DEVELOPMENT OF AN *AGROBACTERIUM VITIS* TRANSFORMATION SYSTEM FOR GRAPEVINE

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

Agrobacterium tumefaciens-mediated transformation technology has been used in a variety of applications throughout the fields of cellular and molecular plant biology as well as plant physiology. Research is conducted in order to extend this application range and overcome some of the intrinsic limitations of the *Agrobacterium* transformation system. Predominantly, these limitations can be attributed to the host range specificity of *A. tumefaciens*, as well as adverse effects induced on explant tissue by active plant defence mechanisms, triggered by the plant-pathogen-interaction. Typically, this active defence mechanism culminates in the hypersensitive response (HR), characterised by localised cell death and necrosis.

Not all *Agrobacterium* species, however, share the same host range and some have evolved the ability to infect plant species not normally considered hosts of *A. tumefaciens*. This host range specificity can be exploited to extend the application of existing *Agrobacterium* transformation systems. In an attempt to establish an efficient transformation system for *Vitis vinifera* which, has proven very difficult to transform with *A. tumefaciens*, indigenous *A. vitis* strains have been evaluated as possible host-specific transformation agents. Strains of *Agrobacterium vitis* should be suitable for this type of endeavour, since they have evolved several unique characteristics directly linked to the infection of their hosts. These include the ability to utilise, tartrate, a host abundant carbon source, as well as the production of an acid polygalacturonase that could play a role during the infection process. The proposition that the evolution of *A. vitis* is a fairly recent event is also confirmed by the relatively little divergence observed between *A. tumefaciens* and *A. vitis*.

In this study, a selection of *A. vitis* strains were evaluated in screenings designed to accentuate desirable traits in strains such as good infectivity of grapevine material (presumably an indicator of an efficient mechanism of gene transfer to be exploited in an engineered transformation system) as well as a favourable reaction (causing no necrosis) on grapevine somatic embryos. Two strains produced large tumours on grapevine cuttings and caused little necrosis on the somatic embryos. Significant variation in infectivity as well as callus necrosis was observed between the strains as well as in a genotype-specific manner on the host material. This genotypic-specific effect of either host or pathogen

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could be an indication of the degree of specialisation developed by plant pathogens to infect specific hosts. On the basis of these results, it was possible to select an *A. vitis* strain for further biochemical and genetic characterisation.

Simple biochemical analysis classified the strain as an octopine strain. DNA-DNA hybridisation techniques combined with a plasmid walking technique resulted in the partial characterisation of the T-DNA of the selected *A. vitis* strain. A partial restriction enzyme map of the T-DNA was constructed and the T-DNA and flanking areas were cloned. Significant differences, most notably, the absence of a TB-area as well as the absence of the agrocinopine (*acs*) gene from the 5' area of the T-DNA, were observed. Partial sequencing data indicated the presence of at least four conserved T-DNA genes located on the TA-DNA, as well as the presence of three bacterial insertion (IS) elements flanking the region. Two of these IS elements, both related to the IS*110* family of IS elements have not yet been reported in *A. vitis.* In fact, these two elements seem to be the 5' and 3' ends of a disrupted element and could therefore have played an evolutionary role in the development of this strain.

This study provides fundamental background for the development of a more efficient transformation system specific for grapevine, exploiting some of the unique characteristics of one of its pathogens, *A. vitis.*

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OPSOMMING

Agrobacterium tumefaciens-gebaseerde transformasiesisteme word in 'n wye reeks van toepassings in die velde van sellulêre- en molekulêre plantbiologie asook plantfisiologie aangewend. Navorsing word voortdurend onderneem om die inherente beperkinge van die *Agrobacterium*-transformasiesisteem te oorkom en sodoende die toepassingsveld van die sisteem verder te verbreed. Die beperkinge tipies aan dié sisteem kan hoofsaaklik toegeskryf word aan die gasheerspesifisteit van *A. tumefaciens*, asook die negatiewe reaksies op eksplantmateriaal wat deur die plant se aktiewe verdedigingsmeganisme, soos ontlok deur die plant-patogeen interaksie, veroorsaak word. Hierdie aktiewe verdedigingsmeganisme lei gewoonlik tot 'n hipersensitiewe respons (HR) in die plant, wat deur gelokaliseerde selafsterwing en nekrose gekenmerk word.

Alle *Agrobacterium*-spesies het egter nie almal dieselfde gasheerreeks nie en sommige rasse het as gevolg van evolusionêre ontwikkelings die vermoë verkry om plantspesies wat normaalweg buite die gasheerreeks van *A. tumefaciens* val, te infekteer. Hierdie tipe gasheerspesifisiteit kan uitgebuit word om die toepassingsmoontlikhede van bestaande *Agrobacterium*-transformasiesisteme te verbreed. In 'n poging om 'n effektiewe transformasiesisteem vir *Vitis vinifera*, 'n moeilik transformeerbare gewas, te ontwikkel, is inheemse rasse van *Agrobacterium vitis* ondersoek as moontlike gasheerspesifieke transformasie-agente. Rasse van *A. vitis* behoort uiters geskik te wees vir so 'n toepassing, aangesien hulle verskeie unieke eienskappe, wat direk aan die infeksie van die gasheer gekoppel is, vertoon. Van hierdie eienskappe is onder meer die vermoë om tartraat, 'n koolstofbron volop in druifplante, te benut. *A. vitis* produseer verder ook 'n suur poligalaktorunase wat vermoedelik 'n rol in die infeksieproses speel. Die voorstel dat die evolusionêre ontwikkeling van *A. vitis* 'n redelike onlangse gebeurtenis is, word onderskryf deur die betreklike homogenisiteit met *A. tumefaciens*.

In hierdie studie is 'n groep *A. vitis*-rasse met behulp van siftingsprosedures wat daarop gemik is om gesogte eienskappe in rasse uit te wys, beoordeel. Die vermoë om druifplantmateriaal te infekteer (wat vermoedelik 'n aanwyser van 'n effektiewe meganisme van geenoordraging is wat in 'n gemanipuleerde transformasiesisteem benut kan word), sowel as 'n gunstige reaksie (d.w.s geen nekrose) op druifplant somatiese embrio's is van die gesogte eienskappe waarvoor gesoek word. Twee rasse het groot tumors op

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druifplant-stingelsegmente veroorsaak terwyl hulle bykans geen weefselskade op somatiese embrio's geïnduseer het nie. Betekenisvolle verskille in infektiwiteit en in kallusnekrose is tussen die rasse sowel as in 'n genotipe-spesifieke-verhouding waargeneem. Hierdie genotipe-spesifieke effek, kenmerkend van óf die gasheer óf die patogeen, kan aanduidend wees van die vlak van spesialisasie wat heers by die infeksie van spesifieke gashere. Na aanleiding van bogenoemde resultate was dit moontlik om 'n *A. vitis*-ras te selekteer wat verder aan biochemiese en genetiese analises onderwerp kon word.

Eenvoudige biochemiese analises het dit moontlik gemaak om die ras as oktopien te klassifiseer. DNA-DNA hibridisasietegnieke gekombineerd met 'n unieke plasmied-wandeltegniek het gelei tot die gedeeltelike karakterisering van die geselekteerde *A. vitis*-ras. 'n Gedeeltelike restriksie-ensiem (RE) kaart van die T-DNA kon gevolglik opgestel word. Die T-DNA en die aangrensende gedeeltes is boonop gekloneer. Betekenisvolle verskille, spesifiek die afwesigheid van 'n TB area, sowel as die afwesigheid van die agrosinopien-sintasegeen (*acs*) aan die 5'-kant van die T-DNA, is waargeneem. Gedeeltelike basispaaropeenvolgingsdata het egter die teenwoordigheid van minstens vier gekonserveerde T-DNA-gene, asook die teenwoordigheid van drie bakteriese invoegingselemente (IS) aan weerskante van die area, geïdentifiseer. Twee van hierdie elemente, wat beide homologie vertoon met die IS*110* familie van IS elemente, is nog nie vantevore in *A. vitis* aangetref nie. Dit wil boonop blyk of dié twee elemente die 5'- en 3'-areas van 'n onderbroke element vorm, wat dus 'n moontlike aanduiding is van hul potensiële rol in die evolusionêre ontwikkeling van die ras.

