500 mg of amoxicillin for a total of 800mg. ¶ Timentin is a mixture of 17mg of clavulanic acid for 500 mg of ticarcillin.

Chemical company locations: Ayerst, Montreal, Canada; Beecham, Pointe Claire, Canada; Boehringer-Manheim, Dorval, Canada; Roussel, Montreal, Canada; Sigma, St-Louis, USA; Terochem, Toronto, Canada; Warner Lambert, Ann Arbor, Michigan, USA.

TABLE II.2. Herbicides, Characteristics and Sources

<u>Chemical name</u>	<u>Code name</u>	<u>Commercial name</u>	<u>M.W.</u>	<u>% A.I.</u>	<u>Source</u>
<u>Sulfonylureas</u>					
Chlorsulfuron	DPX W4189	Glean ^a	357.78	99.9	DuPont, Wilmington, Delaware, USA
Thiamethuron methyl	DPX-M6316	Harmony	387.4	75	idem
--	DPX-A7881	--	410.4	75	idem
--	DPX-L5300	--	395.39	75	idem
Triasulfuron	CGA-131036	Amber	401.83	75	Ciba-Geigy Canada Ltd. , Cottam Ontario, Canada
<u>Imidazolinones</u>					
Imazapyr	AC243,997	Arsenal	261.3	98.75	American Cyanamid Co, Willowdale Ontario, Canada
Imazethapyr	AC263,499	Pursuit	289.3	98.5	idem
Imazaquin	AC252,214	Scepter	311.3	97.1	idem

Stock solutions of herbicides were made in water and with the pH adjusted at 9.5

a. Glean is not chlorsulfuron but a commercial formulation containing chlorsulfuron in combination with another herbicide. Technical grade chlorsulfuron was used in this work.

NaCl, pH=7.2) and A. rhizogenes strains in TYC medium (5g/L tryptone, 2g/L yeast extract, 0.6mM CaCl₂, pH=7). Both media were sterilized by autoclaving at 121°C for 17 min. The media were solidified by adding 15g/L agar prior to autoclaving. The antibiotics were added after sterilization. Growth in liquid medium was performed by shaking at 37°C for E. coli and at 29°C for Agrobacterium.

II.B.3 List of bacterial species, strains and plasmids

The Table II.3 gives the bacterial species, strains and plasmids used in this study and Table II.4 indicates the antibiotic concentrations used with A. tumefaciens, A. rhizogenes or E. coli.

II.B.4 Bacterial conjugation

Bacterial conjugation was used to transfer intermediate vectors from E. coli to A. tumefaciens or Ti plasmids between A. tumefaciens strains. The matings were performed according to the method of Van Haute et al. (1983). Overnight bacterial suspensions, grown without antibiotics, of the receptor A. tumefaciens strain, the E. coli strain containing the helper plasmid (pRK2013 or pBRK4013) and the E. coli or A. tumefaciens donor strain containing the plasmid to be transferred were mixed together in equal proportions and the bacteria were collected by vacuum filtration on a 0.21µM sterile filter (Sartorius Filters Inc., Hayward, California, USA). The filter with the bacteria side up was placed on a LB agar plate for 12-16 hrs and the bacterial on the filter were collected in 10 mL of NaCl (0.85% w/v). Appropriate dilutions were made and plated on LB agar plates containing the required antibiotics. No dilution was made when selecting for

TABLE II.3. Bacterial Species, Strains and p'smids

Strain	Plasmid(T)	Antibiotic marker*	Plant marker**	Reference (Source)
<u>Agrobacterium tumefaciens</u>				
C58	pTiC58		H.A., NOS	Holsters et al. 1980
B6S3	pTiB6S3		H.A., OCS	Petit and Tempe 1978
T37	pTiT37		H.A., NOS	Sciaky et al. 1978
TT-2	pTiB6S3	Rif, Sm	H.A., OCS	Charest and Dion 1985
A348	pTiA6	Rif	H.A., OCS	Sciaky et al. 1978
Ach5	pTiAch5		H.A., OCS	Sciaky et al. 1978
A281	pTiB0542	Rif, NaI	H.A., AGS	Sciaky et al. 1978
GV3101	PGV3850 (C)	Rif, Cb	NOS	Zambryski et al. 1983
GV3101	PM90 (B)	Rif, Gen		Koncz and Schell 1986
LBA4404	PAL4404 (B)	Rif, NaI		Ooms et al. 1982
C58C9	PAL1050 (B)	Cb, Km		Hcekema et al. 1983
A348	pTiA6/PSM1	Cb, Km	H.A., OCS	Stachel and Nester 1986
A348	pTiA6/PSM30	Cb, Km	H.A., OCS	Stachel and Nester 1986
A348	pTiA6/PSM202	Cb, Km	H.A., OCS	Stachel and Nester 1986
A348	pTiA6/PSM219	Cb, Km	H.A., OCS	Stachel and Nester 1986
A348	pTiA6/PSM304	Cb, Km	H.A., OCS	Stachel and Nester 1986
A348	pTiA6/PSM358	Cb, Km	H.A., OCS	Stachel and Nester 1986
A348	pTiA6/PSM363	Cb, Km	H.A., OCS	Stachel and Nester 1986
A348	pTiA6/PSM379	Cb, Km	H.A., OCS	Stachel and Nester 1986
<u>Agrobacterium rhizogenes</u>				
A4	pR1A4	Rif	H.A., AGS	White and Nester 1980
<u>Escherichia coli</u>				
HB101				Boyer and Roulland-Dussoix 1969
DH5a				Bethesda Research Laboratories
DH5a	pBR322	Cb, Tc		Bolivar et al. 1977
HB101	PRK2013	Km		Figurski and Hellinski 1979
DH5a	pBRK4013	Cb		Hamel 1987
DH5a	PGH1	Cb	ALSR	Haughn et al. 1988
DH5a	PTZ19R-ALSW	Cb	ALSW	J. Hattori and J. DeMoor
				unpublished

TABLE II.3. Bacterial Species, Strains and Plasmids (Continued)

Strain	Plasmid(T)	Antibiotic marker*	Plant marker**	Reference (Source)
DH5a	pMON38	Cb	NOS	Monsanto Co., unpublished
JM101	pMON273 (C)	Spc	NOS, 35S-NPTII-NOS	Sanders et al. 1987
DH5a	pMON806 (C)	Spc	NOS, NOS-DHFR-NOS	Monsanto Co., unpublished
H8101	pNCAT4 (C)	Cb, Km	NOS-CAT-NOS	De Block et al. 1984
H8101	pLGV1103neo (C)	Cb, Km	NOS-NPTII-OCs	Hahn et al. 1985
H8101	pBIN19 (B)	Km	NOS-NPTII-NOS	Bevan, 1984
H8101	pBCAT1 (B)	Km	NOS-NPTII-NOS, 35S-CAT-NOS	M. Bevan, Unpublished
H8101	pVU1011 (B)	Km	NOS-NPTII-NOS, 35S-HPT-NOS	M. Bevan, Unpublished
DH5a	pB1121.1 (B)	Km	NOS-NPTII-NOS, 35S-GUS-NOS	Jefferson et al. 1987
DH5a	pBINCH (B)	Km	NOS-NPTII-NOS, ALSR	This work
DH5a	pKALSR (C)	Km	35S-NPTII-NOS, ALSR	This work
DH5a	pKALSW (C)	Km	35S-NPTII-NOS, ALSW	This work
DH5a	p35SALSR (C)	Km	35S-NPTII-NOS, 35S-ALSR	This work
DH5a	p35SALSW (C)	km	35S-NPTII-NOS, 35S-ALSW	This work

(T) = the letter in parenthesis indicates the type of vector for *A. tumefaciens* transformation; (B) = binary vector, (C) = cointegrating vector. * antibiotic resistance genes expressed in the bacteria; Cb=carbenicillin resistance, Km=kanamycin resistance, Str=streptomycin resistance, Spc= spectinomycin resistance, Rif=rifampicin resistance, Nal= nalidixic acid resistance, Gen=gentamicin resistance, Tc=tetracycline resistance. **genes that are expressed in the transformed plant cell; H.A.= hormone autotrophy, NOS= nopaline synthase, OCS= octopine synthase, AGS= agropine synthase, CAT= chloramphenicol acetyltransferase, NPTII= neomycin phosphotransferase II, ALSW= wild type acetolactate synthase gene, ALSR= mutant acetolactate synthase gene, GUS= b-glucuronidase, HPT= hygromycin phosphotransferase, DHFR= dihydrofolate reductase. The construction of the chimeric genes are PROMOTER-CODING SEQUENCE-TERMINATOR. Maps of most of the plasmids are given in appendices.

TABLE II.4. Concentration of Antibiotics Used with E. coli and A. tumefaciens
or A. rhizogenes

Antibiotics	Concentration (ug/mL)	
	<u>E. coli</u>	<u>A. tumefaciens</u> or <u>rhizogenes</u>
Carbenicillin	50	150
Chloramphenicol	50	100
Gentamycin	--	100
Kanamycin	50	150
Nalidixic acid	50	200
Rifampicin	--	150-500
Spectinomycin	--	200
Streptomycin	--	300
Tetracycline	15	25

cointegration and dilutions up to 10^8 were made when selecting for the transfer of a binary vector. Typically, cointegration frequencies of 1 transconjugants per 10^6 to 10^8 receptor bacteria were obtained whereas 1 transconjugants per 10 to 100 receptor bacteria was obtained for the transfer of binary vectors.

II.C Plant species and growth conditions

II.C.1 List of plant species

The Table II.5 gives the plant species used in this thesis, including the cultivars and varieties, the common names and the sources of seeds.

II.C.2 Soil mixture and fertilization

The soil mixture for all the plant species consisted of soil:peat moss:sand:perlite (1:2:1:1) with traces of lime and superphosphate. Plants were fertilized with Nutricote (N:P:K = 14-14-14, 3Kg/m^3) which is a slow release fertilizer (nutrients released in 40 days) and by watering with Hoagland's solution and a solution of N-P-K (20-20-20) once a week. The plants were watered twice daily.

II.C.3 Growth conditions

The growth chambers were set for 16 hrs photoperiod with 20°C day and 15°C night. The illumination was provided by fluorescent and incandescent lights with a light intensity of $400\text{uEm}^{-2}\text{sec}^{-1}$. The relative humidity was kept at 70%.

The greenhouse temperature varied between 20°C - 35°C in the summer and maintained at approximately 22°C in the other seasons.

TABLE II.5. Plant Species and Sources

Plant species	Common name	Cultivar or variety	Source
<u>Avena fatua</u> L.	wild oat	NA*	D.Derksen, Agriculture Canada, Saskatoon, Canada
<u>Arabidopsis thaliana</u> (L.) Schurr		Columbia type	C.Somerville, U. of Michigan, East Lansing, USA
<u>Brassica campestris</u> L.	turnip rape	line GH50	idem
<u>Brassica carinata</u> (A.) Brown	abyssinian mustard	Candle and Tobin	R.K.Downey, Agriculture Canada, Saskatoon, Canada
<u>Brassica hirta</u> L.	yellow mustard	Rapid Cycling	P.Williams, U. of Wisconsin, Madison, USA
<u>Brassica juncea</u> (L.) Czern et Coss	brown mustard	ochre	R.K.Downey, Agriculture Canada, Saskatoon, Canada
<u>Brassica kaber</u> (Dc.) Wheeler	wild mustard	line ZEM, Zaria	R.K.Downey, Agriculture Canada, Saskatoon, Canada
<u>Brassica napus</u> (L.)	rapeseed	NA	D.Derksen, Agriculture Canada, Saskatoon, Canada
		Westar, Altex	R.K.Downey, Agriculture Canada, Saskatoon, Canada
		Jet Neuf, Midas,	
		Oro, Target, Topas,	
		Atow, Zephyr,	
		Global	
<u>Brassica nigra</u> (L.) Koch	black mustard	Rapid Cycling	P.Arnison, Paladin Hybrids, Brampton, Canada
<u>Brassica oleracea</u> L.		Rapid Cycling	P.Williams, U. of Wisconsin, Madison, USA
<u>Crambe maritima</u> L.	crambe	NA	P.Williams, U. of Wisconsin, Madison, USA
<u>Glycine max</u> (L.) Merr	soybean	Maple Presto	G.Baillargeon, Agriculture Canada, Ottawa, Canada
<u>Hordeum vulgare</u> L.	barley	Argyle	Seed Biology Lab., Agriculture Canada, Ottawa, Canada
<u>Lycopersicon esculentum</u> (L.) Miller	tomato	Ohio 7285	V.Poysa, Agriculture Canada, Harrow, Canada
<u>Medicago sativa</u> L.	alfalfa	Rangelander	D.Brown, Agriculture Canada, Ottawa, Ontario
<u>Nicotiana tabacum</u> L.	tobacco	Petit Havana SR1	B.Bowen, Agrigenetics, Madison, USA
<u>Raphanus sativus</u> L.	wild radish	Rapid Cycling	P.Williams, U. of Wisconsin, Madison, USA
<u>Setaria viridis</u> L. (Braun)	green foxtail	NA	D.Derksen, Agriculture Canada, Saskatoon, Canada
<u>Thlaspi arvensis</u> L.	stinkweed	NA	idem
<u>Triticum aestivum</u> L.	wheat	Katepwa	Seed Biology Lab., Agriculture Canada, Ottawa, Canada

*NA= not applicable since these species are weeds

The artificial lights were set for 16 hrs photoperiod with a $60 \mu\text{Em}^{-2}\text{sec}^{-1}$ light intensity to supplement sunlight.

II.C.4 In planta tumourigenesis

B. napus and B. juncea plants (8 week old) were used for in planta induction of tumourigenesis by Agrobacterium. A. tumefaciens grown on LB agar medium and A. rhizogenes grown on TYC agar medium were inoculated with a syringe needle by scratching the stem about 2 mm deep and 1 mm wide for a length of 10mm in three parallel lines at each internodes (4 to 6 internodes/plant). The wound site was filled with Agrobacteria. Tumour formation was scored regularly up to 4 months.

II.C.5 Assessment of herbicide resistance with soil grown seedlings

Flats (20cm X 17cm X 7cm) of self pollinated seeds (approximately 500 seeds per flat) were sown in the greenhouse under the conditions described in section II.C.3. Ten days after sowing, young germinating seedlings were sprayed with 112.5mg active ingredient (a.i.)/flat of DPX A7881 or 56.25mg a.i./flat DPX M6316 or with 562.5mg a.i./flat of AC 263,499. These quantities correspond respectively to 30, 15 and 150 g a.i./ha. Thirty mL of solution was used for each treatment and the results were scored 23 and 40 days after treatment.

II.D Tissue culture

II.D.1 Media composition

MS medium (Murashige and Skoog 1962) with B5 vitamins (Gamborg et al. 1968) at pH 5.8 was used for the growth of seedlings from various species, shoots from B. napus and N. tabacum, thin cell layer explant

from stem epidermal, microspore-derived embryos of B. napus, leaf discs from N. tabacum, stem sections from B. juncea and B. napus and cell suspensions from B. juncea, B. napus and N. tabacum. For seeds, seedlings, shoots and microspore-derived embryos 1% (w/v) sucrose was added and for the others, 3% (w/v) sucrose was added. All media were sterilized by autoclaving at 121°C for 17 min. The microspores from B. napus were grown on NLN medium (Keller et al. 1987) at pH 6 with 13% sucrose and this medium sterilized by filtration with a 0.21 µm filter. The plant media composition and hormone levels are given in Table II.6.

II.D.2 In vitro growth conditions

All plant tissues and cells were grown under continuous illumination provided by fluorescent lights at $400 \mu\text{Em}^{-2}\text{sec}^{-1}$. The temperature was set at 25°C constant. To examine the influence of herbicides on germination, seedlings were germinated at 20°C, 16 hrs day and 15°C night excepted for G. max and cereals which were germinated at 28°C, 16 hrs day and 25°C night.

II.D.3 Seed and explant sterilization

Seeds were wrapped in filter paper bags (Whatman 3MM) and sterilized in a 2L beaker with 70% (v/v) ethanol, followed by 0.2% (w/v) HgCl_2 and finally undiluted Javex (6% (w/v) Na-Hypochlorite; Bristol-Myers, Toronto, Ontario) for various times (see Table II.7). A few droplets of Tween 20 were added to the last two solutions. Paper bags containing seeds were rinsed 3 times with sterile double distilled water and dried overnight under laminar flow. When contamination was high, the sterilization was repeated a few weeks later; however, the HgCl_2 step was omitted from the protocol (Table II.7).

TABLE II.6. Plant Medium composition

Chemical	MS medium + B5 vitamins	NLN medium	B5 medium
<u>Micronutrients</u>	(mg/L)	(mg/L)	(mg/L)
H ₃ BO ₃	6.2	10	3
MnSO ₄ ·4H ₂ O	22.3	25	
ZnSO ₄ ·7H ₂ O	8.6	10	2
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.25
CuSO ₄ ·5H ₂ O	0.025	0.025	0.025
CoCl ₂ ·6H ₂ O	0.025	0.025	0.025
MnSO ₄ ·H ₂ O			10
<u>Macronutrients</u>			
NH ₄ NO ₃	1650		
KNO ₃	1900	62.5	2500
MgSO ₄ ·7H ₂ O	370	62.5	250
KH ₂ PO ₄	170	62.5	
CaCl ₂ ·2H ₂ O	440		147
KI	0.83		0.83
Ca(NO ₃) ₂ ·4H ₂ O		250	
Sequestrene (Fe-330)	40		40
(NH ₄) ₂ SO ₄			134
NaH ₂ PO ₄ ·H ₂ O			150
<u>Vitamins</u>			
biotin		0.05	
folic acid		0.5	
glycine	2	2	
L-glutamine		800	
glutathione		30	
myo-inositol	100	100	100
nicotinic acid	0.5	5	1
pyridoxine-HCl	0.5	0.5	1
L-serine		100	
thiamine HCl	0.1	0.5	10

MS (Murashighe and Skoog 1962), B5 (Gamborg *et al.* 1968), NLN (Lichter 1982, Keller *et al.* 1987)

TABLE II.6. Plant Media Composition (Continued)

Plant media	Growth regulators (mg/L)			Code name
	BAA	NAA	2,4-D	
<u>Brassica</u> cell suspension	-	-	1	MS2D
<u>N. tabacum</u> cell suspension	-	-	1	MS2D
<u>B. napus</u> thin cell layer explants	10	0.5	-	Eg
<u>B. napus</u> stem section explants	1	-	-	EM
<u>N. tabacum</u> leaf disc explants	1	0.1	-	TLD

All other plant tissues did not need hormones in the medium. All the plant tissue culture media were solidified with 0.6% agarose (SeaKem ME, Mandel Scientific Co., Richmond, Ont.). BAA= 6-benzylaminopurine, NAA= 1-naphthaleneacetic acid, 2,4-D= (2,4-dichlorophenoxy) acetic acid.

TABLE II.7. Evaluation of Seed Sterilization Protocols

Species	treatment in min.			contamination		germination
	Ethanol	HgCl ₂	Javex			
<u>A. fatua</u> *	1	25	20 + 20	++	+-	--
<u>A. thaliana</u>	1	0	20	--		++
<u>B. campestris</u>	1	25	20	+-		++
<u>B. carinata</u> *	1	25	20 + 20	++	+-	--
<u>B. hirta</u> *	1	25	20 + 15	++	--	--
<u>B. juncea</u>	1	25	20	--		++
<u>B. kaber</u>	1	25	20	+-		++
<u>B. napus</u>	1	25	20	--		++
<u>B. nigra</u>	1	25	20	+-		+-
<u>B. oleracea</u>	1	25	20 + 15	++	+-	+-
<u>C. maritima</u>	1	25	20	--		--
<u>G. max</u>	1	30	25	+-		++
<u>H. vulgare</u> *	1	30	40	--		--
<u>M. sativa</u>	1	25	20	--		++
<u>N. tabacum</u>	1	0	25	--		++
<u>R. sativus</u>	1	25	25	+-		++
<u>S. viridis</u>	1	25	20	--		++
<u>T. arvensis</u>	1	30	25	--		++
<u>T. aestivum</u>	1	30	40	+-		+-

Varieties used were Tobin and Candle for B. campestris, Zaria for B. juncea, Westar for B. napus, Ochre for B. hirta and Rapid Cycling for B. oleracea, B. nigra and B. carinata. A. thaliana was Columbia type. *Indicates species difficult to sterilize. Estimations of contamination are ++=strong, +=low, --=none. Germination efficiency was; --= 20%, +- 20 to 70%, ++= 70%. Data collected by J. Gabard.

Stem internodes of B. napus used for the production of thin cell layer and stem section explants and stem internodes of B. juncea used to produce stem sections were sterilized in at least 2L of Javex with a few drops of Tween 20 for 40-50 min and rinsed 2 times with 2L of sterile double distilled water for 1 hr. The internodes were sterilized with a home made apparatus consisting of a plastic reservoir closed at each end with a nylon screen and with a handle to submerge and recover the apparatus. This allowed complete immersion of tissues in liquid with shaking and easy transfer from each solution. This apparatus was critical to improve the sterilization procedure; not more than 20% of the explants were contaminated using this protocol compared to nearly 100% without these improvements.

Buds for microspore preparation were sterilized in 7% (w/v) Ca-hypochlorite for 15 min. followed by 3 rinses with sterile double distilled water.

II.D.4 Stem explant culture of B. napus cv. Westar

Thin cell layers from stems were prepared from 8 week old B. napus cv. Westar plants according to the method of Klimaszewska and Keller (1985). The three or four terminal internodes of early flowering plants were sterilized as indicated in section II.D.3. Thin cell layers were peeled from the stem internodes and cut into 5 mm sections of 1 mm thick. The epidermis of the explant was put side up on the Eg medium (see section II.D.1).

Stem cross sections (Kantha et al. 1974) were prepared by slicing the stem internodes in 2-3 mm thick sections after sterilization as described in II.D.3. The sections were put on the EM medium (section II.D.1) without regard to orientation.

II.D.5 Microspore preparation from B. napus cv. Topas

Microspores were prepared according to the method of Keller et al. 1987. B. napus cv. Topas plants were grown in growth chambers under 16 hrs photoperiod with a light intensity of $300 \mu\text{Em}^{-2}\text{sec}^{-1}$ obtained with fluorescent and incandescent lights. Day/night temperatures were $10^{\circ}\text{C}/5^{\circ}\text{C}$. Inflorescences were harvested at the beginning of blooming and buds of 3-4 mm with a short pedicel were chosen. The sterilization of the buds was performed as described in section II.D.3. After sterilization, the buds were taken in groups of 25 in 5-10 mL of B5 medium with 13% (w/v) sucrose (Gamborg et al. 1968). The buds were macerated with a glass pestle to release the microspores. The mixture was filtered through a 44 μm nylon filter and pelleted by centrifugation (100g, 3min). The pellet was resuspended in 10 mL of B5 medium with 13% (w/v) sucrose and the washing step repeated three times. After the washes, microspores were resuspended to a density of about 10^4 microspores/mL in NLN medium. All the microspore preparations were done by J. Grainger from Dr. W. Keller's laboratory (Agriculture Canada, Ottawa, Ontario).

The microspores were incubated at 32.5°C for 4 days to induce embryogenesis and transferred to 25°C in absence of light for culture. The embryos derived from the microspores were transferred to MS medium with B5 vitamins and 1% (w/v) sucrose and grown under the conditions described in II.D.2.

II.D.6 Antibiotic toxicity tests

B. napus cv. Westar seeds and thin cell layer explants from stems,

and cv. Topas microspores and microspore-derived embryos were tested for their sensitivity to various antibiotics (Clavulin 250, carbenicillin, cefotaxime, Claforan, Timentin, kanamycin, paromomycin, geneticin, chloramphenicol, hygromycin, bleomycin, streptomycin, tunicamycin and methotrexate). Except for the thin cell layer explants from stem epidermal strips, all the antibiotics were added to the tissue culture medium immediately. The thin cell layer from stem epidermal strips were precultured 4 days, cocultivated with A. tumefaciens (strain GV3101, ampicillin sensitive derivative) for a period of 2 days and transferred to the medium with carbenicillin and the selective antibiotic. Clavulin 250, carbenicillin, cefotaxime, Claforan and Timentin were assessed for the efficiency to eliminate A. tumefaciens after cocultivation and used at 500 mg/L. All the tissue culture media and growth conditions were described in previous sections. Weights of the tissues and final observations were taken after 6 weeks.

II.D.7 Agrobacterium-explant cocultivation

II.D.7.a N. tabacum leaf disc cocultivation

N. tabacum cv. Petit Havana SR1 leaf discs of 10 mm diameter were excised from in vitro grown plantlets and put on a nurse culture consisting of 3 mL of a 5 day old N. tabacum cell suspension on the surface of TLD medium. The cells were covered by two layers of sterile whatman #2 filter paper to separate the leaf discs, deposited epidermis facing down, from the nurse culture.

After a precocultivation period of two days, leaf discs were dipped in an overnight Agrobacterium suspension for 30-60 sec and

blotted on a sterile filter paper. They were returned to the nurse culture for a cocultivation period of 2 days and transferred to TLD medium containing cefotaxime (500 mg/L) and the selective agent (see Table II.8 for level of antibiotics used in N. tabacum cocultivation experiments).

II.D.7.b B. napus explant cocultivation

B. napus explants were inoculated after a 4 day precocultivation period by dipping them in an overnight Agrobacterium suspension for 30-60 sec followed by blotting on sterile filter paper. During precocultivation and cocultivation, the explants were cultured epidermal side down on a nurse culture consisting of 3 mL of 5 days old N. tabacum or B. napus cell suspension spread on top of the Eg (triple cell layer explants) or the EM (stem section explants) covered by two layers of sterile Whatman #2 filter paper. The cocultivation period was two days unless otherwise indicated. The explants were put on medium with Clavulin 250 (500 mg/L) plus the selective agent (see Chapter IV for the levels used).

II.D.8 Shoot culture

Aseptically grown shoots of N. tabacum cv. Petit Havana line SR1 were obtained from B. Bowen (Agrigenetics Corp., Madison, Wisconsin). They were propagated by transferring terminal or axillary buds with a stem piece to fresh MAH medium (see section II.D.1). These plantlets were used as a source of N. tabacum leaf discs (section II.D.7.a).

Shoots (about 5-10 mm) arising from N. tabacum leaf disc after cocultivation were excised and transferred to MAH medium containing 500

2

Microlith

TABLE II.8. Concentration of Antibiotics Used as Seletive Agent in
N. tabacum Cocultivation Experiments

Antibiotic	Concentration (ug/mL)
Chloramphenicol	20
Hygromycin	5
Kanamycin sulfate	100
Methotrexate	2

ug/mL cefotaxime and the selective agent at the concentration specified in Table II.8.

Shoots (about 5-10 mm) developing from B. napus explants after cocultivation were excised and transferred to MAH medium containing 500 mg/L Clavulin 250 and the selective agent as discussed in Chapter IV.

II.D.9 Transfer of in vitro grown shoots to growth chamber or greenhouse

Well developed shoots of N. tabacum or B. napus with well formed root systems were transferred to soil in growth chambers or greenhouse. They were covered with a plastic bag for a week to insure proper adaptation of the plantlets.

II.D.10 Seedling germination in the presence of herbicides in vitro

Ten to 50 sterile seeds were grown aseptically in GA-7 Magenta boxes (Magenta Corporation, Chicago, Illinois, USA) or in 100X20 mm Petri dishes for A. thaliana and N. tabacum. Fifty or 20 mL of MAH medium were used respectively. Herbicide levels were 0,10,30,100,300 ug/L for sulfonylureas and 0,10,30,100,300 and 1000 nM for imidazolinones. Except for A. fatua, H. vulgare, B. hirta and C. maritima, 10 seedlings at each herbicide level were picked randomly and weighed after 2 to 4 weeks. The average fresh weight was calculated at each herbicide concentration and each measurement was duplicated.

II.D.11 Protoplast preparation for chloramphenicol acetyltransferase assays

Protoplasts of B. napus cv. Westar were prepared from thin cell

layer explants as described by Klimaszevska and Keller (1987). Thin cell layers were prepared from sterile internodes (section II.D.3) by peeling the epidermis plus several layers of subepidermal cells. These strips were precultured for 16-24 hrs in MS medium with B5 vitamins and 2% (w/v) sucrose at pH 5.8 and incubated in an enzyme mixture containing 13% (w/v) mannitol, 1% (w/v) macerozyme and 1% (w/v) cellulase "Onozuka R-10" at pH 5.8 for 15-18 hrs in darkness with shaking (26 strokes/min) at 25°C. After digestion, the protoplasts were filtered through a 44µM nylon mesh and centrifuged (100g, 3min) and washed 3 times in MS liquid medium with B5 vitamins plus 13% (w/v) mannitol at pH 5.8. The protoplasts were then homogenized with 50 strokes of a Dounce homogenizer in the assay buffer (see section II.E.2).

Protoplasts from cell suspension cultures of B. napus cv. Jet Neuf were prepared by digestion of cells for 18 hrs in MS medium with B5 vitamins containing 13% (w/v) mannitol, 2% (w/v) cellulase and 0.5% (w/v) macerozyme at pH 5.8. The protoplasts were filtered through a 44 µM nylon mesh and centrifuged (100g, 3min). They were washed 3 times in the same medium without enzymes and resuspended in the CAT assay buffer and treated as described in the previous paragraph.

II.D.12 Cell suspension sources and culture

B. napus cv. Jet Neuf and Topas cell suspensions were obtained from Dr. W.A. Keller (Agriculture Canada, Ottawa, Ontario, Canada). N. tabacum and A. thaliana columbia type line GH50 cell suspensions were obtained for Dr S. Gledzie (Agriculture Canada, Ottawa, Ontario, Canada). All the cell suspensions were grown in liquid MS2D medium

(see section II.D.1) in 125 mL flasks containing 25 to 40 mL of culture medium. They were transferred weekly or bi-weekly. The cells were allowed to settle for about 15 min and the supernatant removed. A volume of about 5-10 mL of cells was transferred to fresh medium and the cell suspensions were grown in the same conditions as described before with shaking at 150 rpm.

II.E Enzymatic assays

These assays were used to identify and characterize transgenic plants and to assess the expression of the introduced genes in transgenic plants.

II.E.1.a Opine synthase assays

The opine synthase assays are modified from Holbrook et al. (1986). From 10 to 200 mg of leaf material frozen in liquid nitrogen were ground in a 1.5 mL ppendorf tube with ice-cold extraction buffer (3uL/1 mg of plant material) with a pinch of washed sand. The extraction buffer was prepared fresh from: stock A (0.25M Tris, 1.25M sucrose, stored at 4°C, sterile), stock B (0.5% (w/v) ascorbic acid, 0.5% (w/v) cystein, stored at -20°C) and stock C (250mg/mL Bovine serum albumin (BSA), 60ug/mL leupeptine, stored at -20°C). Two volumes of solution A were mixed with 1 volume of solution B and 2 volumes of solution C. To this, 25mg/mL of nicotinamide adenine dinucleotide (NADH) was added and the pH adjusted with concentrated hydrochloric acid (HCl) to 8.

The tissue extracts were centrifuged (10 000g, 30 sec). Four uL of supernatant were mixed with 3 uL of reaction buffer and the mixture

incubated at room temperature (22°C) for 1 hr. The reaction buffer for the nopaline synthase assay was prepared from: stock D (240mM α -ketoglutarate, stored at -20°C), stock E (1M Na-Phosphate, pH 7.5, stored at room temperature and stock F (40 to 60mCi/mmol $[^{14}\text{C}]$ -arginine, stored at -20°C). Thirty six μL of solution D were mixed with 27 μL of solution E, 45 μL of solution F and 25mg/mL of NADH added. The pH was adjusted with potassium hydroxide (KOH, 5N). For the octopine synthase assay, 36 μL of 240mM pyruvic acid (stored at -20°C) were added to solutions E and F.

After incubation, 4 μL of the reaction mixture was spotted on MN-214 chromatographic paper (19X25 cm). The samples were spotted 6 cm from the bottom and 12 to 14 samples could be applied to one sheet of paper. Once dried, the samples were ran to the anode in running buffer (5% v/v formic acid 15% v/v acetic acid, 80% v/v distilled water) for 1 hr at a constant voltage of 400 V.

After electrophoresis, the paper was dried in a fume hood with a hair dryer or air dried. Forty mL of 2,5-diphenyloxazole (PPO 8% w/v) in ether were poured evenly on the paper. The paper was dried and exposed to XAR-5 film (Eastman Kodak Co., Rochester, New York, USA) in an X-ray cassette for 24 to 48 hrs at -80°C . Plate II.1 shows typical results from a nopaline synthase assay (Plate II.1.a) and an octopine synthase assay (Plate II.1.b).

II.E.1.b Opine detection

For opine detection, the extraction buffer was different from the enzyme assay extraction buffer. It did not contain leupeptine and BSA (solution C). The tissue extract (4 μL) was spotted directly on the

Plate II.1 Opine synthase assays.

A) nopaline synthase assay; arg=[^{14}C]arginine, nop= [^{14}C]nopaline, n= negative control, p= positive control, other lanes= transformed plant tissue samples. B) octopine synthase assay; arg= [^{14}C]arginine, oct= [^{14}C]octopine, n= negative control, p= positive control, other lanes= transformed tumour tissues.

chromatographic paper and run as indicated in section II.E.1.a.

Staining was done by spraying the dry paper with a solution containing 16 mL of absolute alcohol, 4 mL of water, 1 g of sodium hydroxide (NaOH) and 2 mg of phenanthrenequinone. It was dried and viewed under UV light. Plate II.2 shows typical results from a nopaline (Plate II.2.a) and an octopine detection (Plate II.2.b).

II.E.2 Chloramphenicol acetyltransferase (CAT) assay

The CAT assay was modified from the method of Gorman *et al.* (1982). 100 mg (fresh weight) of plant tissue were ground in 100 μ L of extraction buffer (0.2M Tris-HCl pH 8, 0.5M sucrose, 0.1% w/v ascorbic acid, 1mM DL-dithiothreitol, 0.8 mM phenylmethylsulfonyl fluoride) with a pinch of washed sand at 4°C. The debris was spun down (10 000g, 5 min). Fifty μ L of supernatant was removed and combined with 0.5 μ L of [14 C]-chloramphenicol (40 to 50 Ci/mmol, Dupont NEN) and 5 μ L of 16mM acetyl CoA. The mixture was incubated at 37°C for 30 min. The chloramphenicol together with its acetylated derivatives were extracted by vortexing with 1 mL of ethyl acetate for 15 sec. The organic phase was collected and evaporated. The residue was resuspended in 40 μ L of ethyl acetate and samples were spotted on thin layer chromatography plates (Redi-Plate, Silica gel GF, Fisher Scientific Co.). Ascending chromatography was performed with a 95:5 mixture of chloroform:butanol. The TLC plates were dried and exposed directly to Kodak XAR-5 films overnight. The percent conversion of [14 C]-chloramphenicol to its acetylated forms was determined by collecting the silica matrix from the TLC plate and counting the radioactivity in a Beckman scintillation counter (model LS7800) using Omnifluor (Dupont NEN) dissolved in toluene as a scintillation cocktail.

Plate II.2 Opine detection assays.

A) octopine detection assay; arg= arginine, oct= octopine, lanes 1-4= extracts from octopine Ti tumours, lane 5= negative control.

B) nopaline detection assay; arg= arginine, nop= nopaline, lanes 1-3= extracts from nopaline Ti tumour tissues, lanes 4-5= negative control.

Inhibition of CAT activity in Brassica species was determined by assaying CAT activity under the following conditions. Extracts from N. tabacum and B. napus (50 uL) were mixed with 25 units of CAT enzyme (Pharmacia Canada, Montreal, Canada). Cell free extracts of transgenic N. tabacum were mixed in various proportions (50:0, 40:10, 30:20, 25:25, 20:30, 10:40 uL of N. tabacum:uL of B. napus) with B. napus extracts. Transgenic N. tabacum extracts (30uL) were mixed with extracts (20uL) from non-transformed N. tabacum, A. thaliana, G. max, L. esculentum and B. juncea.

Cell free extracts of B. napus were also digested with proteinase K (Boehringer-Manheim, Montreal, Canada) for 30 min at 37°C and the CAT assay performed on the digested extract as described above. B. napus cell free extract (200 uL) was also dialyzed against 1L of CAT assay reaction buffer for 2 hours and CAT assay performed on 50 uL of the dialyzed extract.

II.E.3 Neomycin phosphotransferase (NPT II) assay

The NPT II assay was performed according to the method of McDonnell et al. (1987). Plant tissue frozen in liquid nitrogen was ground and cold extraction buffer added (1 uL per mg of tissue). The extraction buffer consisted of 20% (v/v) glycerol, 10% (v/v) b-mercaptoethanol, 0.2% (w/v) sodium dodecyl sulfate and 125mM tris-HCl pH 6.8. After mixing with a vortex for 45 to 60 sec and centrifuging (10 000g, 5 min) at 4°C, 15 uL of supernatant was mixed with 15 uL of the incubation buffer (prepared as follows; 5mL of Tris-Maleate (67mM), pH 7.1, MgCl₂ (42mM) and NH₄Cl (0.4M) were mixed with 5 uL of adenosine triphosphate (sodium salt, 10mM), 7 uL of neomycin sulfate (22mM), 50

uL of NaF (10mM) and 10 uL of [32 P]-g-ATP (10 uCi/uL, Amersham Canada Ltd., Oakville, Ontario). The reaction mixture was incubated at 37°C for 30 min.

After incubation, the reaction mixture was spun down (10 000g, 5 min) and the supernatant (20 uL) was spotted on cellulose phosphate paper (Whatman P81) previously soaked in a solution of ATP (20mM) and pyrophosphate (100mM) and dried. Each spot was allowed 4 cm² for spreading. After the spots were dried, the paper was washed in a Na-Phosphate buffer (10mM, pH 7.5) at 80°C for 10 min. The washes were repeated 3 to 5 times and the paper allowed to dry. Once dry, the paper was exposed to Kodak XAR-5 film overnight. Each square was also counted as described in section II.E.2 for the CAT assay.

II.E.4 Acetolactate synthase assay

Plant material was harvested 2 to 5 weeks after germination depending on the growth rate of the different species. One gram of fresh young growing leaves was frozen in liquid nitrogen and stored at -70°C for up to 10 months. Frozen leaves were ground to a powder in a mortar containing liquid nitrogen. The powder was poured into 3 mL of extraction buffer supplemented with 250 mg polyclar AT per gram fresh weight. For A. thaliana GH50 and columbia wild type cell suspensions, 150 mg of the cell suspension culture was collected 4 days after subculture and homogenized in a motor driven glass tissue grinder on ice for 1 min in 1 mL of extraction buffer containing 40 mg of Polyclar-AT. The homogenate was cooled for 1 min and the procedure was repeated 3 times.

The ALS activity was measured using the method described by Chaleff and Ray (1984) modified by Haughn and Somerville (1986). The extraction buffer consisted of KH_2PO_4 (100mM, pH 7.5), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5mM), sodium-pyruvate (1mM), thiamine pyrophosphate (TPP, 0.5M), flavin adenine dinucleotide (FAD, 10uM) and 10% (v/v) glycerol and was made fresh from a stock solution containing the first three elements, and separate stock solutions for all the other elements.

The crude tissue extract was partially purified. After centrifugation (10 000g, 15 min) at 4°C, the supernatant was mixed with equal volume of saturated ammonium sulfate solution to precipitate the proteins for 1 hr. During that time, Sephadex G-25 (DNA grade, NAP-10 or NAP-15, Pharmacia Canada, Montreal, Canada) columns were prepared by rinsing them 4 times with a total of 20-25 mL of base extraction buffer (without TTP, FAD and glycerol).

The protein precipitate was spun down (10 000g, 5 min) and resuspended with 1 to 2 mL of base extraction buffer. The sample was applied to the column and eluted with 2 to 5 mL of base extraction buffer.

The enzyme assay was done by mixing 100 uL of eluant, 400uL of reaction buffer (56mM KH_2PO_4 pH 7.5, 50mM Na pyruvate, 12.5mM MgCl_2 , 250uM TPP, 23uM FAD) and the herbicide stock if required. The mixture was incubated for 90 min at 30°C and the reaction was stopped by adding 250 uL of H_2SO_4 (6N). The zero reaction was obtained by adding the eluant after adding the acid. Each reaction was done in triplicate. The mixture was then incubated 10 min at 55°C. After incubation, 127 uL of NaOH (50% w/v) was added to one tube and the pH

checked with pH 6.5-10.0 ColorpHast paper (BDH Inc, Toronto, Ontario). By varying the volume 1 uL at a time, the amount of NaOH required to obtain pH 7-11 was determined and added to all the samples. Next, 150 uL of creatine (0.5% w/v) and 150 uL of α -naphthol (5% w/v dissolved in 2.15M NaOH, prepared just before use) were sequentially added. The mixture was incubated 10 min at 55°C and the tubes centrifuged (10 000g, 5 min) at room temperature. The optical density was read at 530nm with the reaction buffer as the blank.

Herbicide levels used were 0, 0.05, 0.5, 1.5, 5, 50, 500uM for AC 263,499 and AC 243,97. With AC 252,214, the maximum concentration used was 250 uM. Concentrations of 0, 1, 3, 1, 30, 100 and 300ug/L were used for DPX M6316, DPX A7881 and DPX L5300. For chlorsulfuron the levels used were 3 to 100 ug/L. ALS measurements were made in triplicate and the percent inhibition of ALS activity at each concentration of herbicide was determined.

II.F Plant DNA analysis

II.F.1 Plant DNA isolation

Plant DNA isolation was performed using the method of Lichtenstein and Draper (1982). Freeze-dried plant leaf tissues (1g) were mixed with 4g of aluminium oxide (Sigma, type 305) and ground to a very fine powder with a pestle and mortar. The powder was put in 50 mL polypropylene centrifuge tube and mixed with 20 mL of extraction buffer (50mM Tris-HCl pH 8, 0.7M NaCl, 10mM ethylene diaminetetraacetic acid(EDTA), 1% w/v cetyltrimethylammonium bromide (CTAB), 1% w/v β -mercaptoethanol). The powder was homogeneously dispersed and the mixture incubated at 60°C for 30-60 min with occasional shaking.

The incubation mixture was allowed to cool to room temperature and extracted with 20 mL of chloroform:octanol (24:1 v/v). The debris was pelleted and the organic phase separated from the aqueous phase by centrifugation (2 000g, 15 min) at room temperature. The top aqueous phase was recuperated and mixed with an equal volume of isopropanol by inversion. The precipitated DNA was either spooled or centrifuged (2 000g, 5 min) and resuspended in TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). The DNA was then put on a CsCl gradient for banding.

II.F.2 CsCl DNA banding

For 9.2 mL of DNA resuspended in TE buffer, 9.2 g of CsCl and 1 mL of ethidium bromide (10 mg/mL in TE) were added. The mixture was put in a "quick seal" ultracentrifuge tube (Beckman Instruments Inc, Palo Alto, California, USA) and sealed by heating. The tubes were centrifuged (150 000g, at least 36 hrs) at 20°C. After centrifugation the DNA band was visualized under UV illumination and taken out with a syringe using a #21 hypodermic needle.

The ethidium bromide was extracted with an equal volume of 1-butanol saturated with distilled water. This was repeated 3 to 5 times until the pink color was eliminated. After extraction, the aqueous phase was dialyzed against 3-4L of TE for at least 5 hrs. The DNA in TE was precipitated by adding 1/10 volume of Na-acetate (3M) and 2 volumes of ethanol (95% v/v). The precipitate was spun down (3 000g, 20 min) and resuspended in TE to concentrations varying from 0.1 to 1 µg/mL. The DNA concentration was determined by measuring the absorbance at 260nm and the purity evaluated by absorbances at 280nm and 360nm.

II.F.3 Plant DNA digestion

Ten or 20 ug of plant DNA in TE were digested at 37°C with 5 units/ug of restriction enzyme (Hind III, Bam HI, Eco RI, Pst I and Xba I). The incubation buffer (10X) provided by the supplier, 10 uL of BSA (1 mg/mL, DNAase free, Pharmacia Canada, Montreal, Canada), 10 uL of spermidine tetrahydrochloride (50mM pH 7.5) were brought to a total volume of 400 uL with sterile double distilled water. The reaction was stopped by adding 1/10 volume of Na acetate (3M) and 2 volumes of cold ethanol (95% v/v). The precipitate was centrifuged (10 000g, 30 min) at 4°C and resuspended in 50 uL of TE.

II.F.4 Southern Blotting

The Southern blotting was performed according to the method of Bernatzsky and Tanskley (1986).

II.F.4.a DNA electrophoresis

Digested plant DNA (10 or 20 ug) was separated on a 1% (w/v) agarose gel (SeaKem ME, Mandel Scientific Co., Richmond, Ontario) in TAE buffer (0.04M Tris-acetate, 0.001M EDTA, 300mL, 20 X 25 cm) and run at 60V overnight until the tracking dye was 15 cm from the wells. The loading buffer consisted of bromophenol blue (0.25% w/v), xylene cyanol (0.25% w/v) and Ficoll (Type 400, 25% w/v) dissolved in double distilled water. For 50 uL of DNA solution, 20 uL of loading buffer was added. The molecular weight standard consisted of 5 ug of phage DNA (Boehringer Mannheim, Montreal, Canada) digested with Hind III and Eco RI.

II.F.4.b Gel staining with ethidium bromide

The gel was immersed for 30 min at room temperature in TAE buffer containing 0.5 ug/mL ethidium bromide. The DNA was visualized directly after staining on a UV transilluminator. The Plate II.3 shows a typical DNA digest on an agarose gel.

II.F.4.c DNA transfer to the nylon membrane

The gel was treated in dilute acid (0.25N HCl) for 10 min with gentle shaking and rinsed with distilled water. Subsequently, the gel was soaked with gentle shaking for 20 to 30 minutes to denature DNA in NaOH (0.5M) and NaCl (0.5M). The gel was then neutralized by a solution of Tris HCl (0.5M) and NaCl (1M) for 20 to 30 min. The gel was then deposited on a sponge covered by 2 sheets of Whatman 3MM paper. The sponge was previously put in a reservoir containing the blotting buffer (10X SSC; 1.5M NaCl, 1.5M Na-Citrate pH 7.0). The nylon membrane (Nytran from Schleicher and Schuell, Keene, New Hampshire, USA or Zeta-Probe from Bio-Rad, Mississauga, Ontario, Canada) was put on top of the gel and covered by two sheets of Whatman 3MM paper soaked in blotting buffer. A stack of blotting paper (about 10 cm) was placed on top with a 500 g weight. The transfer was done for 12 to 16 hrs.

After transfer, the membrane was washed for 20 min in 1X SSC (0.15M NaCl, 0.15M Na-citrate pH 7.0) at 42°C with shaking. The membrane was dried at 80°C for 2 hrs and UV irradiated for 5 minutes wrapped in "Saran-wrap" (DCP Canada Inc., Paris, Ontario) DNA face down on a UV transilluminator. The filter were then stored until used at -4°C.

Plate II.3 Plant DNA digest with Bam HI and Hind III on a 1% agarose gel.

The lane on the right has the molecular weight ladder (phage lamda digested with Eco RI and Hind III) and the sizes of the bands are given in base pairs in the right margin.

II.F.4.d Probe preparation

Fragments of plasmids (see section II.G.3) were used for nick translation using the Nick translation kit of Bethesda Research Laboratories (Burlington, Ontario). 100 to 500 ng of DNA were labeled with [^{32}P]- α -dCTP to give a specific activity of about 1×10^8 cpm/ μg . After nick translation, the reaction was stopped by adding an equal volume of stop buffer (50 μL , not from the kit, 125mM EDTA, 10% w/v SDS, 0.25% w/v bromophenol blue, 0.25% w/v blue dextran). The mixture was passed through a Nick column (Sephadex G-50 DNA grade, Pharmacia Canada, Montreal Canada) equilibrated with EDTA (125mM) and SDS (10% w/v) and the first blue phase collected. This separated the non incorporated nucleotides from the labeled fragments. The total radioactivity was counted by blotting 5 μL of the eluant on a GF/B glass fiber filter (Whatman Int. Ltd., Maidstone, England). The filter was then put in a scintillation vial containing Omnifluor dissolved in toluene and the radioactivity counted on a Beckman (model L5700) scintillation counter.

II.F.4.e DNA filter hybridization

The nylon membranes with the transferred DNA were washed in NaOH (0.4N) for 30 sec and then rinsed in 0.2M Tris-HCl pH 7.5, 2X SSC (0.3M NaCl, 0.3M Na citrate pH 7.0) for 5 minutes. The membranes were prehybridized for 6 hrs at 68°C in a solution containing 5X SSC (0.75M NaCl, 0.75M Na-citrate pH 7.0), NaPO_4 (50mM pH 7.2), SDS (0.4% w/v), 5X Denhardt's solution, EDTA (2.5mM) and 100 $\mu\text{g/mL}$ of denatured salmon sperm DNA (Sigma chemical Co, St-Louis, USA). The 1X Denhardt's solution consisted of Ficoll (0.02% w/v), polyvinylpyrrolidone

(0.02% w/v) and bovine serum albumin (0.02% w/v). The prehybridization and hybridization were done in "Rubbermaid trays" and a plastic sheet was put on top of the hybridization mixture to avoid evaporation.

The hybridization was done by adding the denatured probe (heated at 100°C for 10 min and rapidly cooled on ice) to the prehybridization mixture. The hybridization was for 12 hrs at 68°C without shaking.

II.F.4.f Nylon membrane washing after hybridization

After hybridization, the membranes were washed twice in 2X SSC (0.75M NaCl, 0.75M Na citrate pH 7.0) with SDS (0.1% w/v) for 20 min at 68°C; twice in 1X SSC (0.15M NaCl, 0.15M Na-citrate pH 7.5) and SDS (0.1% w/v) for 10 min at 68°C and rinsed in 0.5X SSC (75mM NaCl, 75mM Na-citrate pH 7.5) at 68°C. The membranes were placed in plastic bags and exposed to Kodak XAR-5 films for autoradiography at -70°C.

II.F.4.g Removal of the probe from hybridized filters

The probe was stripped from nylon membranes by washing in NaOH (0.1N) at 42°C for 30 min and rinsing in Tris-HCl (100mM pH 7.5), 0.1X SSC (0.15M NaCl, 0.15M Na-citrate pH 7.5) and SDS (0.1% w/v) for 20 min at 42°C. The membranes were kept wet to allow probe removal.

II.6 Plasmid isolation from E. coli, digestion and fragment recovery

II.6.1 Plasmid isolation

The plasmid DNA isolation method was from Maniatis et al. (1982) as modified by Tartof and Hobbe (1987). Overnight cultures of E. coli (0.4 to 1L) were grown at 37°C in TB medium (12g/L tryptone, 2g/L

yeast extract, 4mL/L glycerol, 100mL/L of (0.17M KH_2PO_4 + 0.72M K_2HPO_4) with shaking. The cells were harvested by centrifugation at 3 000g, 10 min and resuspended in 20 mL of lysozyme (5 mg/mL), glucose (50mM), Tris-HCl (25mM pH 8.0) and EDTA (10mM) per 500 mL of cell suspension. After incubation for 5 min at room temperature, 40 mL of NaOH (0.2N) and SDS (1% w/v) was added and mixed. The mixture was incubated 10 min on ice and 30 mL of potassium acetate (5M pH 5) was added. After centrifugation (27 000g, 30 min), the supernatant was recovered and mixed with 0.6 volumes of isopropanol. After 15 min at room temperature, the mixture was centrifuged (3000g, 30 min) at room temperature and the pellet washed with 70% (v/v) ethanol. The pellet was centrifuged (3000g, 30 min), dried and resuspended in 10 mL of TE (see section II.F.1). After this step, the DNA was purified on a CsCl gradient by banding as described for plant DNA in section II.F.2.

II.6.2 Plasmid DNA restriction digests

Plasmid DNA was digested by adding 1unit/ug of restriction enzyme, the incubation buffer (10X) provided by the supplier, bovine serum albumin and the total volume raised to 4 to 20 uL per ug of DNA. The digestion were performed at 37°C for the enzymes used in this study.

II.6.3 Plasmid fragment isolation

Digested plasmids were run on a 1% agarose gel (SeaKem ME, Mandel Scientific Co., Richmond, Ontario), (mini-gel, 5X7.5 cm) in TAE buffer (see section II.F.4.a) at 80V for 2 hrs. The loading buffer was as described in section II.F.4.a and used the same way. After electrophoresis and ethidium bromide staining of the gel (as described in II.F.4.b), the fragment of interest was cut from the gel and

extracted from the agarose with the "Geneclean" kit (BIO 101 Inc, La Jolla, California, USA). The fragment was redissolved in TE (see section II.F.1) and its concentration evaluated with a TK-100 mini-fluorometer (Hoeffer Scientific Instruments, San Francisco, California, USA). Volumes of DNA solution as low as 1 μ L were used and mixed with 2 mL of working dye solution (10mM Tris-HCl pH 7.4, 1mM EDTA, 0.1M NaCl, 0.1 μ g/mL Hoechst 33258).

II.H Intermediate vector construction

Five intermediate vectors were constructed specifically for this thesis. The binary vector pBINCH was made for the initial herbicide resistance and cross resistance work in N. tabacum. The intermediate vectors for cointegration p35SALSW, p35SALSR, pKALSW and pKALSR were made to complete that study and to engineer B. napus. These last vectors were made by J. Hattori from Dr. B. Miki's laboratory.

II.H.1 Schemes of construction and maps of the vectors constructed

II.H.1.a Construction of pBINCH

The vector pBINCH was constructed by isolating the 5.8 kb Xba I fragment from the vector pGH1 (Haughn et al. 1988) and by ligating it into the Xba I site of the binary vector pBIN19 (Bevan 1984). The positive clones were screened by the inactivation of the b-galactosidase gene of the multiple cloning site. The maps of these two vectors are given in the appendices.

II.H.1.b Construction of p35SALSW, p35SALSR, pKALSW and pKALSR

The Figure II.1 illustrates the scheme that was followed to construct the intermediate vectors.

This set of vectors has been constructed for cointegration with the modified T-DNA of pGV3850 (Zambryski et al. 1983) which contains pBR322 sequences. For this reason, the plasmid pBR322 was chosen as the cloning vehicle for the intermediate vector constructions. The first step was to introduce the 0.8kb Hind III-Bam HI fragment of pBI121.1 (Jefferson et al. 1987) containing the 35S promoter of the Cauliflower mosaic virus in the Hind III-Bam HI sites of pBR322. Then, the resulting plasmid (p35S) was cut with Eco RI and ligated to the 1.7kb Eco RI fragment from pMON273 which contains the plant marker gene NPT II (Sanders et al. 1987). This chimeric gene was composed of the neomycin phosphotransferase gene II of Tn5 fused to the 35S promoter of CAMV, and the nopaline synthase gene termination sequences. This plasmid was digested with Cla I, the protruding ends filled in and the kanamycin resistance GeneBlock (1.5kb; Pharmacia Canada, Montreal, Canada) introduced by blunt end ligation. The resulting plasmid is shown as pK in Figure II.1.

To construct the vectors with the complete acetolactate synthase gene (ALS; promoter-coding sequences-terminator), the Pst I-Xba I 5.8kb fragment containing the ALS gene was isolated from pGH1 (Haughn et al. 1988) for the mutant ALS gene (ALSR) and from pTZ-ALSW (J. DeMoor and J. Hattori, unpublished) for the wild type ALS gene (ALSW) and ligated to p35S previously cut with Xba I and partially with Pst I. The resulting plasmid (P1) and the pK plasmid were cut with Sal I and partially with Pst I. The fragment of the plasmid P1 containing the

Figure II.1 Schemes of the intermediate vector construction (pKALSR, p35SALSR, pKALSW, p35SALSW). The description of the different steps is given in section II.H.1. The sources of the plasmids are given in Table II.3 and their maps are in appendices.

ALS^R (Pstl) Material (or ALS^R)
from PZ ALS^R for PUC ALS^R

PK-ALS^R (or PK ALS^R)

355 (Pstl) Material (or)
from 355 ALS^R

355-ALS^R (or 355 ALS^R)

B : BamHI
E : EcoRI
N : NcoI
P : PstI
S : SalI
X : XbaI
(X/N) the site destroyed

ALS gene and the fragment of pK containing the NPT II and kanamycin resistance genes were ligated together to give the vector pKALSR or pKALSW depending on which ALS gene was used.

To construct the vectors with the ALS genes combined with the 35S promoter, the plasmid pGH1 or pTZ-ALSW were digested with Nco I and Pst I and the Nco I site of the ALS gene fragment was filled in. The 35S promoter fragment from pBI121.1 was recovered and the Pst I and Xba I ends filled in. The 35S fragment (Pst I-blunt end) was ligated into the Pst I-blunt end sites of the digested plasmid to give the intermediate plasmid P2.

From this plasmid (P2), the Pst I and Xba I fragment containing the chimeric ALS gene was cut out and ligated in the Pst I -Xba I sites left by the removal of the 35S promoter from p35S. The Pst I-Sal I fragment of the resulting plasmid (P3) was ligated into the Pst I-Sal I sites of pK resulting from the removal of the 35S promoter. The intermediate vectors p35SALSR and p35SALSW were obtained from both the wild type and the mutant ALS genes.

The 4 intermediate vectors are illustrated in Figure II.1.

II.H.2 Ligation of DNA fragments

Ligation of DNA fragments was done in a volume of 15 to 20 μ L at 12⁰C for 14-16 hrs in the ligase buffer (66mM Tris-HCl pH 7.5, 1mM EDTA, 10mM MgCl₂, 10mM DTT, 10mM ATP, 5% w/v PEG 8000) and the T4 DNA ligase (0.13 unit /15-20 μ L for sticky ends and 1 unit/15-20 μ L for blunt end).

II.H.3 Transformation of E. coli competent cells

Transformation of E. coli competent cells obtained from Bethesda Research Laboratories (strains DH5 a or HB101) was performed according to the suppliers instructions. The competent cells were removed from the -70°C freezer and thawed on ice in a cold room. The DNA was added to the cells and the mixture shaken for 5 sec. The cells with the DNA were incubated on ice for 30 min and subsequently heat shocked for 45 sec in a 42°C water bath without shaking. They were placed on ice for 2 min and mixed with 0.9 mL of room temperature SOC (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl_2 , 10mM MgSO_4 , 20mM glucose, pH 7.0). The mixture was shaken at 37°C for 1 hour and the cells plated on LB (see section II.B.2) with X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) for strain DH5a and the appropriate antibiotics or without X-gal for HB101. Dilutions of 10^{-1} , 10^{-2} , 10^{-3} were done to obtain discrete colonies.

II.H.4 Minipreparations of plasmid DNA

To verify the construction of plasmid vectors, restriction digests were performed from minipreparations of plasmids. An overnight culture of transformed E. coli was grown in 10 mL TB (see section II.G.1) containing the appropriate antibiotics and 1 mL was centrifuged (10 000g, 5 min). The supernatant was discarded and the pellet dried. The pellet was then resuspended in LiCl lysis buffer (50mM Tris-HCl pH 7.5, 63mM EDTA, 2.5M LiCl, 0.4% v/v Triton X-100) and 10 μL of lysozyme (10 mg/mL) was added. The mixture was boiled for 1 min in a water bath and chilled on ice for 5 min. After centrifugation

(10 000 g, 5 min), the supernatant was recovered and 2 volumes of ethanol 95% (v/v) added. After 30 min at room temperature, the precipitate was pelleted by centrifugation (10 000g, 10 min) and washed with 1 mL of ethanol 70% (v/v). The pellet was air dried and resuspended in 50 uL of TE. The plasmid DNA was digested as described in II.G.2 and the restriction patterns determined as described in II.G.3.

II.1 VIR promoter/Lac Z fusion induction experiments

II.1.1 Growth of A. tumefaciens merodiploid strains containing the VIR promoter/lac Z fusion plasmids

All the merodiploid strains were grown on AB minimal medium (Chilton et al. 1974). Its composition is given in Table II.9. The medium was also supplemented with kanamycin (150 ug/mL) and carbenicillin (150 ug/mL).

II.1.2 Plant tissues and extracts for VIR induction experiments

Sterile stem sections of 4 day old B. napus and B. juncea were sterilized as described in section II.D.3 and grown on Eg medium (section II.D.1) to perform VIR induction experiments. N. tabacum leaf discs grown on TLD for 2 days were also used. In all cases, feeder layer culture was not used.

Extracts were prepared from these explants by incubating them for four days in liquid MSBS medium (section II.D.1) and then filter sterilizing the conditioned medium through a 0.21 um filter.

TABLE II.9. Composition of AB Minimal Medium

	Per liter
K_2HPO_4	3g
NaH_2PO_4	1g
$MgSO_4 \cdot 7H_2O$	0.3g
KCl	0.15g
$CaCl_2$	0.01g
$FeSO_4 \cdot 7H_2O$	2.5mg

-These chemicals are put in solution together,
boiled for 10 min and sterilized by autoclaving

-The pH is adjusted to 7.2 prior to autoclaving

NH_4Cl	1g
Glucose	5g

-These two chemicals are sterilized separately and
added to the medium after autoclaving

II.I.3 VIR induction experiments

The induction medium consisted of 1 mL of liquid MSB5 medium mixed with 1 mL of AB minimal medium, 150 ug/ml of carbenicillin, 150 ug/mL of kanamycin. Explants were put directly on the induction medium according to their weight (0.6g of B. napus and B. juncea stem section explants and 0.4g of N. tabacum leaf disc explants). Acetosyringone (50uM) was used as an positive control for induction. A. tumefaciens merodiploid strains were added to an O.D. of 0.1 of bacteria. The mixture was incubated 12-14 hrs and the b-galactosidase assay performed.

The X-gal induction was performed under the same conditions but X-gal (120 ug/mL) was added at the beginning of the incubation period and the plant extracts were used (1 mL) instead of the AB medium. The incubated mixture was then filtered on 0.21 um filter to collect the Agrobacteria and the intensity of the blue color evaluated qualitatively. This method was adapted from Okker et al. (1984).

II.I.4 b-galactosidase assay

The assay was performed according to the method of Miller (1972). The cell density at the end of the incubation period was first recorded by measuring the absorbance at 600 nm. Then, 0.5 mL of induced bacterial culture was added to 0.5 mL of assay buffer (0.06M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.04M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.01M KCl, 0.001M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05M b-mercaptoethanol, pH 7.0). To this mixture, 10 uL of chloroform and 10 uL of SDS (0.1% w/v) were added. The mixture was vortexed 10 sec to disrupt the cell membrane and the reaction was

started by adding 0.2 mL of o-nitrophenol-b-D-galactoside (ONPG, 4mg/mL) and shaken briefly. The reaction was incubated at 29°C for 30 min and stopped by adding 0.5 mL of Na₂CO₃ (1M). The optical density of the reaction mixture was read at 420 nm after spinning down the debris (10 000g, 10 min). The results were expressed in units of b-galactosidase as calculated by the following formula:

$$U = \frac{1000 \times OD_{420}}{30 \text{ min} \times 0.5 \text{ mL} \times OD_{600}}$$

CHAPTER III
BRASSICA NAPUS TRANSFORMATION WITH
ONCOGENIC AGROBACTERIUM TUMEFACIENS

III.A Introduction

The genetic engineering of B. napus involves numerous steps. To execute these effectively, knowledge of the transformation method is an essential prerequisite. Towards this goal, tumourigenesis in B. napus in planta was studied to determine the most virulent strains of Agrobacterium. Furthermore, experiments were conducted to define some of the important factors affecting tumourigenesis in B. napus. Finally, explants of B. napus were used in cocultivation experiments to correlate in planta results with those obtained through tissue culture.

III.A.1 Brassica species transformation with oncogenic Agrobacterium

Tumour induction by Agrobacterium was studied in Brassica well before the discovery of the transfer of T-DNA to the plant genome (Chilton et al. 1977). The first report of Agrobacterium tumourigenesis in Brassica was published in 1935 by Schilberszky. Unfortunately, most of the information published before the discovery of the Ti plasmid in 1975 is of little use for genetic engineering

studies. The major point from these reports was that B. oleracea, B. campestris var. rapifera, B. nigra, B. napus and B. hirta are potential hosts for A. tumefaciens. No conclusions could be drawn about strain selectivity since in most cases strains were not specified (Table III.1).

Surprisingly, very little work on host-range of various strains of Ti or Ri plasmids with Brassica species was done after 1975. The most extensive work published included B. oleracea, B. nigra, B. campestris, B. napus, B. hirta and the use of 5 A. tumefaciens strains (Holbrook and Miki 1985). Except B. napus which was not transformed by octopine Ti plasmids, differences revealed through in planta inoculation were not observed between the nopaline strain (T37, C58) and the octopine strains (B6806, A6S, B6T) which induced tumours on all the Brassica species studied; however, this study did not make any distinction between the different A. tumefaciens strains and plant cultivars used. B. napus was also reported to be transformed by strain T37 but at a lower frequency (50% vs 100%; Ooms et al. 1985). B. juncea was a good host for this same T37 strain (Matthews et al. 1985, 1986).

In the last few years, systematic work has been done using A. rhizogenes. B. napus was transformed by A. rhizogenes strains A4 (Guerche et al. 1987c) and 1855 (Ooms et al. 1985) and differences were observed among the cultivars used (Ooms et al. 1985). B. oleracea was transformed with strains A4, TR101, TR7, 15834 and 8196 (David and Tempe 1988). B. campestris was transformed by strain A4 (Tanaka et al. 1985).

Summarizing these studies (Table III.1), Brassica species appear to be generally good hosts for Agrobacterium species. However,

TABLE III.1. Genetic Transformation of Brassica Species by Oncogenic Agrobacterium

	Strain (frequency)	References
<u>A. tumefaciens</u>		
<u>B. campestris</u>	C58, T37 (100%) B6806, A6S, B6T	Holbrook and Miki 1985
var. rapifera	ID135 ? (66%) B6	Kado <u>et al.</u> 1972 Hoerner <u>et al.</u> 1945 Galsky <u>et al.</u> 1981
<u>B. hirta</u>	? C58, T37 (100%) B6806, A6S, B6T	Lopatin 1936 Holbrook and Miki 1985
<u>B. juncea</u>	T37	Matthews <u>et al.</u> 1985
<u>B. napus</u>	T37 (pTiT37.14a/a) ?	Matthews <u>et al.</u> 1986 Schilberszky 1936
cv. Jet Neuf	C58, T37 (100%) B6806, A6S, B6T (0 %)	Holbrook and Miki 1985
<u>B. nigra</u>	T37 (50%) Chr IIb	Ooms <u>et al.</u> 1985 Tamm 1954
<u>B. oleracea</u>	C58, T37 (100%) B6806, A6S, B6T	Holbrook and Miki 1985
var. botrytis	? ?	Schilberszky 1936 Lopatin 1936
var. capitata	? (100%) C58	Hoerner <u>et al.</u> 1945 Ondrej 1983
var. gongylodes	? (100%) C58	Hoerner <u>et al.</u> 1945 Ondrej 1983
var. capitata and italica	C58, T37 (100%) B6806, A6S, B6T	Holbrook and Miki 1985
<u>A. rhizogenes</u>		
<u>B. campestris</u> var. rapifera	A4	Tanaka <u>et al.</u> 1985
<u>B. napus</u>	A4	Guerche <u>et al.</u> 1987
cv. Willi	1855 (10%)	Ooms <u>et al.</u> 1985
cv. Rifal	1855 (70%)	
cv. Jet Neuf	1855 (50%)	
cv. Brutor	1855 (50%)	
<u>B. oleracea</u> var. botrytis	A4, TR101, TR7 15834, 8196	David and Tempe 1988

* The number in parenthesis indicate the frequency of tumour or root formation obtained in the reported experiments. ? = no indication of the strain used.

differences in efficiency were observed depending on the Agrobacterium strains and plant cultivars. The information accumulated in the literature is not sufficient to deduce the Agrobacterium species and strains that would give the best tumour frequency. Assuming that tumour frequency is an indicator of transformation frequency, that information is important for designing an efficient gene transfer system. The variation in tumourigenesis among B. napus cultivars has not been adequately explored and needs to be examined to determine the optimal conditions for achieving transformation of specific cultivars.

III.B RESULTS

III.B.1 Host selectivity of tumourigenesis with oncogenic Agrobacterium

Inoculations of B. napus and B. juncea plants with oncogenic Agrobacterium strains were done initially to identify the most efficient wild type strain by examining the frequency of tumour induction on the stems of plants. The results were expressed in terms of "tumour frequency" which was defined as the percentage of wound inoculation sites that developed tumours. Transformation by A. rhizogenes was evaluated the same way but results were expressed as "root frequency". Genetic transformation of the tumours was confirmed by detecting the corresponding opine or the enzymatic activity of the corresponding opine synthase as described in Chapter II (Section II.E.1). Agropine or agropine synthase detection was not performed. B. juncea was used as a control plant for tumour induction because of the rapid formation of tumours on this plant. A. tumefaciens strains containing nopaline (pTiC58, pTiT37), octopine (pTiB6S3, pTiA6, pTiAch5) and agropine (pTiBo542) Ti plasmids and A. rhizogenes containing agrocinopine (pRiA4) Ri plasmids were evaluated on B. napus and B. juncea.

All the wild type strains of A. tumefaciens and native Ti plasmids

induced high tumour frequencies on B. juncea. Tumour development was very fast in B. juncea and tumours were distinguishable within a week of inoculation and reached full size within 3 to 4 weeks. The A. rhizogenes strain A4(pRiA4) induced very small tumours at the wound sites with a few short roots. In B. napus, the nopaline strains C58 and T37 induced 96% and 100% tumour frequencies respectively. The A. rhizogenes strain A4 induced 100% root frequency on B. napus (Table III.2). The agropine strain A281 induced a lower tumour frequency (72%) and tumours were significantly smaller. The octopine strains B6S3, TT-2, A348 and Ach5 gave the lowest tumour frequencies, all being under 50% (50,36,50 and 17% respectively). The development of tumours was also delayed with the octopine plasmids. Tumours appeared within 4 weeks with the nopaline Ti plasmids whereas the octopine Ti plasmids induced tumours after 8 to 12 weeks from inoculation. The tumours were consequently smaller (Table III.2, Plate III.1).

Tumour development with octopine strains was also delayed in B. juncea relative to nopaline strains; however, the delay was more difficult to distinguish. By monitoring tumour development closely, it was possible to observe this difference in the first 2 or 3 weeks. Tumour development was initially uneven at the wound site with octopine strains leaving some parts of the wound without cell proliferation (Plate III.2). In contrast, the nopaline strains induced cell proliferation throughout the entire wound site. The tumours induced by octopine Ti plasmids were significantly smaller than the nopaline Ti plasmid induced tumours up to 4 weeks after inoculation (Plate III.2). After that period, the differences were not significant.

Inoculations of B. napus cv. Westar on various parts of the plant

TABLE III.2. Tumorigenesis Induced by A. tumefaciens and A. rhizogenes on B. napus and B. juncea Plants

Strain designation	chromosomal background	plasmid	B. napus		B. juncea	
			Tumour frequency	Relative tumour size	Tumour frequency	Relative tumour size
Wild type vectors						
C58	C58	pTiC58	96 + 10	+++	100 + 0	+++
B6S3	B6S3	pTiB6S3	50 + 12	++	100 + 0	+++
TT-2	C58	pTiB6S3	36 + 25	++	100 + 0	+++
A348	C58	pTiA6	50 + 20	+	100 + 0	+++
Ach5	Ach5	pTiAch5	17 + 23	+	100 + 0	+++
A281	C58	pTiBo542	72 + 21	+	100 + 0	+++
A4	A4	pTiA4	100 + 0	+++	100 + 0	+
T37	T37	pTiT37	100 + 0	+++	100 + 0	+++
Engineered vectors						
GV3101	C58	pAL1050				
		pGV3850	100 + 0	++++	100 + 0	+++
GV3101	C58	pAL1050				
		pMP90	100 + 0	++++	100 + 0	+++
LBA4404	Ach5	pAL1050				
		pAL4404	7 + 10	+	16 + 10	+
LBA4404	Ach5	pMP90				
		pAL4404	50 + 35	++	100 + 0	+++
No bacteria			0		0	

pGV3850=disarmed pTiC58 with T-DNA borders, pMP90=disarmed pTiC58 without T-DNA, pAL1050= T_R-DNA clone from Ach5, pAL4404= disarmed pTiAch5 without T-DNA, The tumour frequency is given ± the standard deviation, +, ++, +++, +++++, and +++++ correspond to the relative size of tumours from the smallest to the biggest.

Plate III.1 Tumour formation on B. napus and B. juncea stems induced by A. tumefaciens.

Tumours on B. napus stems induced by A) 1= strain C58, 2= strain B6S3, 3= strain GV3101 (pAL1050, pGV3850), 4= strain LBA4404 (pAL1050, pAL4404). B) = strain GV3101 (pAL1050, pMP90). Photos taken after 2 months. C) Tumours on B. juncea stems 1= strain C58, 2= strain B6S3, 3= strain LBA4404 (pAL1050, pAL4404), 4= strain GV3101 (pAL1050, pGV3850). Photo taken after 1 month.

Plate III.2 Comparison of tumourigenesis on B. juncea stems induced by strains TT-2 and C58.

A) tumours after 1 week, B) and C) tumours after 3 weeks, 1= strain C58, 2= strain TT-2.

with strain C58 gave tumours on the stem only. Leaves, petioles and flower buds did not show any tumour development. Roots were not inoculated; however, at a very low frequency (5 plants out of about 200 plants inoculated), tumours developed at the crown of B. napus plants inoculated with A. tumefaciens (Plate III.3).

Interestingly, tumour morphology and texture varied with the type of Ti or Ri plasmid used. Most of the Ti plasmids and Agrobacterium strains used induced smooth tumours (Plate III.3) on B. napus and B. juncea. Moreover, the pTiC58 (strain C58) occasionally induced teratomas on the surface of the smooth tumours of B. napus (Plate III.3). The Ti plasmids A6 (strain A348) and B0542 (strain A281) induced rough tumours on B. napus (Plates III.3 and III.4) and the pTi B0542 also induced rough tumours on B. juncea. This last Ti plasmid induced small short roots on the stem of both B. napus and B. juncea tumours (Plate III.4). The Ri plasmid A4 induced hairy roots on the stems of B. napus (Plate III.4) and small tumours on the stem of B. juncea (Plate III.4).

These results showed that Agrobacterium tumefaciens strains containing octopine Ti plasmids gave a lower tumour frequency than the nopaline strains. The agropine strain used was intermediate between these two groups. Moreover, tumour morphology varied with the type of plasmid used.

III.B.2 Host selectivity of tumourigenesis within the species B. napus

Seven different cultivars of B. napus were tested for tumourigenesis induced by pTiC58 and pTiB6S3 in the same chromosomal background (C58) to evaluate the variation of tumour frequencies within

Plate III.3 Tumour morphology on B. napus stems.

- A) crown gall (arrows) induced by stain C58. B) top= smooth tumour induced by strain C58, bottom= rough tumour induced by strain A348. C) teratomas (arrows) developing on smooth tumour induced by stain C58. D) smooth tumour induced by strain B6S3. Photos taken after 2 months.

Plate III.4 Root and tumour formation on B. napus and B. juncea stems induced by Agrobacterium.

A) tumour and roots induced by A. tumefaciens strain A281 on B. napus.

B) roots induced by A. tumefaciens strain A281 on B. juncea. C) hairy

roots induced on B. napus by A. rhizogenes strain A4. D) small

tumours (arrows) induced on B. juncea by A. rhizogenes strain A4.

Photos taken after 1 month for A,B and C and 2 months for D.

this species. All the B. napus cultivars (Altex, Global, Midas, Topas, Tower, Atow and Westar) yielded approximately the same tumour frequencies with pTiC58 (80 to 100%, Table III.3). The differences were not significant. However, the octopine pTiB6S3 induced lower tumour frequencies (under 46%) which varied widely among the different cultivars. The cultivars Global, Midas, Topas and Atow yielded very low tumour frequencies with pTiB6S3 (8 to 15%) whereas the other cultivars showed tumour frequencies between 30 and 46%.

III.B.3 Factors responsible for low tumour frequencies of pTiB6S3 in B. napus

III.B.3.a Results of in planta inoculations

The differences observed in tumour frequencies between pTiB6S3 (and other octopine Ti plasmids) and the pTiC58 (and pTiT37) were quite dramatic, therefore, attempts were made to alter the chromosomal background, the T-DNA and the VIR regions in an attempt to identify the basis of this difference. In the first experiment, pTiB6S3 in a C58 chromosomal background (Charest and Dion 1985) was compared directly with pTiC58 (strain C58). The different bacterial chromosomal backgrounds were not correlated with different tumour frequencies in B. napus (36% for pTiB6S3 strain C58 and 50% with strain B6S3). The differences observed were within experimental variation (Table III.2). Therefore, the differences observed were due solely to the different Ti plasmids.

The next step was to use a T-DNA binary vector (pAL1050, Hoekema et al. 1983) from the octopine pTiAch5 to examine the virulence of disarmed Ti plasmids from pTiC58 and pTiAch5. When the disarmed pTi

TABLE III.3. Genetic Transformation of *B. napus* Cultivars with pTiB6S3 and pTiC58

Cultivar	Tumour frequency	
	pTiC58	pTiB6S3
Altex	88 $\pm$ 14 (++)	39 $\pm$ 23 (++)
Global	100 $\pm$ 0 (++++)	8 $\pm$ 10 (+)
Midas	88 $\pm$ 18 (+++)	15 $\pm$ 19 (+)
Topas	80 $\pm$ 16 (++)	12 $\pm$ 9 (+)
Tower	91 $\pm$ 15 (+++)	46 $\pm$ 12 (+)
Atow	100 $\pm$ 0 (+++)	15 $\pm$ 18 (+)
Westar	95 $\pm$ 10 (+++)	30 $\pm$ 14 (+)

Both Ti plasmids were in the strain C58. The tumour frequency is given $\pm$ the standard deviation. +, ++, +++, ++++ = the relative size of tumours given in parenthesis from the smallest to the biggest.

plasmids pGV3850 (contains the T-DNA borders but no oncogenes, Zambryski et al. 1984) or pMP90 (T-DNA deleted, Koncz and Schell 1987), which are both derived from pTiC58, were used with pAL1050, they induced tumour frequencies of 100% in both B. napus and B. juncea. The tumours were also bigger than with the original pTiC58. When pAL1050 was combined with the disarmed plasmid pAL4404 (T-DNA deleted, Ooms et al. 1982) derived from Ach5, the tumour frequency was reduced to levels obtained with the original pTiAch5 on B. napus. With B. juncea, the tumour frequency was even lower (16%, Table III.2) than with the original pTiAch5 (100%).

The disarmed Ti plasmid pMP90 was transferred by bacterial conjugation to the Ach5 chromosomal background and combined with pAL1050. This resulted in a reduction of tumour frequency to 50% in B. napus indicating that the bacterial chromosomal genes of the strain Ach5 were not optimal for B. napus transformation with nopaline vectors.

III.B.3.b Coinfection experiments for in planta tumourigenesis

Coinfection with a mixture of A. tumefaciens strains has been used to complement avirulent strains and to obtain tumourigenesis (Otten et al. 1984, 1985). Coinfection was attempted to increase the tumour frequencies induced by octopine Ti plasmids. The pTiB6S3 (strain C58) and pTiAch5 (strain Ach5) were mixed with the strain GV3101 containing the disarmed Ti plasmid pMP90. In both cases, a significant increase in tumour frequency was observed (89% for pTiB6S3 (strain C58) and 60% for pTiAch5 (strain Ach5)) compared with the two strains inoculated alone (36% for pTiB6S3 (strain C58) and 17% for pTiAch5 (strain Ach5))(Table III.4).

TABLE III.4. Tumourigenesis induced by coinfecting A. tumefaciens strains

Plasmid (tumourigenic) (strain)	+ Plasmid (coinfecting) (strain)	Tumour frequency	size
pTiB6S3 (C58)	---	36 $\pm$ 25	++
pTiAch5 (Ach5)	---	17 $\pm$ 23	+
pTiB6S3 (C58)	pMP90 (GV3101)	89 $\pm$ 16	++
pTiAch5 (Ach5)	pMP90 (GV3101)	60 $\pm$ 28	+
pTiB6S3 (C58)	acetosyringone	63 $\pm$ 32	++

Tumour frequencies are given $\pm$ the standard error. The tumour size is given according to the scale +, ++, +++, ++++ and +++++ from the smallest to the biggest.

Attempts to increase the virulence of the octopine pTiB6S3 with acetosyringone were partially successful. An increase in tumour frequency was observed when the acetosyringone was added two hours before inoculation to the bacterial medium (63% tumour frequency). Adding the acetosyringone when the A. tumefaciens was inoculated in the culture medium (14-16 hrs before infection on the plant) or adding it to the wound site with cheese cloth moisturized with an acetosyringone solution did not increase tumour frequency.

III.B.3.c Induction of pTiA6 VIR promoter/lacZ gene fusions with explants or explant extracts

To assess induction of octopine Ti plasmid VIR genes by B. napus, B. juncea and N. tabacum, the VIR::lacZ insertion system of Stachel and Nester (1986) was used. It consisted of the octopine pTiA6 plasmid in the strain A348 (C58 chromosomal background) in which cosmids containing the VIR promoters of pTiA6 fused to the b-galactosidase gene were used to monitor VIR induction. Each bacterial strain was a merodiploid strain for each of the VIR promoter (VIR A, B, C, D, E, F; see Table III.5 for list of strains). The strains were incubated with stem section explants of B. napus and B. juncea and leaf discs of N. tabacum and the b-galactosidase activity assayed. Acetosyringone was used as a positive control.

The VIR A and VIR G promoters were expressed constitutively (Table III.5) except when the VIR G merodiploid strain was incubated with N. tabacum leaf discs where a strong induction was observed. All the other promoters but VIR C were induced strongly by acetosyringone and N. tabacum leaf discs. The VIR C promoter was induced to a lower

TABLE III.5. Induction of pTiA6 VIR Promoter/lac Z Fusion Genes with Explants
of N. tabacum, B. napus and B. juncea (ONPG* test)

Cosmid	VIR promoter	Non induced	Acetosyringone	N. tabacum	B. napus	B. juncea
pSM1	B	32 ± 2	222 ± 33	124 ± 24	34 ± 10	26 ± 2
pSM202	A	98 ± 12	104 ± 22	112 ± 21	106 ± 16	106 ± 36
pSM219	F	22 ± 4	284 ± 46	256 ± 20	58 ± 18	58 ± 8
pSM304	D	24 ± 4	192 ± 33	142 ± 54	26 ± 2	24 ± 4
pSM358	E	96 ± 21	356 ± 55	228 ± 36	98 ± 32	110 ± 8
pSM363	G	162 ± 42	174 ± 16	392 ± 48	146 ± 52	166 ± 70
pSM379	C	18 ± 4	48 ± 16	58 ± 8	18 ± 2	18 ± 2
pTiA6	-	20 ± 2	20 ± 2	19 ± 2	18 ± 2	18 ± 2

the numbers represent the average of 4 experiments, the units are in b-galactosidase
units = $(A_{420} \times 1000) \div (A_{600} \times 30\text{min})$. The units are given ± the standard deviation.
ONPG= o-nitrophenyl-b-D-galactopyranoside.

level. With B. napus and B. juncea stem sections, VIR F was induced slightly and all the other VIR promoters were not induced significantly. The strain A348 (pTiA6) alone did not produce significant b-galactosidase activity.

N. tabacum leaf discs were compared with stem sections for VIR induction (Table III.6). Some differences were observed between stem and leaf disc explants but all the VIR merodiploid strains tested (VIR B, C, D, F) were induced to higher levels than those obtained with the Brassica explants (Table III.5) with both stem sections and leaf discs.