Hierdie studie verskaf basiese inligting wat daartoe kan lei dat 'n doeltreffender transformasiesisteem spesifiek vir druifplante ontwikkel word deur van die unieke kenmerke van een van sy patogene, *A. vitis,* uit te buit.

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This thesis is dedicated to my parents.

Hierdie tesis is opgedra aan my ouers.

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BIOGRAPHICAL SKETCH

Dirk Albert Joubert was born in Pretoria, South Africa on 18th of May 1973. He matriculated at Huguenot High School, Wellington in 1991. Albert enrolled at the University of Stellenbosch in 1993 and obtained a B.Sc. degree, majoring in Microbiology, Biochemistry and Genetics, in 1996. The degree Hons.B.Sc. (Wine Biotechnology) was subsequently awarded to him, whereafter he enrolled for a M.Sc. degree in Wine Biotechnology.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and data from Chapter 3 will form part of an article to be submitted to the Journal of Bacteriology for publication.

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Chapter 2 **LITERATURE REVIEW**

Development of an *Agrobacterium vitis* transformation system for grapevine

Chapter 3 **RESEARCH RESULTS**

Characterisation of an *Agrobacterium vitis* strain as a potential transformation agent for *Vitis vinifera*

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CHAPTER ONE

INTRODUCTION AND PROJECT AIMS

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1.1. INTRODUCTION

The genetic transformation and manipulation of plant species have become common phenomena and have found application in both scientific and economic endeavours (Birch, 1997). The first successful transformation of tobacco, more than fifteen years ago, (De Block *et al*, 1984) served as a catalyst for what could only be described as a "biotechnology revolution" that followed (Ortiz, 1998). DNA transfer systems have been developed for numerous plant species (Brink *et al*, 1998) and, for each of these, several unifying aspects can be observed. These aspects include: i) the availability of regenerable plant tissue, ii) the availability of appropriate gene transfer cassettes, and iii) a reliable method for the transfer of foreign DNA to host cells (Songstad *et al*, 1995). Each of these factors should be considered carefully before a plant transformation experiment is attempted, especially since the explant type, gene transfer cassette, and DNA transfer method used, are dictated by the plant species to be transformed (Birch, 1997).

Several methods of DNA transfer have been developed to accommodate the huge diversity found within the plant environment (Offringa *et al*, 1990). DNA transfer to protoplasts is facilitated by chemical treatment or electroporation (Zimmerman, 1986; Negrutiu *et al*, 1987) whereas intact cells can be transformed via microprojectile bombardment (Klein *et al*, 1987), silicon carbide fibres (Keappler *et al*, 1990), microinjection (Neuhaus and Spangenberg, 1990), as well as electroporation (Hansch *et al*, 1996). In addition to these direct methods of gene transfer, however, the most commonly used procedures are based on the natural transformation system of *Agrobacterium* (Offringa *et al*, 1990).

1.2. *AGROBACTERIUM*-MEDIATED DNA TRANSFER TO PLANT CELLS

Agrobacterium is a Gram negative phytopathogen of the family *Rhizobiaceae* that predominantly infects wounded dicotyledonous plant species through a process of genetic transformation (Zambryski, 1988; Hooykaas and Schilperoort, 1992). They are soil-borne bacteria and can, in association with plant rests, be present in infected soil for many years (Burr *et al*, 1995). *Agrobacterium* has developed an innovative and effective infection process that includes the following essential steps: bacterial colonisation and attachment to wounded plant cells, induction of the bacterial virulence system, the generation of a

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transfer DNA (T-DNA) complex, T-DNA transfer, and the integration of T-DNA into the plant genome (De la Riva *et al*, 1998).

The T-DNA is a discrete fragment of bacterial DNA (bordered by two 24 bp repeats) found on a large extrachromosomal plasmid, the tumour inducing (Ti) plasmid. During the infection process this T-DNA between the border repeats is transferred to the plant genome, where it integrates and is expressed (Winans, 1992). Specific genes located on the Ti plasmid, designated the virulence (*vir*) genes, operate in *trans* and facilitate the transfer of the T-DNA copy during infection. In wild type strains, the expression of the T-DNA genes normally leads to the formation of crown galls in the infected host (Zambryski, 1988).

Genetic analysis of the infection process and the material involved indicated that no physical linkage is required between the T-DNA-genes and the remainder of the Ti plasmid for successful infection (Hooykaas and Schilperoort, 1992). Furthermore, it was shown that the T-DNA genes play no role in the DNA transfer process itself and that any DNA placed between the T-DNA borders will be transferred to the host (Klee *et al*, 1987). This was the breakthrough finding that showed it is possible to exploit and extend this transformation mechanism of *Agrobacterium* to transfer any DNA of interest to host plant cells (Zambryski, 1992). Although this transformation method has successfully been applied to a large variety of mostly dicotyledonous plants, some plant species, e.g. most agronomically important crop species (Gelvin, 1998), remain difficult to transform with the system. Specific problems encountered are the fact that *Agrobacterium tumefaciens* has a limited host range and most monocotyledonous plants fall outside this range, and adverse effects after co-cultivation with the bacterium such as the induction of an active defence response in the infected plant. (Perl *et al*, 1996; Birch, 1997)

A. vitis, a pathogen causing crown gall disease of *Vitis vinifera*, was isolated a number of years ago, but has only recently been recognised as a separate *Agrobacterium* species (Ophell and Kerr, 1990). Although strains of these Gram negative biotype III bacteria were isolated a number of years ago, only a few strains have been fully characterised (Otten *et al*, 1996). Based on isolated characterised strains, only four opine types have been identified to date in this species, namely octopine, octopine/cucumopine (o/c), nopaline (nop) and a novel vitopine (vit) type (Paulus *et al*, 1989). These bacteria infect mainly grapevine and are, unlike their *A. tumefaciens* counterparts, frequently able to utilise

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tartrate, a carbohydrate commonly found in grapevine, as sole carbon source (Otten *et al*, 1995).

1.3. SPECIFIC AIMS

Successful grapevine transformation has been achieved using *A. tumefaciens, A. rhizogenes* and a combination of biolistic bombardment and *Agrobacterium* infection (Guellec et al, 1990; Martinelli and Mandolino, 1994; Scorza *et al*, 1996). The induction of an active defence response in grapevine by *A. tumefaciens* has seriously hampered any transformation experiments attempted with this system due to the massive necrosis of the target material (Baribault *et al*, 1989). The ability of *A. vitis* strains to infect grapevine, however, is indicative of the fact that *A. vitis* has managed to circumvent the active defence response in grapevine and might prove useful in a transformation system specific for grapevine. As a working hypothesis, it is assumed that an indigenous disarmed *A. vitis* strain will enhance the transformation frequency of its host plant, *V. vinifera*, without causing severe tissue necrosis due to the hypersensitive response. This study forms part of a multidisciplinary research objective in the Institute for Wine Biotechnology, which focuses on the genetic improvement of grapevine through recombinant DNA technology. Specific aims of this study included:

i) the selection of an indigenous *A. vitis* strain, capable of efficiently infecting cultivars of *V. vinifera*;

ii) determining the suitability of the *A. vitis* strain as a possible transformation agent specific for grapevine by exposing somatic embryogenic cultures of various cultivars to the selected strain(s);

iii) characterising the selected strain(s) with regard to opine utilisation and host range;

iv) characterising the T-DNA area of the selected strain(s) by mapping studies and the construction of a restriction enzyme map. The T-DNA and T-DNA flanking areas will also be cloned to be potentially used in a disarmament strategy.

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CHAPTER TWO

LITERATURE REVIEW:
An overview of plant transformation with specific emphasis on the role of *Agrobacterium*

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2.1. INTRODUCTION

Since the first reports of the introduction and expression of foreign genes in tobacco more than fifteen years ago (De Block *et al*, 1984), plant transformation has experienced tremendous growth and progress. More than 120 species, in at least 35 families, have been successfully transformed. This has led to over 3000 field trials in at least 30 countries. Successes have been achieved in most agronomically important crops, including trees, vegetables, ornamental-, medicinal-, fruit-, and pasture plants. The process of expanding and refining transformation techniques for greater convenience, higher efficiency, broader genotype range and desired molecular characteristics of transformants, will enable scientists to broaden applications even further (Birch, 1997). Plant transformation techniques have made possible the study of the underlying molecular mechanisms of many physiological processes not previously understood. Many of these physiological processes, e.g. sugar metabolism, may play a direct role in future plant improvement strategies. It is clear that genetic engineering has become a powerful tool in gaining fundamental knowledge of many of the complex processes involved during plant development, enabling us to manipulate specific physiological, genotypical and thus phenotypical characteristics.

Gene transfer systems rely predominantly on three essential requirements: i) availability of target tissues, specifically cells competent for plant regeneration; ii) ppropriate gene constructs that facilitate the selection of transgenic plants as well as the expression of transgenes, either constitutively or differentially; and iii) a method to introduce DNA into regenerable cells in a way that minimises cell damage and allows for the stable expression of transgenes (Birch, 1997; Songstad *et al*, 1995). Practically, this implies the establishment of a suitable tissue culture system to maintain a continuous supply of explants at the correct developmental stage (enabling regeneration) and a DNA transfer system that is applicable to a wide variety of cultivars, and is efficient, economical, simple and safe. This review will focus mainly on DNA delivery methods, emphasising the role of *Agrobacterium*.

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2.2. MANIPULATING PLANT MATERIAL TO ACCEPT AND INCORPORATE FOREIGN GENETIC MATERIAL

The *in vitro* cultivation of plant cells may serve many purposes: e.g. clonal propagation of disease-free plant material, elimination of viruses and scientific studies on physiological and biochemical processes dependent on totipotent cells (Lindsey and Jones, 1989). Totipotent cells can generally be divided into three groups; i) fertilised eggs (zygotes), ii) developing meristematic cells and iii) male and female gametes and haploid cells (Lindsey and Jones, 1989; Warren, 1989). The use of a specific totipotent explant type will be governed by the intended application, since certain characteristics are linked to these cells. Although zygotes have great developmental potential and can develop further into seed, they are not favoured for rapid clonal propagation. Drawbacks include the fact that their phenotype generally remains unknown due to chromosomal crossing over, which lead to genetic recombination as well as random segregation of chromosomes during meiosis and gametogamy. For the purpose of clonal propagation, developing meristematic cells, such as those in developing stems, leaves, etc. have the most complete totipotency. Their visible phenotype enables the selection of superior qualities and they can be regenerated into large numbers of plantlets. Regeneration of plants from single cells can be obtained through adventitious somatic embryogenesis and separate root and shoot formation in processes of organogenesis (Fig. 1) (Warren, 1989). These processes are intensely studied for their application in genetic transformation protocols for different plant species (Birch, 1997). Protoplast regeneration can also be utilised for plant regeneration in a process similar to that of embryogenesis and will be discussed as such (Songstad *et al*, 1995).

2.2.1. The processes, principles and manipulation of organogenesis

In the *in vitro* cultivation of plant cells, organogenesis is a more widespread and easily controllable process than embryogenesis. Precise hormonal switches determine the developmental pathway followed, leading to the induction of meristems or primordia to form roots or shoots, depending on the media components and growth factors administered. Root and shoot formation, also known as caulogenesis and rhizogenesis, are in essence facets of the same process.

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The process of organogenesis can be divided into three phases (Burrit and Leung, 1996). In phase I, the explant gains the ability to respond to an organic induction, that is, it acquires "competence". This competence can be achieved on shoot-, root-, or callus-inducing media. Phase II entails organic induction, mediated by a growth regulator. In this phase competent cells are induced to follow a particular developmental pathway leading to a specific development, constituting phase III. This development can, once initiated, continue without the influence of growth regulators (Sugiyama, 1999).

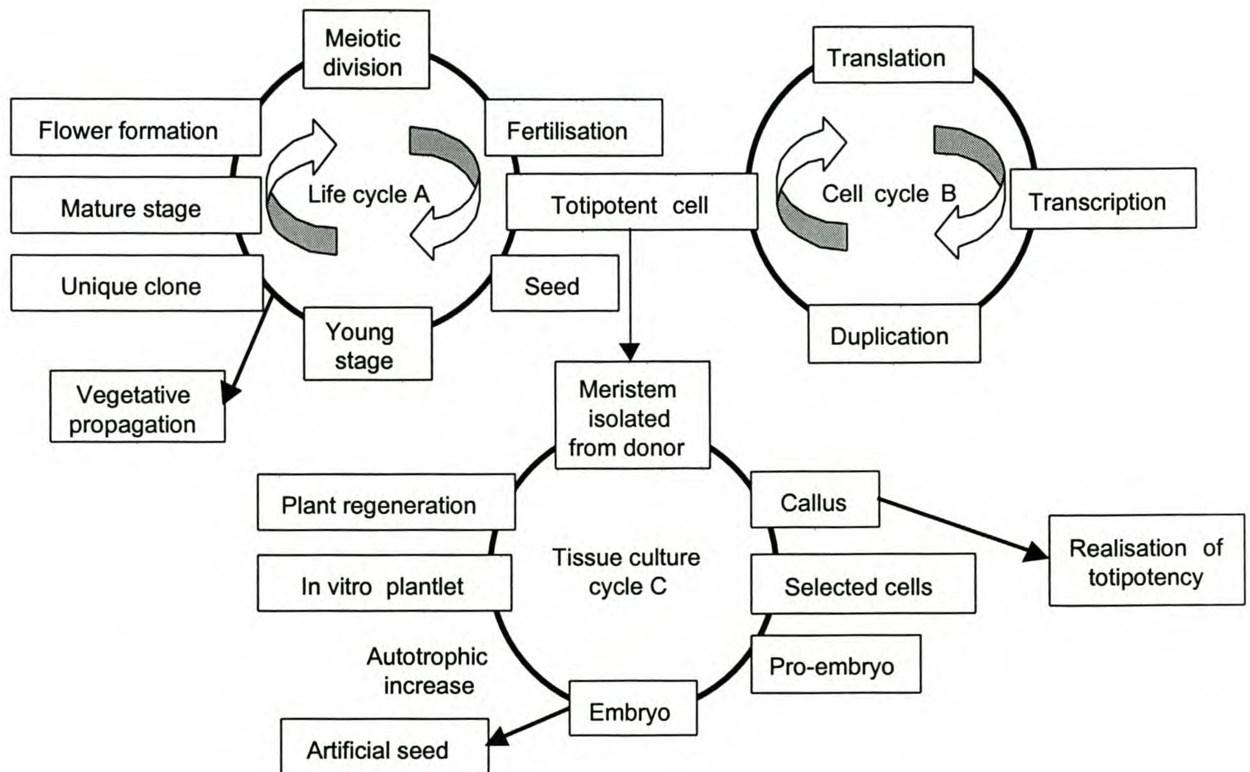

Fig. 1. Generation of totipotent cells in the plant body (adapted from Warren, 1989). Cycle A indicates the life cycle, cycle B shows the nucleoplasmic cycle determined by the cell and cycle C depicts the tissue culture cycle.

Adventitious primordia develop from two general areas of explants, either in close proximity to the vascular tissue, or from the epidermis or sub-epidermal layers. Shoot and root formation does not often occur directly in the same explant and may develop from different cell types. As mentioned earlier, rhizo- and caulogenesis are controlled and

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regulated by several growth regulators or plant hormones. It is interesting to note that these growth regulators are atypical hormones, since they exhibit several abnormal hormonal characteristics; e.g. they can exert effects in the same cell in which they are produced. They do, however, display many true hormonal characteristics, i.e. they function at low concentrations and exert an action from a distance (Burrit and Leung, 1996; Gray and Estelle, 1998). There is growing evidence that plant hormones constitute a pool of intra- and intercellular messengers that can be utilised according to the demands of the cell or the tissue at a particular stage, and therefore exhibit a changing spectrum of partially overlapping functions (Warren, 1989). It is practically impossible to describe general principles underlying hormone action, or as yet, to identify the primary molecular mechanism involved. It is unlikely that a simple relationship exists between the concentration of a hormone added to a specific tissue, and the actual effective concentration at the site of action (Schell *et al*, 1993). Some of the most important growth regulators added to plant cell cultures are shown in Table 1.

Although the exact underlying molecular events are not understood, there are a few important principles recognised as influencing the relationship between added and perceived hormone concentration. First, exogenous hormones can be stored or modified by the plant cell, and second, feedback control of endogenous hormone synthesis can occur. Third, the intra- and intercellular "carry over" of hormones from one treatment to the other can occur, whereas cultured cell clusters or tissues can also regulate their internal hormonal environments (Warren, 1989).