That B. napus and B. juncea explants did not induce VIR promoters could be related to problems of detection using ONPG as a substrate. To investigate this, the substrate X-gal was used during the incubation with the explant instead of ONPG which is used at the end of the incubation period. This approach gives qualitative results since the blue color was not released to the incubation medium from the bacteria. The Agrobacteria were collected on a 0.22 μ m filter and the intensity of the blue color on the filter was evaluated visually. It was found that VIR A, VIR G and VIR E promoters were expressed constitutively and all the other VIR promoters were induced by the explant extracts of N. tabacum, B. napus and B. juncea (Table III.7). Some of the VIR promoters (VIR C and D) appeared to be induced weakly with B. napus explant extracts. The qualitative nature of the assay made the results difficult to assess accurately.

III.B.4 Transformation of tissue explants by wild type Agrobacterium

Oncogenic Agrobacterium strains were used to inoculate explants of B. napus, B. juncea and N. tabacum to correlate the results obtained in

TABLE III.6. Induction of pTiA6 VIR Promoter/lacZ Fusion Genes with Stem and Leaf Discs Explants of N. tabacum (ONPG* test)

Cosmid	Vir promoter	Non induced	Acetosyringone	Leaf	Stem
pSM1	B	30	420	156	84
pSM219	F	26	218	200	124
pSM304	D	34	276	84	78
pSM379	C	12	68	54	90

The experiment was repeated twice and the values did not varied more than 30%.

ONPG= o-nitrophenyl-b-D-galactopyranoside.

TABLE III.7. Induction of pTiA6 VIR Promoter/lac Z Fusion Genes with Explant Extract of N. tabacum, B. napus and B. juncea (X-gal* test)

Cosmid	VIR promoter	Non induced	Acetosyringone	N. tabacum	B. napus	B. juncea
pSM1	B	+	++++	+++	+++	++++
pSM202	A	+++	+++	+++	+++	+++
pSM219	F	-	-	+++	++	++
pSM304	D	+	++++	++	+	++++
pSM358	E	+++	++++	+++	+++	++++
pSM363	G	++++	++++	+++	++++	++++
pSM379	C	-	+	++	+	+++

Color coding: -=no blue color, +=light blue color, ++++=strong blue color, ++ and +++=intermediate blue color. X-gal= 5-bromo-4-chloro-indolyl-b-D-galactoside.

planta with those obtained in tissue culture. Thin cell layer explants from stems (Klimaszewska and Keller 1985) and stem cross-sections (Kantha et al. 1974) were inoculated according to a protocol derived from the N. tabacum leaf disc cocultivation method of Horsch et al. (1985). Tissue culture conditions for transformation of B. napus will be described and discussed in Chapter IV. The "transformation frequency" is defined here as the percentage of explants that were positive for the nopaline synthase assay. The selection for transformed tissues was based on the hormone autotrophic growth characteristic of crown gall tumours. Preconditioning thin cell layer explants for 4 days gave optimum transformation frequencies for both pTiC58 (100%; strain C58) and pTiB6S3 (50%; strain B6S3, Table III.8). Without preconditioning the transformation frequencies dropped to 50% with pTiC58 and eliminated transformation with pTiB6S3 (Table III.8). A cocultivation period of 2 days gave higher transformation frequencies than a cocultivation period of 1 day with both pTiC58 (100% vs 16%) and pTiB6S3 (48% vs 0%) when combined with a 4 day preconditioning period. Stem sections yielded the same transformation frequencies when a preconditioning period of 4 days was combined with a cocultivation period of two days. Under the same conditions, the A. rhizogenes strain A4 generated a transformation frequency of 11%.

B. juncea thin cell layer explants and stem sections responded very well to A. tumefaciens cocultivation and over 80% frequencies of transformation were obtained. N. tabacum leaf discs used as a control gave 100% transformation frequencies with pTiC58(strain C58), pTiB6S3 (strain B6S3), pTiA6 (strain A281) and pRiA4 (strain A4).

Using the binary system, pGV3850 in combination with pAL1050,

TABLE III.8. Transformation Frequencies of Explants of B. napus, B. juncea and N. tabacum

Plasmid (strain)	<u>B. napus</u>						<u>B. juncea</u>		<u>N. tabacum</u>	
	<u>Preconditioning - Cocultivation</u>						<u>4 - 2</u>		<u>2 - 2</u>	
	<u>0 - 2</u>	<u>4 - 2</u>	<u>4 - 1</u>	<u>4 - 1</u>	<u>4 - 1</u>	<u>4 - 1</u>	<u>4 - 2</u>	<u>4 - 2</u>	<u>2 - 2</u>	<u>2 - 2</u>
<u>-Thin cell layer explants</u>										
pTiC58 (C58)	50	100	16				89		NT	NT
pTiB6S3 (B6S3)	0	48	0				85		NT	NT
pGV3850, pAL1050	NT	79 (68)	NT				85 (100)		NT	NT
<u>-Stem sections</u>										
pTiC58 (C58)	NT	100	NT				100		NT	NT
pTiB6S3 (C58)	NT	50	NT				80		NT	NT
pRiA4 (A4)	NT	11	NT				NT		NT	NT
pTiB6S3 (C58) cocultivated on										
<u>N. tabacum</u> leaf discs	NT	100	NT				NT		NT	NT
pAL4404, pAL1050 (LBA4404)	NT	NT	NT				NT		NT	NT
<u>-Leaf discs</u>										
pTiC58 (C58)	NT	NT	NT				NT		100	100
pTiB6S3 (C58)	NT	NT	NT				NT		100	100
pTiA4 (A4)	NT	NT	NT				NT		100	100
pAL4404, pAL1050 (LBA4404)	NT	NT	NT				NT		30	30

a Transformation frequencies were measured as the percentage of infected explants that demonstrated nopaline synthase (pTiC58) or octopine synthase (pTiB6S3, pAL1050) activities

b The explants were cocultivated with the A. tumefaciens strains for 2 days without preconditioning (0-2) or after preconditioning for 4 days (4-2). Explants were also cocultivated for 1 day after preconditioning for 4 days (4-1). N. tabacum explants were preconditioned 2 days and cocultivated 2 days. N.T. = not tested.

* the number in parenthesis indicate the percentage of the tissues that were octopine synthase positive (pAL1050) and also nopaline synthase positive (pGV3850).

**The transformation frequencies for pRiA4 were determined by the number of explants showing hairy root development.

transformation frequencies of 79% in B. napus and 85% in B. juncea were obtained based on the presence of the octopine synthase gene located on the selected pAL1050 T-DNA. Co-transformation frequencies based on the expression of the nopaline synthase gene of the disarmed pGV3850 T-DNA (non-selected) were 68% for B. napus and 100% for B. juncea. These numbers have to be examined with caution since tumour tissues are chimeric in nature.

Interestingly, when B. napus stem sections were cocultivated with strain TT-2 (pTiC58) and were deposited on top of N. tabacum leaf discs, a 100% transformation frequency was obtained (Table III.8).

Some of the transformed tissues obtained in vitro are shown in Plate III.5.

Plate III.5 Transformed tissues from cocultivated B. napus explants with Agrobacterium.

A) calli from A. tumefaciens strain C58. B) teratomas from A. tumefaciens strain C58. C) calli from A. tumefaciens strain B6S3. D) hairy roots from A. rhizogenes strain A4.

III.C DISCUSSION

III.C.1 Agrobacterium-mediated tumourigenesis in B. napus and B. juncea

Tumourigenesis of B. napus and B. juncea by Agrobacterium has not been the subject of extensive studies; probably because Agrobacterium is not a serious pathogen of Brassica species (Martens et al. 1984, Canola growers manual 1988). With the use of Agrobacterium-mediated transformation in the production of transgenic plants, the study of tumourigenesis has become more relevant. A survey was undertaken to determine the transformation frequencies in planta with Ti and Ri plasmids from which disarmed vectors have been derived or transgenic plants obtained (see Chapter I, section I.C.2). This survey confirmed that B. napus is a good host for A. tumefaciens nopaline Ti plasmids and that it is only partially recalcitrant to octopine Ti plasmids. Results showed that 17 to 50% tumour frequencies could be obtained with octopine Ti plasmids compared to no tumours as observed by Holbrook and Miki (1985). Two factors contributed to that difference. First, the plants were grown in growth chambers where the environmental conditions were tightly controlled as opposed to greenhouses where the conditions vary depending on the season. Generally, greenhouses are dryer and warmer in the summer which can be

detrimental to tumour development (Charest and Dion 1983). Second, the stem inoculation procedure was modified to enhance tumour formation. Instead of a single puncture of the stem, three parallel streaks of 0.5 cm were made and covered by an Agrobacterium paste.

No previous studies evaluated differences among octopine Ti plasmids or B. napus cultivars. Slight differences in tumour frequencies were seen with the different octopine Ti plasmids, pTiAch5 inducing the lowest frequency and pTiB6S3 the highest frequency. Bacterial chromosome could be a major factor. As outlined in the literature review, several chromosomal genes (section 1.B.3.a.(3)) are needed for efficient tumour induction. This was found to be the case for strain Ach5 which was very inefficient with B. napus and with other plants. The pTiB6S3 gave equivalent tumour frequencies in either a C58 or B6S3 chromosomal background. The difference in tumour frequencies observed between these octopine and nopaline strains was governed by the Ti plasmid. The results obtained with the octopine pTiA6 in a C58 chromosomal background also supported this conclusion.

The agropine pTiBo542 (in a C58 chromosomal background) generated tumour frequencies between those obtained with octopine and nopaline Ti plasmids. The difference observed can be associated directly with pTiBo542 since all the Ti plasmids were in the same chromosomal background. Finally, the agrocinopine pRiA4 induced a tumour frequency of 100% on B. napus which verified observations made by others that B. napus was a good host for A. rhizogenes (Guerche et al. 1987c, Ooms et al. 1985); however, the root frequency obtained (100%) with the cultivar Westar was much higher than previously reported with other cultivars (10 to 70%; Ooms et al. 1985).

To determine if the difference in tumour frequencies between pTiB6S3 (and other octopine pTi) and pTiC58 was cultivar dependant, 7 B. napus cultivars were inoculated. All the cultivars responded similarly to the nopaline pTiC58 with 80 to 100% tumour frequencies whereas a greater variation was observed with pTiB6S3. The cultivar Global gave the lowest tumour frequency (8%) and the cultivar Tower the highest (46%). These results may be significant when designing transformation strategies for the different cultivars of B. napus.

Previous observations in our laboratory (Charest, Holbrook and Miki, unpublished) had shown that B. juncea was also an excellent host for Agrobacterium tumour induction. The present work confirmed these observations and extended them. Matthews et al. (1985, 1986) also showed that pTiT37 was efficient in the transformation of B. juncea. The data in this thesis showed that B. juncea was highly susceptible to a broad range of Agrobacterium Ti or Ri plasmids and Agrobacterium strains (100% tumour frequencies). However, tumours were smaller when induced by octopine Ti plasmids. The A. rhizogenes strain A4 did not induce as many roots on B. juncea as seen on B. napus. small tumours were seen instead of roots. This change from roots to tumour with plants inoculated with A. rhizogenes has been observed in Nicotiana glauca (White et al. 1982) and may be related to the hormonal balance of the inoculated plant. Generally, B. juncea is an excellent plant for studies in Agrobacterium tumorigenesis or hairy root experiments because tumours can be detected within a week and it is susceptible to a wide variety of strains and Ti or Ri plasmids.

III.C.2 Tumour morphology

T-DNA from various Ti and Ri plasmids have been found to induce distinct tumour morphology on Kalanchoe tubiflora (Ream *et al.* 1983, Charest and Dion 1985), Kalanchoe daigremontiana (Hooykaas *et al.* 1977, 1979) and Kalanchoe fedtschenkoi (Iyer *et al.* 1982). These variations have not been reported in other species. B. napus and B. juncea tumours induced by A. tumefaciens were of two types as described for K. daigremontiana (Hooykaas *et al.* 1982). The pTiA6 and pTiBo542 induced tumours with rough surface on B. napus and on B. juncea with pTiBo542. All the other pTi induced smooth tumour surfaces. Tumours induced with pTiBo542 also had roots differentiating from the basal regions of the tumours in B. napus and B. juncea. Roots were also seen in K. daigremontiana with agropine strains (Hooykaas *et al.* 1982) and K. tubiflorae with octopine strains (Charest *et al.* 1985). With A. rhizogenes, abundant hairy roots were observed in B. napus but small tumours and very few short roots were generated on B. juncea.

Teratomas are common on tumours induced by nopaline strains such as T37 and C58 but not on all plant species (Turgeon, 1982). B. oleracea, Beta vulgaris, L. esculentum, numerous Nicotiana species, and other produced teratomas when inoculated with A. tumefaciens nopaline strains. In our study, B. napus tumours induced by nopaline pTi frequently showed teratomas whereas B. juncea tumours did not.

These variations in tumour morphology reflect different hormone levels modulated by the expression of the T-DNA genes and the hormone level in the plant (Weiler and Schroder 1987, Akiyoshi *et al.* 1983). The presence of teratomas reflects a higher level of cytokinins relatively to auxins in the tumour (Hooykaas *et al.* 1982, Akiyoshi *et*

al. 1983). This is also associated with the smooth surface of nopaline tumours in Kalanchoe (Hooykaas et al. 1982). Octopine strains induce a lower cytokinin/auxin ratio and produce a rough tumour surface in Kalanchoe. The same would apply to agropine tumours. It follows that tumours on B. napus and B. juncea having a smooth morphology and teratomas would contain the highest cytokinin/auxin ratios and those with the rough morphology and the roots the lowest ratios. The hairy roots induced by A. rhizogenes Ri plasmid would result from an even lower cytokinins/auxins ratio. Presumably this differs for B. juncea since no teratomas were observed and small tumours were present instead of roots on wound sites inoculated with A. rhizogenes.

III.C.3 Localization of the region responsible for lower tumour frequencies on octopine Ti plasmid

The lower tumour frequencies and the smaller sizes of B. napus tumours induced by octopine Ti plasmids was due to the Ti plasmid and not the bacterial chromosome (section III.C.2). At least two regions on the Ti plasmid may be responsible for this phenomenon, the T-DNA with its hormone genes and the VIR region with its virulence genes (Yanofsky et al. 1985). The first step in discriminating between these was to combine disarmed nopaline pTi derived from pTiC58 (pMP90, pGV3850) with the T-DNA binary vector from pTiAch5 (pAL1050). This vector includes the TL-TDNA of Ach5, the overdrive sequences and some sequences to the left of the TL-DNA (Hoekema et al. 1983, Peralta et al. 19886, Gielen et al. 1984). All these sequences are important for efficient transformation (Peralta et al. 1986, Wang et al. 1987a). When pAL1050 was used in combination with either pMP90 or pGV3850, 100%

tumour frequencies were obtained. Both disarmed Ti plasmids were in a C58 chromosomal background indicating that the octopine T-DNA was as efficient as nopaline T-DNA in generating tumours. Moreover, when pAL1050 was combined with its natural VIR region (pAL4404), a very low tumour frequency of 7% was obtained which is lower than for the original pTiAch5 (17%). A dramatic decrease in tumour frequency in B. juncea (16% vs 100%) was also observed. The combined data suggested that regions other than the T-DNA on the Ti plasmid were involved in the host selectivity of octopine Ti plasmids.

When pAL1050, pAL4404 (strain LBA4404) was used to transform N. tabacum leaf discs (Table III.8), a 30% transformation frequency was obtained rather than the expected 100% indicating that this phenomenon was not restricted to Brassica species. This could have been related to the absence of the TR-DNA which may be needed for efficient transformation in plants or to the chromosome of the bacterial strain used. The disarmed plasmid pMP90 was transferred by mating to LBA4404 thus evicting the plasmid pAL4404 since these two plasmids are in the same incompatibility group and cannot coexist in the same Agrobacterium (Nester et al. 1984). When used with pAL1050, a reduction in tumour frequency was observed in B. napus (50% vs 100% as expected). In B. juncea, only a significant decrease in tumour size was detected. These results demonstrated that factors encoded by the Ach5 chromosome did not interact well with Brassica in particular B. napus. This did not rule out the possibility that the TR-DNA was also a factor but it is known that pTiB6S3 is almost identical to pTiAch5 (DeVos et al. 1981); only two restriction sites have been found to be different. This would suggest that the pTiAch5 should have the same comportment as pTiB6S3 and that the observation was dependant on the bacterial chromosome.

To summarize, the T-DNA of octopine Ti plasmids is not responsible for the lower tumour frequencies in B. napus; the Ach5 chromosome in Agrobacterium strains is suboptimal in generating tumours induced by Ti plasmids whereas the C58 chromosome is very effective and the difference between octopine and nopaline Ti plasmids appears to be located outside the T-DNA. This leaves the VIR region as a likely candidate to explain that difference.

Experiments were conducted with the octopine pTiA6 (in a C58 chromosomal background) containing cosmids with the VIR promoters fused to the lac Z gene. Essentially, VIR A and VIR G promoters were found to be constitutively expressed in the presence of B. napus, B. juncea and N. tabacum extracts or explants. VIR G was induced with N. tabacum leaf discs only. The other VIR promoters (VIR B, C, D, E, F) were induced with N. tabacum leaf discs and acetosyringone but B. napus and B. juncea induction of the VIR promoters was not detected with ONPG except for VIR F. X-gal allowed detection of VIR induction of all the VIR promoters and confirmed induction by B. napus or B. juncea explants. Vir induction with N. tabacum and acetosyringone gave a lower level of induction than previously reported (Stachel and Nester 1986). This is probably due to longer incubation times with the ONPG and different b-galactosidase assays in our experiments.

It can be postulated that B. napus and B. juncea wound substances are not very effective in activating the VIR G gene products of octopine Ti plasmids mediated by VIR A. It is conceivable that, the phenolics released from B. napus and B. juncea may be different from those found in N. tabacum. Such factors may contribute to the lower tumour frequencies and smaller tumour sizes on B. napus induced by

octopine Ti plasmids and to a lesser extent to the smaller tumour sizes in B. juncea. VIR promoter induction with acetosyringone prior to inoculation also increased the tumour frequency (63%) but the optimal conditions were probably not reached.

This does not rule out the involvement of other factors or genes located on the octopine Ti plasmids outside the T-DNA that are intervening in the transformation process. Coinfection experiments were designed to further support the hypothesis that the VIR region of octopine Ti plasmids is inefficient in generating tumours in B. napus. It has been demonstrated that VIR genes in one Agrobacterium strain can be complemented by other VIR genes located in another Agrobacterium (Otten et al. 1984, 1985). The pTiB6S3 (strain C58) was mixed with pMP90 (strain GV3101) and B. napus plants infected. A higher tumour frequency was observed (89%). This indicated complementation by the nopaline Ti plasmid pMP90. This helper effect has been found to be associated with VIR E or VIR F (Otten 1985) which are involved in the T-DNA processing and protection and the host range respectively (section I.B.3.a.(2)). The same complementation phenomenon was observed to a lower level when pTiAch5 (strain Ach5) was used in combination with pMP90.

The complementation of VIR regions and the VIR induction experiments supported the involvement of the VIR region of octopine Ti plasmid as being responsible for lower tumour frequencies in B. napus.

III.C.4 Implications of the in planta results for genetic engineering and correlation with ex planta results

Basic information needed for the application of A. tumefaciens or

A. rhizogenes as vectors for Brassica genetic engineering has been obtained. Although tumour frequencies and size reflect rate of tumour growth in addition to the frequency of transformation events (number of cells transformed) (Parke et al. 1987); they provide an indication of transformation efficiency that can be obtained easily and relatively quickly. For B. napus, nopaline Ti plasmid such as pTiC58 and pTiT37 are most effective at inducing tumorigenesis; whereas, octopine Ti plasmids and agropine Ti plasmid pTiBo542 were not as good. Disarmed pTiC58 (pGV3850 and pMP90) were as efficient as the wild type pTiC58. Therefore derivatives of the nopaline pTiC58 or pTiT37 should be the first choice for B. napus genetic engineering.

Explants of B. napus, B. juncea and N. tabacum inoculated with pTiB6S3, pTiC58, pRiA4 and pGV3850 combined with pAL1050 with various preconditioning and cocultivation periods in B. napus gave essentially the same results (Table III.8). A preconditioning period of four days combined with a cocultivation period of two days with Agrobacterium gave the optimum transformation frequencies. The cocultivation of N. tabacum leaf discs in contact with B. napus stem sections increased the transformation frequency of the octopine pTiB6S3 to 100% further indicating that N. tabacum metabolites may be more effective in the induction of the VIR region of octopine Ti plasmids.

Interestingly, B. juncea was not transformed to 100% when explants were used (85 to 89%) whereas N. tabacum leaf discs were transformed with all the Ti and Ri plasmids. These data suggest that VIR promoters of octopine Ti plasmid are not induced by B. juncea as well as with N. tabacum.

CHAPTER IV

GENETIC ENGINEERING OF BRASSICA NAPUS

IV.A.1 Introduction

The genetic engineering of a plant species demands certain prerequisite technologies. These include a transformation method, a tissue culture system through which plants can be transformed and regenerated and an effective selection system for the recovery of the transformed tissues. This chapter will deal with the use of B. napus explants for A. tumefaciens-mediated transformation, the sensitivity of B. napus tissues to potential antibiotic resistance markers, the recovery of transgenic plants and the expression of the introduced genes.

IV.A.2 B. napus tissue culture

Outside the Solanaceae, B. napus is probably the species that has been best studied for its tissue culture. Regenerable explant systems include flower buds (Margara and Leydecker 1978), root segments (Lazzeri and Dunwell 1984), cotyledons (Narasimhulu and Chopra 1988, Murata and Orton 1987), hypocotyls (Murata and Orton 1987, Dietert et al. 1982), leaf discs (Dunwell 1981, Stringam 1979) and stem explants

(Stringam 1977, Kartha et al. 1974). Regeneration of plants has also been achieved through protoplast culture (Kartha et al. 1974, Li and Kohlenbach 1982, Thomas et al. 1976, Pelletier et al. 1983, Lu et al. 1982, Chuong et al. 1985, Glimelius 1984, Xu et al. 1982, Kohlenbach et al. 1982, Barsby et al. 1986), anther culture (Keller and Armstrong 1977, 1981), isolated microspore culture (Chuong and Beversdorf 1985, Thomas and Wenzel 1975, Swanson et al. 1987, Keller et al. 1987). These cell and tissue culture systems may act as potential hosts for genetic transformation and thus provide mechanisms through which transgenic plants may be regenerated.

IV.A.3 Genetic engineering of Brassica species

Transgenic plants have been obtained with Brassica species by A. rhizogenes-mediated transformation. The pRi T-DNA was not altered to dissociate the symptoms of hairy root disease. Transgenic plants were recovered based on the capacity of hairy roots to regenerate whole plants as demonstrated in N. tabacum by Ackermann (1977). Transgenic B. napus plants displayed wrinkled leaves, short internodes and plagiotropic roots (Guerche et al. 1987c, Ooms et al. 1985). These symptoms were less severe with B. oleracea var. Botrytis (David and Tempe 1988). Transgenic B. napus plants obtained with A. rhizogenes were reported to be fertile (Guerche et al. 1987c). The disease symptoms were transmitted to the progeny and the fertility was reduced. The disease characters are a major impediment to the practical use of A. rhizogenes-mediated transformation for production of transgenic B. napus.

Direct gene transfer by electroporation has been used to transform B. napus protoplasts with a chimeric NPT II gene (Guerche et al. 1987b).

Two plants were regenerated from 4×10^6 protoplasts electroporated. Resistance to paromomycin in the calli and kanamycin in the progeny of the transgenic plants was demonstrated and presence of the NPT II sequences was shown. However, the experiment was difficult to repeat. B. campestris protoplasts were also used for the PEG-induced direct uptake of CAMV and NPT II DNA (Paszkowski et al. 1986). Calli resistant to kanamycin were recovered; however, transgenic plants were not. Liposome delivery of CAMV DNA was also attempted (Mahmood-Hussain et al. 1985) but no proof of transformation shown. Methods dependant on protoplasts may be useful in transient gene expression studies but they have not been efficient for producing transgenic Brassicas

Microinjection has been used successfully with B. napus microspore-derived embryoids to obtain transgenic plants containing a NPT II chimeric gene. Regeneration through secondary embryogenesis was necessary because of the chimeric nature of the transformants. The transformation frequencies ranged from 27 to 51% and large numbers of plants regenerated. Transmission of the introduced gene to the progeny was not demonstrated therefore the practical value of this approach cannot be assessed. Microinjection of microspore derived embryos appears to be the most reliable system among the direct transformation methods. It depends upon excellent tissue culture which is available for only a few cultivars and on sophisticated equipment. Although, a limited number of embryos can be microinjected in one experiment, the large numbers of transgenic plants produced demands that this approach be considered seriously (Neuhaus et al. 1987).

Disarmed A. tumefaciens Ti plasmids have been used in several laboratories, including our group, to obtain transgenic B. napus

plants. The use of pTiT37 derived vectors with a chimeric mutant mouse dihydrofolate reductase gene coupled to 35S promoter sequences (Pua et al. 1987) and a chimeric NPT II gene coupled to a 35S promoter as selectable marker genes have been reported (Fry et al. 1987). In vitro grown stem sections were used in combination with the drug methotrexate to obtain a frequency of 10 transgenic plants per 100 explants inoculated with A. tumefaciens in the first case. The methotrexate resistance gene was transmitted to the progeny. In the second case, stem segments from greenhouse grown plants were used in combination with the antibiotic kanamycin to recover 48 kanamycin resistant shoots from 555 explants (9% frequency). Resistance was transmitted to the progeny. In a third report, the agropine Ti plasmid pTiB0542 has been used in combination with a binary vector carrying a NPT II gene coupled to a 35S promoter and a napin gene promoter linked to a dihydrofolate reductase gene (Radke et al. 1988). Hypocotyl explants were cocultivated and transformation frequencies of 0.4 to 2.5% were obtained. The napin gene promoter was shown to be regulated in a tissue specific manner and the transformation was verified by Southern blotting. These studies are similar to our work in which thin cell layer explants from stem epidermal strips were also used in combination with kanamycin and chloramphenicol to recover transgenic plants after transformation with a NPT II gene and a CAT gene coupled with nopaline promoter sequences (Charest et al. 1988). A frequency of 2 transgenic plants per 100 explants inoculated was obtained. The kanamycin resistance trait was transmitted to the progeny and proof of integration of the genes given. Details of the procedure and comparisons with the two other studies will be discussed in this chapter.

The advantage of Agrobacterium-mediated transformation with disarmed Ti plasmids is the relative ease of the methodologies for transformation and regeneration of normal plants.

IV.B RESULTS

IV.B.1 B. napus explant culture and cocultivation

B. napus thin cell layer explants (Klimaszewska and Keller 1985) were evaluated as hosts for A. tumefaciens-mediated transformation. The uninoculated explants were highly regenerable in culture producing on average, 5 shoots per explant with 80% of the explants producing shoots within 3-4 weeks. Exposure to A. tumefaciens strains following the procedure for leaf disc transformation (Horsch et al. 1985) induced a severe hypersensitive response that completely prohibited the recovery of viable tissues (Plate IV.1.a, e). The tissue necrosis was found to be strictly due to A. tumefaciens. Immersion of the explants in bacterial culture medium without A. tumefaciens did not cause any hypersensitive reaction. Adding acetosyringone increased the hypersensitive reaction, thus reduced further the tissue viability. The procedures for inoculation and culture of explants were therefore modified to minimize tissue necrosis (Plate IV.1.b,d,f,g) and to enhance transformation rates (Table III.8).

The inoculation of the explants with A. tumefaciens was a critical step in the recovery of healthy tissues. With suspensions of 10^9 bacteria per mL, inoculations could not exceed 30 sec because

Plate IV.1 Thin cell layer explants cocultivated with A. tumefaciens strain GV3101.

a,e Explants prepared according to Klimaszewska and Keller (1985) and cocultivated using the leaf disc transformation procedure (Horsch et al. 1985) were chlorotic and brown after 3 weeks. b,f Viable explants with green meristematic regions after 3 weeks were recovered using the modified conditions for inoculation and culture. d,g Shoot differentiation was achieved by six weeks. c The frequency of shoot differentiation was reduced on media solidified in 0.8% agar.

rapid bacterial growth during cocultivation could not be inhibited by carbenicillin, cefotaxime, Claforan or Clavulin 250 at 500 ug/mL. Shorter inoculations permitted cocultivation periods up to 2 days and subsequent inhibition of bacterial growth by carbenicillin or Clavulin 250. Cefotaxime and Claforan were toxic to the explants under these conditions of culture. In the recovered tissues, necrosis was still evident and the frequency of shoot differentiation was suppressed to about 0.6 shoots per explant (Plate IV.1.c).

A dramatic reversal of tissue necrosis was achieved by replacing agar in the media with 0.7% agarose (Plate IV.1.b,d,f,g). Shoot differentiation was restored to levels of 3-5 shoots per explants (Plate IV.1.d,g); however, this was delayed by 2-3 weeks relative to uninoculated controls. Preconditioning of explants prior to inoculation with feeder layers of B. napus or N. tabacum cell suspension cultures and during cocultivation did not alter these results significantly but appeared to be associated with greater consistency among experiments in the recovery of viable tissues. Under these improved culture conditions, any of the antibiotics carbenicillin, cefotaxime, Claforan or Clavulin 250 were effective in the control of bacterial growth without significant effect on plant tissue growth (Fig. IV.1). Carbenicillin had a slight positive effect on shoot differentiation. Another antibiotic, Timentin (Ticarcillin + clavulanic acid) was tested but did not control A. tumefaciens growth effectively.

Other types of explants were also evaluated such as leaf discs (Dunwell 1981, Stringam 1979), flower buds (Margara and Leydecker 1978) and stem sections (Stringam 1977, Kartha et al. 1974). In our hands,

Figure IV.1 Influence of b-lactam and cephalosporin antibiotics on E. napus thin cell layer explants. At least 12 explants (fresh weight 10 ± 2 mg) cocultivated with A. tumefaciens strains GV3101 were incubated on media with antibiotics at 500 ug/mL. The average fresh weights after six weeks were expressed as a percentage of the average fresh weight of explants incubated without antibiotics (601 ± 32 mg). Bars indicate standard errors.

leaf discs and flower buds did not give satisfactory shoot regeneration (<1 shoot/explant). However, stem section explants used as described for the thin cell layer explants but with the hormone levels recommended by Fry *et al.* (1987) gave similar results for shoot regeneration and transformation (Plate IV.3, Table III.8). Moreover, stem section explants appeared to have a lower hypersensitive response to *A. tumefaciens*.

IV.B.2 Toxicity of antibiotics to *B. napus* tissues

For *Agrobacterium*-mediated transformation, different antibiotics are used either to eliminate *Agrobacterium* after cocultivation or to select transformed cells that express chimeric antibiotic selectable marker genes. The use of such markers is dependant on the sensitivity of the cocultivated plant tissue to the antibiotics. Once transformed tissues and transgenic plants are recovered, it is also important to assess the expression of the introduced gene in the progeny to avoid artifacts generated in tissue culture. A survey was undertaken to evaluate the sensitivity of *B. napus* thin cell layer explants, seeds, embryos derived from microspores and microspores to potential selective agents which have been demonstrated useful in other studies (see section I.C.2). These were kanamycin, geneticin, paromomycin, chloramphenicol, hygromycin, bleomycin and methotrexate. Another antibiotic, tunicamycin, was used in cell-cycle studies (Ettlinger *et al.* 1986, Loschiaro *et al.* 1986) and has the potential to be used as a genetic transformation marker.

All the antibiotics were toxic to *B. napus* tissues tested. Microspores were the most sensitive to antibiotic action and their

Plate IV.2 Comparison of B. napus explants.

Thin cell layer explants (A) on nurse culture after 4 days and (B) 1 month after cocultivation with A. tumefaciens. Stem sections (C) on nurse culture after 4 days and (D) 1 month after inoculation with A. tumefaciens

sensitivity was 10 to 100 times higher than the other tissues (Fig. IV.2.a,b) with levels from 0.01 to 10 ug/mL preventing any embryo development. Thin cell layer explants were the most resistant in terms of tissue growth (Fig. IV.3.a,b) but shoot differentiation was generally prevented at a lower level than what was needed to kill seedlings (Table IV.1; from 0.1 to 100 ug/mL); however, higher levels of tunicamycin, methotrexate and paromomycin were needed to prevent shoot differentiation from explants than to kill the seedlings. Microspore-derived embryos possessed antibiotic sensitivities between microspores and thin cell layer explants (0.005 to 100 ug/mL) except with methotrexate, tunicamycin and bleomycin which were toxic at the same level as for microspores (Fig. IV.4.a,b,c). The toxic levels for seedlings were comparable or lower than the levels that prevent tissue growth in the thin cell layer explants (0.005 to 100 ug/mL, Table IV.1). The antibiotic toxicities were to a certain extent specific to the tissue tested and general trends were difficult to determine.

Methotrexate was the most toxic chemical used and the levels of toxicity were equivalent in all the tissues (0.005 to 0.01 ug/mL). Tunicamycin, bleomycin and hygromycin were the most potent antibiotics after methotrexate; however, the levels of toxicity varied with the tissue tested. Chloramphenicol and streptomycin were the least toxic. Chloramphenicol toxicity was difficult to assess with seedlings. Although inhibition was seen during the first 2 to 3 weeks on the medium, the seedling seemed to recover even if they were transferred to fresh medium. The 3 aminoglycoside antibiotics (kanamycin, paromomycin and geneticin) had similar toxicities to B. napus tissues; their toxicity levels varied with the tissue tested and they were generally

Figure IV.2 Influence of antibiotics on B. napus cv. Topas microspore development. Two Petri dishes per antibiotic concentration were incubated for 4 weeks. The percentage of microspore that gave embryos is given relatively to the microspores grown without antibiotics. The approximate number of starting microspores was 10^4 per Petri dish giving rise to approximately 600 embryos for the control.

TABLE IV.1.1. Inhibitory Levels of Antibiotics for B. napus Seedling Growth and Shoot Differentiation From Thin Cell Layer Explants

Antibiotic	Inhibitory levels (ug/mL)	
	seedling growth	shoot differentiation
kanamycin	25	35 ^a
paromomycin	20	100
geneticin	50	5
chloramphenicol	80 ^b	20
hygromycin	5	1
bleomycin	50	1
streptomycin	100	50
tunicamycin	1	10 ^c
methotrexate	0.005	0.01

The results were taken after 6 weeks. a: white shoots developed but eventually died. b: toxicity to chloramphenicol was observed at 80 ug/mL but the seedlings seemed to recover after the 6 week period, c: tunicamycin killed all the calli after the observation period; however, 2 shoots came out of 12 explants even if the tissues looked dead.

Figure IV.3 Influence of antibiotics on *B. napus* cv. Westar thin cell layer explant growth. At least 12 explants (fresh weight 10 ± 2 mg) cocultivated with strain GV3101 were incubated on media with antibiotics at each of the concentrations. The average fresh weights after five weeks were expressed as a percentage of the average fresh weight of explants incubated without antibiotics (601 ± 32 mg). Bars indicate standard errors.

Figure IV.4 Influence of antibiotics on *B. napus* cv. Topas microspore-derived embryo growth. At least 20 embryos (fresh weight 3 ± 2 mg) of 2 week old were incubated on media with antibiotics at each of the concentrations. The average fresh weights after five weeks were expressed as a percentage of the average fresh weight of explants incubated without antibiotics (403 ± 46 mg). Bars indicate standard errors.

intermediate between the least toxic antibiotics (chloramphenicol and streptomycin) and the most toxic ones (bleomycin, tunicamycin and hygromycin).

Two types of toxic symptoms were observed with shoots and seedlings. The first type associated with kanamycin and streptomycin, was the appearance of purple leaves that became white after a while. The second type observed with the other antibiotics, was wilting and browning of the leaves and stems.

IV.B.3 Selection and regeneration of transformants

Based on the results obtained with oncogenic Ti vectors, the disarmed pGV3850 was chosen among the vectors available at the time of these experiments. The intermediate vectors pLGV1103neo (Hain et al. 1985) and pNCAT4 (DeBlock et al. 1984) which are cointegrates and pBCAT1 (M. Bevan, unpublished) which is a binary vector were used to recover transgenic plants. Kanamycin and chloramphenicol were the selective agents corresponding to the resistance genes in the intermediate vectors (pLGV1103neo, pBCAT1 - Km; pNCAT4, pBCAT1 -Cm). Detailed toxicity profiles with kanamycin and chloramphenicol for thin cell layer explants after cocultivation with Agrobacterium carrying only pGV3850 displayed an abolition of shoot differentiation at 35 ug/mL of kanamycin and 20 ug/mL chloramphenicol (Fig. IV.5); however, following cocultivation with Agrobacterium carrying pGV3850::pLGV1103neo or pGV3850,pBCAT1, shoots did not differentiate in the presence of 35 ug/mL of kanamycin despite repeated attempts. The same strains were used to recover N. tabacum shoots resistant to 100 ug/mL kanamycin (see section IV.B.6). At 15 ug/mL kanamycin many

Figure IV.5 Detailed profiles of the effects of kanamycin and chloramphenicol on thin cell layer explants of *B. napus* cv. Westar. At least 12 explants (fresh weight 10 ± 2 mg) cocultivated with strain GV3101 were incubated on media with antibiotics at each of the concentrations indicated (Km ●—● ■—■; Cm ○—○ □—□). The average fresh weight after five weeks (●—● ○—○) were expressed as a percentage of the average fresh weight of explants incubated without antibiotics (601 ± 32 mg). Bars indicate standard errors. The frequency of shoot differentiation (■—■ □—□) was expressed as a percentage of the frequency (shoots per explant) measured for explants without antibiotics.

SHOOT PER EXPLANT (% OF CONTROL)

3

Microlin

shoots differentiated but several were gradually lost at later stages of growth and differentiation (Plate IV.3). Typically, these shoots became chlorotic and would not form roots before dying (Plate IV.3.b). Many that survived and formed roots in the presence of 15 ug/mL kanamycin after 1-2 months (Plate IV.3) were successfully transferred to soil after 2-3 months. Approximately one-half of the plants displayed nopaline synthase activity following transformation with pGV3850::pLGV1103neo. Most of them displayed activity after transformation with pGV3850,pBCAT1; however, nopaline synthase activity in these plants reflected transformation by the T-DNA of pGV3850 whereas the NOS-NPTII-NOS and 35S-CAT-NOS genes were carried by pBCAT1. A summary of these experiments is presented in Table IV.2.

With chloramphenicol, resistant shoots could not be recovered at 20 ug/mL after transformation with pGV3850::pNCAT4 or pGV3850,pBCAT1. Reduction of chloramphenicol to 7 ug/mL yielded many shoots but tissues transformed with pGV3850 alone also yielded shoots at this level despite a suppression of tissue growth (Fig. IV.5). About one half of the plants recovered following transformation by pGV3850::pNCAT4 were associated with nopaline synthase activity (Table IV.2).

Most of the plants potentially transformed with pBCAT1 displayed nopaline synthase activity encoded by pGV3850 (Table IV.2). Three of these plants were recovered on kanamycin and 2 on chloramphenicol.

Transformation with A. tumefaciens containing pGV3850 alone also gave transgenic plants based on the activity of the nopaline synthase gene. Three plants were positive out of 10 plants transferred to soil.

The vectors pVU1011 (35S-HPT-NOS; M. Bevan unpublished) and pMON806 (35S-DHFR-NOS; Monsanto Co., unpublished) were also used in

Plate IV.3 Selection of transformed shoots (a) and plantlets (b) on kanamycin.

Thin cell layer explants were cocultivated with A. tumefaciens harbouring pGV3850::pLGV1103neo and cultured on 15 ug/mL kanamycin. a) shoot differentiation occurred in the presence of kanamycin; however, many shoots became chlorotic (s) and died. b) the survivors (r) were excised and roots were induced in the presence of kanamycin. Many of them did not form roots. They became chlorotic and eventually died (s). Others formed roots in the presence of kanamycin (r) and were transferred to soil.

TABLE IV.2. Recovery of Transformed *B. napus* plants

Plasmids	Selection ^a	Explants inoculated	Resistant ^b shoots	Plantlets in soil	Nopaline ^c synthase
pGV3850::pLGV1103neo	Km	600	40/80	26	12
pGV3850::pNCAT4	Cm	600	30/70	19	10
pGV3850,pBCAT1	Km,Cm ^d	200	10/40	6	5
pGV3850	none	60	NA	10	3
PGV3850,pVU1011	Hy	300	none	NA	NA
pGV3850::pMON806	Mtx	400	9/50	lost ^e	NA

a=antibiotic selections were performed at 15 ug/mL Km, 7 ug/mL chloramphenicol, 2 ug/mL hygromycin or 0.005 ug/mL methotrexate. b=the resistant shoots are indicated as a proportion of the total shoots that differentiate in the presence of low levels of antibiotics.

c=nopaline synthase activity was determined for plants established in soil as indicated in section II.D.9. d=explants transformed with pBCAT1 were subjected to selection on Km or Cm. Each of the selections yielded 3 plants. These were analyzed for nopaline synthase activity which was encoded by pGV3850 T-DNA. e=The plantlets were lost during the transfer to the greenhouse. NA=not applicable.

combination with pGV3850 in A. tumefaciens cocultivation of B. napus (Table IV.2). Transgenic plants were not recovered with these two vectors. With the vector pVU1011, shoots resistant to hygromycin (2ug/mL) were not recovered whereas shoots resistant to methotrexate (0.005ug/mL) were recovered with the vector pMON806 but they were subsequently lost when transferred to the greenhouse.

All of the plants that displayed nopaline synthase activity flowered and set seeds within 5 to 6 months. In morphology and fertility, these plants were indistinguishable from control plants derived from seeds or from explants grown under identical conditions (Plate IV.4).

IV.B.4 Analyses of transgenic plants

IV.B.4.a Resistance to kanamycin and expression of the NPT II gene.