Table 1. Important growth regulators used for the induction of organogenesis (Gray and Estelle, 1998; Kakimoto, 1998; Walker and Estelle, 1998; D'Agostino and Kieber, 1999).

Class	Growth Regulator	Characteristic
Auxin	Indole acetic acid (IAA)	Natural, light sensitive
	1-Naphthylacetic acid (NAA)	Synthetic
	2,4-Dichlorphenoxyacetic acid (2,4-D)	Synthetic, high potency
Cytokinin	Zeatin	Natural
	Zeatin riboside	Natural
	Kinetin	Synthetic (prepared from DNA)
	6-Benzyl-aminopurine (BAP)	Synthetic
Gibberelin	GA3	Natural Usually heat labile

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The influence of hormones, either on their own or in combination with others, varies significantly in different plant species and cultivars. Several factors influence these hormonal interactions, but none are more important than the genotype-dependent hormonal requirements as illustrated in the next scenario (Gawel and Robacker, 1990). Intra-specific regeneration inconsistencies have been noted in many crops, including *Zea mays* (Hodges *et al*, 1986), *Oryza sativa* (Abe and Futsuhara, 1986), *Nicotiana tabacum* (Keyes *et al*, 1981) and *Brassica napus* (Julliard *et al*, 1992). Internal stem segments from two cultivars (cv) of *B. napus*, Bienvenu and Darmor, regenerated shoots on a medium without NAA, but regenerated only a hard, whitish callus in the presence of NAA, suggesting that the explant must contain sufficient endogenous auxin. *B. napus* cv Westvar, however, showed a high frequency of shoot regeneration in the presence of NAA, clearly indicating a genotypic effect on hormonal requirements (Julliard *et al*, 1992). Hormonal requirements are not the only source of variation; facets such as somoclonal variation, tissue competence and factors linked to the culture conditions (e.g. exposure to light, temperature, humidity, etc.) are important since they all play a vital role in organogenesis (Birch, 1997; Decout *et al*, 1994).

There are, however, a limited number of hormonal effects imparting differentiation that do show a degree of consistency; i) relatively high auxin concentrations suppress organised growth and promote the formation of meristem-like cells, ii) the ratio of auxin to cytokinin influences the balance between root and shoot formation, iii) cytokinins inhibit root formation, and iv) embryogenesis is stimulated by lowering the auxin concentration (Table 2) (Warren, 1989).

2.2.2. The formation of vegetative embryos through somatic embryogenesis

Somatic embryogenesis has been defined as the formation of a bipolar vegetative structure, the somatic embryo, that is similar in appearance and development to the zygotic embryo (Zimmerman, 1993). Two types of somatic embryogenesis, direct and indirect, have been identified. They share developmental phases: formation of the initiation-cell, and globular-, heart- and torpedo stage embryos (De Jong *et al*, 1993). Direct embryogenesis, in which a single cell commences meristematic growth and all the descendants from this cell form part of the embryo, is a very rare event (Aleith and Richter, 1990). Indirect embryogenesis is observed much more frequently (Wang *et al*, 1990), and

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is characterised by the development of an embryo from one cell in a previously formed meristematic cluster, designated pro-embryonic masses (De Jong *et al*, 1993). These somatic embryos have several characteristics that render them suitable for transformation and regeneration protocols, e.g., their regeneration ability and their predictable genotype (Aleith and Richter, 1990; Warren, 1989; Zimmerman, 1993).

Table 2. Effect of hormonal ratios on plant regeneration (Lindsey and Jones, 1989).

Plant developmental stage	Auxin : Cytokinin	Effect
Morphogenesis	High	Caulogenesis
	Low	Rhizogenesis
Embryogenesis	High	Callus formation
	Low	Embryo formation

2.2.2.1. The induction of somatic embryogenesis

Indirect somatic embryogenesis induction requires the availability of an efficient callus culture system. The removal of auxin or substitution for a less potent auxin in the culture medium can usually attain development of somatic embryos from pre-embryogenic callus. Normally, 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthaleneacetic acid (NAA) are used for this purpose (Wang *et al*, 1990; Zimmerman, 1993; Gonsalves *et al*, 1995). The role of exogenous auxin appears to depend on the nature of the explant used in the experiment. Explants such as petioles, hypocotyls and single cells isolated from established suspension cultures (Zimmerman, 1993), require an extended, high-auxin treatment prior to auxin removal, whereas microcallus cells of alfalfa require only a short pulse of auxin before they are competent to initiate embryogenesis (Dudits *et al*, 1993). Auxin substitution is one of the most commonly used methods for the induction of somatic embryogenesis, but several other methods are also utilised, and one plant species is not necessarily limited to a specific method. Somatic embryogenesis of *B. napus* can, for example, either be induced by heat treatment, and it has been proposed that heat shock proteins (HSPs) may play a role in this induction process (Dong and Dunstan, 1996), or by treatment with colchicine, a microtube-depolymerizing agent (Zhao *et al*, 1996). Some plant species allow for the induction of embryogenesis via both the direct and the indirect pathways. *Freesia refracta* epidermal cells, for example, allow for the direct induction of somatic embryos, as well as indirect embryogenesis via intervening callus. Both pathways

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can be controlled and regulated by varying the concentrations of exogenous hormones (Wang *et al*, 1990).

2.2.2.2. Gene expression during somatic embryogenesis

Somatic embryogenesis can be regarded as the result of the reprogramming of gene expression which involves co-ordinated induction of a large number of genes during hormone-induced cell division and subsequent organised growth (Gyorgyey *et al*, 1991). Two genes from *Arabidopsis* have recently been cloned and provided insight into factors that control embryo formation. The *leafy cotyledon1* (*lec1*) gene encodes for a homologue of a subunit of the CCAAT-box binding transcription factor. This transcription factor functions as a central regulator of both early-and late embryogenesis (Lotan *et al*, 1998). Analysis of the Lec1$^-$ mutant phenotype, as well as gene expression analysis suggests that *lec1* functions exclusively during embryogenesis. Ectopic expression of *lec1* during post-embryogenic growth is sufficient to induce embryo development in vegetative tissues, but supresses post-embryonic development of transgenic plants. These results suggest a critical role for *lec1* in embryo development by directly controlling processes during early and late embryogenesis (Harada, 1999). The *pickle* (*pkl*) gene from *Arabidopsis* may also be important in controlling embryogenesis. Roots of *pkl* mutants accumulate lipids normally found in seeds and express embryo specific genes, and, when cultured on hormone free media, generate somatic embryos. Genetic manipulations of *lec1* and *pkl* gene expression can therefore establish a cellular environment that permits embryo formation to occur in the absence of hormone treatments (Harada, 1999).

The changes in cell metabolism as a result of embryogenesis may generate a need for proteins that function to ensure proper folding or assembly of cellular proteins. HSPs may function as chaperones during cell proliferation and differentiation. This is indicated by both the expression of HSPs in the absence of heat shock conditions and the altered response to high temperature at different developmental stages (Dong and Dunstan, 1996). The most commonly found HSPs are the small or low molecular weight HSPs. The structural features of these proteins suggest that they may have a function in protein aggregation (Gyorgyey *et al*, 1991). The accumulation patterns of *hsp* gene transcripts and HSPs during plant embryogenesis have been shown to be dependent on the specific plant species and the type of *hsp* genes (Prandle *et al*, 1995). The *hsp*18-1 and *hsp*18-2

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alfalfa transcripts accumulate at early embryonic stages and decrease during torpedo and subsequent embryo stages (Gyorgyey *et al*, 1991), whereas in carrot, the mid globular somatic embryos were found to synthesise and accumulate fewer low molecular weight HSP mRNAs and were more sensitive to heat shock than the other embryonic stages (Zimmerman *et al*, 1989). HSPs may therefore be considered as functional components in various assembly processes during embryonic cell differentiation.

Genes involved in somatic embryogenesis of several plant species, including, *Daucus carota* (Aleith and Richter, 1990) and *Zea mays* have been identified (Williams and Tsang, 1991). Several of the encoding transcripts were found during the globular stage of embryogenic development; they seem to be distinct in character and showed a remarkable diversity in primary structure and the putative function of the proteins involved. The functional importance of these genes expressed during the initial stage of somatic embryogenesis could, however, not be determined.