To assess the level of kanamycin that could be tolerated, shoots were cloned from the axillary buds of plants selected on 15 ug/mL kanamycin and from explants that were cocultivated with A. tumefaciens containing pGV3850::pLGV1103neo and pGV3850,pBCAT1. Shoots from each were subcultured on media with 20, 40, 80 and 100 ug/mL kanamycin containing 500 ug/mL Clavulin or Claforan. If the Clavulin 250 was present, the non-transformed shoots grew without toxicity symptoms at 20 ug/mL; however, with cefotaxime or no antibiotics, 20 ug/mL kanamycin was toxic to the non-transformed shoots.

In one transformation experiment where shoots resistant to 15 ug/mL were recovered on cefotaxime containing medium but not screened for nopaline synthase activity, 13 plants were found sensitive to 20 ug/mL kanamycin (Table IV.3). One plant was tolerant to 40 ug/mL but

Plate IV.4 Comparison of regenerated transgenic plants with normal B. napus cv. Westar plants. a) Transgenic plant transformed with pGV3850::pLCV1103neo. c,e) leaf; flower, floral parts, seeds derived from transgenic plant. b) normal B. napus cv. Westar plant grown from seed. d,f) leaf, flower, floral parts, seeds derived from normal plants.

not 80 ug/mL and another plant was resistant to 80 ug/mL but not 100 ug/mL. In another transformation experiment, where the shoots were selected on 15 ug/mL kanamycin and tested positive for nopaline synthase activity in the presence of Clavulin 250, all of the 10 shoots tested (including control) were resistant to 20 ug/mL. Eight nopaline synthase positive shoots were resistant to 40 ug/mL, six were resistant to 80 ug/mL and none to 100 ug/mL kanamycin. The control shoots under these conditions were sensitive at 40, 80, and 100 ug/mL.

The activity of the NPT II gene was assayed according to the protocol of McDonnell et al. (1987) in a few transgenic plants. Significant levels of activity were detected in transgenic plants no. 8 and no. 10. Plants grown in growth chambers had to be used and incubation of the leaf material on 40 ug/mL Km increased the detected NPT II activity (Table IV.4, Plate IV.5) without affecting the background activity of the control. In N. tabacum, the NPT II activity was much higher with the transgenic plants transformed with an NPT II gene linked to a 35S promoter (11 399 cpm) than with transgenic plants transformed with NPT II gene fused to a NOS promoter (2670 cpm; Table IV.4). This latter activity was also higher than what was found in transgenic B. napus (471 cpm; Table IV.4, Plate IV.5).

IV.B.4.b Factors affecting the use of CAT as a marker for B. napus genetic transformation

Experiments for chloramphenicol sensitivity were performed with 8 plants transformed with pGV3850::pNCAT4 and pGV3850,pBCAT1 that were subjected to various levels of chloramphenicol (20, 40, 80, 100 ug/mL); however, the results were inconclusive due to the inherent and variable

TABLE IV.3. Sensitivity to Kanamycin of Recovered Shoots after Cocultivation
Clone #*

	Levels of Km (ug/mL)			
	20	40	80	100
-Not tested for nopaline synthase on claforan				
13 shoots				
#8	S	S	S	S
#10	R	R	R	S
control	R	R	R	S
-Nopaline synthase positive on Clavulin 250				
NEO 6A	R	R	NT	NT
NEO 12A	R	R	R	NT
NEO 8B	R	R	R	NT
NEO 9B	R	R	R	S
NEO11B	R	S	S	S
NEO 12B	R	R	R	NT
BCAT 1A	S	S	S	S
BCAT 1B	R	R	R	NT
BCAT 1C	R	R	R	NT
BCAT 2B	R	R	S	S
Control	R	S	S	S

NT= not tested, *Plants #8, #10 and NEO were recovered after cocultivation
with pGV3850::pLGV1103neo. Plants BCAT were recovered after cocultivation
with pGV3850,pBCAT1.

TABLE IV.4. Expression of the NPT II Gene in Transgenic N. tabacum and B. napus Plants

Plant	Source of leaf tissue		
	<u>In vitro</u>	<u>In planta</u>	<u>In planta</u>
Incubated on Km			
Control <u>N. tabacum</u>	180 $\pm$ 6	177 $\pm$ 11	199 $\pm$ 14
Transgenic <u>N. tabacum</u> (NOSP*)	2670 $\pm$ 61	NT	NT
(35SP**)	11399 $\pm$ 492	NT	NT
Control <u>B. napus</u>	201 $\pm$ 10	159 $\pm$ 5	173 $\pm$ 6
Transgenic <u>B. napus</u> (NOSP*)	282 $\pm$ 26	326 $\pm$ 23	471 $\pm$ 55

The units are in counts per minute, the average of 3 experiments is given with the standard deviation. *NOSP= nopaline synthase promoter,

**35SP= 35S promoter of CAMV. NT= not tested.

Plate IV.5 Neomycin phosphotransferase assay with transformed tissues.

Control N. tabacum= A1,A2,B1; Control B. napus= E1,F1,F2; Transgenic N. tabacum transformed with pGV3850::pLGV1103neo= A3,A4,A5; Transgenic B. napus from greenhouse incubated on kanamycin= B2,B3,B4,B5,C2,C3,C4,C5; Transgenic B. napus grown in vitro= C1,D1,D2,D3; Transgenic B. napus from greenhouse= D4,D5,E2,E3,E4,E5, F3,F4,F5.

tolerance of differentiated B. napus shoots to this antibiotic (see section IV.B.2).

Further experiments were made to explain these results and to pursue the preliminary observations that plant endogenous CAT (chloramphenicol acetyl transferase) activity existed in B. napus (Balazs and Bonneville 1987, Charest et al. 1986).

IV.B.4.b.(1) Plant endogenous CAT activity

A survey on the presence of plant endogenous CAT activity was undertaken to assess its extent in the Brassica genus and in other Cruciferae. All the Brassica species examined revealed high levels of CAT activity (Table IV.5). This included the cultivated diploid species (B. campestris, B. oleracea, B. nigra and B. hirta) and the wild diploid species (B. kaber). The cultivated amphidiploid descendants of the diploid cultivated species (B. juncea, B. carinata and B. napus) also displayed the endogenous CAT activity in leaves. Furthermore, nine different cultivars of B. napus were tested (Altex, Jet Neuf, Midas, Oro, Target, Topas, Atow, Westar and Zephyr) and high levels of plant CAT activity were evident in all (Table IV.5). In comparison with N. tabacum which displayed little CAT activity (0 to 5%; Table IV.6), the Brassica endogenous CAT activity was very prominent (15 to 32%; Table IV.6). In this study, CAT activity is defined as the percent conversion of [^{14}C]chloramphenicol to its acetylated forms. This activity was sensitive to heat denaturation at 100°C for 20 min or digestion with proteinase K for 30 min. at 37°C. Furthermore, it was dependant on acetyl CoA as a substrate suggesting that the reaction was catalysed by endogenous enzymes.

TABLE IV.5. Brassica Species and Cultivars Examined for Endogenous CAT Activity^a

Species	Genomic composition ^b	Cultivars and lines
<u>B. campestris</u>	AA	Candle
<u>B. nigra</u>	BB	
<u>B. oleracea</u>	CC	
<u>B. hirta</u>	DD	
<u>B. kaber</u>	SS	
<u>B. juncea</u>	AABB	ZEM
<u>B. carinata</u>	BBCC	
<u>B. napus</u>	AACC	Altex, Jet Neuf, Midas, Oro, Target, Topas, Atow, Westar, Zephyr

^aEndogenous CAT activity varied between 10 to 30% conversion of the [¹⁴C]chloramphenicol to its acetylated derivatives as measured on selected samples. ^bThe genomic composition indicates the diploid content of the genotype.

TABLE IV.6. CAT Activity in Control and Transgenic N. tabacum and B. napus
Cultivar Westar

		CAT activity	
Type of tissue	Vector	<u>N. tabacum</u>	<u>B. napus</u> buffer ^a
Control			
no additions	--	2-5 %	15-32% --
+25 Units CAT ^b	--	75-95%	1-2% 87-99%
Transformed			
	pBCAT1	95-98%	15-30% --
	pNCAT4	NT ^c	15-30% --

^aAssays performed without cell-free extracts but extraction buffer only.

^bA unit of CAT is defined as the quantity of CAT enzyme needed for the acetylation of one nanomole of chloramphenicol per minute at 37°C, pH 7.8; the CAT enzyme added to the control leaf tissues was produced by a gene identical to the one used to construct pNCAT4 and pBCAT1. ^cNot tested

Endogenous CAT activity detected by autoradiography was found in all of the tissues and cell-types of B. napus cultivars Jet Neuf, Topas and Westar that were surveyed. Leaves, stems and roots from mature plants and immature aseptically grown seedlings each revealed high levels of endogenous CAT activity (Table IV.7). The same observations were made with cells and tissues grown in culture such as cell suspension cultures of cv. Jet Neuf or protoplasts made from them, as well as calli or protoplasts from thin cell layer explants of cv. Westar. Embryos derived from cultured microspores of cv. Topas also displayed high levels of CAT activity (Table IV.7). Incubation of cell suspensions, seedlings and callus in the dark for 1 to 5 days did not influence the proportion of [14 C]chloramphenicol and the acetylated forms monitored by autoradiography (data not shown).

IV.B.4.b.(2) Transgenic bacterial CAT activity and inhibition in B. napus

Transgenic N. tabacum plants transformed with pGV3850, pBCAT1 were used as a positive control for the expression of transgenic bacterial CAT activity. The vector pBCAT1 carries a CAT gene from Tn9 fused to a 35S promoter in addition to an NPT II gene fused to a nos promoter. Transgenic plants were selected on either chloramphenicol (10 ug/mL) or kanamycin (100 ug/mL). Five plants tested yielded very high levels of CAT activity (95 to 98%, Table IV.6). Five transgenic B. napus plants transformed with pGV3850: pNCAT4 were selected on chloramphenicol (7ug/mL). Two plants transformed with pGV3850, pBCAT1 were selected separately on chloramphenicol (7 ug/mL) and on kanamycin (15 ug/mL). They all showed lower but significant CAT activity (15 to 30%, Table

TABLE IV.7. Survey of CAT Activity Detected by Autoradiography
in B. napus Tissues

Cells or Tissues	<u>B. napus</u> Cultivars		
	Westar	Jet Neuf	Topas
Leaf	++ ^a	++	++
Stem	++	++	NT ^b
Root	++	++	NT
Seedling	++	++	NT
Callus ^c	++	++	NT
Cell suspension	NT	++	NT
Microspore-derived embryos	NT	NT	++
Protoplasts ^d	++	++	NT

^aCAT activity was measured on selected samples and 15-30% activity correlated with ++, ^bNT not tested, ^c for cv. Westar callus was derived from epidermal strips; for cv. Jet Neuf callus was derived from cell suspension. ^dfor cv. Westar protoplasts were derived from stem cortex; for cv. Jet Neuf protoplasts derived were from cell suspension.

IV.6); however, this level was indistinguishable from the control B. napus plants (Table IV.6). The integration of the CAT genes in these plants was confirmed by Southern blotting (see following section) and each revealed nopaline synthase activity.

The transgenic bacterial CAT activity appeared to be selectively inhibited in Brassica cell free extracts. CAT activity in extracts of transgenic N. tabacum leaf tissue was completely inhibited by the addition of one fifth volume of B. napus extract (Plate IV.6.a). As shown in Plate IV.6.c, complete inhibition of the transgenic CAT activity in N. tabacum was also achieved with B. juncea extract. The residual activity observed in the N. tabacum-Brassica mixtures were equivalent to the levels of endogenous activity found in the Brassica extracts. Inhibition was not observed with N. tabacum extracts from transformed or non-transformed tissues and from G. max, L. esculentum and A. thaliana (Plate IV.6.c). The inhibitor from B. napus was inactivated by heating at 100°C for 20 min and it was not separated from the extract by dialysis (Plate IV.6.b).

Bacterial CAT enzyme was added to N. tabacum or B. napus leaf tissue extracts. This CAT enzyme (Pharmacia Canada, Montreal, Canada) was identical to the enzyme produced by the CAT gene of transposon Tn9 used to make the vectors pNCAT4 and pBCAT1. The addition of 25 units of bacterial CAT enzyme to leaf tissue extracts yielded activities of 1 to 2% for B. napus and 75 to 95% for N. tabacum (Table IV.6) compared to 87 to 99% when the pure enzyme was assayed in the absence of cell-free extracts (Table IV.6). The relative levels of activity obtained with the enzyme mixed with plant tissues were still stronger than the plant endogenous CAT activity in N. tabacum; however, with B.

Plate IV.6. CAT activities in cell-free extracts of N. tabacum transformed with vector pBCAT1 and detection of an inhibitor in Brassica species.

A. CAT activities in cell-free extracts of transgenic N. tabacum diluted with reaction buffer in the ratios 5:0(lane 1), 4:1(lane 2), 3:2(lane 3), 1:1(lane 4), 2:3(lane 5), 1:4(lane 6) or mixed with B. napus cell free extract in the ratios 1:4(lane 7), 2:3(lane 8), 1:1(lane 9), 3:2(lane 10), 4:1(lane 11). B. CAT activities in cell free extracts of transgenic N. tabacum mixed with B. napus extracts (3:2 ratio) that was heated at 100°C for 20 min (lane 1) or dialysed against extraction buffer (See Material and Methods) (lane 3). CAT activities in cell free extracts of B. napus alone after heating (lane 2) and dialysis (lane 4). CAT activities in cell free extracts of transgenic N. tabacum diluted (3:2 ratio) with reaction buffer (lane 5) or cell free extracts of control untransformed N. tabacum (lane 6). C. Comparison of transgenic CAT activity in N. tabacum (lane 4) with endogenous CAT activities in N. tabacum (lane 1), S. bicolor (lane 2), L. esculentum (lane 3), B. napus (lane 5), B. juncea (lane 6), A. thaliana (lane 7). CAT activity in cell free extracts of transgenic N. tabacum diluted (3:2) with extracts from L. esculentum (lane 8), G. max (lane 9), B. napus (lane 10), B. juncea (lane 11), A. thaliana (lane 12). D. Activity of purified bacterial CAT enzyme mixed with cell free extracts of B. napus (lane 1) and control N. tabacum (lane 3). CAT activity in N. tabacum extracts alone (lane 2).

The CAT assays were performed as outlined in the Material and Methods section. Autoradiographs of TLC of [¹⁴C] chloramphenicol (Cm) acetylated by the CAT enzyme to give 1-acetyl [¹⁴C] chloramphenicol (1-A-Cm), 3-acetyl [¹⁴C] chloramphenicol (3-A-Cm) and 1,3-diacetyl [¹⁴C] chloramphenicol (1,3-A-Cm) are shown. In mixing experiments, extracts from each species were pooled together and then aliquoted to avoid any sample variation.

napus cell free extracts the bacterial CAT activity was absent and the plant endogenous CAT activity was reduced (Table IV.6).

Differential sensitivities of endogenous CAT activity and transgenic CAT activity to heat was not observed. When leaf tissues were heated at 68°C for 40 min, 75°C for 10 in or 80°C for 10 min, N. tabacum transgenic bacterial CAT activity was only slightly reduced by these heat treatments and the level of CAT activity was still well over background. In transgenic and control B. napus, the level of CAT activity was also slightly reduced and indistinguishable from each other.

IV.B.4.c Nopaline synthase expression in transgenic B. napus

Transgenic plants were initially screened for nopaline synthase activity in leaves. Ten leaf pieces were taken randomly on each putative transgenic plant and put on arginine containing MAH medium (see section II.E.1.a) for 2 days and assayed for the conversion of [¹⁴C]arginine to nopaline. Important variations were observed from plant to plant within the 10 samples analyzed (Table IV.8). An index of variation was assigned to each plant from 1 to 4; 1 indicating that no or very little variation was observed and 4 indicating a maximum of variation. It was not possible to detect nopaline synthase activity in transgenic shoots kept in vitro; however, tumour callus grown in vitro always tested positive.

IV.B.4.d Southern blot analysis of transgenic B. napus

For 10 plants that were nopaline synthase positive and transformed with pGV3850::pNCAT4, 7 had sequences hybridizing with the nopaline

TABLE IV.8. Variation of NOS Activity in Transgenic *B. napus* Plants

Clone #*	# of NOS positive/10	Variation index
NEO 8B	10	1
9B	7	4
10B	10	3
11B	8	4
12B	7	4
1C	7	4
7A	3	1
11A	2	2
12A	1	2
1D	4	2
2D	8	3
NCAT 6A	5	4
2B	1	1
3B	2	3
4B	6	3
5B	2	1
7B	5	3
8B	9	3
1C	1	3
4C	3	3
1E	3	4
BCAT 1A	10	2
2A	5	4
1B	10	4
2B	2	3
1C	7	4

The variation index is an indication of the variability of the NOS activity within a plant; it is a reflection of the strength of the conversion of arginine to nopaline. A minimum of variation is indicated by a V.I. of 1 and a maximum by a V.I. of 4.

NEO plants were recovered after cocultivation with pGV3850: :pLGV1103neo, NCAT plants were recovered after cocultivation with pGV3850::pNCAT4, BCAT plants were recovered after cocultivation with pGV3850,pBCAT1.

TABLE IV.9. Summary of the Detection of Integrated Sequences in Transgenic *B. napus*

Plant #	# of sites NOS-RB*	Probe used	
		presence or absence	
		CAT**	NPTII**
NCAT 2B	3	-	
NCAT 3B	3	+	
NCAT 4B	1	+	
NCAT 5B	nd†	+	
NCAT 7B	2	+	
NCAT 8B	2	-	
NCAT 6A	nd	-	
NCAT 1C	1	-	
NCAT 1E	1	+	
BCAT 1A	1	-	-
BCAT 1B	1	+	1§
BCAT 2B	nd	-	-
BCAT 1C	4	+	5§
NEO 11A	4		+
NEO 12A	nd		+
NEO 8B	2		+
NEO 9B	2		+
NEO 10B	nd		-
NEO 11B	1		+
NEO 12B	2		+
NEO #8	nd		+
NEO #10	3		+

*The plant DNA, digested with Hind III and Bam HI, was probed with a 0.995 kb DNA fragment including part of the NOS gene and some sequences left of the right T-DNA border (see appendix A.7). The different sizes of the bands observed were an indication of various integration sites (see Plate IV.7 for examples) due to right border rearrangement. **The CAT and NPT II genes were detected as described in Plate IV.7 with probes from the pNCAT4 plasmid (see appendix A.3) and pLGV1103neo (see appendix A.4). † nd= not detected; however, this does not imply the absence of integration but rather the absence of the probed sequences. § The plant DNA in this case was digested with Hind III which allowed the detection of different band sizes due to the border rearrangement.

synthase (NOS) probe (Table IV.9). Right border usage was evident and the number of independent copies integrated varied from 1 to 3. Four plants out of the 7 that showed the NOS sequences had the specific 0.8 kb Bam HI fragment of the CAT gene (Plate IV.7). One plant had the CAT sequences without hybridizing to the NOS sequences.

For 9 plants that were nopaline synthase positive and transformed with pGV3850::pLGV1103neo, 6 plants displayed sequences hybridizing with the NOS probe (Table IV.9). Right border usage was again evident and the number of independent copies integrated varied from 1 to 4. Six plants out of the six that had DNA sequences homologous to the NOS probe had positive hybridization signals with the NPT II gene. Two plants had DNA hybridizing with the NPT II gene without hybridization to the NOS gene. Four plants showed the expected 1.6 kb Pst I fragment of the NPT II gene (Plate IV.7) whereas four plants showed unexpected fragment sizes (Plate IV.8).

Three out of 4 plants that were nopaline synthase positive and transformed with pGV3850,pBCAT1 showed sequences hybridizing with the NOS gene. The number of independent integration sites was 1 in 2 plants and 4 in the third one. Two of the 3 plants had sequences hybridizing with both the NPT II and the CAT gene sequences. The Eco RI CAT fragment was the expected size of 0.85 kb (Plate IV.8).

IV.B.5 Transmission of the marker gene to the progeny

Genetic analysis performed on the progeny of some of the transgenic plants indicated that the kanamycin resistance gene (Table IV.10, and Plate IV.9) and the nopaline synthase gene (Table IV.11) were transmitted to the progeny and expressed; however, some of the ratios

Plate IV.7. Southern Blot analyses of transgenic plants.

The arrows indicate the minimum fragment size that would encompass the T-DNA junction at the right border of pGV3850 following digestion with Bam HI and Hind III and hybridization with the Bam HI-Sst II fragment from the Hind III 23 fragment of pTiC58 as the probe. DNA from transgenic plant NCAT 7B transformed with pGV3850::pNCAT4 was digested with Bam HI and Hind III (lane 1). The 1.2 kb Bam HI-Hind III fragment of pNCAT4 is indicated. Two T-DNA-plant DNA junction fragments of 4.1 and 2.3 kb were revealed. DNA from transgenic plant NEO 12B transformed with pGV3850::pLGV1103neo was digested with Pst I, Bam HI and Hind III (lane 3). Two T-DNA-plant DNA junction fragments of 3.0 kb and 1.9 kb were revealed in addition to the 1.4 kb Pst I fragment of pLGV1103 neo. DNA from transgenic plant BCAT1B transformed with pGV3850, pBCAT1 digested with Bam HI and Hind III (lane 6). A single T-DNA-plant DNA junction fragment of 1.45 kb was revealed. Comparable bands were not found in DNA from untransformed control plants (lanes 2,4,5). The 1.6 kb Pst I fragment of pLGV1103 neo was used as the probe to reveal the same fragment which contains the coding region of npt II in the DNA of NEO 12B digested with Pst I (lane 7). The coding region of NPT II was also demonstrated in a 7.0 kb fragment from DNA of transgenic plant BCAT 1B digested with Pst I (lane 8). The 0.8 kb Bam HI fragment of pNCAT4 was used as the probe to reveal the same fragment which contains the coding region of chloramphenicol acetyltransferase gene in the DNA of NCAT 7B digested with Bam HI (lane 9). A comparable band is not found in DNA from untransformed control plant (lane 10). The maps of the plasmids containing the DNA probe fragments are given in appendices A7 (for the NOS probe), A4 (for the NPT II probe) and A3 (for the CAT probe).

Plate IV.8 Southern blot analyses for the NPT II and CAT genes.

Part 1- Lanes A to H, the 1.6 kb Pst I fragment of pLGV1103neo was used as a probe to reveal the same fragment which contains the coding region of NPT II in the DNA of transgenic plants digested with Pst I (plant NEO 8B, lane B; plant NEO #10, lane E; plant NEO 11B, lane G). In some transgenic plants, bands of unexpected sizes were detected (plant NEO 9B, lane C; plant NEO 11A, lane F; plant NEO 12A, lane H). Comparable bands were not found in DNA from untransformed control plant (lane A). Part 2- Lanes I and J, the 0.8 kb Bam HI fragment of pNCAT4 was used as the probe to reveal an expected fragment of 0.85 kb in the DNA of BCAT 1B plant digested with Eco RI (lane I). A comparable band was not found in DNA from untransformed control plant (lane J). 20 ug of DNA were loaded into each lane. The map and location of the DNA fragment used as a probe is given in appendix A4.

TABLE IV.10. Inheritance of Kanamycin Resistance in the Progeny of
B. napus Transgenic Plants

Clone #*	Levels of kanamycin(ug/mL)		
	20	40	80
	R:S**	R:S	R:S
#8	--	13:5	37:17
#10	--	--	20:16
NEO 12A	8:5		
NEO 8B	7:4		
NEO 9B	--	69:25	
NEO 10B	5:3		
NEO 11B	2:13		
NEO 12B	36:25	20:26	
NCAT 6A	0:25		
Control	0:25		
BCAT 1A	1:9	0:10	0:10
BCAT 1B	7:8		
BCAT 2B	2:13	0:15	0:15
BCAT 1C	--	65:60	

*NEO, #8 and #10 plants were recovered after cocultivation with pGV3850:
 :pLGV1103neo, NCAT plants were recovered after cocultivation with
 pGV3850::pNCAT4, BCAT plants were recovered after cocultivation with
 pGV3850,pBCAT1. **R= resistant, S= sensitive.

Plate IV.9 Segregation of the kanamycin resistance in the progeny of transgenic B. napus plants.

a) seeds from a self pollinated transgenic plant NEO 12B and b) control B. napus plants were germinated in the presence of 20 ug/mL kanamycin. Sensitive seedlings (s) were chlorotic and growth was inhibited. Resistant seedlings were green and displayed normal growth characteristics.

TABLE IV.11. Segregation of the Nopaline Synthase Marker in the Progeny
of Selected Transgenic Plants

Clone #	NOS+ : NOS-
NCAT 4B	14 : 5
NCAT 3B	5 : 5
NCAT 1E	12 : 4
#8	4 : 6
#10	3 : 4

NCAT plants were recovered after cocultivation with pGV3850::pNCAT4,
#8 and #10 plants were recovered after cocultivation with pGV3850:
:pLGV1103neo.

obtained did not support a single Mendelian pattern of segregation as expected from the Southern blot analyses. Others such as NCAT 4B and NCAT 1E revealed single T-DNA integration in Southern blot and 3:1 segregation for the nopaline synthase gene. These were less frequent. Results with chloramphenicol were inconclusive.

IV.B.6 Recovery of transgenic B. napus plants from A. rhizogenes cocultivation

Transgenic B. napus plants from A. rhizogenes-mediated transformation were recovered to compare them with transgenic plants transformed with A. tumefaciens containing disarmed Ti plasmid. Shoots were recovered by culture of hairy roots from transformed stem sections on medium without hormones (MAH). After approximately 2 months, shoots developed at a low frequency (2 or 3 shoots per petri dish covered with hairy roots). These shoots were excised and grown in Magenta boxes on MAH medium (Plate IV.10). Five plantlets were grown and all showed the characteristic symptoms of the hairy root disease. The leaves were wrinkled, the internodes were shortened and the roots were plagiotropic. When the plants were grown in soil, the wrinkled leaves and the short internodes were severe compared to normal plants (Plate IV.10). Moreover, the flower buds did not develop normally and senesced prematurely.

IV.B.7 Comparison of B. napus explant and N. tabacum leaf disc cocultivations

N. tabacum leaf disc transformation is considered to be a model system for genetic engineering studies. This system was used for three major reasons: to verify the effectiveness of the disarmed

Plate IV.10 Plant regeneration from A. rhizogenes transformed B. napus tissues.

A) B. napus plantlet regenerated from A. rhizogenes cocultivated explant; wrinkled leaves, short internodes and plagiotropic roots are evident. B) Comparison between a normal B. napus cv. Westar plant (left) and A. rhizogenes transgenic (right) B. napus cv. Westar plant (both are 8 week old). C) Senescing flowers of A. rhizogenes transgenic B. napus cv. Westar plant. D) A. rhizogenes transgenic B. napus cv. Westar plant. E) comparison between (right) normal plant and A. rhizogenes (left) transgenic B. napus plant.

vectors used; to compare the efficiency of the N. tabacum leaf disc system with the B. napus explant system; and was to test genes for expression in plants (see Chapter V).

All the Ti disarmed vectors (cointegrate or binary) used in this study functioned with N. tabacum leaf discs. Shoots resistant to high level of antibiotics were recovered (Table IV.12) and those tested for the expression of the introduced gene were all positive (Table IV.12).

Striking differences were observed between N. tabacum leaf discs (Plate IV.11) and B. napus explants. First, no hypersensitive response to A. tumefaciens was observed with N. tabacum leaf discs; i.e. the explants did not show any tissue darkening after cocultivation. Second, the number of shoots per explant was consistently high (20-30 shoots/explant) and a delay in shoot development was not observed relative to uninoculated tissues. Third, the time required to obtain transgenic tissues or shoots was very short. With kanamycin selection, shoots were seen within 3 weeks and rooted plantlets regenerated in two months compared to 3 to 4 months with B. napus explants. Fourth, high levels of antibiotics were effective in the recovery of transgenic shoots (Table IV.12). Fifth, toxicity to antibiotics such as Clavulin 250, cefotaxime and ceforan was not observed with N. tabacum in contrast to the observations with B. napus. Sixth, transfer of the explants was less frequent with N. tabacum (every 4 to 6 weeks) compared with B. napus (every 2 weeks). Overall, the N. tabacum leaf disc cocultivation protocol was fast and did not require the same effort as B. napus explants.

TABLE IV.12. *N. tabacum* Leaf Disc Cocultivation with *A. tumefaciens* Disarmed Ti Vectors

Plasmids	Selection	Explants	shoots/explant	proportion of positives
	(ug/mL)	inoculated		
pGV3850:				
:pLGV1103neo	Km (100)	30	20-30	5/5 NPT II
pGV3850:				
:pNCAT4	Cm (15)	30	20-30	5/5 CAT
pGV3850,	Km (100)	45	20-30	5/5 NPT II
pBCAT1	Cm (25)	45	20-30	5/5 CAT
pGV3850:				
:pMON806	Mtx (2)	45	20-30	3/3 NOS
pGV3850,				
pVU1011	Hy (50)	45	20-30	4/4 NOS

Selection was done with Km (kanamycin), Cm (chloramphenicol), Mtx (methotrexate), and Hy (hygromycin). A few transgenic resistant shoots were tested for the expression of the introduced genes (% positive), the genes tested were NPT II (neomycin phosphotransferase), CAT (chloramphenicol acetyltransferase), NOS (nopaline synthase).

Plate IV.11 N. tabacum leaf disc cocultivation and regeneration.

- A) N. tabacum leaf discs in culture for 2 day old (left) and 5 day old (right). B) Shoots from cocultivated leaf discs with A. tumefaciens after 5 weeks and selected on kanamycin (100 ug/mL). C) Control leaf discs on kanamycin (100 ug/mL) after 5 weeks. D) Regenerated resistant N. tabacum plantlet transformed with pLGV1103neo::pGV3850 on kanamycin (100 ug/mL).

IV.C DISCUSSION

II.C.1 Comparison of transgenic plants transformed with disarmed A. tumefaciens Ti plasmid and wild type A. rhizogenes Ri plasmid

Transgenic plants obtained with A. tumefaciens containing disarmed Ti plasmid were indistinguishable from control plants (Plate IV.4) and fully fertile. The same results were observed by other. (Pua et al. 1987, Fry et al. 1987, Radke et al. 1988). Transgenic plants obtained with A. rhizogenes displayed strong hairy root symptoms; that is to say wrinkled leaves, short internodes, plagiotropic roots and sterility (Plate IV.10). These abnormalities were too severe to consider these plants for practical or basic studies. Similar observations have also been made previously (Guerche et al. 1987c, Ooms et al. 1985). Since there is no disarmed Ri plasmid available at this time, the use of disarmed Ti plasmid vectors was pursued and the use of Ri plasmids discontinued.

IV.C.2 Recovery of transgenic b. napus plants generated with disarmed Ti plasmids

A prerequisite for the recovery of transgenic plants is the availability of a regenerable tissue culture system. A survey of

methods described for the regeneration of B. napus plants from tissue explants such as leaf discs (Dunwell 1981, Stringam 1979), flower buds (Margara and Leydecker 1978), stem sections (Stringam 1977, Kartha et al. 1974) and thin cell layer from stem explants (Klimaszewska and Keller 1985) revealed that thin cell layer explants and stem sections were particularly regenerable and well suited for the experimental manipulations involved in transformation by A. tumefaciens. A major obstacle encountered was the severe hypersensitive response of the tissue explants to A. tumefaciens and the associated loss of morphogenesis. The same phenomenon was observed with B. napus hypocotyl transformation with A. tumefaciens (Radke et al. 1988). This problem was alleviated somewhat by determining the optimal conditions for inoculating the explants and by modifying the culture conditions to enhance tissue survival. Preconditioning, the use of agarose, feeder layer culture and precise inoculation conditions, all contributed to a more efficient recovery of transformed tissues. The use of agarose and feeder layers was shown to be generally useful for tissue and cell in culture (Holbrook et al. 1985, Horsch and Jones 1980, Adams and Townsend 1983, Shillito et al. 1983).

Another important factor for successful transformation was the use of Agrobacterium strains that interact efficiently with the host plant. The survey of oncogenic Ti and Ri plasmids (Chapter III) indicated that nopaline Ti plasmids were more efficient than octopine Ti plasmids with B. napus as suggested earlier by Holbrook and Miki (1985). Oncogenicity was used as a marker in this study for determining conditions that enhanced the interactions between B. napus explants and Agrobacterium. It was further demonstrated that disarmed nopaline Ti

vectors were as virulent as the oncogenic wild type Ti plasmids. Plasmids such as pGV3850 in particular allowed the use of intermediate vectors of both cointegrate (pNCAT4, pLGV1103neo) and binary (pBCAT1) types.

One of the drawbacks of using pGV3850 was the presence of a bacterial ampicillin resistance gene which prevented the use of carbenicillin to eliminate A. tumefaciens after cocultivation. It limited the choice to cephalosporin antibiotics such as Claforan and cefotaxime or to b-lactam antibiotics containing a b-lactamase inhibitor (clavulanic acid) such as Clavulin 250 and Timentin. The slight toxicity of cefotaxime and its commercial preparation Claforan and the inability of Timentin to control A. tumefaciens left Clavulin 250 as the practical antibiotic for the control of A. tumefaciens. Carbenicillin would have been preferred because it is non-toxic and promoted shoot differentiation.

All of the antibiotics used as selective agents were toxic to B. napus tissues. Differences in toxicity levels were observed depending on the type of tissue and the antibiotic tested. The least toxic antibiotics (aminoglycoside antibiotics such as kanamycin, paromomycin, geneticin, streptomycin and chloramphenicol) were those acting on the ribosomes of mitochondria and chloroplasts, thus interfering in organellar protein synthesis (Davis 1987). The most toxic antibiotics acted on DNA or DNA synthesis (methotrexate, bleomycin; Fling and Elwell 1980, Murray and Martin 1985) or on protein glycosylation (tunicamycin; Ettlinger et al. 1986). An exception was hygromycin which acts on protein synthesis (Gonzales et al. 1978, Moazed and Noller 1987) and was also highly toxic.

Based on toxicity profiles and the availability of intermediate vectors at the time of this study, two cointegrating vectors (pLGV1103neo and pNCAT4) and one binary vector (pBCAT1) were chosen to initiate the research project. The intermediate vectors were transferred to A. tumefaciens by mating and the resulting strains were used to inoculate B. napus explants. The frequency of transgenic plant recovery per number of explants inoculated was much lower in B. napus (2%) than in N. tabacum (2000% i.e. more than 20 transgenic plants per explant). These frequencies corresponded to those obtained in other laboratories (0.4 to 10%) with similar A. tumefaciens transformation systems (Fry et al. 1987, Pua et al. 1987, Radke et al. 1988). The higher frequency of transgenic plant recovery with N. tabacum was easily explained by the absence of the hypersensitive response in leaf discs and the higher frequency of regeneration inherent to that system.

Under our conditions, the stem explant system developed by Kartha et al. (1974) and used for transformation by Fry et al. (1987) gave similar results to the thin cell layer explants. The higher frequency of transgenic plants obtained by Fry et al. (1987) may be due to the use of carbenicillin to eliminate A. tumefaciens.

Surprisingly, cocultivation of B. napus explants with A. tumefaciens containing pGV3850 alone resulted in a very high frequency of transgenic plants (3 plants out of 10 plants tested were nopaline synthase positive) even without selection. The frequency obtained with kanamycin (45%) and chloramphenicol (53%) selections indicated that the low levels of antibiotics only marginally increased the proportion of transgenic plants recovered. With N. tabacum, only 9% of the shoots regenerated after transformation with A. tumefaciens strain containing

pGV3850 alone were transformed (Zambryski et al. 1983) as detected by the presence of nopaline. This implied that transformation of regenerable cells in the B. napus explant occurred very efficiently and that regeneration and selection were the major problems.

Kanamycin sensitivity was manifested in shoots by bleaching after a period of anthocyanosis. Chloramphenicol selection was very poor at differentiated shoot and seedling stages. The high proportion of transgenic shoots with chloramphenicol comparable to kanamycin could be explained by its action at the callus stage whereas differentiated tissues could be less sensitive or even resistant.

IV.C.3 Analyses of transgenic plants

To confirm transformation in the recovered shoots, three approaches were taken: the biochemical detection of enzyme activity coded by the introduced genes, examination of phenotypic changes in the plants and the detection of the gene sequence in the plant genome.

IV.C.3.a The nopaline synthase gene

The initial screening for transgenic plants was done with the nopaline synthase assay (Holbrook et al. 1986) which is a sensitive and reliable marker for B. napus transformation. Since no visible phenotype was associated with this gene, the enzyme activity was the only way to detect its expression. The variation observed in the nopaline synthase activities within a plant could result from differences in expression in the leaf tissue at different stages of development or chimerism in the transgenic plants. Some plants that screened positive did not display any DNA hybridizing with the nopaline

synthase gene. This could indicate instability of the T-DNA or loss of the transformed cells from chimeras. Segregation of the nopaline synthase gene as a single Mendelian character (3:1) was observed for 2 out of the 5 plants tested. The 3 other plants displayed a 1:1 ratio which could be due to the inherent variation observed with the nopaline synthase assay or to the chimerism of the plants tested. The first hypothesis is the most probable since Mendelian pattern of segregation was observed with the NPT II gene with one plant and Southern blotting analysis of various plant tissue samples of the other plants gave consistently positive detection of both the NPT II and the NOS genes.

IV.C.3.b The neomycin phosphotransferase gene

The NPT II gene activity was detected by the enzyme assay in a few transgenic plants; however, the sensitivity of the enzyme assay (McDonnell et al. 1987) and the low level of expression of the chimeric genes rendered its use difficult. The phenotypic expression of the gene was demonstrated by the resistance to kanamycin in the transgenic plants and their progeny. This resistance was variable, a few plants were resistant to high levels of kanamycin (80 ug/mL) but most of the regenerated plants were sensitive to that level. A high proportion (50%) of the plants regenerated were sensitive to 20 ug/mL kanamycin. Weak expression of the NPT II gene in our transgenic plants could be explained by many factors. First, the low level of kanamycin used in selection (15 ug/mL) could have allowed recovery of transgenic plants with low level of expression. Second, the gene construction may not have been adequate for high levels of expression of the NPT II gene: for instance, the NPT II gene from Tn5 possesses an extra AUG codon which

decreased the translational efficiency of the chimeric NPT II RNA (Rogers et al. 1985). This was not significant for the recovery of N. tabacum transgenic plants but in transgenic B. napus, these chimeric genes could be less efficient. Third, some of the plants tested for the presence of NPT II sequences did not show any hybridization signals indicating the absence of transformation, the loss of the gene or the chimeric nature of the transgenic plants. Also, the expected size of the NPT II gene was confirmed in 5 transgenic plants whereas 4 other plants showed unexpected band sizes which could indicate gene rearrangement and loss of NPT II gene expression. Complex integration patterns where gene rearrangement occurred have been shown by others (Fry et al. 1987, Radke et al. 1988).

Kanamycin resistance often segregated as a single Mendelian character; however, more than one integration site was detected by Southern blot analysis in some cases. This apparent discrepancy could be explained by either the lack of expression of some of the integrated NPT II genes or by multiple integration of the T-DNA at a same locus on the plant DNA. The hypothesis of chimerism could be supported by the irregular ratios observed when the progeny of the transgenic plants was tested for kanamycin resistance.

IV.C.3.c The chloramphenicol acetyltransferase gene

The enzymatic and phenotypic expression of the CAT gene in B. napus was impossible to detect. First, the presence of a high plant endogenous CAT activity and of an inhibitor of CAT enzyme did not allow the detection of the enzyme. Second, the inherent tolerance of differentiated B. napus shoots and seedlings prevented the phenotypic

detection of the CAT gene expression. Nevertheless, 5 plants transformed with pNCAT4 out of 8 plants tested and 2 plants transformed with pBCAT1 out of 4 plants tested showed the presence of CAT sequences in transgenic tissues. In contrast with the NPT II gene, rearrangement was not detected and the characteristic fragment size was observed. It is possible that the chloramphenicol selection was effective at one point in the transformation protocol. If so, the probable stage was the callus phase.

IV.C.3.d Factors affecting the use of CAT as a marker for B. napus genetic transformation

Plant CAT activity is strongly expressed in all members of the Brassica genus surveyed. This included ancient (diploid species) cultivated species (B. campestris, B. oleracea, B. nigra and B. hirta) and weed species (B. kaber). Amphidiploid descendants also possessed endogenous CAT activity (B. juncea, B. carinata and B. napus). No differences were observed among nine cultivars of B. napus. Furthermore, the level of this activity was found in all the tissues and developmental stages tested. Attempts to generate variation through cell and tissue culture or growth under different light conditions failed. CAT activity seemed to be limited to the genus Brassica since it was not found in other members of the Cruciferae such as A. thaliana and Orychophragmus violaceus (Balazs and Bonneville 1987).

The presence of CAT activity in Brassica plants raised concerns about the usefulness of the bacterial CAT gene for studies in tissue specific gene expression in transgenic Brassica plants and transient

expression analyses with Brassica protoplasts. Analyses of tissue-specific expression using the CAT gene have been performed in N. tabacum (Herrera-Estrella et al. 1984, Thornburg et al. 1987, Timko et al. 1987); however, no plant CAT activity was found with N. tabacum. Transient expression studies using electroporated protoplasts have been performed in dicots such as Daucus carota, G. max, P. hybrida and N. tabacum (Bradshaw et al. 1987, Callis et al. 1987, Guerche et al. 1987a, Hauptmann et al. 1987) as well as with monocots such as Triticum aestivum, Oryza sativa, Zea mays and Sorghum bicolor (Callis et al. 1987, Hauptmann et al. 1987). Again, no or very little plant endogenous CAT activity has been observed with these plants. In Brassica species, the endogenous CAT activity was present in all of the differentiated tissues examined as well as protoplasts, cells and tissues in culture.

Experiments were conducted to examine transgenic bacterial CAT activity in B. napus using 2 chimeric CAT genes with different promoters. In transgenic N. tabacum, the vector pBCAT1 which carries a CAT gene with a 35S promoter, yielded 95 to 98% conversion of chloramphenicol to the acetylated forms. The vector pNCAT4 which carries a CAT gene with a nopaline synthase promoter has been reported to yield 6% conversion to acetylated chloramphenicol (Timko et al. 1985). Both activities were significantly higher than the background activity found in N. tabacum. In B. napus, the plant endogenous CAT activity was very high (15 to 30%) but higher levels of activity were not found in transgenic B. napus transformed with pNCAT4 or pBCAT1. From the results achieved with N. tabacum, it might have been predicted that CAT activity significantly above background should have been

detected with the vector pBCAT1 since the CAT gene was detected as well as significant level of nopaline synthase activity. The possibilities that the CAT genes were not expressed or the transgenic CAT activity was not detectable were therefore considered.