It is clear that no simple, uniform tissue culture systems exist for all plant species. The conditions for induction and maintenance of somatic embryogenesis may vary significantly from species to species, hampering the use of recombinant DNA technology for the improvement of many agronomically important species (Gawel and Robacker, 1990). The optimisation of an effective culture system before any genetic manipulation is attempted, is therefore of vital importance to ensure effective transformation and regeneration of explants. The genetic unravelling of the underlying process will undoubtedly aid in our understanding of the complex processes of embryogenesis.

2.3. CONSTRUCTION OF GENE CASSETTES FOR PLANT TRANSFORMATION EXPERIMENTS

The success of plant transformation experiments is largely dependent on the choice of promoters and selective agents used to select for transformation in the plant tissue. Typical features of gene cassettes include: i) appropriate transcriptional promoters and enhancers, ii) introns, iii) transcriptional terminators and 3' enhancers, iv) polyadenylation signals, v) codon usage, vi) transit sequences for subcellular localisation and stability of the gene product and vii) the appropriate reporter and selection genes enabling selection of transformed tissue (Birch, 1997). Integration patterns and transgene expression vary significantly among transformants, reflecting the influence of different sequences flanking

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the integration sites upon the expression of the transgene (Peach and Velten, 1991). Of even greater concern is the gene silencing phenomenon that has been attributed mainly to multicopy transgenes (Finnegan and McElroy, 1994). Certain promoter-reporter constructs have, however, been found to be silenced at high frequency, irrelevant of copy number and it would be important to determine which sequences in these constructs trigger gene silencing (Birch, 1997).

2.3.1. Promoter and enhancer elements: Driving the expression of the transgene

The first aspect to be considered in the construction of transgene transfer cassettes is the use of appropriate promoter and enhancer sequences. Early attempts to express transgenes in plants failed due to the inability of the plant transcriptional machinery to recognise foreign gene control sequences, particularly promoter sequences of many bacterial genes. Subsequently, control sequences of *Agrobacterium* (octopine promoter and terminator) as well as that of the cauliflower mosaic virus (35S promoter and terminator), known to be expressed in plants, were used to drive transgene expression (Birch, 1997). The need, however, for tissue-specific, developmental, environmental as well as growth regulator controlled regulation, prompted the identification of new enhancers and promoters, increasing the available repertoire for tailoring transgene expression to specific needs (Gallie, 1998). Several advances have been made in the development of promoters for the transformation of monocotyledonous plants. New promoters isolated include: the tissue specific CM3 promoter from barley (Coleman *et al*, 1997), the ethylene-inducible promoter of the maize hydroxyproline-rich glycoprotein gene (Menossi *et al*, 1997), and the maize ubiquitin promoter combined with an intron from the maize alcohol dehydrogenase (*Adh*) gene (Oritz *et al*, 1997). The role of *cis*-regulatory elements are also being elucidated and they are increasingly found in locations other than the 5' flanking region (Gallie, 1998). Furthermore, increasing evidence shows that cloned promoters generally retain the expression profiles of their native genes, both in the original species and in the transgenic plant species (Komari *et al*, 1998). These findings accentuate the importance of selecting the correct promoter and enhancer sequences when attempting to express transgenes in plants, since activity of promoters may be different from one plant species to the next.

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2.3.2. The selection of transformants

The selection of transformants in any transformation protocol is one of the key components and has the potential to be a serious pitfall in plant transformation experiments. Not only are the substances used inhibitory to plant regeneration to varying degrees, but their intrinsic nature, e.g. antibiotic or herbicidal, might also be undesirable to consumers of intended modified foodstuffs. A few types of selective agents are currently in use in plant transformations, although antibiotic and herbicide marker-genes are the most prevalent.

2.3.2.1. Antibiotic and herbicide-based resistance

Dominant genes encoding for either antibiotic or herbicide resistance are normally used when developing a selection system for plant transformation. These antibiotics, however, usually have negative effects on proliferation and differentiation. Differentiation of adventitious shoots is slowed down and some plants are insensitive or tolerant to selective agents. The marker genes remain in transgenic plants and may pose a health risk and their products therefore need to be assessed for safety as well as environmental impact. Introducing a second gene into transgenic plants already containing selectable marker genes also poses problems (Ebinuma *et al*, 1997).

The neomycin phosphotransferase gene *(nptII)* of the bacterial transposable element *Tn5* encodes for neomycin phosphotransferase (NPT) which is active against a limited group of aminoglycoside antibiotics, including kanamycin, geneticin, neomycin and paromycin (Novelli and Aldwinckle, 1993). It inactivates kanamycin by phosphorilating the 3'-OH of the 6-deoxy-6-aminoglucose-1-alpha sugar residue (Yoshikuro, 1989). Several other bacterial antibiotic resistance genes are available which produce enzymes that modify antibiotics to non-toxic forms. One class of these bacterial genes encodes gentamycin-3-N-acetyltransferase [AAC(3)] (Hayford *et al*, 1988). It inactivates certain aminoglycoside compounds (e.g. gentamycin) by acetylation of the amino group at position three of the 2-deaxystreptomine moiety. Two enzymes, AAC(3) type III and AAC(3) type IV, both with broad substrate specificity, were isolated independently and are functionally similar. It modifies gentamycin, kanamycin, tobramycin, neomycin and paromycin, whereas AAC type IV also inactivates opramycin and G418 (Brau *et al*, 1984). Normally a strong, constitutively expressed plant transcriptional promoter, such as the

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cauliflower mosaic virus (CaMV35S) promoter is used for the expression of these genes in plants. Both the *AAC* genes are used to confer resistance to gentamycin, and can be used for the retransformation of plants previously transformed with the *nos/nptII/nos* vector system (Novelli and Aldwinckle, 1993).

Another antibiotic, hygromycin B, is an aminocyclitol antibiotic frequently used as a selectable marker in plants. It is produced by *Streptococcus hygroscopicus* and inhibits protein synthesis by interfering with ribosomal translocation and aminoacyl-tRNA recognition, causing misreading of mRNA in pro- and eukaryotes (Zheng *et al*, 1994). The hygromycin phosphotransferase (*hph*) gene from *Escherichia coli* detoxifies hygromycin B through phosphorylation and the gene was successfully used as a selectable marker for the transformation of *E. coli, Saccharomyces cerevisiae* (Gritz and Davies, 1983), mammalian (Asselsberg and Pronk, 1993) and plant cells (Rothstein *et al*, 1987; Hayashimoto *et al*, 1990).

Herbicide resistance is widely used as selectable marker genes in plant transformation systems and as a trait in agronomically important crops (Gallo-Meagher and Irvine, 1996; Wehrman *et al*, 1996). Much attention has been focussed on the molecular mechanism involved in the enzymatic modification of herbicides. Resistance against a broad spectrum of phosphinotrichin (4-[hydroxy-(methyl)-phosphinoyl]-D,L-homoalanine (L-PPT) based herbicides can be obtained by introducing the *bar* gene (bialophos cloned from *Streptomyces hygroscopicus),* (Thompson *et al*, 1987), or the *pat* gene (phosphinotrichin acetyltransferase from *Streptomyces viridochromogenes*), (Strauch *et al*, 1988), into plants. These two genes encode phosphinotrichin acetyl transferases, consisting of 1183 amino acid polypeptides and exhibit an overall identity of 85% and 87% at amino acid and nucleotide level, respectively (Wohlleben *et al*, 1988). Both proteins are rapidly inactivated at temperatures higher than 45°C and are immediately digested in human gastric fluid, making them reasonably safe for human consumption.

2.3.2.2. Other selection strategies

Methotrexate resistance. Methotrexate (Mtxp), is one of the less frequently used selection agents. It is an antimetabolite and powerful inhibitor of pro- and eukaryotic dihydrofolate reductase (Dhfrp), ultimately preventing biosynthesis of glycine, thymine and purines (Cella *et al*, 1984; Rogers *et al*, 1988). Although plant cells and tissues are very

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sensitive to methotrexate, carrot cell lines showing up to 30x higher resistance than that of the parent line have been isolated (Cella *et al*, 1984). Methotrexate has nevertheless successfully been used for the selection of transformed tobacco (Rogers *et al*, 1988) as well as petunia (Eichholtz *et al*, 1989) cells.

Selection of marker free transgenic plants. The array of problems experienced with antibiotic and herbicide marker genes, led to the development of the multi-auto-transformation (MAT) vector system (Ebinuma *et al*, 1997). In essence it consists of a chimeric isopentyl transferase (*ipt*) gene, inserted into the maize transposable element, *Ac*. Plants transformed with this cassette exhibit an extreme shooty phenotype (ESP) due to the overproduction of cytokinin (Klee *et al*, 1987) and can easily be selected. However, during the *Ac* translocation process, about 10% of the *Ac* elements do not re-insert (Belzile and Yoder, 1992), effecting the loss of the *ipt* gene and subsequent loss of the ESP. Marker-free transgenic plants have the added advantage that hey can be visually selected (Fig. 2). The selection of single copy inserts are also possible, since the excision of one copy from a multi-copy insertion event would not eliminate the *ipt* gene and therefore not affect the ESP. These vector systems have been successfully used to transform tobacco as well as pine trees (Ebinuma *et al*, 1997).

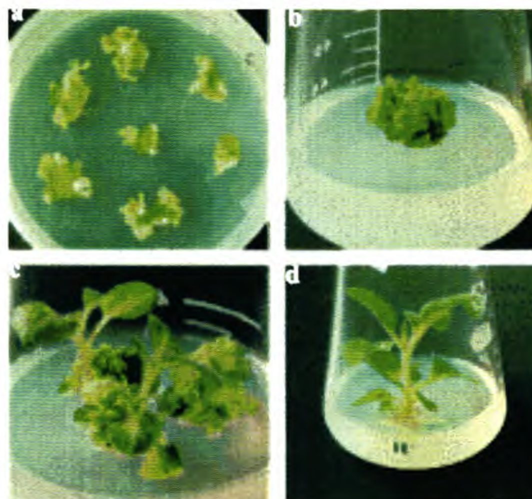

Fig. 2. ESP and loss of apical dominance due to *ipt* expression in tobacco (taken from Ebinuma *et al*, 1997). a) Leafdisks transformed with the MAT vector, b) ESP due to *ipt* expression, c) loss of ESP due to *Ac* excision, and d) regeneration of normal plants.

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2.3.3. The use of reporter genes in plant transformation experiments

Several reporter systems have been developed to facilitate the expression of transgenes in various plant organs, during different developmental stages and under various environmental conditions. These include the GUS (β-glucuronidase) gene fusion system (Jefferson, 1989), firefly luciferase (Gould and Subramani, 1988) as well as the green fluorescent protein (GFP) system (Haselhof *et al*, 1997) (Table 3). The GUS reporter system is one of the most commonly used systems, mainly due to the fact that it relies on a simple and cost effective assay system (Otha *et al*, 1990). The assay, however, is destructive and *in vivo* assaying of transgene expression patterns is not possible. The firefly luciferase and GFP reporter systems are more sensitive than the GUS system and have the further advantage of non-destructive *in vivo* assays (Barnes, 1990, Leffel *et al*, 1997). *In vivo* assaying using the luciferase system is, however, limited due to a dependence on the efficient delivery of luciferin, the substrate for luciferase, into plant cells (Gould and Subramani, 1988). To date, the GFP system is the only truly useful *in vivo* reporter system in plants, and with the development of better forms of the GFP gene, its usefulness should increase even further (Leffel *et al*, 1997).

Table 3. Comparison of commonly used reporter gene systems.

Reporter system	Effect/Assay	Advantages	Disadvantages	Reference
1. GUS	Histochemical assay. Intense blue coloration	Simple assay	Destructive assay Mostly *in vitro* applications Insensitive	Jefferson, 1989
2. Luciferase	Fluorescent assay	Non destructive *in vivo* assay	Sensitive equipment needed Luciferin difficult to absorb	Barnes, 1990
3. GFP	Fluorescent assay	Non destructive *in vivo* assay Sensitive Direct-visualisation Rapid screening	GFP-tag affects function of fused protein Signal-amplification difficult GFP folding is slow	Haselhof *et al*, 1997; Leffel *et al*, 1997

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2.4. The transfer of DNA to target plant tissue

Several methods of DNA transfer to plant cells have been developed in order to overcome many of the intrinsic hurdles posed by the diversity of agronomically important crop plants (Songstad *et al*, 1995). Methods can be divided into direct or *in vitro* DNA transfer as well as *in vivo* DNA transfer (Gelvin, 1998a). *In vitro* transfer methods include the more commonly used protoplast and biolistic transformation, as well as the lesser used silicon carbide method, microinjection and electroporation of intact cells (Hayashimoto *et al*, 1990; Keappler *et al*, 1990; Neuhaus and Spangenberg, 1990; Herbèrt *et al*, 1993; Hansch *et al*, 1996). *In vivo* transfer methods include the use of viral vectors that will result in transient but not stable transformation, as well as the well known *Agrobacterium tumefaciens* mediated transformation system. The selection of a DNA transfer system for plant transformation experiments is normally based on the plant species, types of explant and experimental layout. The use of direct transfer methods normally results in high transgene copy numbers as well as rearranged integration patterns. Most plants, however, are susceptible to these transformation methods, although they normally result in a low number of stably transformed cells (Birch, 1997). *A. tumefaciens* mediated transformation is, for many species, relatively efficient and low copy numbers of non-rearranged transgenes are frequently integrated into the plant genome. The system, however, is at best inefficient for many important crop species (such as many cereals and soybean) (Hiei *et al*, 1994; Gelvin, 1998a).

2.4.1. Direct DNA delivery techniques

2.4.1.1. Protoplast mediated DNA delivery techniques

In principle, protoplasts are considered ideal cells for DNA delivery and selection of transgenic events (Songstad *et al*, 1995). The most commonly used protoplast isolation methods involve the digestion of intact plant material and suspension cultures with mixtures of purified fungal cellulases, pectinases and hemicellulases in high osmotic solutions (Cocking, 1960; Lindsey and Jones, 1989; Koop *et al*, 1996). Culture conditions enabling the formation of cell walls, sustained mitosis and plant regeneration have been developed for several plant species including tobacco (Takebe *et al*, 1971), rice (Abdullah *et al*, 1986) and oyster mushrooms (Peng *et al*, 1993). The most commonly used procedure for direct DNA delivery into plant protoplasts involves treatment with

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polyethylene glycol (PEG). This treatment results in the alteration of plasma membrane properties effecting reversible permeabilisation that enables exogenous macromolecules to enter the cytoplasm (Lindsey and Jones, 1989). This technique involves incubating protoplasts with naked DNA in the presence of PEG and $CaCl_2$ and has been applied to dicotyledonous plants as well as a number of monocotyledonous plants, including ryegrass (Potrykus *et al*, 1985) and wheat (Lorz *et al*, 1985). More recently, chemical treatments of protoplasts using polybrene increased transformation frequencies significantly and it is likely that future modifications in protoplast culture systems and agents could enhance the transformation frequency even more (Antonelli and Stadler, 1990).

Another technique used in protoplast transformation involves subjecting protoplasts to high field strength electrical pulses to cause reversible permeabilisation of the plasmamembrane, enabling macromolecule delivery (Zimmerman, 1986). Crops transformed with the electroporation method include tobacco (Negrutiu *et al*, 1987), maize (Rhodes *et al*, 1988) and rice (Shimamoto *et al*, 1989). The primary advantages of electroporation over PEG or other chemical-mediated treatments are reproducibility of high frequency DNA delivery and the simplicity of the technique (Jones *et al*, 1987). Electroporation and PEG/chemical-mediated protoplast transformation suffer from the drawbacks and limitations inherent of protoplast cultures (Songstad *et al*, 1995). The most serious limitation involves determining optimal culturing and regeneration protocols for protoplasts (Peng *et al*, 1993). These conditions are species-specific and need to be optimised for every transformation experiment (Gelvin, 1998a). In order to avoid this drawback, transformation methods involving intact plant tissues were developed.