The possibility that the selection protocols favoured the recovery of transgenic B. napus plants that expressed low levels of CAT could not be eliminated. It has been generally accepted that chloramphenicol is a poor selective agent (De Block et al. 1984). In this study, 7 ug/mL of chloramphenicol were used to recover plants which is close to what was used for N. tabacum (10 ug/mL, De Block et al. 1984). At this level, only a 50% reduction in B. napus explant growth was achieved; however, transgenic plants could not be recovered at higher levels. The endogenous CAT activity in Brassica did not seem to confer a higher level of tolerance to chloramphenicol on B. napus. The same vectors produced transgenic N. tabacum plants with high levels of transgenic CAT activity despite selection with low levels of chloramphenicol or with kanamycin alone. In other studies, the use of CAT as a reporter gene was clearly not dependant on selection for resistance to chloramphenicol (Timko et al. 1985, Hauptmann et al. 1987, Thornburg et al. 1987).

Clear evidence was found for the presence of an inhibitor of transgenic CAT activity in B. napus and B. juncea. A complete inhibition of transgenic CAT activity in N. tabacum extracts was observed when only one fifth volume of B. napus or B. juncea crude extract was added to the assay mixture. Curiously, the endogenous Brassica CAT activity was still observed under conditions in which transgenic CAT activity was completely inhibited. The CAT inhibitor

was not found in the other species outside the Brassica genus (A. thaliana, G. max, L. esculentum) that were examined. The addition of bacterial CAT enzyme to extracts of B. napus confirmed the presence of this inhibitor.

The bacterial CAT activity has been reported to be resistant to heat treatments (Shaw, 1975, 1983). In B. napus, heat treatments reduced equally the CAT activity in both transgenic and non transformed plants therefore, it was still not possible to distinguish between the two CAT activities. Evidence for selective inactivation of the inhibitor by heat was not observed. It appeared that manipulation with temperature did not assist in the detection of transgenic CAT activity.

Attempts to biochemically discriminate between the CAT activities were unsuccessful. DTNB (5,5'-dithio-bis-2 nitrobenzoic acid) inhibits types II and III CAT but not type I which is used in the vectors pNCAT4 and pBCAT1 (Shaw, 1983); however, in our experiments, all CAT activities in both transgenic N. tabacum and B. napus were eliminated. Protease treatments of the samples also eliminated all CAT activities in both species despite the observation that type I CAT appeared to be protease resistant (Young et al. 1985).

To summarize, the CAT gene has been used successfully in numerous transformation studies; however, for Brassica species, it is not a practical marker. The high levels of plant endogenous CAT activity found at all developmental stages and in all tissues is only one obstacle. A major impediment is the presence of an unidentified inhibitory factor in Brassica species which appears to be selective for bacterial CAT activity.

CHAPTER V
TOWARDS THE GENETIC ENGINEERING OF HERBICIDE
RESISTANCE IN BRASSICA NAPUS

V.A.1 Introduction

Genetic engineering for herbicide resistance in a crop plant includes diverse research areas that have to be covered prior the completion of such a project. The first step is to assess the potential use of a given herbicide or family of herbicides against common weed species present in the crop. Second, a strategy for engineering the herbicide resistance into the crop plant has to be developed and tested. Last, the actual engineering of herbicide resistance in the crop plant must be functional and achieved in a way that is not deleterious to the agronomic properties of the crop.

In this thesis, the sulfonylurea and imidazolinone herbicides acting on the ALS enzyme have been evaluated for their use with B. napus and their effectiveness on common weeds present in this crop. Two strategies to obtain sulfonylurea and imidazolinone resistance, i.e. the use of a mutant ALS gene resistant to chlorsulfuron (gene csr1-1 from A. thaliana; Haughn and Sommerville 1986) and the elevated expression of the wild type ALS gene have been tested in N. tabacum. The use of herbicide resistance as selectable marker has also been

evaluated in detail. Finally, the genetic engineering of sulfonylurea and imidazolinone resistance has been initiated in B. napus.

V.A.2 Biochemistry of herbicides acting on the acetolactate synthase enzyme

The acetolactate synthase (ALS or AHAS, EC 4.1.3.18) enzyme catalyses the first step in the biosynthesis of isoleucine (Fig. V.1.a), leucine and valine (Fig. V.1.b) which are essential amino acids (Schloss et al. 1988, LaRossa and Schloss 1984). This enzyme is the target of at least three classes of structurally different herbicides: the sulfonylureas, the imidazolinones and the sulfonanilides (Schloss et al. 1988, LaRossa and Schloss 1984, Shaner et al. 1984). The chemical structures of representative herbicides from each class are given in Fig. V.2. These herbicides are peculiar since they do not share homology with the enzyme substrates (pyruvate, α -ketobutyrate, thiamine pyrophosphate, FAD and magnesium) or its products (valine, isoleucine and leucine). It is postulated that the site of action is a vestigial quinone binding site originating from the quinone binding site of pyruvate oxidase (Schloss et al. 1988).

V.A.3 Use of the herbicides acting on the ALS enzyme

The sulfonylureas are principally used to control broad leaf weeds (Table V.1) in cereal crops; sulfometuron methyl is non selective and is used on non-crop lands (Information from the manufacturer data sheets and the Herbicide handbook of the weed society of America). The sulfonylurea herbicide DPX A7881 is a new compound that has been developed but not registered yet for Canola crops (Dupont Co. data

TABLE V.1. Use of Sulfonylurea and Imidazolinone Herbicides in Crop Plants

Herbicide			Field dose
Code name	Chemical name	Crop	g a.i.*/ha
<u>Sulfonylureas</u>			
DPX M6316	thiamethuron-methyl	wheat, barley	17.5-70
DPX L5300	--	wheat, barley	8.75-70
DPX A7881	--	canola	10-120
DPX W4189	chlorsulfuron	cereals	15-35
DPX T5648	sulfometuron-methyl	non selective	n.a.
CGA 131036	triasulfuron	wheat, barley	5-40
<u>Imidazolinones</u>			
AC 243,997	imazapyr	non selective	n.a.
AC 252,925	--	non selective	400-3000
AC 263,499	imazethapyr	legumes	30-300
AC 222,293	imazamethabenz	wheat, barley, rye	500-2000
AC 252,214	imazaquin	legumes, tobacco	
		hardwood trees	70-280

n.a. not available, a.i.= active ingredient.

Figure V.1 Reactions catalyzed by the acetolactate synthase enzyme

Figure V.2 Three classes of herbicides acting on the acetolactate synthase enzyme; sulfonylureas, imidazolinones, sulfonanilides.

SULFONYLUREAS

Sulfometuron-methyl

Chlorsulfuron

Triasulfuron

Thiameturon-methyl

IMIDAZOLINONES

Imazapyr

Imazaquin

Imazethapyr

Imazamethabenz

SULFONANILIDES

2-NO₂-6-Me-sulphonanilide

sheets). The imidazolinones are mainly used in legumes (AC 263,499 and AC 252,214) and cereals (AC 222,243) but some of them are non selective and used on non-crop lands (AC 243,997 and AC 252,925). These imidazolinone herbicides are registered for use in the United States only.

V.A.4 Resistance to herbicides that act on ALS

Natural tolerance has been found in some plant species and resistance has been generated or engineered in other plant species. The natural tolerance of cereal crops to sulfonylureas, imidazolinones and sulfonanilides is due to the metabolic detoxification of the herbicides by the plants. Chlorsulfuron has been demonstrated to be metabolized to an inactive derivative in small grain crops (Sweetser et al. 1982, Hutchison et al. 1984). The herbicide imazamethabenz (AC 222,293) used in small grain crops is also metabolized to inactive derivatives (Shaner et al. 1982). Tolerance to chlorsulfuron has been observed in grassy weeds such as Avena fatua and annual blue grass and some broad leaf plants such as Solanum nigrum and Linum usitatissimum (Hutchison et al. 1984, Sweetser et al. 1982). Moreover, tolerance has been reported with DPX A7881 in B. napus, imazethapyr (AC 263,499) in legumes and with imazaquin (AC 252,214) in legumes, hardwood trees and N. tabacum (Information from Dupont and American Cyanamid data sheets).

Resistance has been obtained to chlorsulfuron by induced mutations in L. usitatissimum (Jordan and McHughen 1987), Datura innoxia (Saxena and King 1988, Xiao et al. 1987), G. max (Sebastian and Chaleff 1987), N. tabacum (Chaleff and Ray 1984, Chaleff and Bascomb 1987), A. thaliana (Haughn and Somerville 1986) and B. napus (Swanson et al.

1988). The ALS wild type and mutant genes from N. tabacum (Mazur et al. 1987a, 1987b) and A. thaliana (Haughn et al. 1988) have been isolated and sequenced. The A. thaliana mutant gene (csrl-1) showed a cytosine to thymidine transition predicting a proline to serine substitution (Mazur et al. 1987a) whereas a proline to alanine substitution at the same site for a N. tabacum mutant gene (SurRB, Mazur et al. 1987b). The same substitution to serine was previously found at an identical position of the ALS gene of a Saccharomyces cerevisiae mutant (Yadav et al. 1986) suggesting an essential role for this region in herbicide binding.

Genetic engineering has been used to transfer chlorsulfuron resistance in plants as discussed in I.D.4 for L. esculentum and N. tabacum.

V.B RESULTS

V.B.1 Potential use of sulfonylurea and imidazolinone herbicides with Brassica species

Limited data were available on the effects of the sulfonylurea and imidazolinone herbicides on Brassica crop species and their weeds. These two classes of herbicides are not registered for use with Brassica species. Surveys of the inhibition of ALS activity in 19 species by 4 sulfonylurea and 3 imidazolinone herbicides was completed as well as a comparison of the toxicity of these herbicides on in vitro seedling germination and growth.

V.B.1.a Inhibition of ALS enzymatic activity

The effect of the sulfonylurea and imidazolinone herbicides on the target acetolactate synthase enzyme was examined in some details. For all species tested, young growing leaves were needed to detect ALS activity in cell free extracts. Little or no activity was detected in extracts from cotyledons or from mature expanded leaves. The inhibition of ALS activities by herbicides are shown in Tables V.2 and V.3 by the level of herbicide needed to obtain 50% inhibition of ALS activity (I_{50}) and by the maximum level of inhibition reached in the presence of the highest concentration of herbicide (I_{max}).

TABLE V.2. Inhibition of ALS Activity by Sulfonylurea Herbicides.

Species	Herbicides§							
	DPX L5300		DPX A7881		DPX M6316		Chlorsulfuron	
	I50	Imax	I50	Imax	I50	Imax	I50	Imax
<u>B. campestris</u>	44	89	19	94	3	98	19	100
<u>B. nigra</u>	16	90	18	97	6	100	70	98
<u>B. oleracea</u>	100	85	20	90	10	98	16	100
<u>B. juncea</u>	6	100	4	100	3	100	7	100
<u>B. napus</u>	55	89	6	97	5	98	26	95
<u>B. carinata</u>	20	100	50	100	6	100	13	93
<u>B. hirta</u>	61	79	8	85	5	86	28	95
<u>B. kaber*</u>	13	82	11	79	8	81	105	96
<u>R. sativus</u>	3	100	2	100	4	100	29	95
<u>C. maritima</u>	73	85	2	94	4	97	12	95
<u>T. arvensis</u>	14	97	11	100	4	100	74	100
<u>A. thaliana</u>	18	87	11	85	3	83	11	100
<u>M. sativa</u>	54	87	4	99	4	98	11	98
<u>G. max</u>	150	72	13	88	14	94	90	87
<u>N. tabacum</u>	47	84	8	100	4	100	44	100
<u>S. viridis</u>	75	83	1	95	2	91	150	89
<u>A. fatua</u>	50	83	16	85	16	93	18	100
<u>H. vulgare</u>	45	81	14	95	8	100	28	96
<u>T. aestivum</u>	135	83	15	95	6	95	26	80

§=I50 represents the level of herbicide necessary to obtain 50% inhibition of ALS activity (ug/mL) and Imax the maximum level of inhibition reached in presence of the highest concentration of herbicide (%). *=bad experiment. Data collected by J. Gabard and G. Sunohara.

TABLE V.3. Inhibition of ALS Activity by Imidazolinone Herbicides.

Species	Herbicides§					
	AC 243,997		AC 263,499		AC 252,214	
	I50	Imax	I50	Imax	I50	Imax
<u>B. campestris</u>	.54	100	.29	100	.17	100
<u>B. nigra</u>	.80	100	.45	100	.16	100
<u>B. oleracea</u>	.55	100	.35	100	.30	100
<u>B. juncea</u>	3.53	70	.35	100	.50	80
<u>B. napus</u>	1.15	86	.43	100	.40	95
<u>B. carinata</u>	.50	72	.50	90	1.10	73
<u>B. hirta</u>	.94	90	.38	100	.40	92
<u>B. kaber</u>	13.30	68	.61	87	.50	85
<u>R. sativus</u>	.39	90	.43	90	.40	94
<u>C. maritima</u>	.97	92	.32	95	.50	97
<u>I. arvensis</u>	2.50	87	1.20	100	.30	100
<u>A. thaliana</u>	.47	100	.29	100	.26	100
<u>M. sativa</u>	10.45	94	8.68	85	1.96	94
<u>G. max</u>	9.10	85	3.20	92	2.50	88
<u>N. tabacum</u>	0.47	90	.20	100	.33	92
<u>S. viridis</u>	2.60	66	.35	94	.53	82
<u>A. fatua</u>	.63	100	.39	100	.26	96
<u>H. vulgare</u>	.80	100	.30	100	.26	100
<u>I. aestivum</u>	2.60	74	.55	89	.47	92

§=I50 represents the level of herbicide necessary to obtain 50% inhibition of ALS activity (mg/mL) and Imax the maximum level of inhibition reached in presence of the highest concentration of herbicide (%). Data collected by J. Gabard and G. Sunohara.

A comparison of the inhibition of ALS activities by sulfonylureas in different species is shown in Table V.2. The sulfonylurea herbicides DPX M6316 and DPX A7881 were the strongest inhibitors of ALS activity. The range of I_{50} values were 2-16 ug/L and 1-50 ug/L respectively. The level of inhibition was 79-100% at 300 ug/L of DPX A7881 and 81-100% for DPX M6316. Natural resistance to either of these herbicides was not observed. Results obtained with DPX L5300 and chlorsulfuron revealed a higher range of I_{50} values for ALS inhibition. For instance, the I_{50} value ranged from 3 to 150 ug/L for DPX L5300 and 7-150 ug/L for chlorsulfuron. The I_{max} values were less variable; for instance, 81-100% for DPX L5300 and 80-100% for chlorsulfuron. These results were comparable to those obtained with DPX M6316 and DPX A7881. Generally, species that were tolerant to specific sulfonylureas in growth did not exhibit ALS activities that were resistant to the herbicide.

A comparison of the inhibition of ALS activities by imidazolinones is shown in Table V.3. The range of I_{50} values were again broad; for instance, 0.39-13.30 mg/L for AC 243,997, 0.20-8.68 mg/L for AC 263,499 and 0.16-2.50 mg/L for AC 252,214. The range of I_{max} values were 66-100%, 85-100% and 73-100% respectively. ALS activities that were naturally resistant to the imidazolinones were not observed; however, it was noted that the ALS activities of M. sativa and G. max were particularly tolerant to all 3 imidazolinones (Table V.3). In each case, however, the I_{max} values revealed extensive inhibition of ALS activities at high herbicide levels (Table V.3).

V.B.1.b Inhibition of germination in vitro

The effect of the sulfonylurea and imidazolinone herbicides on the germination and aseptic growth of seedlings was examined in detail (Plate V.1). Toxicity was measured by the concentration needed to inhibit growth by 50% (I_{50}) and by the maximum level of inhibition reached (I_{max}) (Tables V.4 and V.5). Under the conditions described in II.C.5, DPX L5300 was not effective (Table V.4); however, each of the others performed generally as expected. In several cases, herbicides at concentrations below toxic levels enhanced the growth of the seedlings (Plate V.1).

Within the Brassica genus, B. campestris, B. napus and B. carinata were very tolerant to the sulfonylurea DPX A7881 (Plate V.1, Table V.4). Other crucifers such as R. sativus were marginally tolerant but I. arvensis and A. thaliana were very sensitive. In contrast, all of the crucifers tested were sensitive to both chlorsulfuron and DPX M6316. Similarly, all were sensitive to each of the imidazolinones tested; however, I. arvensis and R. sativus displayed a marginal tolerance to AC 243,997 (Table V.5).

The two legumes, G. max and M. sativa, were both very tolerant to each of the imidazolinones examined but sensitive to the sulfonylureas (Plate V.1, Tables V.4 and V.5). The grassy weed species, S. viridis, was relatively sensitive to each of the herbicides. N. tabacum, the only Solanaceae examined, was very sensitive to each herbicide (Tables V.4 and V.5).

Plate V.1 In vitro germination and growth of B. napus cv. Westar, B. kaber, M. sativa and G. max on herbicides.

The herbicides used were DPX A7881, chlorsulfuron and Pursuit (AC 263,499). The herbicide concentrations were 300, 100, 30, 10, 1, 0 ug/L chlorsulfuron or DPX A7881 (left to right) and 289 (1000), 87 (300), 29 (100), 8.7 (30), 2.9 (10) and 0 ug/L (nM) of AC 263,499.

The photographs were taken after 2 weeks on aseptic growth media by J. Gabard.

	Sulfonylurea		Imidazolinone	
	Chlorsulfuron	A 7881	Pursuit	
B. napus
B. kaber
M. sativa
G. max

TABLE V.4. Inhibition of Seedling Growth by Sulfonylurea Herbicides in vitro.

Species	Herbicides§							
	Chlorsulfuron		DPX A7881		DPX M6316		DPX L5300	
	I50	Imax	I50	Imax	I50	Imax	I50	max
<u>B. campestris</u>	10	88	NR	25	6	95	NR	NI
<u>B. nigra</u>	7	86	23	89	1	93	26	89
<u>B. oleracea</u>	.9	80	95	59	8	66	NR	NI
<u>B. juncea</u>	7	81	190	75	4	88	NR	30
<u>B. napus</u>	20	84	NR	7	7	86	100	73
<u>B. carinata</u>	30	66	NR	NI	55	79	NR	NI
<u>B. hirta</u>	nd		I		I		NR	NI
<u>B. kaber</u>	3	93	10	90	7	93	NR	NI
<u>R. sativus</u>	10	62	200	59	80	61	NR	NI
<u>C. maritima</u>	7	88	nd		nd		nd	nd
<u>T. arvensis</u>	7	77	20	79	10	87	NR	NI
<u>A. thaliana</u>	.7	100	7	85*	.8	100*	NR	41*
<u>M. sativa</u>	55	55	10	64	30	69	NR	NI
<u>G. max</u>	20	90	NR	10	5	80	NR	NI
<u>N. tabacum</u>	8	98	210	66	22	94	NR	NI
<u>S. viridis</u>	NR	42	100	66	NR	43	NR	NI
<u>A. fatua</u>	NR	NI	NR	NI	NR	NI	NR	NI
<u>H. vulgare</u>	NR	NI	NR	NI	NR	NI	NR	NI
<u>T. aestivum</u>	NR	NI	NR	NI	NR	NI	NR	NI

§=I50 represents the level of herbicide necessary to obtain 50% inhibition of the seedling growth (ug/mL) and Imax the maximum level of inhibition reached in presence of the highest concentration of herbicide (%). nd= not done. NI= no inhibition, NR= 50% inhibition not reached. *=maximum herbicide concentration used was 30 instead of 300 ug/L. I=inhibitor but contaminations, or too low germination for measurement.

Data collected by J. Gabard and G. Sunohara.

TABLE V.5. Inhibition of Seedling Growth by Imidazol'none Herbicides
In Vitro.

Species	Herbicides§					
	AC 243,997		AC 263,499		AC 252,214	
	I50	I _{max}	I50	I _{max}	I50	I _{max}
<u>B. campestris</u>	52	83	69	77	9	90
<u>B. nigra</u>	24	77	61	79	23	79
<u>B. oleracea</u>	52	66	20	73	27	68
<u>B. juncea</u>	13	94	42	96	7	99
<u>B. napus</u>	131	67	26	84	16	87
<u>B. carinata</u>	14	83	13	75	9	88
<u>B. hirta</u>		nd		nd		nd
<u>B. kaber</u>	22	85	36	88	3	88
<u>R. sativus</u>	NR	34	245	53	23	70
<u>C. maritima</u>	I		I		I	
<u>I. arvensis</u>	NR	36	188	52	202	65
<u>A. thaliana</u>	21	96	5	98	5	98
<u>M. sativa</u>	NR	NI	NR	7	NR	20
<u>G. max</u>	NR	NI	NR	NI	NR	NI
<u>N. tabacum</u>	68	93	25	82	31	69
<u>S. viridis</u>	222	59	43	77	53	76
<u>A. fatua</u>	NR	NI	NR	NI	NR	NI
<u>H. vulgare</u>	NR	NI	NR	NI	NR	NI
<u>I. aestivum</u>	NR	NI	NR	NI	NR	NI

§=I50 represents the level of herbicide necessary to obtain 50% inhibition of the seedling growth (ug/mL) and I_{max} the maximum level of inhibition reached in presence of the highest concentration of herbicide (%). nd= not done. NI= no inhibition, NR= 50% inhibition not reached. I=inhibitor but contaminations, or too low germination for measurement. Data collected by J. Gabard and G. Sunohara.

V.B.2 Introduction of sulfonylurea resistance in N. tabacum and study of herbicide cross resistance

V.B.2.a Transformation with the csrl-1 ALS mutant gene

Transgenic N. tabacum SR1 lines were obtained by A. tumefaciens-mediated transformation of leaf discs using the disarmed Ti plasmid pGV3850 (strain GV3101, Zambryski et al. 1983) in combination with the binary vector pBINCH (derivative of pBIN19, Bevan 1984, appendix A5) which includes the mutant A. thaliana ALS gene csrl-1 (Haughn and Somerville 1986, Haughn et al. 1988, appendix A12). The selection was done directly on chlorsulfuron without kanamycin. From 60 leaf discs, all gave rise to callus and shoots resistant to 80 ng/mL (225 nM) chlorsulfuron (Plate V.2.a). An average of 10 shoots per explant was observed which was comparable to selection for kanamycin resistance; however, shoot differentiation was delayed by 4 to 6 weeks and the shoot growth was also delayed. The use of chlorsulfuron as a selectable marker will be described in section V.B.3. The five best growing shoots were propagated in vitro and transferred to the same medium lacking chlorsulfuron but supplemented with 100 ug/mL kanamycin. Each clone grew and formed roots on chlorsulfuron or kanamycin at levels that inhibited the growth of the control shoots (Plate V.2.b). Two of these clones named CHL-1 and CHL-2 were chosen for further studies and transferred to the greenhouse.

V.B.2.b Analysis of the transgenic N. tabacum

Southern blot analyses of CHL-1 and CHL-2 total DNA is shown in Plate V.3. Digestion with XbaI generated a single 5.8 kbp fragment that hybridised with the radiolabelled 5.8 kbp XbaI fragment from pGH1

Plate V.2 Use of chlorsulfuron for the selection and genetic analysis of transgenic N. tabacum plants.

Selection was performed directly on 80 ng/ml (225 nM) chlorsulfuron without kanamycin. A) Shoot differentiation from leaf discs inoculated with A. tumefaciens strain GV3101 carrying the gene csrl-1 in a Ti plasmid vector (right) and from leaf discs that were not inoculated (left), after 3 months. B) In the presence of chlorsulfuron, resistant shoots formed roots and developed normally (right) whereas control shoots died (left). C) The segregation of chlorsulfuron resistance was examined in the presence of 100 ng/mL (280 nM) chlorsulfuron with the progeny of CHL-2 self pollinated seeds.

Plate V.3 Southern blot analysis of transgenic N. tabacum.

SR1 (lanes 1 and 4), CHL-1 (lane 3) and CHL-2 (lane 2) DNA digested with Xba I and hybridized with the radiolabelled Xba I 5.8 kb fragment from pGH1 (Haughn et al. 1988). The 5.8 kb Xba I fragment is indicated by the arrow.

1 2 3 4

>

which was used as the probe. This fragment carried the complete csr1-1 gene including flanking regions of A. thaliana genomic DNA (Haughn et al. 1988). The hybridization was performed under stringent conditions that allowed detection of only A. thaliana DNA. Resident ALS genes of N. tabacum were not detected under these conditions in control or transgenic tissues (Plate V.3).

Genetic analysis of CHL-1 and CHL-2 is shown in Table V.6. Segregation (Plate V.2.c) ratios of selfpollinations (3:1) and backcrosses with control SR1 (1:1), indicated that only one integrated ALS mutant gene was expressed and acted as a dominant Mendelian character. The same results were obtained for the chimeric nptII gene. This hypothesis was supported by the chi-square values obtained (Table V.6). The close linkage of csr1-1 and NPTII genes was demonstrated by transferring 62 kanamycin resistant seedlings of selfpollinated CHL-2 progeny from kanamycin to chlorsulfuron containing medium. All of them were able to maintain normal growth and ability to form roots after the transfer. The growth characteristics of CHL-1 and CHL-2 on kanamycin differed consistently during analyses of seedlings from selfpollination and backcrosses suggesting that these were separate N. tabacum clones.

V.B.2.c Cross resistance to sulfonylurea herbicides in transgenic

N. tabacum

To assess the resistance of the mutant csr1-1 ALS gene in transgenic N. tabacum and mutant A. thaliana line GH50, ALS assays were performed with the 4 sulfonylurea herbicides chlorsulfuron, DPX A7881, DPX M6316 and DPX L530 and with the three imidazolinones herbicides AC 252,214, AC 263,499 and AC 243,997. Inhibition of ALS activity by

TABLE V.6. Genetic Analysis of Transgenic N. tabacum

Clone	Crosses	kanamycin				chlorsulfuron			
		R	S	ratio	χ^2	p	R	S	ratio χ^2 p
CHL-1	self X	137	43	3:1	0.15	0.7	109	25	3:1 1.44 0.2
	X SR1	31	43	1:1	3.05	0.1	21	17	1:1 0.44 0.5
CHL-2	self X	87	32	3:1	3.05	0.1	89	27	3:1 0.24 0.6
	X SR1	53	54	1:1	0.01	0.9	45	33	1:1 1.85 0.2

Sterile seeds were plated onto the surface of Petri dishes containing MAH medium supplemented with either 200 ug/mL kanamycin or 100 ng/L (280 nM) chlorsulfuron. Two weeks after sowing, they were scored for their resistance (R) or sensitivity (S) to each of these chemicals. Chi-square values were calculated with 1 degree of freedom. Data collected with help from J. Gabard.

Figure V.3 Inhibition of ALS activity in A. thaliana and N. tabacum by sulfonylurea and imidazolinone herbicides. ALS activity was measured at each herbicide concentration and expressed as a percentage of the uninhibited control. Control A. thaliana wt (A,E). A. thaliana mutant line GH50 (B,F). Control N. tabacum SR1 (C,G). Transgenic N. tabacum line CHL-2 (D,H). Chlorsulfuron (○—○), DPX A7881 (□—□), DPX M6316 (△—△), DPX L5300 (▽—▽), CGA 131,036 (◇—◇), AC 252,214 (▲—▲), AC 263,499 (●—●), AC 243,977 (■—■). Data collected by J. Gabard and G. Sunohara.

herbicides is shown in Figure V.3 for the wild type N. tabacum and A. thaliana, the transgenic N. tabacum and the mutant A. thaliana line GH50. The results confirmed an earlier finding (Haughn and Somerville 1986) that ALS of mutant line csrl-1 is not inhibited by chlorsulfuron. Cross resistance to the 3 other sulfonylurea herbicides DPX A7881, DPX M6316 and DPX L5300 was also observed (Fig. V.3.b). In contrast, A. thaliana wild type ALS was almost completely inhibited with each of these sulfonylureas at 300 ug/mL (Fig. V.3.a). The ALS activity of the mutant line GH50 was weakly inhibited by low levels of CGA 131036 but at higher levels, the activity was inhibited to the same degree as the control (Fig. V.3.b). Both csrl-1 and wild type A. thaliana ALS were clearly inhibited at 50 uM imidazolinones (Fig. V.3.e), csrl-1 ALS retained a residual activity of 10 to 15% in the presence of equivalent higher levels of imidazolinones (Fig. V.3.f). This partial cross resistance was most pronounced with AC 243,997 (Fig. V.3.f).

In transgenic N. tabacum carrying the mutant ALS gene csrl-1, cross-resistance of ALS activity to DPX M6316, DPX A7881 and DPX L5300 was again observed. This was shown at high concentrations of the herbicides, as a residual activity of 20 to 40% of the total that was clearly resistant to each of the sulfonylureas (Fig. V.3.d). The profiles for the inhibition of ALS by these sulfonylureas differed significantly from those for the A. thaliana mutant line GH50 in which 70-100% ALS activity was retained (Fig. V.3.b) and control N. tabacum SR1 in which ALS activity was essentially completely inhibited (Fig. V.3.c). ALS of both control SR1 and transgenic line CHL-2 were completely inhibited at 1000 ug/L CGA 131036 (Fig. V.3.c and V.3.d) but CHL-2 displayed a 10-20% higher activity in presence of lower

concentrations (Fig. V.3.d). This was significantly different from the resistant activity observed for the other sulfonylureas. In the presence of imidazolinones no significant differences among inhibition profiles for the ALS activity of control N. tabacum SR1 (Fig. V.3.g), transgenic line CHL-2 (Fig. V.3.h) and A. thaliana wild type (Fig. V.3.e) were observed. Fig. V.3 illustrates the variability that exists among the herbicides in their capacity to inhibit ALS activity. Generally DPX L5300 and chlorsulfuron were the weakest sulfonylurea inhibitors, especially with N. tabacum ALS (Fig. V.3.c and V.3.d). AC 243,97 was the least efficient inhibitor among the imidazolinones tested (Fig. V.3.e and V.3.f).

V.B.2.d Comparison of herbicide effects on germination and growth in vitro

The germination and growth of progeny from selfpollination of transgenic N. tabacum line CHL-1 and CHL-2 in vitro was examined in the presence of the herbicides. Results with DPX M6316, DPX A7881 or AC 252,214 are shown in Figure V.4 and Plate V.4 for CHL-2. Two weeks after sowing, the germination of control SR1 seedlings was completely inhibited at 300 ug/L of DPX M6316 or DPX A7881. (Plate V.4.a and V.4.c) and the inhibition of growth was significantly greater with DPX M6316 (Plate V.4.a). The I_{50} values were 22 ug/L DPX M6316 and 210 ug/L of DPX A7881 (Plate V.4.a). The growth of CHL-2 seedlings was not inhibited at 300 ug/L of DPX A7881 or DPX M6316 (Plate V.4.a); however, at 300 ug/L DPX M6316 a 25% reduction in growth of CHL-1 was observed. Occasionally, a slight reduction in CHL-2 growth was also observed on DPX M6316 (Plate V.4.d). It was noticed that DPX A7881 enhanced

Figure V.4 Inhibition of seedling germination and growth in the presence of herbicides in vitro. Seedlings of control *N. tabacum* SR1 (——) and transgenic line CHL-2 (----) were germinated in vitro at various herbicide concentrations. Average fresh weights and standard errors were determined after 2 weeks on A) the sulfonyleurea herbicides DPX A7881 (□—□) and DPX M6316 (△—△) and B) the imidazolinone herbicide AC 252,214 (▲—▲). Data collected by J. Gabard.

HERBICIDE CONCENTRATION

Plate V.4 Inhibition of seedling germination and growth in vitro.
Seedlings of control N. tabacum SR1 (A,C,E) and transgenic line CHL-2
(B,D,F) were germinated and grown aseptically for 2 weeks in the
presence of 300, 100, 30, 10, 1, and 0 ug/L DPX A7881 (A,B) and DPX
M6316 (C,D) and 100, 300, 100, 30, 10, 0 nM AC 252,214 (E,F). Data
collected by J. Gabard.

control N. tabacum SR1 seedling growth below the levels that were toxic for this herbicide (Plate V.4.a). This growth enhancement has been observed for several other species with both sulfonylurea and imidazolinone herbicides. DPX 15300 was not toxic for control SR1 at the levels used despite the observation that this herbicide inhibited ALS activity (Plate V.4.C).

Generally the growth of control SR1 and transgenic CHL-2 were both sensitive to the imidazolinones (Plate V.4.e and V.4.f). At low imidazolinone concentrations CHL-2 grew more actively than control SR1 but at higher levels this apparent difference was lost (Plate V.4.b, V.4.e and V.4.f). In both cases germination occurred at the highest levels of imidazolinone tested (Plate V.4.e and V.4.f). This differed from the inhibition of germination in control SR1 observed with the highest levels of sulfonylureas (Plate V.4.a and V.4.c).

V.B.2.e Herbicide resistance in vivo

Control SR1 and transgenic CHL-2 progeny of selfpollination were sprayed with DPX M6316, DPX A7881 or CGA 131036 at levels equivalent to 15, 30 and 10 g ai/ha respectively, after 7 to 10 days in soil. As shown in Plate V.5.a, CHL-2 remained green and leaf development in the presence of DPX A7881 was normal, 23 and 40 days after the treatment. Growth was slightly reduced and delayed by DPX M6316, 23 days after the application. Occasionally, leaf elongation was abnormal. Development of CHL-2 seedlings was greatly affected by CGA 131036. A significant reduction in size and leaf number as well as an altered morphology of the first and second true leaves was observed. After germination, control SR1 development was completely inhibited at the cotyledon stage

Plate V.5. Analysis of resistance under greenhouse conditions.

One week old seedlings of control of N. tabacum SR1 (B,D,F,H) and transgenic line CHL-2 (A,C,E,G) were sprayed with 30 g a.i./ha DPX A7881 (A,B), 15 g a.i./ha DPX M6316 (C,D), 10 g a.i./ha CGA 131036 (E,F) and 15 g a.i./ha AC 263,499 (G,H). Photographs were taken after 23 days (C-H) or 40 days (A-B). Data collected by J. Gabard

A

B

C

D

E

F

G

H

Plate V.6. Effect of chlorsulfuron on mature *N. tabacum* plants.
Mature *N. tabacum* SR1 plant (C) and transgenic line CHL-2 plant (T)
were sprayed with 1.4 mg/L chlorsulfuron at the flowering stage.

by each of the herbicides. Severe bleaching in areas and finally death of the plantlets followed. Root development was also retarded characteristically displaying reduced size and lacking branching.

Seedlings of both control SR1 and transgenic CHL-2 treated with AC 263,499 at a level equivalent to 150 g ai/ha were equivalently retarded in growth and rarely developed beyond the cotyledon stage. After 23 days control SR1 and transgenic CHL-2 seedlings were dead (Plate V.5). The inhibition of growth induced by the imidazolinones was delayed compared with the sulfonylureas.

Mature plants were also sprayed with 1.4mg/L chlorsulfuron. The control plants at the flowering stage had lost all their flowers and had severe damage on leaves, stem and axillary buds after 2 weeks from the treatment. Transgenic *N. tabacum* at the same stage had slight leaf burns; however, flowers and capsules developed normally (Plate V.6).

V.B.3 Use of chlorsulfuron resistance as a selectable marker for transformation in *N. tabacum*

The use of herbicide resistance as a selectable marker for plant genetic transformation has not been documented in the literature. This part of the thesis will describe the use of chlorsulfuron resistance (with the gene csr1-1, Haughn and Somerville 1986) as a selectable marker in *N. tabacum* leaf disc transformation. For this purpose, 4 intermediate vectors were constructed for cointegration with pGV3850 (Zambryski et al. 1983) from the *A. thaliana* wild type and mutant ALS csr1-1 genes. These vectors (Fig. V.5) were constructed with the wild type gene (pKALSW) with its own promoter and with the 35S promoter from the cauliflower mosaic virus (p35SALSW), or with the mutant csr1-1 gene

Figure V.5 The four intermediate vectors used in N. tabacum and B. napus transformation experiments. Description of the vector construction in section II.H.1. Vector construction and gene isolation by J. Hattori and J. DeMoor.

(pKALSR) with its own promoter and with the 35S promoter of CAMV (p35SALSR). The section 11.H.1 covers the construction of these vectors. Following N. tabacum leaf disc cocultivation and selection on kanamycin (100 ug/mL) , comparable numbers of shoots developed (on average 20 per leaf disc) with all four vectors. The percentage of escapes calculated as the proportion of shoots transferred to kanamycin after excision from leaf discs and that showed kanamycin sensitivity varied from 5% to 24%. Kanamycin inhibited shoot development on the uninoculated control (Plate V.7, Table V.7).

Selection on chlorsulfuron gave different results. First, the uninoculated control leaf discs as well as leaf discs transformed with pKALSW gave rise to shoots at a low frequency (3 shoots per explant). The other vectors (pKALSR, p35SALSW and p35SALSR) gave frequencies of shoot regeneration similar to kanamycin selection (20 shoots per explant). The percentage of escapes on chlorsulfuron calculated by the proportion of shoots excised from leaf discs selected on chlorsulfuron that showed sensitivity to chlorsulfuron on transfer was very high (82 and 87% for p35SALSW and p35SALSR). Moreover, chlorsulfuron delayed shoot differentiation on N. tabacum leaf discs by 4 to 6 weeks and the recovery of resistant plantlets was also delayed by 3 to 4 weeks relative to plantlets grown on kanamycin. In the presence of kanamycin, shoots appeared on the leaf discs after 3 weeks and were excised after 6 weeks. On chlorsulfuron, shoots appeared after 7 to 9 weeks and were excised after 11 to 14 weeks.

Reciprocal experiments were conducted in which shoots excised from leaf discs selected on chlorsulfuron were transferred to kanamycin and vice versa. Following selection on kanamycin, 27% and 31% of the

Plate V.7 Comparison of chlorsulfuron and kanamycin as selectable transformation markers.

A) Control N. tabacum leaf discs; without selection (1), with 330 nM chlorsulfuron (3), with 440 nM chlorsulfuron (4) and with 100 ug/mL kanamycin (2). Photographs after 2 months. B) Selection on kanamycin (100 ug/mL) for leaf discs cocultivated with p35SALSW (1), with p35SALSR (2) and control leaf discs (3). Photograph after 1 month. C) Selection on chlorsulfuron (330 nM) for leaf discs cocultivated with p35SALSW (1), with p35SALSR (2) and control leaf discs (3). Photograph after 2 months.

TABLE V.7. N. tabacum Leaf Disc Transformation with the Acetolactate Synthase Gene

Vectors	control	pKALSW	pKALSR	p35SALSW	P35S ALSR
<u>Selection on kanamycin (100ug/mL)</u>					
# of discs inoculated	10	45	45	60	60
# of shoots per disc	0	20	20	20	20
# of plantlets transferred to Km	0	42	20	36	34
# of resistant plantlets	0	32 (76%)	19 (95%)	28 (78%)	29 (85%)
<u>Selection on chlorsulfuron (330nM)</u>					
# of discs inoculated	10	45	45	60	60
# of shoots per disc	3	3	20	20	20
# of plantlets transferred to Chl.	--	--	--	22	16
# of resistant plantlets	--	--	--	4 (18%)	2 (13%)

The numbers in parenthesis indicate the % of resistant plantlets. Selection on chlorsulfuron delays the appearance of shoots from 4 to 6 weeks, the growth of resistant shoots is also delayed on chlorsulfuron.

TABLE V.8. Selection at the Plantlet Level after Initial Selection on Kanamycin and Chlorsulfuron

Vectors	% of sensitive plantlets	
	p35SALSW	p35SALSR
Plantlets selected on kanamycin		
transferred on chlorsulfuron	31%	27%
Plantlets selected on chlorsulfuron		
and transferred on kanamycin	87%	79%

shoots were sensitive to chlorsulfuron (Table V.8). The highest frequency (31%) was seen with the wild type gene fused to the 35S promoter. Selection on chlorsulfuron and subsequent transfer of shoots to kanamycin resulted in a generally higher proportion of shoots (79 to 87%) that were sensitive to kanamycin. In both types of experiments, the kanamycin selection was better than chlorsulfuron selection in that less escapes were observed. The escapes in this experiment included both non-transformed shoots and the transformed shoots that did not express the genes at a level sufficient for resistance.

V.B.4 The engineering of herbicide tolerance and resistance by manipulation of the acetolactate synthase gene in transgenic *N. tabacum*

For each vector (pKALSR, p35SALSR, pKALSW and p35SALSW), ten plants were regenerated using selection for kanamycin resistance. Transformation was confirmed by the expression of the NPT II gene in transgenic tissues (Plate V.8). The plants were analyzed for the level of ALS activity in transgenic tissues to assess the proportion of resistant enzyme coded by the different intermediate vector constructs. Transgenic plants transformed with the vectors p35SALSW and pKALSW did not possess forms of ALS enzyme resistant to the herbicide chlorsulfuron. At the highest level of chlorsulfuron (1000 ug/L), no ALS activity was observed with plants transformed with the vector pKALSW (Table V.9, Fig. V.6) and 4% of the total activity was active with p35SALSW (Table V.9, Fig. V.6). One plant transformed with p35SALSW out of 7 plants tested gave 22% ALS activity at 1000 ug/L chlorsulfuron with the vector p35SALSW.

Plants with the mutant ALS gene with its own promoter possessed a

4

Micro-D

Plate V.8 Expression of the NPT II gene in transgenic N. tabacum.
Transgenic N. tabacum (A1-5, B1-5, C1-5), non transformed N. tabacum
(D1,2,4,5). Transgenic B. napus callus transformed with
pKALSR::pGV3850 (D3).

TABLE V.9. Average Level of ALS Activity Obtained with the 4 Intermediate Vectors (p35SALSR, p35SALSW, pKALSR, pKALSW) at 1000 ug/L Chlorsulfuron in Transgenic N. tabacum

Intermediate vector	% of ALS activity	number of plants tested
pKALSR	26 $\pm$ 4	5
p35SALSR	72 $\pm$ 15	5
pKALSW	0 $\pm$ 0	4
p35SALSW	4 $\pm$ 8	7

The percent of activity is given $\pm$ the standard deviation.

Data collected with assistance from G. Sunohara.

Figure V.6 Inhibition profiles of ALS activity in transgenic *N. tabacum* transformed with the ALS gene constructs. ALS activity was measured at each herbicide concentration and expressed as a percentage of the uninhibited control. Transgenic *N. tabacum* transformed with the vector p35SALSR (○—○); pKALSR (▲—▲); p35SALSW (●—●) and control (■—■).

resistant form of ALS which accounted for an average 25% of the total ALS activity (Table V.9). The 35S promoter linked to the mutant ALS gene (p35SALSR) conferred much higher levels of ALS activity resistant to chlorsulfuron (average 72% at 1000 ug/L; Fig. V.6, Table V.9).

To correlate the enzyme inhibition studies with effects on growth, plantlets were transferred to media containing toxic levels of chlorsulfuron (220nM) and imazapyr (AC 243,997, 400 ng/L). Transgenic plants obtained with pKALSW did not show resistance to either of the two herbicides. Transgenic plants obtained with pKALSR showed resistance to chlorsulfuron but not to imazapyr. In contrast, plants transformed with p35SALSR were resistant to chlorsulfuron (Table V.10) and most of them (8 out of 10) were also tolerant to imazapyr (Table V.10, Plate V.9). Tolerance is defined by the survival of plantlets at toxic levels of herbicide in the growth medium; however, some toxicity symptoms and growth inhibition may be apparent. Resistance is defined here by the complete absence of symptoms and faster growth rate than the tolerant plantlets (Plate V.9). The plantlets transformed with p35SALSW were all tolerant to various degrees to chlorsulfuron and most of them (7 out of 9) tolerant but to a lesser extent to imazapyr (Table V.10). The control plantlets were sensitive to both chlorsulfuron and imazapyr.

V.B.5 Engineering herbicide resistance in B. napus

After evaluating the sulfonylurea and imidazolinone herbicides for their potential use in B. napus and after assessing the feasibility of engineering resistance to these two classes of herbicides in N. tabacum, most of the basic information for engineering B. napus was

TABLE V.10. Resistance and Tolerance to Sulfonylurea and Imidazolinone Herbicides in Transgenic *N. tabacum*

Transgenic plant #	Chlorsulfuron (20 nM)	Imazapyr (200 ng/L)
<u>p35SALSR</u>		
#1	RR	TTT
#2	RR	TTT
#9	R	S
#10	R	TTT
#11	R	TTT
#12	R	TTT
#15	R	T
#16	R	S
#17	R	TTT
#18	R	T
<u>p35SALSW</u>		
#3	T	TTT
#4	TT	T
#5	TTT	T
#6	TTT	TTT
#7	T	S
#8	T	S
#13	TTT	T
#14	TT	NT
#15	T	T
#19	TT	TTT
Control	S	S

R= resistance to the herbicide, S=sensitive to the herbicide
 T= tolerance to the herbicide. The letter repetition indicates
 a higher level of tolerance or resistance. NT= not tested.

Plate V.9 Resistance to chlorsulfuron in transgenic N. tabacum.

A) Transgenic N. tabacum transformed with pGV3850::p35SALSR, B) and C) Transgenic N. tabacum transformed with pGV3850::p35SALSW. D) Control N. tabacum line SR1. Herbicide concentration 220 nM. Photographs taken after 4 weeks.

assembled. To extend these data, more detailed analyses of the sensitivity of B. napus ALS enzyme to sulfonylurea and imidazolinone herbicides was performed and the toxicity of these herbicides to various B. napus tissues was examined. Finally, the use of chlorsulfuron as a selectable marker in B. napus transformation and the expression of the csrl-1 mutant gene in transgenic B. napus tissues was studied.

V.B.5.a Toxicity of sulfonylurea and imidazolinone herbicides to B. napus tissues in vitro

The engineering of herbicide resistance in B. napus presupposes that this plant species is sensitive to the herbicides being used. Very few data are available on the toxicity of sulfonylureas to B. napus ALS enzyme and B. napus tissues. Basic information was therefore compiled on both aspects to further extend the work done in section V.B.1. The B. napus ALS enzyme was sensitive to all imidazolinones (Fig. V.7.b) and sulfonylureas tested (Fig. V.7.a).

To complete this information, thin cell layer explants from stem epidermal strips, seeds, microspores and embryos derived from microspores were grown on medium containing increasing amounts of chlorsulfuron. Essentially, all the tissues were sensitive to this sulfonylurea herbicide (Fig. V.8) and, as noted with antibiotics, the microspores were the most sensitive. The herbicide was very toxic and all tissues were killed at a concentration of 10nM (3 ug/mL) chlorsulfuron.

Figure V.7 Inhibition profiles of ALS activity by sulfonylurea and imidazolinone herbicides in B. napus. ALS activity was measured at each herbicide concentration and expressed as a percentage of the uninhibited control. (A) Sulfonylurea herbicides: Chlorsulfuron (●—●); DPX A7881 (◆—◆); DPX M6316 (■—■); DPX L5300 (▲—▲); CGA 131,036 (◇—◇); (B) Imidazolinone herbicides: AC 252,214 (□—□); AC 263,499 (△—△); AC 243,977 (○—○). Data collected with help from J. Gabard.

Figure V.8 Growth inhibition of *B. napus* microspores, microspores-derived embryos and thin cell layer explants by chlorsulfuron. For thin cell layer explants (○—○) and microspore-derived embryos (●—●), at least 12 explants (fresh weight 10 ± 2 mg) and 20 embryos (fresh weight 3 ± 2 mg) were incubated on media with chlorsulfuron. The average fresh weights after 5 weeks were expressed as a percentage of the average fresh weight of explants (601 ± 32 mg) or embryos (403 ± 46 mg) incubated without chlorsulfuron. Bars indicate standard errors. For microspores (△—△), two Petri dishes per chlorsulfuron concentration were incubated for four weeks. The percentage of microspores that gave embryos is given relatively to Petri dishes not containing antibiotics. The approximate number of starting microspores was 10^4 per Petri dish giving rise to approximately 600 embryos in the control plate. For microspores and microspores-derived embryos, the cultivar Topas was used. For explants, the cultivar Westar was used.

V.B.5.b Use of chlorsulfuron resistance as a selectable marker in B. napus

The use of chlorsulfuron resistance as a selectable marker for B. napus transformation was evaluated in experiments similar to those described in section V.B.3. Three intermediate vectors were used: pKALSR, p35SALSW and p35SALSR. Selection was applied after cocultivation of B. napus stem sections using kanamycin (40 ug/mL) or chlorsulfuron (20 nM) with pKALSR and p35SALSW (Table V.10). With chlorsulfuron, no resistant shoots were recovered with any of the vectors tested. With kanamycin, 1.2 to 5% of calli produced resistant shoots (Table V.10) and the frequencies were similar with the three vectors.

V.B.5.c Resistance to sulfonylureas in transgenic B. napus tissues

During the preparation of this thesis, experiments had not progressed enough for the recovery of a complete set of transgenic plants, therefore analysis for the expression of the introduced genes was not completed; however, calli and one transgenic B. napus plant from cocultivated tissues with A. tumefaciens containing the vector pKALSR were analyzed for inhibition of ALS activity by the sulfonylurea herbicides chlorsulfuron and DPX A7881 in the calli and with chlorsulfuron in the transgenic plant. The calli were resistant to 20nM chlorsulfuron. The enzymatic assay revealed that the ALS enzyme was also resistant to chlorsulfuron (46% activity at 1000 ug/mL, Fig. V.9) and to DPX A7881 (48% activity at 100 ug/mL) demonstrating expression of the mutant gene in the resistant calli. The proportion of ALS enzyme resistant to chlorsulfuron was 23% in the transgenic B. napus plant (Fig. V.10).

TABLE V.11. Evaluation of the Acetolactate synthase gene as a selectable marker gene for B. napus

Vector	pKALSR	p35SALSW	p35SALS R
<u>Selection on kanamycin (40 ug/mL)</u>			
# of explants inoculated	1156	778	4242
# of shooting explants	37	12	46
# of resistant shoots	57 (5%)	21 (2.7%)	52 (1.2%)
<u>Selection on chlorsulfuron (20 nM)</u>			
# of explants inoculated	1084	734	1200
# of shooting explants	0	0	0
# of resistant shoots	0	0	0

The numbers in parenthesis indicate the percentage of resistant shoots recovered from 100 explants. Data collected with assistance of H. Labbe.

Figure V.9 Inhibition profiles of ALS activity in transgenic B. napus callus by sulfonylurea herbicides. ALS activity was measured at each herbicide concentration and expressed as a percentage of the uninhibited control. Transgenic B. napus callus with chlorsulfuron (○—○) and DPX A7881 (△—△); control B. napus leaf tissue on chlorsulfuron (●—●) and DPX A7881 (▲—▲).

Figure V.10 Inhibition profiles of ALS activity in transgenic B. napus plant by chlorsulfuron. ALS activity was measured at each herbicide concentration and expressed as a percentage of the uninhibited control. Transgenic B. napus leaf tissue (○—○) and control B. napus leaf tissue (●—●).

V.C DISCUSSION

V.C.1 Potential use of ALS genes for genetic engineering of Brassica species

This survey covered the potential use of 4 sulfonylureas (chlorsulfuron, DPX A7881, DPX M6316 and DPX L5300) and 3 imidazolinones (AC 252,214, AC 263,499 and AC 243,997) on some Brassica species (B. campestris, B. hirta, B. nigra, B. oleracea, B. juncea, B. napus, B. carinata and R. sativus) and the most common weeds affecting canola production (B. kaber, C. maritima, I. arvensis, S. viridis and A. fatua). Other crops that are tolerant to some of these herbicides were surveyed for their possible use in rotation with genetically altered Brassica crop species (G. max, M. sativa, H. vulgare and I. aestivum). Model plants (N. tabacum and A. thaliana) employed in experimental genetic manipulations were also included. Two complementary aspects of herbicide resistance were tested: the activity of the target enzyme and the germination and growth of seeds both in the presence of the 7 herbicides.

Forms of ALS enzyme that were naturally resistant to the sulfonylureas or imidazolines were not found in the plant species tested. These results are shown in Tables V.2 and V.3 as indicated by

the $I_{\max}$ values. As shown by others (Ray 1985), the I_{50} values for the sulfonylureas varied greatly among species but were not correlated with the natural tolerance that certain species exhibit to specific herbicides. Similar results were obtained with the imidazolinones although it was observed that the ALS activities of G. max and M. sativa were tolerant to the herbicides. The insertion of mutant genes coding for resistant ALS enzyme to one or both classes of herbicide could be potentially effective, depending on the pattern of natural tolerance that exists for each species.

The biochemical basis for natural tolerance to these herbicides appears to involve metabolic pathways for detoxification. For instance, the rapid hydroxylation of chlorsulfuron followed by glycosylation has been described for Graminae, S. nigrum and L. usitatissimum (Sweetser et al. 1982, Hutchinson et al. 1984). Natural tolerance to specific herbicides is dependant on a number of factors under field conditions such as soil type, microbial activity, moisture, climate, movement of water in soil (Blair and Martin 1988). In this study, the in vitro conditions used for the germination of seedlings in the presence of herbicides provided a uniform basis for comparison of herbicidal activity. The data paralleled observations made in field evaluations; however, obvious differences were also noted. The sulfonylurea DPX L5300 which has been shown to control species such as I. arvensis, B. kaber and other Brassica species (Ferguson et al. 1985) was generally ineffective in vitro although inhibition of ALS activity by the herbicides was confirmed in biochemical assays. This observation is explained by the short half life of this chemical in vitro (C.J. Mauvais, Dupont Co., Delaware, USA). As expected, all of

the Brassica species and related weeds were sensitive to chlorsulfuron and DPX M6316 whereas the cereals were tolerant to the sulfonylureas. The short residual sulfonylurea DPX A7881 was of particular interest because it is being evaluated for use with B. napus. Clear tolerance was observed for B. campestris, B. napus and B. carinata. Various degrees of sensitivity to DPX A7881 were observed for other Brassica species indicating that the tolerance of B. napus is not a general characteristic of the genus. All Brassica species and related weeds were sensitive to the imidazolinones whereas the legumes and cereals were tolerant as expected.

These data support the potential use of genetic manipulation to engineer resistance in Brassica crops to sulfonylureas such as chlorsulfuron, DPX M6316 and DPX L5300 and to imidazolinones such as AC 252,214, AC 263,499 and AC 243,997. The natural resistance of Brassica crop species to DPX A7881 will probably be used for the application of this herbicide in the field; however, the engineering for the other sulfonylurea and imidazolinone herbicides would extend the array of herbicides that could be used in Brassica crop species. The finding that forms of ALS enzyme exist in G. max and M. sativa which are naturally more tolerant to the imidazolinones than ALS of Brassica species raises the possibility of using these enzymes to obtain tolerance to the imidazolinone herbicides by overexpressing the target enzyme. This was demonstrated with transgenic P. hybrida and A. thaliana where elevated expression of EPSP synthase using the 35S promoter of CAMV was sufficient to achieve resistance to glyphosate (Shah et al. 1986, Klee et al. 1988).

V.C.2 Gene csr1-1 as a selectable marker for plant genetic transformation

Selectable marker genes have been used in plant genetic transformation studies to recover transformed tissues. The most widely used selectable marker is the bacterial gene neomycin phosphotransferase II which confers resistance to kanamycin, paromomycin, neomycin and geneticin (Fraley et al. 1983, Herrera-Estrella et al. 1983a, Guerche et al. 1987a). Other antibiotic resistance genes have also been used with success in different plant species (Waldron et al. 1985, Eichholtz et al. 1987, Jones et al. 1987). For most of them, selectivity is based on a detoxification mechanism encoded by the gene such as the enzyme neomycin phosphotransferase II which phosphorylates antibiotics such as kanamycin (Fraley et al. 1983). Herbicide resistance genes have been considered to be potentially good selectable markers for plant transformation because they are specific to plants and some of them are also of plant origin. Other mechanisms for resistance such as mutations within or overexpression of the target enzyme (Haughn et al. 1988, Mazur et al. 1987a, 1987b, Klee et al. 1987, Shah et al. 1987) have been exploited. To make a proper comparison between the NPT II gene and the csr1-1 gene as selectable markers, a set of 4 intermediate vectors (pKALSR, p35SALSR, pKALSW, p35SALSW) were constructed with both genes on the same vector. In N. tabacum, selection on kanamycin gave similar results with all vectors. If shoots were taken directly from leaf discs and further selected on kanamycin, a low proportion of escapes were observed (between 5 and 24%) and after one transfer on kanamycin containing medium, the escapes were eliminated. Moreover, kanamycin selection did

not allow the development of shoots on the control leaf discs. With chlorsulfuron selection, the number of shoots per explant was similar to kanamycin selection (20 shoots/explant) but high proportions of escapes were observed. Moreover, shoots regenerated from the control leaf discs and those transformed with pKALSW in the presence of chlorsulfuron. The escapes were eliminated after two transfers to media containing chlorsulfuron. The major problem with chlorsulfuron selection was the lengthy delay in shoot regeneration and plantlet growth. Reciprocal experiments where the alternate selective agent was used to transfer shoots excised from leaf discs confirmed the data obtained previously and supported the finding that chlorsulfuron resistance was a poor selection system in N. tabacum.

With B. napus, chlorsulfuron selection did not allow the recovery of resistant shoots at the level of selection used (20 nM). Chlorsulfuron was far too toxic for the non-transformed tissues. This in turn probably prevented the survival of the transformed cells. Kanamycin has the advantage of being less toxic and allows the survival of non transformed tissues as shown by the white shoots developing on the explants.

These experiments were only done with the herbicide chlorsulfuron as a selective agent; however, it is possible that the use of one of the other short residual sulfonylurea herbicides would have improved the selection. Moreover, results could have been different with herbicide acting on a different target enzyme (such as glyphosate or phosphinotricin) or with a different mechanism of resistance such as detoxification.

V.C.3 Engineering sulfonylurea herbicide resistance in transgenic N. tabacum

Herbicide resistance has been engineered in plants using 3 different mechanisms. Resistance was obtained by using mutant genes encoding a resistant target protein (Haughn et al. 1988, Mazur et al. 1987a, 1987b, Lee et al. 1988, Comai et al. 1985), by introducing a gene overexpressing the target enzyme (Shah et al. 1986, Klee et al. 1987) or by introducing a gene coding for a detoxifying enzyme (De Block et al. 1987). With the herbicides acting on the ALS enzyme, two approaches were taken to engineer resistance in transgenic plants: the use of the mutant csrl-1 gene (Haughn et al. 1988) and the overexpression of the wild type and mutant genes.

V.C.3.a Cross resistance to sulfonylurea herbicides in transgenic N. tabacum

Genetic engineering with 2 ALS genes isolated from chlorsulfuron resistant mutant N. tabacum lines have been used to recover both transgenic N. tabacum (Lee et al. 1988) and L. esculentum (Mazur et al. 1987a, 1987b). The mutant ALS gene csrl-1 from the A. thaliana mutant line GH50 resistant to chlorsulfuron was also transferred to N. tabacum (Haughn et al. 1988). In each case resistance to chlorsulfuron was obtained showing that the plant ALS mutant genes function in heterologous as well as homologous systems. The results that are discussed in this section are aimed at extending these findings to a broader analysis of the selective resistance to the sulfonylurea or imidazolinone herbicides in transgenic N. tabacum plants obtained with the csrl-1 ALS mutant gene and to compare it with the resistance in the

original GH50 A. thaliana mutant line. Moreover, assessment of potential cross resistance to short residual sulfonylureas, useful for crop plants, has been made.

Transgenic N. tabacum plants that have integrated in their genome the mutant ALS gene of the GH50 A. thaliana mutant line had segregation patterns consistent with the presence of the ALS and kanamycin gene at a single locus. Biochemical assays performed on young transgenic plants revealed the presence of a chlorsulfuron resistant form of ALS that accounted for about 30% of the total activity. This form corresponded to the A. thaliana GH50 ALS enzyme whereas the sensitive form corresponded to resident N. tabacum ALS enzyme. N. tabacum has an allotetraploid genome with at least 4 ALS alleles (Mazur *et al.* 1987b).

When mature plants were sprayed with chlorsulfuron, at the flowering stage the controls had lost all their flowers and severe damage on leaves, stem and axillary buds were visible 2 weeks after treatment. Transgenic N. tabacum had slight leaf burns; however, flowers and capsules developed normally, indicating that the residual resistant ALS form was sufficient to offset the chlorsulfuron toxicity.

Cross-resistance to all sulfonylurea herbicides tested was observed biochemically in transgenic plants. For DPX M6316, DPX A7881 and DPX L5300, the resistant activity accounted for 20 to 25% of the total. This level of activity was sufficient to allow normal germination, growth and development in the presence of DPX A7881 or DPX M6316. Unlike the other sulfonylureas, tolerance to CGA 131036 was detected only at low concentrations. In the greenhouse, the development of transgenic CHL-2 seedlings was unaffected by DPX A7881; slightly delayed by DPX M6316 and significantly reduced by CGA 131036.

Growth of control SR1 seedlings was completely blocked at the cotyledon stage. These results clearly demonstrated the feasibility of cross resistance as a mechanism for employing other short residual sulfonylureas with transgenic plants.

As expected (Haughn and Somerville 1986), the specific mutation in the csrl-1 gene did not extend resistance to the imidazolinones, in the A. thaliana mutant GH50 or transgenic N. tabacum line CHL-2. A slightly better growth for CHL-2 was observed with AC 252,214 in vitro and with AC 263,499 in the greenhouse. The ALS profiles also reflected this slight resistance but the level was insufficient for practical considerations.

V.C.3.b Genetic manipulation of the ALS gene

The previous sections demonstrated the feasibility of engineering herbicide resistance in B. napus by using a mutant ALS gene. The manipulation of the mutant csrl-1 and wild type forms of the ALS gene with the strong constitutive promoter 35S of CAMV (Odell et al. 1985) aimed to evaluate the use of elevated gene expression to obtain herbicide resistance.

Plants regenerated after transformation with the mutant gene with its own promoter (pKALSR) or with the 35S promoter were resistant to chlorsulfuron. The enzymatic assays revealed that the mutant gene with the 35S promoter gave a much higher proportion of enzyme resistant to chlorsulfuron (72%) than with the ALS promoter (26%). These results were supported by Northern blotting in which more ALS mRNA was detected with the 35S promoter (data not shown). These observations strongly suggest that the 35S promoter leads to a stronger level of

transcription than the ALS gene promoter. This was expected since the 35S promoter has been shown to be a strong constitutive promoter giving higher levels of expression than promoters such as the nopaline synthase promoter (Sanders *et al.* 1987) and the 19S promoter of CAMV (Harpster *et al.* 1988).

Plants transformed with the wild type gene under the control of the 35S promoter did not yield enzyme resistant to the herbicide chlorsulfuron; however, tolerance of *in vitro* grown plantlets was observed to chlorsulfuron and imazapyr (AC 243,997). Tolerance to imazapyr was also observed with plantlets transformed with the mutant ALS gene under the control of the 35S promoter. Tolerance to chlorsulfuron and cross tolerance to imazapyr obtained when the ALS gene was under the control of a 35S promoter demonstrated the feasibility of engineering herbicide resistance by overexpression of the target ALS enzyme.

The words tolerance and resistance are often confusing. In this thesis, resistance refers to the growth of plant or plant tissues without any symptoms of toxicity; however, a delay in growth can be observed when compared to non treated control plants. Tolerance indicated the growth of plant or plant tissues with toxicity symptoms but without their death.

V.C.4 Genetic engineering with the ALS gene in B. napus

Potentially, both sulfonylureas and imidazolinones can be used with B. napus; however, this species is resistant to the sulfonylurea herbicide DPX A7881. Genetic engineering can be used to introduce resistance to these two classes of herbicides in B. napus. The use of

the mutant ALS gene csr1-1 to genetically modify N. tabacum (Haughn et al. 1988) and L. esculentum (Mazur et al. 1987a, 1987b) had demonstrated the possibility of introducing chlorsulfuron resistance in plants. The data in this thesis confirmed these results and furthermore showed that this mutant gene gives resistance to other sulfonylureas in transgenic plants but not to imidazolinones. Moreover, the use of the ALS gene (mutant or wild type) with a 35S promoter was shown to give tolerance to the imidazolinones in transgenic plants.

For B. napus, three intermediate vectors were used (pKALSR, p35SALSR, p35SALSW) to attempt the engineering of herbicide resistance. Resistant shoots to kanamycin were obtained and a few plants have been regenerated. Data obtained with B. napus transgenic calli following transformation with pKALSR showed that a high proportion of ALS enzyme was resistant to both chlorsulfuron and DPX A7881. The calli was resistant to chlorsulfuron in vitro. In one transgenic B. napus plant tested, a form of ALS resistant to chlorsulfuron was also observed. These results demonstrated the expression of the introduced csr1-1 gene and the feasibility of recovering resistant B. napus transgenic plants. More data is needed to confirm the practical use of the mutant gene or the wild type ALS gene to obtain field level herbicide resistance.

CHAPTER VI

GENERAL DISCUSSION

VI.A Introduction

When this research thesis was initiated in 1984, a system for producing transgenic B. napus did not exist and genes of agronomic relevance were not isolated. Motivated by the dramatic advances that were made with N. tabacum and P. hybrida during this era and the perception that such technology would greatly benefit Canola (B. napus), the project was undertaken with uncertain prospects of success. Rather than a "shotgun" approach, we decided to develop reliable technology in a slow systematic manner hoping that in the end genes relevant for study in transgenic plants would become available as the field of plant molecular biology developed. In retrospect, we can conclude that our decisions were correct. Indeed, the transformation and genetic engineering of B. napus has become the focus of much attention worldwide. Although we can take credit for originating many of the approaches and early findings, in the end we did not publish first due to the immense competition in the field and our willingness to share with our competitors. Four papers published in the last 2 years including our own are very similar and 3 of these are based on our early findings.

The frequency of transgenic B. napus plants obtained with our system (c.a. 2%) is in the same range as that published by others with A. tumefaciens-mediated transformation (0.4 to 10%; Pua et al. 1987, Fry et al. 1987, Radke et al. 1988). The transgenic plants were morphologically normal and fertile in all cases. The introduced genes (nopaline synthase and neomycin phosphotransferase II) did not usually segregate in the progeny as a single Mendelian trait (i.e. 3:1). This may be due to the low level of expression of the genes. In a recent study, Deroles and Gardner (1988a, 1988b) demonstrated that in transgenic P. hybrida, a high proportion of plants (45/104) showed non-Mendelian patterns of inheritance for the NPT II gene which was associated with its low expression. Rearrangements and multiple integrations were common and may have also contributed to these findings.

In this work, selection with kanamycin was relatively low (15 ug/mL). Such levels were also used by others (Radke et al. 1988) but, as observed by Fry et al. (1987), increasing the level of selection (from 50 to 100 ug/mL) reduced the number of escapes. Our data clearly showed that the majority of the transformants were kanamycin sensitive and very few of them were resistant to levels above 50 ug/mL. Preliminary data on selection at 40 ug/mL of kanamycin in our laboratory indicated a much lower frequency for the recovery of kanamycin resistant shoots. The inefficiency of the selection did not necessarily imply that other genes within the T-DNA were poorly expressed. For instance, expression of the nopaline synthase gene was used to detect transgenic plants recovered without selection. The implications were that the expression of other genes within the T-DNA

is not certain from our studies. It is possible that the majority of the plants recovered by our system, may not express genes in transgenic plants to levels that are acceptable. Such phenomenon have often been ascribed to position effects governing the inserted genes; however, the molecular basis for such effects is not known. The only practical method for overcoming this obstacle is to produce large numbers of transgenic plants and to identify those with desirable qualities.

VI.B Comparison with other transformation systems

Transgenic plants regenerated following A. rhizogenes-mediated transformation showed the characteristic symptoms of the hairy root disease. The A. rhizogenes symptoms have also been observed in transgenic B. napus by others (Guerche et al. 1987c, Ooms et al. 1985). Unless the Ri plasmid T-DNA were disarmed, the A. rhizogenes approach is not very useful for producing transgenic B. napus plants.

Microinjection of microspore-derived embryos is the only direct gene transfer method where transgenic B. napus plants have been recovered in large numbers and with high transformation frequencies (27-51%, Neuhaus et al. 1987); however, the transgenic plantlets were chimeric and needed a cloning step involving secondary embryogenesis. The number of transgenic plants produced and the speed of recovery exceeded the efficiencies observed with A. tumefaciens-mediated transformation. A critical assessment of this approach cannot be completed because transmission of the introduced character to the progeny was not demonstrated and the characteristics of the plants are unknown. This method is promising but A. tumefaciens-mediated transformation is further advanced at this time. In the future

transformation methodologies for B. napus will no doubt change dramatically. It should be recognized that this is still an exploratory stage in the development of these technologies.

VI.C Comparison of B. napus and N. tabacum transformation with A. tumefaciens

The frequency of transgenic plant recovery in N. tabacum leaf disc transformation with A. tumefaciens is at least 2 orders of magnitude higher than with B. napus. By exploring the basic differences between the two systems, it is possible to point out where improvements could be made to enhance B. napus transgenic plant recovery (Table VI.1).

N. tabacum leaf discs gave higher proportions of explants with shoots, more shoot/explant and a shorter regeneration time than B. napus. These differences are inherent to the tissues themselves but could be improved by media and growth condition manipulations. Moreover, alternative tissue sources must be explored for B. napus in regard to the aspects mentioned previously.

In B. napus, the explants (thin cell layers or stem sections) have a strong adverse response to A. tumefaciens. They exhibited a severe hypersensitive response resulting in a decrease in the shoot frequency and a delay in shoot regeneration. The use of silver nitrate or activated charcoal in the tissue culture medium to offset the hypersensitive response is an avenue that can be explored. Silver nitrate has been shown to reduce tissue necrosis and enhance microspore development (Biddington et al. 1988). The use of carbenicillin instead of clavulin 250 (as used in other studies; Pua et al. 1987, Fry et al. 1988, Radke et al. 1988) could be an improvement because of its

TABLE VI.1. Comparison of N. tabacum and B. napus Explants Used in A. tumefaciens-mediated Transformation

Aspects	<u>N. tabacum</u>	<u>B. napus</u>
<u>Tissue culture</u>		
% of explants giving shoots	100%	20-80%
# of shoot per explant	20-30	5-10
regeneration time	3 weeks	3-4 weeks
time to get a plant in greenhouse	2 months	6 months
<u>Transformation</u>		
Hypersensitive response to <u>A. tumefaciens</u>	NO	YES, strong
Delay in shoot regeneration	NO	YES, 2-3 weeks
Decrease in # of shoot per explant	NO	YES, 3-5 times
Susceptibility to <u>A. tumefaciens</u> pTi	Excellent	Limited*
Performance of transformation markers	no problems	Some limitations**
% of transgenic plants	2000%	0.4% to 10%

* B. napus is better transformed with nopaline Ti plasmids. ** Limitations were found in the use of the CAT gene marker, in some chemical toxicities and in specific gene constructions. § This table was assembled from data obtained in this thesis but from data of other works for B. napus (Radke et al. 1988, Pua et al. 1987, Fry et al. 1987).

beneficial effect (as shown in section IV.B.2) on B. napus explant growth; however, it would involve a major change in the vectoring strategy. The ampicillin resistance gene in the disarmed plasmid pGV3850 prevented the use of carbenicillin. This plasmid was the only nopaline disarmed Ti plasmid available when this work started. Another improvement would be the use of the 35S promoter of CAMV instead of the nopaline synthase promoter.

Transformation markers were less efficient in B. napus and the CAT gene was impractical in this regard. Tissue explants of B. napus were more sensitive to toxic chemicals than N. tabacum leaf discs and a potential herbicide resistance marker coding for the mutant acetolactate synthase gene could not be used as a selectable marker in B. napus. The use of selectable marker genes with higher expression should be explored. Indeed, the need for selection at all must be evaluated correctly. Another improvement would be the use of the 35S promoter of CAMV instead of the nopaline synthase promoter. Using this promoter with our transformation protocol gave high levels of expression of the NPT II gene in transgenic B. napus (preliminary data). The level of expression obtained was comparable to that observed in transgenic N. tabacum.

VI.D Engineering herbicide resistance in B. napus

Chlorsulfuron resistance was an inefficient selectable marker in both N. tabacum and B. napus; however, different methods for employing chlorsulfuron (or other sulfonylureas) were not evaluated. Similar results were obtained with glyphosate selection (R. Horsch, Monsanto Co., personal communication) in B. napus transformation experiments.

In the case of glyphosate, the kanamycin selection was used to recover transgenic plants.

The feasibility of engineering herbicide resistance with the gene csrl-1 of A. thaliana in N. tabacum was confirmed in our work as demonstrated by others (Haughn et al. 1986, Mazur et al. 1987a, 1987b, Lee et al. 1988). Moreover, we described the resistance conferred by the gene to short residual herbicides in addition to chlorsulfuron in the original A. thaliana GH50 line and in transgenic N. tabacum. Tolerance to imidazolinone was obtained with overexpression of the ALS gene.

This work demonstrated the expression of the csrl-1 gene and the presence of an ALS form resistant to chlorsulfuron in transgenic B. napus. In addition to developing herbicide resistance in B. napus, the study of the acetolactate synthase gene is interesting. In B. napus, ALS is encoded by a family of at least 3 genes that could be developmentally regulated and this gene family seems to originate from the diploid ancestors of the species (B. campestris and B. oleracea; R. Rutledge and B. Miki, personal communication).

VI.E Pathway to recover B. napus transgenic plants

This thesis followed closely the pathway illustrated in Figure VI.1. The tissue culture system chosen was the stem explants (thin cell layers or stem sections). This was used with A. tumefaciens disarmed Ti plasmid in cocultivation experiments. The transgenic plants were selected on kanamycin and screened for the expression of the nopaline synthase gene. Whole plants were regenerated, checked for the presence of the chimeric genes and for their transmission to the

Figure VI.1 Flow chart of the pathway followed to complete the recovery of transgenic B. napus plants.

progeny. This technology was used again to obtain transgenic B. napus plants with an introduced ALS gene. This gene was introduced in B. napus in order to obtain herbicide resistance.

VI.F Achievements of this thesis

The wild type Agrobacterium-mediated genetic transformation of B. napus and B. juncea has been studied with 8 wild type Agrobacterium strains and 4 genetically modified strains. The data confirmed the high transformation efficiency of nopaline Ti plasmids in contrast to the low efficiency of octopine Ti plasmids. Disarmed Ti plasmids were shown to be as virulent as the wild type plasmids. The poor tumour induction by octopine Ti plasmids was explained by a weak VIR gene induction with B. napus.

B. juncea was found to be a good test plant for in planta tumourigenesis because of the rapidity of tumour formation and its susceptibility to a wide range of Agrobacterium strains. Tumour morphology varied with the Agrobacterium strains used in both B. napus and B. juncea which makes these two plants as interesting as Kalanchoe species for tumourigenesis studies.

A transformation procedure for B. napus has been developed (see previous section) using disarmed nopaline Ti plasmid pGV3850 which allowed the recovery of morphologically normal and fertile transgenic plants following A. tumefaciens-mediated transformation. This work revealed the inefficiency of the chloramphenicol acetyltransferase gene as a transformation marker for Brassica species and the problems linked to its use.

The mutant ALS gene csr1-1 of A. thaliana was used to engineer

herbicide resistance in N. tabacum. This study demonstrated that the csrl-1 gene conferred the same selectivity of herbicide resistance in both the mutant A. thaliana line GH50 and transgenic N. tabacum. It conferred resistance to chlorsulfuron and also to short residual sulfonylurea herbicides (DPX A7881, DPX L5300, DPX M6331); however, imidazolinone resistance was not obtained unless expression was elevated by the use of the 35S promoter.

The mutant ALS gene and the wild type ALS gene were linked to a 35S promoter from CAMV and transferred to both N. tabacum and B. napus. With these gene constructs, chlorsulfuron resistance was evaluated as a selectable marker for plant genetic transformation and found to be less effective than the neomycin phosphotransferase gene.

VI.6 Further developments with Brassica species

In terms of frequency of transgenic plant recovery, N. tabacum is still the model plant system of choice; however, B. napus has immense potential that could dethrone N. tabacum in the next few years. In addition to the features of the Brassica genus and B. napus described in the introduction chapter of this thesis, this crop plant has enormous capabilities to ally study of crop quality characteristics with fundamental gene regulation and gene expression studies. The production of oil in specialized compartments is one aspect that can be tackled to modify the type of fatty acid produced in response to new market demands (Knauf 1987). The characterization of seed storage protein genes such as napin and cruciferin opened the door to protein quality improvement and the study of developmental gene regulation in B. napus (Crouch 1982, Crouch et al. 1983). Another interesting avenue

with B. napus is the study of self incompatibility and male sterility which could lead to major applications in plant breeding (Barsby et al. 1987, Erickson et al. 1986, Nasrallah et al. 1985). Other important prospects are the study of plant viruses such as CAMV (cauliflower mosaic virus), TuMV (turnip mosaic virus) and TYMV (turnip yellow mosaic virus) that infect B. napus (Walsh and Tomlinson 1985), the examination of the role of compounds such as brassinosteroids which have hormonal effects (Mandava 1988, Suge 1986) and the investigation of the functions of plant mitochondrial plasmids (Erickson et al. 1986). These are a few areas of research in which Brassica species have been used to explore genetic mechanisms that are also relevant to crop quality and productivity. Genetic transformation technology will provide the experimental means for testing these hypotheses and manipulating them for economic gains.

REFERENCES

- ACKERMANN, C. (1977) Pflanzen aus Agrobacterium rhizogenes - tumoren an Nicotiana tabacum. Plant Sci. Lett. 8: 23-30.
- ADAMS, T.L. and J.A. TOWNSEND (1983) a new procedure for increasing efficiency of protoplast plating and clone selection. Plant Cell Rep. 2: 165-168.
- ADOLPHE, D. (1982) Canola - Canada's No. 1 Oilseed. Notes on Agriculture 18: 5-7.
- AKIYOSHI, D.E., R.O. MORRIS, R. HINZ, B.S. MISCHKE, T. KOSUGE, D.J. GARFINKEL, M.P. GORDON and E.W. NESTER (1983) Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. Proc. Natl. Acad. Sci. USA 80: 407-411.
- ALT-MOERBE, J., B. RAK and J. SCHRÖDER (1986) A 3.6 Kbp segment from the VIR region of Ti plasmids contains genes responsible for border sequence-directed production of T region circles in E. coli. EMBO J. 5: 1129-1135.
- AN, G. (1986) Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol. 81: 86-91.
- ANONYMOUS (1985) Situation report. Agriculture Canada, 8 pp.
- ARONSON, A.I., W. BECKMAN and P. DUNN (1986) Bacillus thuringiensis and related insect pathogens. Microbiol. Rev. 50: 1-24.
- ASHBY, A.M., M.D. WATSON and C.H. SHAW (1987) A Ti-plasmid determined function is responsible for chemotaxis of Agrobacterium tumefaciens towards the plant wound product acetosyringone. FEMS Microbiol. Lett. 41: 189-192.
- ASHBY, A.M., M.D. WATSON, G.J. LOAKE and C.H. SHAW (1988) Ti plasmid-specified chemotaxis of Agrobacterium tumefaciens C58C1 toward VIR-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. J. Bacteriol. 170: 4181-4187.
- BABA, A., S. HASEZAWA and K. SYONO (1986) Cultivation of rice protoplasts and their transformation mediated by Agrobacterium spheroplasts. Plant Cell Physiol. 27: 463-471.

- BALAZS, E. and J.M. BONNEVILLE (1987) Chloramphenicol acetyl transferase activity in Brassica spp. *Plant Sci.* 50: 65-68.
- BARSBY, T.L., S.A. YARROW, R.J. KEMBLE and I. GRANT (1987) The transfer of cytoplasmic male sterility to winter-type oilseed rape (Brassica napus L.) by protoplast fusion. *Plant Sci.* 53: 243-248.
- BARSBY, T.L., S.A. YARROW and J.F. SHEPARD (1986) a rapid and efficient alternative procedure for the regeneration of plants from hypocotyl protoplasts of Brassica napus. *Plant Cell Rep.* 5: 101-103.
- BARTON, K.A., A.N. BINNS, A.J.M. MATZKE and M.D. CHILTON (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. *Cell* 32: 1033-1043.
- BARTON, K.A., H.R. WHITELEY and N.S. YANG (1987) bacillus thuringiensis-endotoxin expressed in transgenic Nicotiana tabacum provides resistance to Lepidopteran insects. *Plant Physiol.* 85: 1103-1109.
- BASIRAN, N., P. ARMITAGE, R.J. SCOTT and J. DRAPER (1987) Genetic transformation of flax (Linum usitatissimum) by Agrobacterium tumefaciens: Regeneration of transformed shoots via a callus phase. *Plant Cell Rep.* 6: 396-399.
- BAULCOMBE, D.C., G.R. SAUNDERS, M.W. BEVAN, M.A. MAYO and B.D. HARRISON (1986) Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. *Nature* 321: 446-449.
- BAYER, V.E., K.H. GUGEL, K. HAGELE, H. HAGENMAIER, S. JESSIPOW, W.A. KONIG and H. ZAHNER (1972) Phosphinothricin und phosphinothrycil-alanyl-alanin. *helv. Chim. Acta* 55: 224-239.
- BERNATZKY, R. and S.D. TANKSLEY (1986) Methods for detection of single or low copy sequences in tomato on Southern blots. *Plant Mol. Biol. Rep.* 4: 37-91.
- BEVAN, M. (1984) Binary Agrobacterium vectors for plant transformation. *Nucleic Acids Res.* 12: 8711-8721.
- BEVAN, M., W.M. BARNES and M.D. CHILTON (1983) Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acids Res.* 11: 369-379.
- BIDDINGTON, N.L., R.A. SUTHERLAND and H.T. ROBINSON (1988) Silver nitrate increases embryo production in anther culture of Brussels sprouts. *Ann. Bot.* 62: 181-185.
- BINO, R.J., J. HILLE and J. FRANKEN (1987) Kanamycin resistance during in vitro development of pollen from transgenic tomato plants. *Plant Cell Rep.* 6: 333-336.