2.4.1.2. DNA transfer by microprojectile bombardment

Microprojectile bombardment involves the use of high velocity microprojectiles to deliver foreign genes into plant cells or tissues (Klein *et al*, 1987). Exogenous DNA or RNA molecules can be adsorbed to the surface of microscopic tungsten or gold particles, which are then accelerated to high velocities by a particle gun, enabling them to pierce cell walls and membranes (Songstad *et al*, 1995). Since the culture of protoplasts is not a prerequisite, this technique is preferred to methods that involve electroporation or chemical treatments (Komari *et al*, 1998). Commercialisation of the biolistic method of gene transfer has resulted in the Biolistics^{TM} PDS-1000 gene delivery device (Bio-Rad

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Laboratories, Richmond, California). The primary application of this system has been the transformation of species recalcitrant to the conventional *Agrobacterium* methods, and is widely used in the transformation of monocotyledonous plants (Birch, 1997). Plant species successfully transformed with this technique are numerous and include maize (Brettschneider *et al*, 1997), sugarcane, (Bower *et al*, 1996), rice (Cao *et al*, 1992), and grapevine, (Kikkert *et al*, 1996). Microprojectile transformation techniques normally lead to a low frequency of stably transformed cells with high copy numbers of transgenes, which could lead to homology-dependent suppression (transgene silencing) (Gelvin, 1998b). In an attempt to address this problem, the genes responsible for the generation of a T-DNA copy (*vir*D1 and *vir*D2) in *Agrobacterium tumefaciens* were transformed together with an artificial T-DNA, containing a selectable marker gene, simulating the natural T-DNA integration process of *Agrobacterium* in a so-called "Agrolistic" transformation. This approach resulted in low copy numbers of artificial T-DNA integrated in the plant genome, but transformants were isolated where the *vir*D1 and *vir*D2 genes integrated into the plant genome as well (Hansen and Chilton, 1996). Although microprojectile bombardment normally yields low numbers of stably transformed cells, it is also an excellent system for monitoring transient expression of transgenes, rendering it very attractive for genetic studies such as promoter analyses or *in planta* testing of vector constructs before the more lengthy process of stable transformation is attempted (Birch, 1997).

2.4.1.3. Other DNA delivery methods

Several novel DNA delivery methods have been developed in numerous attempts to transform traditionally recalcitrant plant species. As mentioned, these include the use of silicon carbide fibres, microinjection and electroporation of intact plant tissues. Due to the widespread applications of microprojectile bombardment, these methods are not widely used for direct DNA transfer and will need further research before their application could compete with the more commonly used delivery methods (Songstad *et al*, 1995). Drawbacks include the health risks involved with silicon carbide tissues (Ljungman *et al*, 1994), time consumption (microinjection) (Morikawa and Yamada, 1985), and the lack of standardised equipment for electroporation of intact tissues (Griesbach and Hammond, 1993).

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2.5. A PHYTOBACTERIUM USED FOR THE TRANSFER OF DNA TO PLANT CELLS

2.5.1. Plant bacterial interactions: A general overview.

Plant-bacterial interactions is a very diverse and complex process, most of which falls outside the scope of this review (for excellent reviews, the reader is referred to Baker *et al*, 1997 and Hutcheson, 1998). Here, issues underlying the processes and elements involved in the induction of the hypersensitive response of plants are discussed.

Bacterial plant pathogens have evolved a number of mechanisms to disrupt plant metabolism and cause disease. These mechanisms include the alteration of plant growth regulator balance and the production of toxins, plant cell wall degrading enzymes, and extracellular polysaccharides (Clarke *et al*, 1992). During the initial interaction with plant hosts, bacteria primarily interact with plant cell walls and this interaction appears to determine whether bacterial multiplication will take place (Sequeira, 1985).

Plants have developed a range of strategies to circumvent bacterial infection. The first line of defence (passive defence) is comprised of physical barriers that prevent phytobacteria from penetrating the host cell. These include the cuticle, consisting mainly of cutin and covered in a layer of wax, as well as epidermal cell walls, consisting of cellulose, pectins, hemicelluloses and structural proteins. Some bacteria have, however, developed mechanisms to overcome these barriers, whereas others infect opportunistically when structural damage provides an opening in the cell wall.

Once the structural barriers have been breached by the invading pathogen, the active defence responses of the plant come into play (Sequeira, 1985; Baker *et al*, 1997; Heuck, 1998). The characteristic defence response of plants is termed the hypersensitive response (HR) which often results in localised cell and tissue death at the site of infection, thereby limiting the further spread of the infection. Cell death is an important physiological process in plants and is achieved through a genetically conserved process (Pontier *et al*, 1998). Cell death genes are encoded by the plant genome and regulated by pathogen-related signals. Activation of cell death genes may result in an oxidative burst, or rapid production of active oxygen species (AOS) such as superoxide anions (O^{-2}_2), hydroxy radicals (OH^-) and hydrogen peroxide (H_2O_2). The production of AOS is one of the earliest detectable responses of plants treated with pathogen elicitors and precedes cell death (Kazan *et al*, 1998).

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The HR in turn, often triggers a battery of non-specific defence related processes throughout the plant, collectively known as systemic acquired resistance (SAR). The products of these processes, which include pathogenesis related (PR) proteins, e.g. PR-1 (anti-fungal), PR-2 (acidic and basic ß-1,3-glucanases), PR-3 (chitinase), PR-4 (anti-fungal), PR-5 (thaumatin-like protein) and PR-8 (acidic and basic class III chitinases), provide resistance to a wide range of pathogens for several days (Shirasu *et al*, 1996; Baker *et al*, 1997; Cordeiro *et al*, 1998). Whether a specific bacterial infection leads to plant disease or to a HR and subsequent responses, is determined by the initial recognition events between host and pathogen, of which the genetic basis is known as the gene-for-gene hypothesis (Shirasu *et al*, 1996; Cook, 1998).

During 1930 Flor defined the basic elements of gene-for-gene complementarity wherein single plant disease resistance (*R*) genes, and single complementary or matching avirulence genes (*avr*) in the pathogen account for host recognition, resulting in the HR (Leach and White, 1996). Recognition only occurs between dominant or semi-dominant *R* alleles and dominant *avr* alleles. If either partner lacks a functional allele, recognition and resistance does not occur and the phytopathogen will infect the plant and cause disease (Keen, 1990). It has been proposed that phytopathogen *avr* products function as ligands or signal molecules recognised by host *R* products that function as receptors in a specific interaction leading to plant resistance (Baker *et al*, 1997). Plant *R* genes play a crucial role in this recognition process; they seem to encode receptors that interact directly or indirectly with elicitors produced by pathogen *avr* genes. Isolated *R* gene products share either a leucine rich (LRR) motif or a serine–threonine kinase domain and can be grouped into five classes (Table 4) (Baker *et al*, 1997).

More than 40 *avr* genes have been cloned from bacterial plant pathogens (Hutcheson, 1998). Most of these are monocistronic and/or the activity is dependent on the product of a single open reading frame of an operon (Tamaki *et al*, 1988; Kobayashi, 1990; Leach and White, 1996). The biochemical and physiological function of Avr proteins are unknown. However, there are indications that the Avr proteins themselves are directly functioning as avirulence determinants (Hutcheson, 1998). Analysis of structure-function relationships of *avr* genes is suggestive of an extracellular role for the Avr proteins; phenotypic expression of Avr proteins, however, is not independent of the bacterium and is only associated with pathogenic bacteria (Leach and White, 1996; Hutchenson, 1998).

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The products of several bacterial genes are, either on their own (for non-host plants) or in combination with *avr* gene products (host plants), responsible for a HR. Several of these hypersensitive response and pathogenicity (*hrp*) genes have been identified in Gram-negative phytopathogenic bacteria, including pathovars of *Pseudomonas, Xanthomonas, Ralstonia* and *Erwinia* spp. A large number of isolated *hrp* genes share homology with genes encoding components of type III secretion systems of bacterial animal pathogens (Fenselau *et al*, 1992) that has been proven to be essential for plant and animal pathogenesis. The characteristic phenotype associated with *hrp* mutants is the inability to cause disease on susceptible host plants, as well as the inability to elicit the HR on resistant cultivars of susceptible plants and non-host plants (Lindgren *et al*, 1986) (For a recent review on the role and structure of *hrp* genes during plant-bacterial interactions, see Lindgren, 1997).

Bacteria commonly use type III and IV secretion/transfer systems to deliver protein or protein-DNA complexes into the host plant cell. The Avr proteins from several phytopathogenic *Pseudomonas* and *Xanthomonas* spp., Yop proteins from *Yersinia*, and virulence factors from *E. coli*, are transferred by type III secretion pathways (Baker *et al*, 1997). Proteins injected from type III secretion pathways often resemble eukaryotic factors with signal transduction functions capable of interfering with eukaryotic signalling pathways. These proteins may result in the disarmament of host immune responses by the redirection of cellular signal transduction or may lead to cytoskeletal reorganisation, establishing subcellular niches for bacterial colonisation. In doing so, they facilitate a highly adapted pathogenic strategy of "stealth and interdiction" of host defence communication lines (Baker *et al*, 1997; Hueck, 1998). Although vast differences exist between secreted proteins, the type III secretion pathway is conserved in mammalian pathogens as well as in plant pathogens (e.g. *Yersinia* and *Erwinia*), clearly illustrating the array of diseases depending on a single pathogenicity mechanism (Heuck, 1998).