- BIROT, A.M., D. BOUCHEZ, F. CASSE-DELBART, M. DURAND-TGARDIF, L. JOUANIN, V. PAUTOT, C. ROBAGLIA, D. TEPFER, M. TEPFER, J. TOURNEUR and F. VILAIN (1987) Studies and uses of the Ri plasmids of Agrobacterium rhizogenes. *Plant Physiol. Biochem.* 25: 323-335.
- BLAIR, A.M. and T.D. MARTIN (1988) A review of the activity, fate and mode of action of sulfonylurea herbicides. *Pestic. Sci.* 22: 195-219.
- BOUTRY, M., F. NAGY, C. PAULSEN, K. AOYAGI and N.H. CHUA (1987) Targeting of bacterial chloramphenicol acetyltransferase to mitochondria in transgenic plants. *Nature* 328: 340-342.
- BOLIVAR, R., R.L. RODRIGUEZ, P.J. GREENE, M.C. BETLACH, H.L. HEYNEKER, H.W. BOYER, J.H. CROSA and S. FALKOW (1977) Construction and characterization of new cloning vehicles, II A multipurpose cloning system. *Gene* 2: 95-113.
- BOYER, H.W. and D. ROULAND-DUSSOIX (1969) A complementation analysis of the restriction and modification of DNA in Escherichia coli. *J. Mol. Biol.* 41: 459-472.
- BOYNTON, J.E., N.W. GILLHAM, E.H. HARRIS, J.P. HOSHER, A.M. JOHNSON, A.R. JONES, B.L. RANDOLPH-ANDERSON, D. ROBERTSON, T.M. KLEIN, K.B. SHARK and J.C. SANFORD (1988) Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. *Science* 240: 1534-1538.
- BRADSHAW, H.N. Jr., W.W. PARSON, M. SHEFFER, P.J. LIOUBIN, E.R. MULVIHILL and M.P. GORDON (1987) Design, construction and use of an electroporator for plant protoplasts and animal cells. *Anal. Biochem.* 166: 342-348.
- BUCHANAN-WOLLASTON, V., J.E. PASSIATORE and F. CANNON (1987) The MOB and ORIT mobilization functions of a bacterial plasmid promote its transfer to plants. *Nature* 328: 172-175.
- BUCHHOLZ, W.G. and M.F. THOMASHOW (1984) Host range encoded by the Agrobacterium tumefaciens tumor inducing plasmid pTiAg63 can be expanded by modification of its T-DNA oncogene complement. *J. Bacteriol.* 160: 327-332.
- BYTEBIER, B., F. DEBOECK, H. de Greve, M. VAN MONTAGU and J.P. HERNALSTREENS (1987) T-DNA organization in tumor cultures and transgenic plants of the monocotyledon Asparagus officinalis. *Proc. Nat. Acad. Sci. USA* 84: 5345-5349.
- CABOCHE, M. and A. DESHAYES (1984) Utilisation de liposomes pour la transformation de protoplastes de mesophylle de tabac par un plasmide recombinant de E. coli leur conférant la résistance à la kanamycine. *C.R. Acad. Sci. Paris, Sér. III* 299: 663-666.

- CALLIS, J., M. FROMM and V.W. WALBOT (1987) Expression of mRNA electroporated into plant and animal cells. *Nucleic Acids Res.* 15: 5823-5831.
- CANGELOSI, G.A., L. JUNG, V. PUVANESARAJAH, G. STACEY, D.A. OZGA, J.A. LEIGH and E.W. NESTER (1987) Common loci for Agrobacterium tumefaciens and Rhizobium meliloti exopolysaccharide synthesis and their roles in plant interactions. *J. Bacteriol.* 169: 2086-2091.
- CATLIN, D., O. OCHOA, S. McCORMICK and C.F. QUIROS (1988) Celery transformation by Agrobacterium tumefaciens: cytological and genetic analysis of transgenic plants. *Plant Cell Rep.* 7: 100-103.
- CHALEFF, R.S. and N.F. BASCOMB (1987) Genetic and biochemical evidence for multiple forms of acetolactate synthase in Nicotiana tabacum. *Mol. Gen. Genet.* 210: 33-38.
- CHALEFF, R.S. and C.J. MAUVAIS (1984) Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. *Science* 224: 1443-1445.
- CHALEFF, R.S. and T.B. RAY (1984) Herbicide-resistant mutants from tobacco cell cultures. *Science* 223: 1148-1151.
- CHANDLER, J.M., A.S. HAMILL and A.G. THOMAS (1984) Crop losses due to weeds in Canada and the United States. *Weed Science Society of America*, Champaign, USA, 22 pp.
- CHAREST, P.J. and P. DION (1985) The influence of temperature on tumorigenesis induced by various strains of Agrobacterium tumefaciens. *Can. J. Bot.* 63: 1160-1167.
- CHAREST, P.J., L.A. HOLBROOK, J. GABARD, V.N. IYER and B.L. MIKI (1988) Agrobacterium-mediated transformation of thin cell layer explants from Brassica napus L. *Theor. Appl. Genet.* 75: 438-445.
- CHAREST, P.J., L.A. HOLBROOK, V.N. IYER et B.L. MIKI (1986) Activité endogène d'une enzyme chloramphenicol acetyltransferase chez Brassica napus. *Ann. ACFAS* 55: 30.
- CHEN, W.H., K.M.A. GARTLAND, M.R. DAVEY, R. SOTAK, J.S. GARTLAND, B.J. MULLIGAN, J.B. POWER and E.C. COCKING (1987) Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. *Plant Cell Rep.* 6: 297-301.
- CHILTON, M.D., T.C. CURRIER, S.H. FARRAND, A.J. BENDICH, M.P. GORDON and E.W. NESTER (1974) Agrobacterium tumefaciens DNA and P58 bacteriophage DNA not detected in crown gall tumors. *Proc. Natl. Acad. Sci. USA* 71: 3672-3676.

- CHILTON, M.D., M.H. DRUMMOND, D.J. MERLO, D. SCIAKY, A.L. MONTOYA, M.P. GORDON and E.W. NESTER (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* 11: 263-271.
- CHILTON, M.D., D.A. TEPFER, A. PETIT, C. DAVID, F. CASSE-DELBART and J. TEMPE (1982) Agrobacterium rhizogenes inserts T-DNA into the genome of the host plant root cells. *Nature* 295: 432-434.
- CHRISTIE, P.J., J.E. WARD, S.C. WINANS and E.W. NESTER (1988) The Agrobacterium tumefaciens vir E2 gene product is a single stranded DNA binding protein that associated with T-DNA. *J. Bacteriol.* 170: 2659-2667.
- CHRISTOU, P., J.E. MURPHY and W.F. SWAIN (1987) Stable transformation of soybean by electroporation and root formation from transformed callus. *Proc. Natl. Acad. Sci. USA* 84: 3962-3966.
- CHRISTOU, P., D.E. McCabe and W.F. SWAIN (1988) Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiol.* 87: 671-674.
- CHUONG, P.V. and W.D. BEVERSDORF (1985) High frequency embryogenesis through isolated microspore culture in Brassica napus L. and corinata Braun. *Plant Sci.* 39: 219-226.
- CHUONG, P.V., K.P. PAULS and W.D. BEVERSDORF (1985) A simple culture method for Brassica hypocotyl protoplasts. *Plant Cell Rep.* 4: 4-6.
- CLOSE, T.J., P.M. ROGOWSKY, C.I. KADO, S.A.C. WINANS, M.F. YANOFSKY and E.W. NESTER (1987) Dual control of Agrobacterium tumefaciens Ti plasmid virulence genes. *J. Bacteriol.* 169: 5113-5118.
- CLOSE, T.J., R.C. TAIT and C.I. KADO (1985) Regulation of Ti plasmid virulence genes by a chromosomal locus of Agrobacterium tumefaciens. *J. Bacteriol.* 164: 774-781.
- COEN, D.M., J. BEDBROOK, L. BOGORAD and A. RICH (1977) Maize chloroplast DNA fragment encoding the large subunit of ribulosebiphosphate carboxylase. *Proc. Natl. Acad. Sci. USA* 74: 5487-5491.
- COMAI, L., D. FACCIOITTI, W.R. HIATT, G. THOMPSON, R.E. ROSE and D.M. STALKER (1985) Expression in plants of a mutant aro A gene from Salmonella typhimurium confers tolerance to glyphosate. *Nature* 317: 741-744.
- CROSSWAY, A.J., V. OAKES, J.M. IRVINE, B. WARD, V.C. KNAUF and C.K. SHEWMAKER (1986) Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. *Mol. Gen. Genet.* 202: 179-185.

- CROUCH, M.L. (1982) Non-zygotic embryos of Brassica napus L. contain embryo-specific storage proteins. Planta 156: 520-524.
- CROUCH, M.L., K.M. TENBARGE, A.E. SIMON and R. FERL (1983) cDNA clones for Brassica napus seed storage proteins: evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor polypeptide. J. Mol. Appl. Genet. 2: 273-283.
- CUOZZO, M., K.M. O'CONNELL, W. KANIEWSKI, R.X. FANG, N.H. CHUA and N.E. TUNER (1988) Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. BioTechnology 6: 549-557.
- DAVID, C. and J. TEMPÉ (1988) Genetic transformation of cauliflower (Brassica oleracea L. var. Botrytis) by Agrobacterium rhizogenes. Plant Cell Rep. 7: 88-91.
- DAVIES, J.W., R. TOWNSEND and J. STANLEY (1987) The structure, expression functions and possible exploitation of gemini virus genomes. In: Plant DNA infectious agents, T. Hohn and J. Schell eds. pp. 31-52.
- DAVIS, B.D. (1987) Mechanism of bactericidal action of aminoglycosides. Microbiol. Rev. 51: 341-350.
- DAVIS, R.D. (1984) Cadmium in sludges used as fertilizer. Experientia 40: 117-126.
- DEAK, M., G.B. KISS, C. KONCZ and D. DUDITS (1986) Transformation of Medicago by agrobacterium-mediated gene transfer. Plant Cell Rep. 5: 97-100.
- DE BLOCK, M., J. BOTTERMAN, M. VANDEWIELE, J. DOCK, C. THOEN, V. GOSSELE, N.R. MO, C. THOMPSON, M. VAN MONTAGU and J. LEEMANS (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6: 2513-2518.
- DE BLOCK, M., L. HERRERA-ESTRELLA, M. VAN MONTAGU, J. SCHELL and P. ZAMBRYSKI (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO J. 3: 1681-1689.
- DE BLOCK, M., J. SCHELL and M. VAN MONTAGU (1985) Chloroplast transformation by Agrobacterium tumefaciens. EMBO J. 4: 1367-1272.
- DE CLEENE, M. (1985) The susceptibility of monocotyledons to Agrobacterium tumefaciens. Phytopath. Z. 113: 81-89.
- DE CLEENE, M. and J. DE LEY (1976) The host range of crown gall. Bot. Rev. 42: 389-466.

- DE CLEENE, M. and J. DE LEY (1981) The host range of infectious hairy-root. *Bot. Rev.* 47: 147-194.
- DE GREVE, J., P. DHAESE, J. SEURINCK, M. LENMERS, M. VAN MONTAGU and J. SCHELL (1983) Nucleotide sequence and transcript map of the Agrobacterium tumefaciens Ti plasmid encoded octopine synthase gene. *J. Mol. Appl. Genet.* 1: 499-511.
- DE LA PENA, A., H. LÖRZ and J. SCHELL (1987) Transgenic rye plants obtained by injecting DNA into young floral tillers. *Nature* 325: 274-276.
- DELLA-CIOPPA, G., S.C. BAUER, M.L. TAYLOR, D.E. ROCHESTER, B.K. KLEIN, D.M. SHAH, R.T. FRALEY and G.M. KISHORE (1987) Targeting a herbicide-resistant enzyme from Escherichia coli to chloroplasts of higher plants. *BioTechnology* 5: 579-584.
- DEPICKER, A., S. STACHEL, P. DHAESE, P. ZAMBRYSKI and H.M. GORDMAN (1983) Nopaline synthase: transcript mapping and DNA sequence. *J. Mol. Appl. Genet.* 1: 561-573.
- DEROLES, S.C. and R.C. GARDNER (1988a) Expression and inheritance of kanamycin resistance in a large number of transgenic petunias by Agrobacterium-mediated transformation. *Plant Mol. Biol.* 11: 355-364.
- DEROLES, S.C. and R.C. GARDNER (1988b) Analysis of the T-DNA structure in a large number of transgenic petunias generated by Agrobacterium-mediated transformation.
- DESHAYES, A., L. HERRERA-ESTRELLA and M. CABOCHE (1985) Lysome-mediated transformation of tobacco mesophyll protoplasts by an Escherichia coli plasmid. *EMBO J.* 4: 2731-2737.
- DE VOS, G., M. DE BEUCKELEER, M. VAN MONTAGU and J. SCHELL (1981) Restriction endonuclease mapping of the octopine tumor-inducing plasmid pTi Ach5 of Agrobacterium tumefaciens. *Plasmid* 6: 249-253.
- DIETERT, M.F., S.A. BARRON and O.C. YODER (1982) Effects of genotype on in vitro culture in the genus Brassica. *Plant Sci. Lett.* 26: 233-240.
- DI RITA, V.J. and S.B. GELVIN (1987) Deletion analysis of the mannopine synthase gene promoter in sunflower crown gall tumors and Agrobacterium tumefaciens. *Mol. Gen. Genet.* 207: 233-241.
- DOUGLAS, C.J., W. HALPERIN and E.W. NESTER (1982) Agrobacterium tumefaciens mutants affected in attachment to plant cells. *J. Bacteriol.* 152: 1265-1275.

- DOUGLAS, C.J., R.J. STANELONI, R.A. RUBIN and E.W. NESTER (1985) Identification and genetic analysis of an Agrobacterium tumefaciens chromosomal virulence region. J. Bacteriol. 161: 850-860.
- DOWNEY, R.K., A.J. KLASSEN and G.R. STRINGAM (1980) Rapeseed and mustard, Chapt. 35 in Hybridization of crop plants, American Soc. Agronomy - Crop Science of America, Madison, USA, pp. 495-509.
- DUNWELL, J.M. (1981) In vitro regeneration from excised leaf discs of three Brassica species. J. Exp. Bot. 32: 789-799.
- ECKES, P., G. DONN and F. WENGENMAYER (1987) Genetic engineering with plants. Am. Gen. Chem. Int. Ed. Eng. 26: 382-402.
- ECKES, P., S. ROSAHL, J. SCHELL and L. WILLMITZER (1986) Isolation and characterization of a light-inducible, organ-specific gene from potato and analysis of its expression after tagging and transfer into tobacco and potato shoots. Mol. Gen. Genet. 205: 14-22.
- EICHHOLTZ, D.A., S.G. ROGERS, R.B. HORSCH, H.J. KLEE, M. HAYFORD, N.L. HOFFMANN, S.B. BRAFORD, C. FINK, J. FLICK, K.M. O'CONNEL and R.T. FRALEY (1987) Expression of mouse dihydrofolate reductase gene confers methotrexate resistance in transgenic petunia plants. Som. Cell Mol. Gen. 13: 67-76.
- ENQUIST, I. and N. STERNBERG (1979) In vitro packaging of Dam vectors and their use in cloning DNA fragments. Methods Enzymol. 68: 281-298.
- ERICKSON, L., I. GRANT and W. BEVERSDORF (1986) Cytoplasmic male sterility in rapeseed (Brassica napus L.). 2. The role of a mitochondrial plasmid. Theor. Appl. Genet. 72: 161-157.
- ETTLINGER, J., J. SCHINDLER and L. LEHLE (1986) Cell-cycle arrest of plant suspension cultures by tumicamycin. Planta 168: 101-105.
- EVERETT, N.P., K.E.P. ROBINSON and D. MASCARENHAS (1987) Genetic engineering of sunflower (Helianthus annuus L.). Biotechnology 5: 1201-1204.
- FACCIOTTI, D., J.K. O'NEAL, S. LEE and C.K. SHEWMAKER (1985) Light-inducible expression of a chimeric gene in soybean tissue transformed with Agrobacterium. BioTechnology 3: 241-246.
- FAN, Z., K.C. ARMSTRONG and W.A. KELLER (1988) Development of microspores in vivo and in vitro in Brassica napus L. Protoplasma. In press).
- FELDMAN, K.A. and M.D. MARKS (1987) Agrobacterium-mediated transformation of germinating seeds of Arabidopsis thaliana: A non-tissue culture approach. Mol. Gen. Genet. 208: 1-9.

- FERGUSON, D.T., S.E. SCHELL, L.H. HAGEMANN, G.E. LEPONE and G.A. CARRARO (1985) DPL-L5300 A new cereal herbicide. Proc. of the 1985 British Crop Protection Conference Weeds. Vol. 1, Brighton Metropole, England, pp. 43-48.
- FIGURSKI, D.H. and D.R. HELINSKI (1979) Replication of an origin-containing derivative of plasmid RK2 dependant on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76: 1648-1652.
- FILLATTI, J.J., J. KISER, R. ROSE and L. COMAI (1987a) Efficient transfer of a glyphosate tolerance gene into tomato using a binary Agrobacterium tumefaciens vector. BioTechnology 5: 726-730.
- FILLATTI, J.A., J. SELLMER, B. McCOUN, B. HAISSIG and L. COMAI (1987b) Agrobacterium-mediated transformation and regeneration of Populus. Mol. gen. Genet. 206: 192-199.
- FIROOZABADY, E., D.L. DEBOER, D.J. MERLO, E.L. HALK, L.N. AMERSON, K.E. RASHKA and E.E. MURRAY (1987). Transformation of cotton (Gossypium hirsutum L.) by Agrobacterium tumefaciens and regeneration of transgenic plants. Plant Mol. Biol. 10: 105-116.
- FRALEY, R.T. (1983) Liposome-mediated delivery of tobacco mosaic virus RNA into petunia protoplast. Plant Mol. Biol. 2: 5-14.
- FRALEY, R.T., S.G. ROGERS and R.B. HORSCH (1986) Genetic transformation in higher plants. CRC Crit. Rev. Plant Sci. 4: 1-45.
- FRALEY, R.T., S.G. ROGERS, R.B. HORSCH, D.A. EICHHOLTZ, J.S. FLICK, C.L. FINK, N.L. HOFFMAN and P.R. SANDERS (1985) The SEV system: a new disarmed Ti plasmid vector system for plant transformation. BioTechnology 3: 629-635.
- FREEMAN, J.P., J. DRAPER, M.R. DAVEY, E.C. COCKING, K.M.A. GARTLAND, K. HARDING and D. PENTAL (1984) A comparison of methods for plasmid delivery into plant protoplasts. Plant Cell Physiol. 25: 1353-1365.
- FROMM, M.E., L.P. TAYLOR and V. WALBOT (1986) Stable transformation of maize after gene transfer by electroporation. Nature 319: 791-793.
- FRY, J., A. BARNASON and R.B. HORSCH (1987) Transformation of Brassica napus with Agrobacterium tumefaciens based vectors. Plant Cell Rep. 6: 321-325.
- FULTON, R.W. (1986) Practices and precautions in the use of cross protection for plant virus disease control. Ann. Rev. Phytopathol. 26: 67-81.

- GALSKY, A.G., J.A. SCHEPPLER and M.S. CRANFORD (1981) Crown-gall tumors possess tumor-specific antigenic sites on their cell walls. *Plant Physiol.* 67: 1195-1197.
- GAMBROG, O.L., R.A. MILLER and K. OJIMA (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.
- GARCIA, J.A., J. HILLE and R. GOLDBACH (1986) Transformation of cowpea Vigna unguiculata cells with an antibiotic resistance gene using a Ti-plasmid-derived vector. *Plant Sci.* 44: 37-41.
- GARDNER, R.C. and V.C. KNAUF (1986) Transfer of Agrobacterium DNA to plants requires a T-DNA border but not the VIR E locus. *Science* 231: 725-727.
- GATEHOUSE, A.M.R. and D. BOULTER (1983) Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (Vigna unguiculata) and other legumes on development of the bruchid beetle Callosobruchus maculatus. *J. Sci. Food Agric.* 34: 345-350.
- GATEHOUSE, A.M.R., J.A. GATEHOUSE and D. BOULTER (1980) Isolation and characterization of trypsin inhibitors from cowpea (Vigna unguiculata). *Phytochemistry* 19: 751-756.
- GELVIN, S.B. (1986) Transcription "enhancer-like" sequences in plant genes. *Plant. Mol. Biol.* 7: 229-232.
- GLIMELIUS, K. (1984) High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. *Physiol. Plant.* 61: 38-44.
- GONZALES, A., A. JIMENEZ, D. VAZQUEZ, J.E. DAVIES and D. SCHINDLER (1978) Studies on the mode of action of hygromycin B, an inhibitor of translocation in Eukaryotes. *Bioch. Biophys. Acta* 521: 459-469.
- GORMAN, C.M., L.F. MOFFAT and B.H. HOWARD (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2: 1044-1051.
- GRILL, E., E.L. WINNACKER and M.H. ZENK (1985) Phytochelations: The principal heavy-metal complexing peptides of higher plants. *Science* 230: 674-676.
- GRIMSLEY, N. and D. BISARO (1987) Agroinfection in: Plant DNA infectious agents, T.H. Hohn and J. Schell eds. Springer-Verlag, New York, pp. 88-109.
- GRIST, R. and L. BUTLER (1983) Effect of transforming DNA on growth and frequency of mutation of Streptococcus pneumonias. *J. Bacteriol.* 153: 153-162.

- GRONENBORN, B. (1987) The molecular biology of cauliflower mosaic virus and its application as plant gene vector. In: Plant DNA infectious agents. T. Hohn and J. Schell, eds., pp. 1-29.
- GUERCHE, P., C. BELLINI, J.M. LE MOULLEC and M. CABOCHE (1987a) Use of a transient expression assay for the optimization of direct gene transfer into tobacco mesophyll protoplasts by electroporation. *Biochimie* 69: 621-628.
- GUERCHE, P., M. CHARBONNIER, L. JOUANIN, C. TOURNEUR, J. PASZKOWSKI and G. PELLETIER (1987b) Direct gene transfer by electroporation in Brassica napus. *Plant Sci.* 52: 111-116.
- GUERCHE, P., L. JOUANIN, D. TEPFER and G. PELLETIER (1987c) Genetic transformation of oilseed rape (Brassica napus) by the Ri T-DNA of Agrobacterium rhizogenes and analysis of inheritance of the transformed phenotype. *Mol. Gen. Genet.* 206: 382-386.
- HAGIYA, M., T.J. CLOSE, R.C. TAIT and C.I. KADO (1985) Identification of pTi C58 plasmid-encoded proteins for virulence in Agrobacterium tumefaciens. *Proc. Natl. Acad. Sci. USA* 82: 2669-2673.
- HAIN, R., P. STABEL, A.P. CZERNILOFSKY, H.H. STEINBISS, L. HERRERA-ESTRELLA and J. SCHELL (1985) Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. *Mol. Gen. Genet.* 199: 161-168.
- HAIN, R., H.H. STEINBISS and J. SCHELL (1984) Fusion of Agrobacterium and E. coli spheroplasts with Nicotiana tabacum protoplasts - direct gene transfer from microorganism to higher plants. *Plant Cell Rep.* 3: 60-64.
- HAMEL, A. (1987) Involvement of the chromosomal recA gene of Agrobacterium tumefaciens in the processing of the DNA of its plant tumor-inducing plasmid. M.Sc. Thesis, Carleton University, 100 pp.
- HANISCH TEN CATE, C.H., E. ENNIK, S. ROEST, K. SREE RAMULU, P. DIJKHUIS and B. DE GROOT (1988) Regeneration and characterization of plants from potato root lines transformed by Agrobacterium rhizogenes. *Theor. Appl. Genet.* 75: 452-459.
- HANOLD, D. (1983) In vitro transformation of protoplast-derived Hyoscyamus muticus cells by Agrobacterium tumefaciens. *Plant Sci. Lett.* 30: 177-183.
- HARPSTER, M.H., J.A. TOWSEND, J.O.G. JONES, J. BEDBROOK and P. DUNSHUIK (1988) Relative strengths of the 35 cauliflower mosaic virus, 1¹, 2¹, and nopaline synthase promoters in transformed tobacco sugarbeet and oilseed rape callus tissue. *Mol. Gen. Genet.* 212: 182-190.

- HARRISON, B.D., M.A. MAYO and D.C. BAULCOMBE (1987) Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. *Nature* 328: 799-805.
- HASEZAWA, S., C. MATSUI, T. NAGATA and K. SYONO (1983) Cytological study of the introduction of Agrobacterium tumefaciens spheroplasts into Vinca rosea protoplasts. *Can. J. Bot.* 61: 1052-1057.
- HASEZAWA, S., T. NAGATA and K. SYONO (1981) Transformation of Vinca protoplasts mediated by Agrobacterium spheroplasts. *Mol. Gen. Genet.* 182: 206-210.
- HAUGHN, G.W., J. SMITH, B. MAZUR and C. SOMERVILLE (1988). Transformation with a mutant Arabidopsis acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. *Mol. Gen. Genet.* 211: 266-271.
- HAUGHN, G.W. and C. SOMERVILLE (1986) Sulfonylurea-resistant mutants of Arabidopsis thaliana. *Mol. Gen. Genet.* 204: 430-434.
- HAUPTMANN, R.M., P. OZIAS-AKINS, V. VASIL, Z. TABAEIZADEH, S.G. ROGERS, R.B. HORSCH, I.K. VASIL and R.T. FRALEY (1987) Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species. *Plant Cell Rep.* 6: 265-270.
- HAWES, M.C., L.Y. SMITH and A.J. HOWARTH (1988) Agrobacterium tumefaciens mutants deficient in chemotaxis to root exudate. *Mol. Plant-Microbe Int.* 1: 182-186.
- HELMER, G., M. CASADABAN, M. BEVAN, L. KAYES and M.D. CHILTON (1984) A new chimeric gene as a marker for plant transformation: the expression of Escherichia coli -galactosidase in sunflower and tobacco cells. *Biotechnology* 2: 520-527.
- HEMENWAY, C., R.X. FANG, W.K. KANIEWSKI, N.H. CHUA and N.E. TURNER (1988) Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO J.* 7: 1273-1280.
- HERRERA-ESTRELLA, L., M. DE BLOCK, E. MESSENS, J.P. HERALSTEENS, M. VAN MONTAGU and J. SCHELL (1983a) Chimeric genes as dominant selectable markers in plant cells. *EMBO J.* 2: 987-995.
- HERRERA-ESTRELLA, L., A. DEPICKER, M. VAN MONTAGU and J. SCHELL (1983b) Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* 303: 209-213.
- HERRERA-ESTRELLA, L.G., VAN DEN BROECK, R. MAENHAUT, M. VAN MONTAGU J. SCHELL, M. TIMKO and A. CASHMORE (1984) Light inducible and chloroplast associated expression of a chimeric gene introduced into Nicotiana tabacum using a Ti plasmid vector. *Nature* 310: 115-120.

- HILDER, V.A., A.M.R. GATEHOUSE, S.E. SHEERMAN, R.F. BARKER and D. BOULTER (1987) A novel mechanism of insect resistance engineered into tobacco. *Nature* 330: 160-163.
- HILLE, J., J. VAN KAN and R. SCHILPEROORT (1984) Transacting virulence functions of the octopine Ti plasmid from Agrobacterium tumefaciens. *J. Bacteriol.* 158: 754-756.
- HILLE, J., F. VERHEGGEN, P. ROELVINK, H. FRANSSEN, A. VAN KAMMEN and P. ZABEL (1986) Bleomycin resistance: a new dominant selectable marker for plant cell transformation. *Plant Mol. Biol.* 7: 171-176.
- HINCHEE, M.A., D.V. CONNOR-WARD, C.A. NEWELL, R.E. McDONNELL, S.J. SATO, C.S. GASSER, D.A. FISCHHOFF, O.B. RE, R.T. FRALEY and R.B. HORSCH (1988) Production of transgenic soybean plants using Agrobacterium-mediated DNA transfer. *BioTechnology* 6: 915-922.
- HIROOKA, T. and C.I. KADO (1986) Location of the right boundary of the virulence region on Agrobacterium tumefaciens plasmid pTi C58 and a host specifying gene next to the boundary. *J. Bacteriol.* 168: 237-243.
- HIROOKA, J., P.M. ROGOWSKY and C.I. KADO (1987) Characterization of the VIR E locus of Agrobacterium tumefaciens plasmid pTi C58. *J. Bacteriol.* 169: 1529-1536.
- HOEKEMA, A., B.S. DE PATER, A.J. FELLINGER, P.J.J. HOOYKAAS and R.A. SCHILPEROORT (1984) The limited host range of an Agrobacterium tumefaciens strain extended by a cytokinin gene from a wide host range T-region. *EMBO J.* 3: 3043-3047.
- HOEKEMA, A., P.R. HIRSCH, P.J.J. HOOYKAAS and R.A. SCHILPEROORT (1983) A binary plant vector strategy based on separation of VIR- and T-region of the Agrobacterium tumefaciens Ti-plasmid. *Nature* 303: 179-180.
- HOERNER, G.R. (1945) Crown gall of hops. *Plant Dis. Rep.* 29: 98-111.
- HOLBROOK, L.A., M. HAFFNER and B.L. MIKI (1986) A sensitive fluorographic method for the detection of nopaline and octopine synthase activities in Brassica crown-gall tissues. *Biochem. Cell Biol.* 64: 126-132.
- HOLBROOK, L.A. and B.L. MIKI (1985) Brassica crown gall tumorigenesis and in vitro culture of transformed tissue. *Plant Cell Rep.* 4: 329-332.
- HOLBROOK, L.A., T.J. REICH, V.N. IYER, M. HAFFNER and B.L. MIKI (1985) Induction of efficient cell division in alfalfa protoplasts. *Plant Cell Rep.* 4: 229-232.

- HOLSTERS, M., B. SILVA, F. VAN VLIET, C. GENETELLO, M. DE BLOCK, P. DHAESE, A. DEPICKER, D. INZE, G. EMGLER, R. VILLAROEEL, M. VAN MONTAGU and J. SCHELL (1980) The functional organization of the nopaline, Agrobacterium tumefaciens pTi C58. *Plasmid* 3: 212-230.
- HOOD, E.E., R.T. FRALEY and M.D. CHILTON (1987) Virulence of Agrobacterium tumefaciens strain A281 on legumes. *J. Bacteriol.* 83: 529-534.
- HOOD, E.E., G.L. HELMER, R.T. FRALEY and M.D. CHILTON (1986) The hypervirulence of Agrobacterium tumefaciens A281 is encoded in a region of pTi B0542 outside of T-DNA. *J. Bacteriol.* 168: 1291-1301.
- HOOYKAAS, P.J.J., G. OOMS and R.A. SCHILPEROORT (1982) Tumors induced by different strains of Agrobacterium tumefaciens. In: *Molecular biology of plant tumors*, G. Kahl and J.S. Schell eds. Academic Press, New York, pp. 373-390.
- HORSCH, R.B., J.E. FRY, N.L. HOFFMANN, D.S. EICHHOLTZ, S.G. ROGERS and R.T. FRALEY (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229-1231.
- HORSCH, R.B. and G.E. JONES (1980) A double filter paper technique for plating cultured plant cells. *In Vitro* 16: 103-108.
- HUSSAIN, M.M., G. MELCHER and R.C. ESSENBERG (1985) Infection of evacuated turnip protoplasts with liposome-package cauliflower mosaic virus. *Plant Cell Rep.* 4: 58-62.
- HUTCHINSON, J.M., R. SHAPIRO and P.B. SWEETSER (1984) Metabolism of chlorsulfuron by tolerant broadleaves. *Pestic. Biochem. Physiol.* 22: 243-247.
- IMAI, S., H. SETO, T. SASAKI, T. TSUMOKA, H. OGAWA, A. SATOH, S. INOUE, T. NIIDA and N. OTAKE (1985) Studies on the biosynthesis of bialophos (SF-1293) 6. Production of N-acetyl-deme-thylphosphotricin and N-acetylbialaphos by blocked mutants of Streptomyces hygroscopicus SF 1293 and their roles in the biosynthesis of bialophos. *J. Antibiot.* 38: 687-690.
- IYER, V.N., H.J. KLEE and E.W. NESTER (1982) Units of genetic expression in the virulence region of a plant tumor-inducing plasmid of Agrobacterium tumefaciens. *Mol. Gen. Genet.* 188: 418-424.
- JANSSENS, A., C. GENETELLO, M. VAN MONTAGU and P. ZAMBRYSKI (1986) Plant cells induce transcription of the Agrobacterium tumefaciens nopaline pTi C58 virulence region. *Plant Sci.* 47: 185-193.

- JAYASWAL, R.K., K. VELUTHAMBI, S.B. GELVIN and J.L. SLIGHTON (1987) Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in Escherichia coli by a VIR D-encoded border specific endonuclease from Agrobacterium tumefaciens. J. Bacteriol. 169: 5035-5045.
- JEFFERSON, R.A., T.A. KAVANAGH and M.W. BEVAN (1987) Gus fusions: -glucoronidase as a sensitive and versatile gene marker in higher plants. EMBO J. 6: 3901-3907.
- JIN, S., T. KOMARI, M.P. GORDON and E.W. NESTER (1987) Genes responsible for the supervirulence phenotype of Agrobacterium tumefaciens A281. J. Bacteriol. 169: 4417-4425.
- JOHNSTON, S.A., P.Q. AMZIANO, K. SHARK, J.C. SANFORD and R.A. BUTOW (1988) Mitochondrial transformation in yeast by bombardment with microprojectiles. Science 2540: 1538-1541.
- JONES, J.D.G., C. DEAN, D. GIDONI, D. GILBERT, D. BOND-NUTTER, R. LEE, J. BEDBROOK and P. DUNSMUIR (1988) Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. Mol. Gen. Genet. 212: 536-542.
- JONES, J.D.G., P. DUNSMUIR and J. BEDBROOK (1985) High level expression of introduced chimaeric genes in regenerated transformed plants. EMBO J. 4: 2411-2418.
- JONES, J.D.G., K.L. GRADY, T.V. SUSLOW and J.R. BEDBROOK (1986) Isolation and characterization of genes encoding two chitinase enzymes from Serratia marcescens. EMBO J. 5: 467-473.
- JONES, J.D.G., Z. SVAK, E.C. HARPER, C.D. HURWITZ and P. MALIGA (1987) A dominant nuclear streptomycin resistance marker for plant cell transformation. Mol. Gen. genet. 210: 86-91.
- JORDAN, M.C. and A. MCHUGHEN (1987) Selection for chlorsulfuron resistance in flax (Linum usitatissimum) cell cultures. J. Plant. Physiol. 131: 333-338.
- JORDAN, M.C. and A. MCHUGHEN (1988) Glyphosate tolerant flax plants from Agrobacterium-mediated gene transfer. Plant Cell Rep. 7: 181-184.
- JOUANIN, L. (1984) Restriction map of an agrofine-type Ri plasmid and its homologies with Ti plasmids. Plasmid 12: 91-102.
- JUNKER, B., J. ZIMMY, R. LUHRS and H. LÖRZ (1987) Transient expression of chimaeric genes in dividing and non-dividing cereal protoplasts after PEG-induced DNA uptake. Plant Cell Rep. 6: 329-332.

- KADO, C.I., M.G. HESKETT and R.A. LANGLEY (1972) Studies on Agrobacterium tumefaciens: characterization of strains 1D135 and B6, and analysis of the bacterial chromosome, transfer RNA and ribosomes for tumor-inducing ability. *Physiol. Plant. Path.* 2: 47-57.
- KARTHA, K.K., O.L. GAMBORG and F. CONSTABEL (1974) In vitro plant formation explants of rape (Brassica napus cv. Zephyr). *Physiol. Plant.* 31: 217-220.
- KARTHA, K.K., M.R. MICHAYLUK, K.N. KAO, O.L. GAMBORG and F. CONSTABEL (1974) Callus formation and plant regeneration from mesophyll protoplasts of rape plants (Brassica napus L. cv. Zephyr). *Plant Sci. Lett.* 3: 265-271.
- KELLER, W.A. and K.C. ARMSTRONG (1977) Embryogenesis and plant regeneration in Brassica napus anther cultures. *Can. J. Bot.* 55: 1383-1388.
- KELLER, W.A. and K.C. ARMSTRONG (1978) High frequency production of microspore-derived plants from Brassica napus anther cultures. *Z. Pflanzenzüchtg.* 80: 100-108.
- KELLER, W.A., Z. FAN, P. PECHAN, N. LONG and J. GRAINGER (1987) An efficient method for culture of isolated microspores of brassica napus. *Proc. 7th Intl. Rapeseed Congr., Poznan, Poland.*
- KLEE, H.J., Y.M. MUSKOPF and C.S. GASSER (1987) Cloning of an Arabidopsis thaliana gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Mol. Gen. Genet.* 210: 437-442.
- KLEE, H.J., F.F. WHITE, V.N. IYER, M.P. GORDON and E.W. NESTER (1983) Mutational analysis of the virulence region of an Agrobacterium tumefaciens Ti plasmid. *J. Bacteriol.* 153: 879-883.
- KLEIN, T.M., M. FROMM, A. WEISSINGER, D. TOMES, S. SCHAAF, M. SLETTEN and J.C. SANFORD (1988) Transfer of foreign genes into intact maize cells with high-velocity micro-projectiles. *Proc. Natl. Acad. Sci. USA* 85: 4305-4309.
- KLEIN, T.M., T. GRADZIEL, M.E. FROMM and J.C. SANFORD (1988) Factors influencing gene deliver into Zea mays cells by high velocity microprojectiles. *BioTechnology* 6: 559-563.
- KLEIN, T.M., E.D. WOLF, R. WU and J.C. SANFORD (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327: 70-73.
- KLIMASZEWSKA, K. and W.A. KELLER (1985) High frequency plant regeneration from thin cell layer explants of Brassica napus. *Plant Cell Tissue Organ Cult.* 4: 183-197.

- KLIMASZEWSKA, K. and W.A. KELLER (1986) Plant regeneration from stem cortex protoplasts of Brassica napus. Plant Cell Tissue Organ Cult. 8: 225-233.
- KNAUF, V.C. (1987) The application of genetic engineering to oilseed crops. Trends Biotech. 5: 40-47.
- KNAUF, V., M. YANOFSKY, A. MONTOYA and E. NESTER (1984) Physical and functional map of an Agrobacterium tumefaciens tumor-inducing plasmid that confers a narrow host range. J. Bacteriol. 160: 564-568.
- KOHLNBACH, H.W., M. KORBER and L.C. LI (1982) Cytodifferentiation of protoplasts isolated from a stem embryo system of Brassica napus tracheary elements. Z. Pflanzenphysiol. 107: 367-371.
- KOHLNBACH, H.W., G. WENZEL and F. HOFFMANN (1982) Regeneration of Brassica napus plantlets in cultures from isolated protoplasts of haploid stem embryos as compared with leaf protoplasts. Z. Pflanzenphysiol. 105: 131-142.
- KOHLER, F., C. GOLZ, S. EAPEN, H. KOHN and O. SCHIEDER (1987a) Stable transformation of moth bean Vigna aconitifolia via direct gene transfer. Plant Cell Rep. 6: 313-317.
- KOHLER, F., C. GOLZ, S. EAPEN and O. SCHIEDER (1987b) Influence of plant cultivar and plasmid-DNA on transformation rates in tobacco and moth bean. Plant Sci. 53: 87-91.
- KONCZ, C., O. OLSSON, W.H.R. LANGRIDGE, J. SCHELL and A.A. SZALAY (1987) Expression and assembly of functional bacterial luciferase in plants. Proc. Natl. Acad. Sci. USA 84: 131-135.
- KONCZ, C. and J. SCHELL (1986) The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Mol. Gen. Genet. 204: 383-396.
- KOUKOLIKOVA-NICOLA, Z., R.O. SHILLITO, B. HOHN, K. WANG, M. VAN MONTAGU and P. ZAMBRYSKI (1985) Involvement of circular intermediates in the transfer of T-DNA from Agrobacterium tumefaciens to plant cells. Nature 313: 191-196.
- KOZIEL, M.G., T.L. ADAMS, M.A. HAZLET, D. DAMM, J. MILLER, D. DAHLBECK, S. JAYNE and B.J. STASKAWICZ (1984) a cauliflower mosaic virus promoter directs expression of kanamycin resistance in morphogenic transformed plant cells. J. Mol. Appl. Genet. 2: 549-562.
- KRUGER-LEBUS, S. and I. POTRYKUS (1987) A simple and efficient method for direct gene transfer to Petunia hybrida without electroporation. Plant Mol. Biol. Rep. 5: 289-294.

- LANGRIDGE, W.H.R., B.J. LI and A.A. SZALAY (1985) Electric field mediated stable transformation of carrot protoplasts with naked DNA. *Plant Cell Rep.* 4: 355-359.
- LAROSSA, R.A. and J.V. SCHLOSS (1984) The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in Salmonella typhimurium. *J. Biol. Chem.* 259: 8753-8757
- LAWTON, M.A., M.A. TIERNEY, I. NAKAMURA, E. ANDERSON, Y. KOMEDA, P.L. DUBÉ, N. HOFFMAN, R.T. FRALEY and R.N. BEACHY (1987) Expression of a soybean -conglycinin gene under the control of the cauliflower mosaic virus 35S and 19S promoters in transformed petunia tissues. *Plant Mol. Biol.* 9: 315-324.
- LAZZERI, P.A. and J.M. DUNWELL (1984) In vitro shoot regeneration from seedling root segments of Brassica oleracea and Brassica napus cultivars. *Ann. Bot.* 54: 341-350.
- LEE, K.Y., J. TOWNSEND, J. TEPPERMAN, M. BLACK, C.F. CHUI, B. MAZUR, P. DUNSMUIR and J. BEDBROOK (1988) The molecular basis of sulfonylurea herbicide resistance in tobacco. *EMBO J.* 7: 1241-1248.
- LEFEBVRE, D.D., B.L. MIKI and J.F. LALIBERTÉ (1987) Mammalian metallothionein functions in plants. *BioTechnology* 5: 1053-1056.
- LEROUX, B., M.F. YANOFSKY, S.C. WINANS, J.E. WARD, S.F. ZIEGLER and E.W. NESTER (1987) Characterization of the VIR A locus of Agrobacterium tumefaciens: a transcriptional regulator and host range determinant. *EMBO J.* 6: 849-856.
- LI, L.C. and H.W. KOHLENBACH (1982) Somatic embryogenesis in quite a direct way in cultures of mesophyll protoplasts of Brassica napus L. *Plant Cell Rep.* 1: 209-211.
- LICHENSTEIN, C. (1986) Bacterial-plant conjugation: a bizarre vegetal bestiality. *Nature* 322: 682-683.
- LICHENSTEIN, C. (1987) Bacteria conjugate with plants. *Nature* 328: 176.
- LICHTER, R. (1982) Induction of haploid plants from isolated pollen of Brassica napus. *Z. Pflanzenphysiol.* 105: 427-434.
- LIPPINCOTT, B.B. and J.A. LIPPINCOTT (1969) Bacterial attachment to a specific molund site as an essential stage in tumor initiation by Agrobacterium tumefaciens. *J. Bacteriol.* 97: 620-628.
- LIPPINCOTT, J.A., B.B. LIPINCOTT and M.P. STARR (1981) The Genus Agrobacterium. In: *The prokaryotes: a handbook on habitats, isolation and identification of bacteria*. M.P. Starr, H. Stolp, H.G. Truper, A. Balons and H.G. Schlegel, eds. Springer-Verlag, New York, pp. 842-855.

- LLOYD, A.M., A.R. BARNASON, S.G. ROGERS, M.C. BYRNE, R.T. FRALEY and R.B. HORSCH (1986) Transformation of Arabidopsis thaliana with Agrobacterium tumefaciens. Science 234: 464-466.
- LOAKE, G.L., A.M. ASHBY and C.H. SHAW (1988) Attraction of Agrobacterium tumefaciens C58C1 towards sugars involves a highly sensitive chemotaxis system. J. Gen. Microbiol. 134: 1432.
- LOESCH-FRIES, L.S., D. MERLO, T. ZINNEN, L. BURHOP, K. HILL, K. KRAHN, N. JARVIS, S. NELSON and E. HALK (1987) Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance. EMBO J. 6: 1845-1851.
- LOPATIN, M.I. (1936) The susceptibility of plants to Bacterium tumefaciens, the causative agent of the root cancer of plants. Mikrobiologia (Moscow) 5: 716-724.
- LÖRZ, H., B. BAKER and J. SCHELL (1985) Gene transfer to cereal cells mediated by protoplast transformation. Mol. Gen. Genet. 199: 178-182.
- LOSCHIARO, F., L.A. QUESADA-ALUE and Z.R. SUNG (1986) Tunicamycin affects somatic embryogenesis but not cell proliferation of carrot. Plant Sci. 44: 65-71.
- LU, D.Y., D. PENTAL and E.C. COCKING (1982) Plant regeneration from seedling cotyledon protoplasts. Z. Pflanzenphysiol. 107: 59-63.
- LUNDQUIST, R.C., T.J. CLOSE and C.I. KADO (1984) Genetic complementation Agrobacterium tumefaciens Ti plasmid mutants in the virulence region. Mol. Gen. Genet. 193: 1-7.
- MACHIDA, Y., S. USAMI, A. YAMAMOTO, Y. NIWA and I. TAKEBE (1986) Plant-inducible recombination between the 25 bp border sequences of T-DNA in Agrobacterium tumefaciens. Mol. Gen. Genet. 204: 374-382.
- MAITI, I.B., A.G. HUNT and G.J. WAGNER (1988) Seed transmissible expression of mammalian metallothionein in transgenic tobacco. Biochem. Biophys. Res. Comm. 150: 640-647.
- MANDAVA, N.B. (1988) Plant growth-promoting brassinosteroids. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39: 23-52.
- MANIATIS, T., E.F. FRITSCH and J. SAMBROOK (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory 545 pp.
- MANNERS, J.M. (1987) Transformation of Stylosanthes spp. using Agrobacterium tumefaciens. Plant Cell rep. 6: 204-207.
- MANNERS, J.M. (1988) Transgenic plants of the tropical pasture legume Stylosanthes humilis. Plant Sci. 55: 61-68.

- MANNERS, J.M. (1988) Transgenic plants of the tropical pasture legume Stylosanthes humilis. Plant Sci. 55: 61-68.
- MARGARA, J. and M.T. LEYDECKER (1978) Différents types d'organogénèse observés chez le Colza, Brassica napus L. var. oleifera Metzg. Application à la multiplication végétative in vitro. C.R. Acad. Sci. Paris, Sér. D 287: 17-20.
- MARTENS, J.W., W.L. SEAMAN and T.G. ATKINSON (1984) Diseases of canola, rapeseed and mustard. In: Diseases of field crops in Canada, pp. 116-121.
- MATTHEWS, H., P.S. RAO and C.R. BHATIA (1986) Transformation of Brassica juncea by Agrobacterium tumefaciens harboring plasmid pTiT37 and its rooty mutant pTiT37.14a/a. J. Genet. 65: 37-44.
- MATTHEWS, V.H., C.R. BHATIA, R. MITRA, T.G. KRISHNA and P.S. RAO (1985) Regeneration of shoots from Brassica juncea (Linn) Czern and Coss cells transformed by Agrobacterium tumefaciens and expression of nopaline dehydrogenase genes. Plant Sci. 39: 49-54.
- MATSU, C., S. HASEGAWA, N. TANAKA and K. SYONO (1983) Introduction of E. coli cells and spheroplasts in Vinca protoplasts. Plant Cell Rep. 2: 30-32.
- MATTHYSSE, A.G. (1983) Role of bacterial cellulose fibrils in Agrobacterium tumefaciens infection. J. Bacteriol. 154: 906-915.
- MATTHYSSE, A.G. (1987) Characterization of nonattaching mutants of Agrobacterium tumefaciens. J. Bacteriol. 169: 313-323.
- MATTHYSSE, A.G. and R.H.G. GURLITZ (1982) Plant cell range for attachment of Agrobacterium tumefaciens to tissue culture cells. Physiol. Plant. Path. 21: 381-387.
- MATTHYSSE, A.G., K.V. HOLMES and R.H. GURLITZ (1981) Elaboration of cellulose fibrils by Agrobacterium tumefaciens during attachment to carrot cells. J. Bacteriol. 145: 583-595.
- MAZUR, B.J., C.F. CHUI and J.K. SMITH (1987a) Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol. 85: 1110-1117.
- MAZUR, B.J., S.C. FALCO, S. KNOWLTON and J.K. SMITH (1987b) Acetolactate synthase, the target enzyme of the sulfonylurea herbicides. In: Plant Molecular Biology, D. Von Wettstein and N.H. Chua eds., Plenum Press, New York.
- MURATA, M. and T.J. ORTON (1987) Callus initiation and regeneration capacities in Brassica species. Plant Cell Tissue Organ Cult. 11: 111-123.

- MURRAY, V. and R.F. MARTIN (1985) The sequence specificity of bleomycin-induced DNA damage in intact cells. *J. Biol. Chem.* 160: 10389-10291
- NAGATA, T., K. OKADA, T. KAWAZU and I. TAKEBE (1987) Cauliflower mosaic virus 35S promoter directs S phase specific expression in plant cells. *Mol. Gen. Genet.* 207:242-244.
- NARASIMHULU, S.B. and V.L. CHOPRA (1988) Species specific shoot regeneration response of cotyledonary explants of Brassicas. *Plant Cell Rep.* 7:104-106.
- NASRALLAH, J.B., T.H. KAO, M.L. GOLDBERG and M.E. NASRALLAH (1985) A cDNA clone encoding an S-locus specific glycoprotein from Brassica oleracea. *Nature* 318: 263-267.
- NEGRUTIU, I., R. SHILLITO, I. POTRYKUS, F.G. BIASINI and F. SALA (1987) Hybrid genes in the analysis of transformation conditions. 1. Setting up a simple method for direct gene transfer in plant protoplasts. *Plant Mol. Biol.* 8; 363-373.
- NELSON, R.S., S.M. MCCORMICK, X. DELANNAY, P. DUBÉ, J. LAYTON, E.J. ANDERSON, M. KANIEWSKA, R.K. PROKSCH, R.B. HORSCH, S.G. ROGERS, R.T. FRALEY and R.N. BEACHY (1988) Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. *BioTechnology* 6: 403-409.
- NESTER, E.W., M.P. GORDON, R.M. AMASINO and M.F. YANOFSKY (1984) Crown gall: a molecular and physiological analysis. *Ann. Rev. Plant Physiol.* 35: 387-413.
- NESTER, E.W. and T. KOSUGE (1981) Plasmids specifying plant hyperplasias. *Ann. Rev. Microbiol.* 35: 531-565.
- NEUHAUS, G., G. SPANGENBERG, O. MITTELSTENSCHIED and H.G. SCHWEIGER (1987) Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids. *Theor. Appl. Genet.* 75: 30-36.
- NODA, T., N. TANAKA, Y. MANO, S. NABESHIMA, H. OHKAWA and C. MATSUI (1987) regeneration of horseradish hairy roots incited by Agrobacterium rhizogenes infection. *Plant Cell Rep.* 6: 283-286.
- ODELL, J.T., F. NAGY and N.H. CHUA (1985) Identifying of DNA sequences required for activity of the cauliflower mosaic virus 35 promoter. *Nature* 313: 810-812.
- OHGARAWA, T., H. UCHIMIYA and H. HARADA (1983) Uptake of liposome-encapsulating plasmid DNA by plant protoplasts and molecular fate of foreign DNA. *Protoplasma* 116: 145-148.

- OKKER, R.J.H., H. SPAINK, J. HILLE, T.A.N. VAN BRUSSEL, B. LUGTENBERG and R.A. SCHILPEROORT (1984) Plant-inducible virulence promoter of the Agrobacterium tumefaciens Ti plasmid. *Nature* 312: 564-566.
- ONDREJ, M. (1983) Detection of nopaline dehydrogenase and lysopinedehydrogenase activities in crown gall tumors of different plant species. *Biol. Plant.* 25: 378-384.
- OOMS, G., P.J.J. HOOYKAAS, R.J.M. VAN VEEN, P. VAN BEELEN, T.J.G. REGENSBURG-TRINK and R.A. SCHILPEROORT (1982) Octopine Ti plasmid deletion mutants of Agrobacterium tumefaciens with emphasis on the right side of the T-region. *Plasmid* 7: 15-29.
- OOMS, G., A. BAINS, M. BURRELL, A. KARP, D. TWELL and E. WILCOX (1985) Genetic manipulation in cultivars of oilseed rape (Brassica napus) using Agrobacterium. *Theor. Appl. Genet.* 71: 325-329.
- OOMS, G., M.M. BURRELL, A. KARP, M. BEVAN and J. HILLE (1987) Genetic transformation in two potato cultivars with T-DNA from disarmed Agrobacterium. *Theor. Appl. Genet.* 73: 744: 744-750.
- OTTEN, L., G. POTRAVIK, P. HOOYKAAS, M. DUBOIS, E. SZEGEDI and J. SCHELL (1985) Identification of an Agrobacterium tumefaciens pTiB6S3 VIR region fragment that enhances the virulence of pTiC58. *Mol. Gen. Genet.* 199: 189-193.
- OU-LEE, T.M., R. TURGEON and R. WU (1986) Expression of a foreign gene linked to either a plant-virus or a Drosophila promoter, after electroporation of protoplasts of rice, wheat, and sorghum. *Proc. Natl. Acad. Sci. USA* 83: 6815-6819.
- OW, D.W., K.V. WOOD, M. DE LUCA, J.R. DEWET, D.R. HELINSKI, S.H. HOWELL (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* 234: 856-859.
- PARKE, D., L.N. ORNSTON and E.W. NESTER (1987) Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in Agrobacterium tumefaciens. *J. Bacteriol.* 169: 5336-5338.
- PASZKOWSKI, J., B. PISAN, R.D. SHILLITO, T. HOHN, B. HOHN and I. POTRYKUS (1986) Genetic transformation of Brassica campestris var. rapa protoplasts with an engineered cauliflower mosaic virus genome. *Plant Mol. Biol.* 6: 303-312.
- PASZKOWSKI, J., R.D. SHILLITO, M. SAUL, V. MANDAK, T. HOHN, B. HOHN and I. POTRYKUS (1984) Direct gene transfer to plants. *EMBO J.* 3: 2717-2722.
- PEAREN, R. and W.D. BEVERSDORF (1982) Rapeseed - Manipulating the crop. *Notes on Agriculture* 18: 3-4.

- PELLETIER, G., C. PRIMARD, F. VEDEL, P. CHERIT, R. REMY, P. ROUSSELLE and M. RENARD (1983) Intergeneric cytoplasmic hybridization in Cruciferae by protoplast fusion. Mo. Gen. Genet. 191: 244-250.
- PERALTA, E.G., R. HELLMISS and W. REAM (1986) Overdrive, a T-DNA transmission enhancer on the A. tumefaciens tumour-inducing plasmid. EMBO J. 5: 1137-1142.
- PETIT, A., C. DAVID, G.A. DAHL, J.M. ELLIS, P. GUYON, F. CASSE-DELBART and J. TEMPE (1983) Further extension of the opine concept: plasmids in Agrobacterium rhizogenes cooperate for opine degradation. Mol. Gen. Genet. 204-214.
- PETIT, A., J. STOUGAARD, A. KUHLE, K.A. MARCKER and J. TEMPE (1987) Transformation and regeneration of the legume Lotus corniculatus: a system for molecular studies of symbiotic nitrogen fixation. Mol. Gen. Genet. 207: 245-250.
- PETIT, A. and J. TEMPE (1978) Isolation of Agrobacterium Ti-plasmid regulatory mutants. Mol. Gen. Genet. 166: 147-155.
- POON, C.P.C. (1984) Removal of cadmium from wastewaters. Experientia 40: 127-136.
- PORTER, S.G., M.F. YANOFSKY and E.W. NESTER (1987) Molecular characterization of the VIR D operon from Agrobacterium tumefaciens. Nucleic Acids Res. 15: 7503-7517.
- POTRYKUS, I., J. PASZKOWSKI, R.D. SHILLITO and M.W. SAUL (1987) Direct gene transfer to plants. In: Plant DNA infectious agents. T. Hohn and J. Schell eds., Springer Verlag, New York, pp. 230-248.
- POWELL-ABEL, P., R.S. NELSON, B. DE, N. HOFFMANN, S.G. ROGERS, R.T. FRALEY and R.N. BEACHY (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232: 738-743.
- PRAKASH, S. and K. HINATA (1980) Taxonomy, cytogenetics and origin of crop Brassicas, a review. Opera Botan. 55: 1-57.
- PUA, E.C., A. MEHRA-PALTA, F. NAGY and N.H. CHUA (1987) Transgenic plants of Brassica napus L. BioTechnology 5: 815-817.
- PUERTAS, M.J., A. DE LA PENA, B. ESTADES and F. MERINO (1984) Early sensitivity to colchicine in developing anthers of rye. Chromosoma 89: 121-126.
- RADKE, S.E., B.M. ANDREWS, M.M. MOLONEY, M.L. CROUCH, J.C. KRIDL and V.C. KNAUF (1988) Transformation of B. napus L. using Agrobacterium tumefaciens: developmentally regulated expression of a reintroduced napin gene. Theor. Appl. Genet. 75: 685-694.

- RAY, T.B. (1986) Sulfonylurea herbicides as inhibitors of amino acid biosynthesis in plants. *trends Biochem. Sci.* 11: 180-183.
- RAY, T.B. (1985) The site of action of the sulfonylurea herbicides. *Proc. of the 1985 British Crop Protection Conference Weeds*. Vol. 1. Brighton Metropole, England, pp. 131-138.
- REAM, L.W., M.P. GORDON and E.W. NESTER (1983) Multiple mutations in the T region of the Agrobacterium tumefaciens tumor-inducing plasmid. *Proc. Natl. Acad. Sci. USA* 80: 1660-1664.
- REICH, T.J., V.N. IYER, M. HAFFNER, L.A. HOLBROOK and B.L. MIKI (1986a) The use of fluorescent dyes in the microinjection of alfalfa protoplasts. *Can. J. Bot.* 64: 1259-1267.
- REICH, T.J., V.N. IYER and B.L. MIKI (1986b) Efficient transformation of alfalfa protoplasts by the intranuclear microinjection of Ti plasmids. *BioTechnology* 4: 1001-1004.
- REICH, T.J., V.N. IYER, B. SCOBIE and B.L. MIKI (1986c) A detailed procedure for the intranuclear microinjection of plant protoplasts. *Can. J. Bot.* 64: 1255-1258.
- RHODES, C.A., D.A. PIERCE, I.J. METTLER, D. MASCARENHAS and J.J. DETMER (1988) Genetically transformed maize plants from protoplasts. *Science* 240: 204-207.
- RIKER, A.J., W.M. BANFIELD, W.H. WRIGHT, G.W. KEITT and H.E. SAGEN (1930). Studies on infections hairy-root of nursery apple tree. *J. Agric. Res.* 41: 507-540.
- ROBERTSON, J.L., T. HOLLIDAY and A.G. MATTHYSSE (1988) Mapping of Agrobacterium tumefaciens chromosomal genes affecting cellulose synthesis and bacterial attachment to host cells. *J. Bacteriol.* 170: 1408-1411.
- ROGERS, S.G., R.T. FRALEY, R.B. HORSCH, A.D. LEVINE, J.S. FLICK, L.A. BRAND, C.L. FINK, T. MOZER, K. O'CONNELL and P.R. SANDERS (1985) Evidence for ribosome scanning during translation initiation of mRNAs in transformed plant cells. *Plant Mol. Biol. Rep.* 3: 111-116.
- ROGERS, S.G. and H. KLEE (1987) Pathways to plant genetic manipulation employing Agrobacterium. In: *Plant DNA infectious agent*, T. Hohn and J. Schell eds. Springer-Verlag, New York, pp. 179-204.
- ROGOWSKY, P.M., T.J. CLOSE, J.A. CHIMERA, J.J. SHAW and C.I. KADO (1987) Regulation of the VIR genes of Agrobacterium tumefaciens. *J. Bacteriol.* 169: 5101-5102.

- ROSENBERG, N., A.E. GAD, A. ALTMAN, N. NAVOT and H. CZOSNEK (1988) Liposome-mediated introduction of the chloramphenicol acetyl transferase (CAT) gene and its expression in tobacco protoplasts. *Plant Mol. Biol.* 10: 185-191.
- RISUELO, G., BATTISTONI and P. COSTANTINO (1982). Regions of homology between tumorigenic plasmids from Agrobacterium rhizogenes and Agrobacterium tumefaciens. *Plasmid* 7: 45-51.
- SANDERS, P.R., J.A. WINTER, A.R. BARNASON, S.G. ROGERS and R.T. FRALEY (1987) Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. *Nucleic Acids Res.* 15: 1543-1558.
- SAXENA, P.K. and J. KING (1988) Herbicide resistance in Datura innoxia: cross resistance of sulfonylurea-resistant cell lines to imidazolinones. *Plant Physiol.* 86: 863-867.
- SCHAFER, W., A. GORZ and G. KAHL (1987) T-DNA integration and expression in a monocot crop plant after induction of Agrobacterium. *Nature* 327: 529-532.
- SCHENCK, H.R. and F. HOFFMANN (1979) Callus and root regeneration from mesophyll protoplasts of basic Brassica species: B. campestris, B. oleracea and B. nigra. *Z. Pflanzenzüchtg.* 82: 354-360.
- SCHILBERSZKY, K. (1935) Beitrage zur biologie von Pseudomonas tumefaciens. *Z. Pfl. Krankh. Pfl. Schutz* 45: 146-159.
- SCHLOSS, J.V., L.M. CISKANIK and D.E. VAN DYK (1988) Origin of the herbicide binding site of acetolactate synthase. *nature* 331: 360-362.
- SCHOCHER, R.J., R.D. SHILLITO, W.M. SAUL, J. PASKOWSKI and I. POTRYKUS (1986) Cotransformation of unlinked foreign genes into plants by direct gene transfer. *EMBO J.* 4: 1093-1096.
- SCIACKY, D., A.L. MONTOYA and M.D. CHILTON (1978) Fingerprints of Agrobacterium Ti plasmids. *Plasmid* 1: 238-253.
- SEBASTIEN, S.A. and R.S. CHALEFF (1987) Soybean mutants with increased tolerance for sulfonylurea herbicides. *Crop Sci.* 27: 948-952.
- SHAH, D.M., R.B. HORSCH, H.J. KLEE, G.M. KISHORE, J.A. WINTER, N.E. TURNER, C.M. HIRONAKA, P.R. SANDERS, C.S. GASSER, S. AYKENT, N.R. SIEGEL, S.G. ROGERS and R.T. FRALEY (1986) Engineering herbicide tolerance in transgenic plants. *Science* 233: 478-481.
- SHAHIN, E.A. and R.B. SIMPSON (1986) Gene transfer system for potato. *HortScience* 21: 1199-1201.

- SHAHIN, E.A., A. SPIELMANN, K. SUKHAPINDA, R.B. SIMPSON and M. YASHAR (1986a) Transformation of cultivated alfalfa using disarmed Agrobacterium tumefaciens. *Crop Sci.* 26: 1235-1239.
- SHANIN, E.A., K. SUKHAPINDA, R.B. SIMPSON and R. SPIVEY (1986b) Transformation of cultivated tomato by a binary vector in Agrobacterium rhizogenes: transgenic plants with normal phenotypes harbor binary vector T-DNA, but no Ri plasmid T-DNA. *Theor. Appl. Genet.* 72: 770-777.
- SHANER, O.L., P.C. ANDERSON and M.A. STIDHAM (1984) Imidazolinones: potent inhibitors of acetoxyacid synthase. *Plant Physiol.* 76: 545-546.
- SHANER, O.L., P.D. SIMCOX, P. ROBSON, D.R. CIARLANTE, G. MANGELS, B. REICHERT and M. LOS (1982) Translocation and metabolic selectivity of AC 222, 293. *Proc. 1982 British Crop Protection Conf. Weeds* 1: 333-339.
- SHAW, C.H., A.M. ASHBY, A. BROWN, C. ROYAL, G.T. LOAKE and C.H. SHAW (1988) VIR A and VIR G are the Ti plasmid functions required for chemotaxis of Agrobacterium tumefaciens towards acetosyringone. *Mol. Microbiol.* 2: 413-417.
- SHAW, C.H., G.H. CARTER, M.D. WATSON and C.H. SHAW (1984) A functional map of the nopaline synthase promoter. *Nucleic Acids Res.* 12: 7831-7846.
- SHAW, W.V. (1983) Chloramphenicol acetyltransferase: enzymology and molecular biology. *CRC Crit. Rev.* 14: 1-46.
- SHAW, W.V. (1975) Chloramphenicol acetyltransferase from chloramphenicol resistant bacteria. *Methods Enzymol.* 53: 737-754.
- SHILLITO, R.D., J. PASZKOWSKI and I. POTRYKUS (1983) Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. *Plant Cell Rep.* 2: 244-247.
- SHILLITO, R.D., M.W. SAUL, J. PASZOWSKI, M. MULLER and I. POTRYKUS (1985) High efficiency direct gene transfer to plants. *BioTechnology* 3: 1099-1103.
- SIMONSEN, C.C. and A.D. LEVINSON (1983) Isolation and expression of an altered mouse dihydrofolate reductase cDNA. *Proc. Natl. Acad. Sci. USA* 80: 2495-2499.
- SIMPSON, J., J. SCHELL, M. VAN MONTAGU and L. HERRERA-ESTRELLA (1986) Light-inducible and tissue-specific pea LHCP gene expression involves an upstream element combining enhancer- and silencer-like properties. *Nature* 323: 551-554.

- SLIGHTON, J.L., L. JOUANIN, F. LEACH, R.F. DRONG and D. TEPFER (1985) Isolation and identification of T₁-DNA/plant junctions in Convolvulus arvensis transformed by Agrobacterium rhizogenes strain A4. EMBO J. 4: 3069-3077.
- SMITH, E.F. and C.O. TOWNSEND (1907) A plant tumor of bacterial origin. Science 25: 671-673.
- SPANIO, L., D. MARIOTTI, M. PEZZOTTI, F. DAMIANI and S. ARCIONI (1987) Hairy root transformation in alfalfa (Medicago sativa L.). Theor. Appl. Genet. 73: 523-530.
- STACHEL, S.E., G. AN, C. FLORES and E.W. NESTER (1985) A TN3 lac Z transposon for the random generation of -galactosidase gene fusions: application to the analysis of gene expression in Agrobacterium. EMBO J. 4: 891-898.
- STACHEL, S.E., E. MESSENS, M. VAN MONTAGU and P. ZAMBRYSKI (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in Agrobacterium tumefaciens. Nature 318: 624-629.
- STACHEL, S.E. and E.W. NESTER (1986) The genetic and transcriptional organization of the VIR region of the A6 Ti plasmid of Agrobacterium tumefaciens. EMBO J. 5: 1445-1454.
- STACHEL, S.E., E.W. NESTER and P.C. ZAMBRYSKI (1986a) A plant cell factor induces Agrobacterium tumefaciens VIR gene expression. Proc. Natl. Acad. Sci. USA 83: 379-383.
- STACHEL, S.E., B. TIMMERMAN and P. ZAMBRYSKI (1986b) Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from Agrobacterium tumefaciens to plant cells. Nature 322: 706-712.
- STACHEL, S.E., B. TIMMERMAN and P. ZAMBRYSKI (1987) Activation of Agrobacterium tumefaciens VIR gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5' VIR D gene products. EMBO J. 6: 857-863.
- STACHEL, S.E. and P.C. ZAMBRYSKI (1986) Agrobacterium tumefaciens and the susceptible plant cell: a novel adaptation of extra cellular recognition and DNA conjugation. Cell 47: 155-157.
- STEINRUCKEN, H.C. and N. AMRHEIN (1980) The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimate acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94: 1207-1212.
- STEINRUCKEN, H.C., A. SCHULZ, N. AMRHEIN, C.A. PORTER and R.T. FRALEY (1986) Overproduction of 5-enolpyruvylshikimate-3-phosphate synthase in a glyphosate-tolerant Petunia hybrida cell line. Arch. Biochem. Biophys. 244: 169-178.

- STRINGAM, G.R. (1977) Regeneration in stem explants of haploid rapeseed (Brassica napus L.). *Plant Sci. Lett.* 9: 115-119.
- STRINGAM, G.R. (1979) Regeneration in leaf-callus cultures of haploid rapeseed (Brassica napus L.). *Z. Pflanzenphysiol.* 92: 459-462.
- STOUGAARD JENSEN, J., K.A. MARCKER, L. OTTEN and J. SCHELL (1986) Nodule-specific expression of a chimeric soybean leghaemoglobin gene in transgenic Lotus corniculatus. *Nature* 321: 669-674.
- STOUGAARD, J., N.S. SANDALS, A. GRON, A. KUHLE, and K.A. MARCKER (1987) 5' analysis of the soybean leghaemoglobin LBC₃ gene: regulatory elements required for promoter activity and organ specificity. *EMBO J.* 6: 3565-3569.
- SUGE, H. (1986) Reproductive development of higher plants as influenced by brassinolide. *Plant Cell Physiol.* 27: 199-205.
- SWANSON, E.B., M.P. COUMANS, G.L. BROWN, J.D. PATEL and W.D. BEVERSDORF (1988) The characterization of herbicide tolerant plants in Brassica napus L. after *in vitro* selection of microspores and protoplasts. *Plant Cell Rep.* 7: 83-87.
- SWEETSER, P.B., G.S. SCHOW and J.M. HUTCHINSON (1982) Metabolism of chlorsulfuron by plants: Biological basis for selectivity of a new herbicide for cereals. *Pestic. Biochem. Physiol.* 17: 18-23.
- TACHIBANA, K., T. WATANABE, Y. SEKIZAWA and T. TAKEMATSU (1986). Accumulation of ammonia in plants treated with bialophos. *J. Pesticide Sci.* 11: 33-37.
- TAMM, B. (1954) Experimentelle untersuchungen über das Auftreten von sekundärtumoren. *Archiv. Mikrobiol.* 20: 273-292.
- TANAK., N., M. HAYAKAWA, Y. MANO, H. OHKAWA and C. MATSUI (1985) Infection of turnip and radish storage roots with Agrobacterium rhizogenes. *Plant Cell Rep.* 4: 74-77.
- TANAKA, N., M. IKEGAMI, T. HOHN, C. MATSUI and I. WATANABE (1984) E. coli spheroplast-mediated transfer of cloned cauliflower mosaic virus DNA into plant protoplasts. *Mol. Gen. Genet.* 195: 378-380.
- TARTOF, K.D. and C.A. HOBBS (1987) Improved media for growing plasmid and cosmid clones. *Focus* 9: 12.
- THOMAS, E., F. HOFFMANN, I. POTRYKUS and G. WENZEL (1976) Protoplast regeneration and stem embryogenesis of haploid androgenetic rape. *Molec. Gen. Genet.* 145: 245-247.
- THOMAS, E. and G. WENZEL (1975) Embryogenesis from microspores of Brassica napus. *Z. Pflanzenzüchtg.* 74: 77-81.

- THOMASHOW, M.F., J.E. KARLINSEY, J.R. MARKS and R.E. HURLBERT (1987) Identification of a new virulence locus in Agrobacterium tumefaciens that affects polysaccharide composition and plant cell attachment. *J. Bacteriol.* 169: 3209-3216.
- THOMPSON, C.J., N.R. MOVVA, R. TIZARD, R. CRAMERI, J.E. DAVIS, M. LAUWEREYS and J. BOTTERMAN (1987a) Characterization of the herbicide-resistance gene bar from Streptomyces hygroscopicus. *EMBO J.* 6: 2519-2523.
- THOMPSON, D.V., L.S. MELCHERS, K.3. IDLER, R.A. SCHILPEROORT and P.J.J. HOOYKAAS, (1988) Analysis of the complete nucleotide sequence of the Agrobacterium tumefaciens VIR B operon. *Nucleic Acids Res.* 16: 4621-4636.
- THOMPSON, G.A., W.R. HIATT, D. FACCIOTTI, D.M. STALKER and L. COMAI (1987b) Expression in plants of a bacterial gene coding for glyphosate resistance. *Weed Sci.* 35: 19-23.
- THORNBURG, R.W., G. AM, T.E. CLEVELAND, R. JOHNSON and C.A. RYAN (1987) Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA* 84: 744-748.
- TIMKO, M.P., A.P. KAUSCH, C. CASTRESANA, J. FASSLER, L. HERRERA-ESTRELLA, G. VAN DEN BROECK, M. VAN MONTAGU, J. SCHELL and A.R. CASHMORE (1985) Light regulation of plant gene expression by an upstream enhancer-like element. *Nature* 318: 579-582.
- TORIYAMA, K., Y. ARIMOTO, H. UCHIMIYA and K. PINATA (1988) Transgenic rice plants after direct gene transfer into protoplasts. *BioTechnology* 6: 1072-1074.
- TRINH, T.H., S. MANTE, E.C. PUA and N.H. CHUA (1987) Rapid production of transgenic flowering shoots and F₁ progeny from Nicotiana glauca epidermal peels. *BioTechnology* 5: 1081-1084.
- TRULSON, A.J., R.B. SIMPSON and E.A. SHAHIN (1986) Transformation of cucumber (Cucumis sativus L.) plants with Agrobacterium rhizogenes. *Theor. Appl. Genet.* 73: 11-15.
- TUMER, N.E., K.M. O'CONNELL, R.S. NELSON, P.R. SANDERS, R.N. BEACHY, R.T. FRALEY and D.M. SHAH (1987) Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. *EMBO J.* 6: 1181-1188.
- U, N. (1935) Genome analysis in Brassica with special reference to the experimental formation of B. napus and peculiar mode of fertilization. *Japan. J. Bot.* 7: 389-452.

- UCHIMIYA, H., T. FUSHIMI, H. HASIMOTO, H. HARADA, K. SYONO and Y. SUGAWARA (1986) Expression of a foreign gene in callus derived from DNA-treated protoplasts of rice (Oryza sativa L.). Mol. Gen. Genet. 204: 204-207.
- UMBECK, P., G. JOHNSON, K. BARTON and Q. SWAIN (1987) Genetically transformed cotton (Gossypium hirsutum L.) plants. Biotechnology 5: 263-266.
- UNGER, L., S.F. ZIEGLER, G.A. HUFFMAN, V.C. KNAUF, R. PEET, L.W. MOORE, M.P. GORDON and E.W. NESTER (1985) New class of limited host range Agrobacterium mega- tumor-inducing plasmids lacking homology to the transferred DNA of a wide host range, tumor-inducing plasmid. J. Bacteriol. 164: 723-730.
- USAMI, S., S. MORIKAWA, I. TAKEBE and Y. MACHIDA (1987) Absence in monocotyledonous plants of the diffusible plant factors inducing T-DNA circularization and VIR gene expression in Agrobacterium. Mol. Gen. Genet. 209: 221-226.
- VAECK, M., A. REYNAERTS, H. HÖFTE, S. JANSSENS, M. DE BEUCKELEER, C. DEAN, M. ZABEAU, M. VAN MONTAGU and J. LEEMANS (1987) Transgenic plants protected from insect attack. Nature 328: 33-37.
- VAN DUN, C.M.P., L. VAN VLOTEN-DOTING and J.F. BOL. (1988) Expression of alfalfa mosaic virus cDNA 1 and 2 in transgenic tobacco plants. Virology 163: 572-578.
- VAN HAAKEN, M.J.J., J.T. PRONK, R.A. SCHILPEROORT and P.J.J. HOOYKAAS (1987) Functional analysis of the Agrobacterium tumefaciens octopine Ti-plasmid left and right T-region border fragments. Plant Mol. Biol. 8: 95-104.
- VAN HAUTE, E., H. JOOS, M. MAES, G. WARREN, M. VAN MONTAGU and J. SCHELL (1983) Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of the Ti plasmids of Agrobacterium tumefaciens. EMBO J. 2: 411-417.
- VAN LAREBEKE, N., C. GENETELLO, J. SCHELL, R.A. SCHILPEROORT, A.K. HERMANS, J.P. HERNALSTEENS and M. VAN MONTAGU (1975) Acquisition of tumour-inducing ability by non-oncogenic Agrobacteria as a result of plasmid transfer. Nature 255: 742-743.
- VELUTHAMBI, K., R.K. JAYASWAL and S.B. GELVIN (1987) Virulence genes A, G, D mediate the double-stranded border cleavage of T-DNA from the Agrobacterium Ti plasmid. Proc. Natl. Acad. Sci. USA 84: 1881-1885.
- VELUTHAMBI, K., W. REAM and S.B. GELVIN (1988) Virulence genes, borders and overdrive generate single-stranded T-DNA molecules from the A6 Ti plasmid of Agrobacterium tumefaciens. J. Bacteriol. 170: 1523-1532.

- WALDRON, C., E.B. MURPHY, J.L. ROBERTS, G.D. GUSTAFSON, S.L. ARMOUR and S.K. MALCOM (1985) Resistance to hygromycin B: A new marker for plant transformation studies. *Plant Mol. Biol.* 5: 103-108.
- WALSH, J.A. and J.A. TOMLINSON (1985) Viruses infecting winter oilseed rape (Brassica napus spp. oleifera). *Ann. Appl. Biol.* 107: 485-495.
- WANG, K., C. GENETELLO, M. VAN MONTAGU and P.C. ZAMBRYSKI (1987a) Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. *Mol. Gen. Genet.* 210: 338-346.
- WANG, K., S.E. STACHEL, B. TIMMERMAN, M. VAN MONTAGU and P.C. ZAMBRYSKI (1987b) Site-specific nick in the T-DNA border sequence as a result of Agrobacterium vir gene expression. *Science* 235: 587-591.
- WARD, J.E., D.E. AKIYOSHI, D. REGIER, A. DATTA, M.P. GORDON and E.W. NESTER (1988) Characterization of the vir B operon from an Agrobacterium tumefaciens Ti plasmid. *J. Biol. Chem.* 263: 5804-5814.
- WATSON, B., T.C. CURRIER, M.P. GORDON, M.D. CHILTON and E.W. NESTER (1975) Plasmid required for virulence of Agrobacterium tumefaciens. *J. Bacteriol.* 123:255-264.
- WEBER, G., S. MONAJEMBASHI, K.O. GEULICH and J. WOLFRUM (1988) Genetic manipulation of plant cells and organelles with a laser microbeam. *Plant Cell Tissue Organ Cult.* 12: 219-222.
- WEI, Z.M., H. KAMADA and H. HARADA (1986) Transformation of Solanum nigrum L. protoplasts by Agrobacterium rhizogenes. *Plant Cell Rep.* 5: 93-96.
- WEILER, E.W. and J. SCHRODER (1987) Hormone genes and crown gall disease. *Trends Biochem. Sci.* 12: 271-275.
- WEN, W. and H. LÖRZ (1986) Transient gene expression in a Gramineae cell line: a rapid procedure for studying plant promoters. *Mol. Gen. Genet.* 202: 471-475.
- WHITE, F.F., G. GHIDOSI, M.P. GORDON and E.W. NESTER (1982) Tumor-induction by Agrobacterium rhizogenes involves the transfer of plasmid DNA to the plant genome. *Proc. Natl. Acad. Sci. USA* 79: 3193-3197.
- WHITE, D.W.R. and D. GREENWOOD (1987) Transformation of the forage legume Trifolium repens L. using binary Agrobacterium vectors. *Plant Mol. Biol.* 8: 461-469.
- WHITE, F.F. and E.W. NESTER (1980a) Hairy root: plasmid encodes virulence traits in A. rhizogenes. *J. Bacteriol.* 141: 1134-1141.
- WHITE, F.F. and E.W. NESTER (1980b) Relationship of plasmids responsible for hairy root and crown gall tumorigenicity. *J. Bacteriol.* 144: 710-720.

- WINANS, S.C., P.R. EBERT, S.E. STACHEL, M.P. GORDON and E.W. NESTER (1986) A gene essential for Agrobacterium virulence is homologous to a family of positive regulatory loci. Proc. Natl. Acad. sci. USA 83: 8278-8282.
- WIRTZ, V., J. SCHELL and A.P). CZERNILOFSKY (1987) Recombination of selectable marker DNA in Nicotiana tabacum. DNA 6: 245-253.
- WULLEMS, G.J., L. MOLENDIJK, G. OOMS and R.A. SCHILPEROORT (1981) Retention of tumor markers in F₁ progeny plants from in vitro induced octopine and nopaline tumor tissues. Cell 24: 719-727.
- XIAO, W., P.K. SAXENA, J. KING and G.H. RANK (1987) A transient duplication of the acetolactate synthase gene in a cell culture of Datura innoxia. Theor. Appl. Genet. 74: 417-422.
- XU, Z.H., M.R. DAVEY and E.C. COCKING (1982) Plant regeneration from root protoplasts of Brassica. Plant Sci. Lett. 24: 117-121.
- YADAV, N., R.E. McDEVITT, S. BERNARD and S.C. FALCO (1986) Single amino acid substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl. Proc. Natl. Acad. Sci. USA 83: 4418-4422.
- YAMAMOTO, A., M. IWAHASHI, M.F. YANOFISKY, E.W. NESTER, I. TAKEBE and Y. MACHIDA (1987) The promoter proximal region in the VIR D locus of Agrobacterium tumefaciens is necessary for the plant inducible circularization of T-DNA. Mol. Gen. Genet. 206: 174-177.
- YAMAYA, J., M. YOSHIOKA, T. MESHI, Y. OKADA and T. OHNO (1988) Expression of tobacco mosaic virus RNA in transgenic plants. Mol. Gen. Genet. 211: 520-525.
- YANOFISKY, M., B. LOWE, A. MONTOYA, R. RUBIN, W. KRUL, M. GORDON and E. NESTER (1985a) Molecular and genetic analysis of factors controlling host range in Agrobacterium tumefaciens. Mol. Gen. Genet. 201: 237-246.
- YANOFISKY, M., A. MONTOYA, V. KNAUF, B. LOWE, M. GORDON and E. NESTER (1985b) Limited host-range plasmid of Agrobacterium tumefaciens: Molecular and genetic analyses of transferred DNA. J. Bacteriol. 163: 341-348.
- YANOFISKY, M.F. and E.W. NESTER (1986) Molecular characterization of a host- range-determining locus from Agrobacterium tumefaciens. J. Bacteriol. 168: 244-250.
- YANOFISKY, M.F., S.G. PORTER, C. YOUNG, L.M. ALBRIGHT, M.P. GORDON and E.W. NESTER (1986) The VIR D operon of Agrobacterium tumefaciens encodes a site-specific endonuclease. Cell 47: 471-477.

YOUNG, S.L., A.E. JACKSON, D. PUETT and M.H. MELNER (1985) Detection of chloramphenicol acetyltransferase activity in transfected cells: a rapid and sensitive HPLC-based method. *DNA* 4: 469-475.

ZAENEN, I., N. VAN LAREBEKE, H. TEUCHY, M. VAN MONTAGU and J. SCHELL (1974) Supercoiled circular DNA in crown gall inducing Agrobacterium strains. *J. Mol. Biol.* 86: 109-127.

ZAMBRYSKI, P., H. JOOS, C. GENETELLO, J. LEEMANS, M. VAN MONTAGU and J. SCHELL (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* 2: 2143-2150.

ZORREGUIETA, A., R.A. GEREMIA, S. CAVAGNAC, G.A. CANGELOSI, E.N. NESTER and R.A. UGALDE (1988) Identification of the product of an Agrobacterium tumefaciens chromosomal virulence gene. *Mol. Plant. Microbe Int.* 1: 121-127.

APPENDICES

- A.1 Disarmed vectors (pGV3850 and pMP90) derived from pTiC58
- A.2 Disarmed vectors (pAL4404) and binary (pAL1050)
derived from pTiAch5
- A.3 Map of pNCAT4 and location of the fragment for the
CAT probe
- A.4 Map of pLGV1103neo and location of the fragment for the
NPT II probe
- A.5 Map of pBIN19
- A.6 Maps of the fragments introduced in pBIN19 to produce
the vectors pBCAT1, pVU1011 and pBI121.1
- A.7 Map of the vector pMON38 and location of the fragment for
the Nopaline synthase probe
- A.8 Map of the vector pMON273
- A.9 Map of the vector pMON806
- A.10 Map of the plasmid pBR322
- A.11 Kanamycin resistance GeneBlock (gene from Tn903)
- A.12 Map of the acetolactate synthase gene of A. thaliana

Abbreviations:

a.a. = amino acids

Ap^r = ampicillin resistance

CAMV = cauliflower mosaic virus

CAT = chloramphenicol acetyltransferase

DHFR = dihydrofolate reductase

HPT = hygromycin phosphotransferase

kb = kilobase

Km^r = kanamycin resistance

LB = left border

Min = minimum

NOS = nopaline synthase

NPT II = neomycin phosphotransferase II

OCS = octopine synthase

ORI = origin of replication

P = promoter sequences

PA = polyadenylation and termination signals

RB = right border

Spc^r = spectinomycin resistance

Tc^r = tetracycline resistance

T_L = left T-DNA

T_R = right T-DNA

UPS = upstream sequences

Z = β-galactosidase gene

pBR322 sequences

A

pBCAT 1 (+ 1.8 kb)

B

pVU1011

C

pBI121.1

pBR322 sequences

- pBR322 sequences

Kanamycin resistance GeneBlock

1.5 kb

ACETOLACTATE SYNTHASE GENE

END

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FIN