Three models have been suggested to describe the interaction between pathogen encoded *avr* and *hrp* genes, and plant *R* genes (Leach and White, 1996). In the simplest model, the primary *avr* product is the elicitor or ligand, which is exported from the cell via the Hrp apparatus and interacts directly with the plant receptor, likely the product of the corresponding *R* gene. In Model 2 the product of the avr gene directs the synthesis of, or modifies a race specific elicitor. Model 3 proposes the transfer of a proteinaceous elicitor

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or enzyme into the plant cell and is based on analogy with the strategies of some mammalian bacterial pathogens (Leach and White, 1996).

Although the HR consists primarily of localised cell death, it is not yet clear if the interaction between plant *R* and bacterial *avr* genes is directly responsible for biochemical or physiological changes resulting in cell death. A mechanistic understanding of this gene-for-gene complementarity promises considerable insight into the basis of disease defence in plants, as well as the probability of improved disease control in practical agriculture. Plant-pathogen interactions, as well as the HR, still remain a complicated and mostly unelucidated process, the understanding of which has great scientific and economic importance.

Table 4. Isolated plant resistance genes (adapted from Baker *et al*, 1997)

Class	R gene	Source plant	Pathogen	Avr gene	Structure
1	*RPS2*	*Arabidopsis*	*Pseudomonas syringae* pv. *tomato*	*avrRpt2*	LZ-NBS-LRR
	RPM1	*Arabidopsis*	*P. syringae* pv. *maculicola*	*avrRpm1*, *avrB*	LZ-NBS-LRR
	Prf	Tomato	*P. syringae* pv. *tomato*	*avrPto*	LZ-NBS-LRR
	N	Tobacco	Tobacco mosaic virus	*TMV* Replicase?	TIR-NBS-LRR
	L^6	Flax	*Melampsora lini*	AL^6	TIR-NBS-LRR
	M	Flax	*M. lini*	*AM*	TIR-NBS-LRR
	RPP5	*Arabidopsis*	*Peronospora parasitica*	*AvrPp5*	TIR-NBS-LRR
	I_2	Tomato	*Fusarium oxysporum*	Unknown	NBS-LRR
2	*Pto*	Tomato	*P. syringae* pv. *tomato*	*avrPto*	Protein kinase
3	*Cf-9*	Tomato	*Cladosporum fulvum*	*Avr9*	LRR-TM
	Cf-2	Tomato	*C. fulvum*	*Avr2*	LRR-TM
	$HS1^{pro-1}$	Sugar beet	*Heterodera schachtii*	Unknown	LRR-TM
4	*Xa21*	Rice	*Xanthomonas oryzae pv. oryzae*	Unknown	LRR, protein kinase
5	*Hm1*	Maize	*C. carbonum*, race 1	None	Toxin reductase

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2.5.2. The genetic transformation process of *Agrobacterium* as it occurs in nature.

2.5.2.1. *Agrobacterium* as a pathogen

Agrobacterium is a Gram-negative soil pathogen of the family *Rhizobiaceae* that naturally infects wounded plant cells in dicotyledonous plants. Well-known species include *A. tumefaciens, A. rhizogenes, A. vitis, A. radiobacter* and *A. rubi*, of which only *A. tumefaciens, A.vitis* and *A. rhizogens* are pathogenic, causing either crown galls, or in the case of *A. rhizogenes*, hairy root disease (Ophell and Kerr, 1990; Hooykaas and Schilperoort, 1992; Yanagi and Yamasato, 1993). *A. tumefaciens* was first indicated as the causative agent of crown galls in 1907 and has since been extensively studied, initially to understand the mechanisms of crown gall tumour induction. It was hoped to apply this knowledge in cancer research, but most of the studies were conducted to understand the bacterium's unique ability to transfer a discrete part of its DNA into plant cells (Smith and Townsend, 1907; De la Riva *et al*, 1998). A large, extra-chromosomal element, the tumour inducing (Ti) plasmid, carries most functions necessary for DNA transfer. *A. tumefaciens* (as well as *A. rhizogenes* and *A. vitis*) transfers a particular segment (T-DNA) of the Ti plasmid into the plant nucleus where it is stably integrated and transcribed, causing the crown gall disease (Zambryski, 1988; Hooykaas and Schilperoort, 1992; Zambryski, 1992). The T-DNA is defined by two 24-bp direct repeats (T-DNA borders), flanking the region. The T-DNA contains genes for growth regulator autonomy and the synthesis of a wide range of opines (Klapwijk *et al*, 1977; Bevan, 1984; Dessaux *et al*, 1988). These opines, produced by the condensation between amino acids and sugars, are excreted by crown gall cells and utilised by *A. tumefaciens* as a carbon and nitrogen source, creating a favourable environmental niche for the bacterium.

The genes encoding the transfer functions of the T-DNA are located outside the T-DNA borders and are comprised of six to eight virulence (*vir*) operons (*vir*A-H) that are either absolutely essential for (i.e. *vir*A, *vir*B, *vir*D, and *vir*G) or that enhance the efficiency of (*vir*C and *vir*E) plant cell transformation (Rogowsky *et al*, 1990). The *vir* region operates in *trans* and is the master switch for the transformation process, since the expression of the *vir* loci is regulated to occur only when *Agrobacterium* is in the presence of susceptible plant cells (Zambryski, 1988).

Several chromosomal loci have been uncovered that play important roles during bacterial attachment to plant cells, as well as the subsequent bacterial colonisation and

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Table 5. Summery of the major steps within the *Agrobacterium*-Plant interaction (refer to Fig. 3 for a more detailed visualisation)

Cellular process	Effect	Bacterial proteins involved	Gene location
Cell-cell recognition	Agrobacteria binds to plant cell surface re-ceptors	ChvA, ChvB, PscA, Att	Bacterial chromosome
Signal transduction	Recognition of plant cell signal molecules and activation of T-DNA transport pathway	ChvE, VirA, VirG,	Bacterial chromosome, *vir* loci of Ti plasmid
Transcriptional activation	Expression of the *vir* genes after phos-phorylation of the transcriptional activator	VirG	*vir* loci of Ti plamid
Conjugal DNA mobilisation	T-DNA border nicking and mobilisation of a single stranded copy of the T-DNA.	VirD1, VirD2, VirC1	*vir* loci of Ti plamid
Intercellular transport	Formation of protein-T-DNA complex and transport into cytoplasm of host plant cell	VirE2, VirE1, VirD2, VirD4, VirB4, VirB7, VirB9, VirB10, VirB11	*vir* loci of Ti plamid
Nuclear import	Interaction with the host cell nuclear localisation signal receptors and transport through the nuclear pore	VirD2, VirE2	*vir* loci of Ti plamid
T-DNA integration	Integration into the plant cell genome, synthesis of the second strand of T-DNA	VirD2, VirE2	*vir* loci of Ti plamid
T-DNA expression and gall formation	Expression of T-DNA genes within the plant genome.	Oncogene proteins	T-DNA of Ti plasmid

are therefore important for the transformation process. The *chv*B encodes a membrane protein of approximately 235 kilodaltons (kDa) that acts as an intermediate in the synthesis of cyclic ß-1-2-glucan, while the *chv*A gene may encode a transport protein (Zambryski, 1988; Cangelosi *et al*, 1989). The *chv*E gene is required for the sugar enhancement of *vir* gene induction and bacterial chemotaxis (Charles and Nester, 1993), while the *cel* locus plays a role during the synthesis of cellulose fibrils (Matthysse, 1983a). Other chromosomal loci of importance are the *pscA* locus involved in the synthesis of cyclic glucan and acid succinoglycan and the *att* locus, involved in the synthesis of cell surface proteins (Tomashaw *et al*, 1987). Several essential steps are involved in the *Agrobacterium* infection process of plant cells (Table 5) (Fig. 3), but bacterial chemotaxis

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and colonisation, induction of bacterial virulence, generation of T-DNA transfer complex, T-DNA transfer and the integration of T-DNA into plant genome will be discussed in more detail.

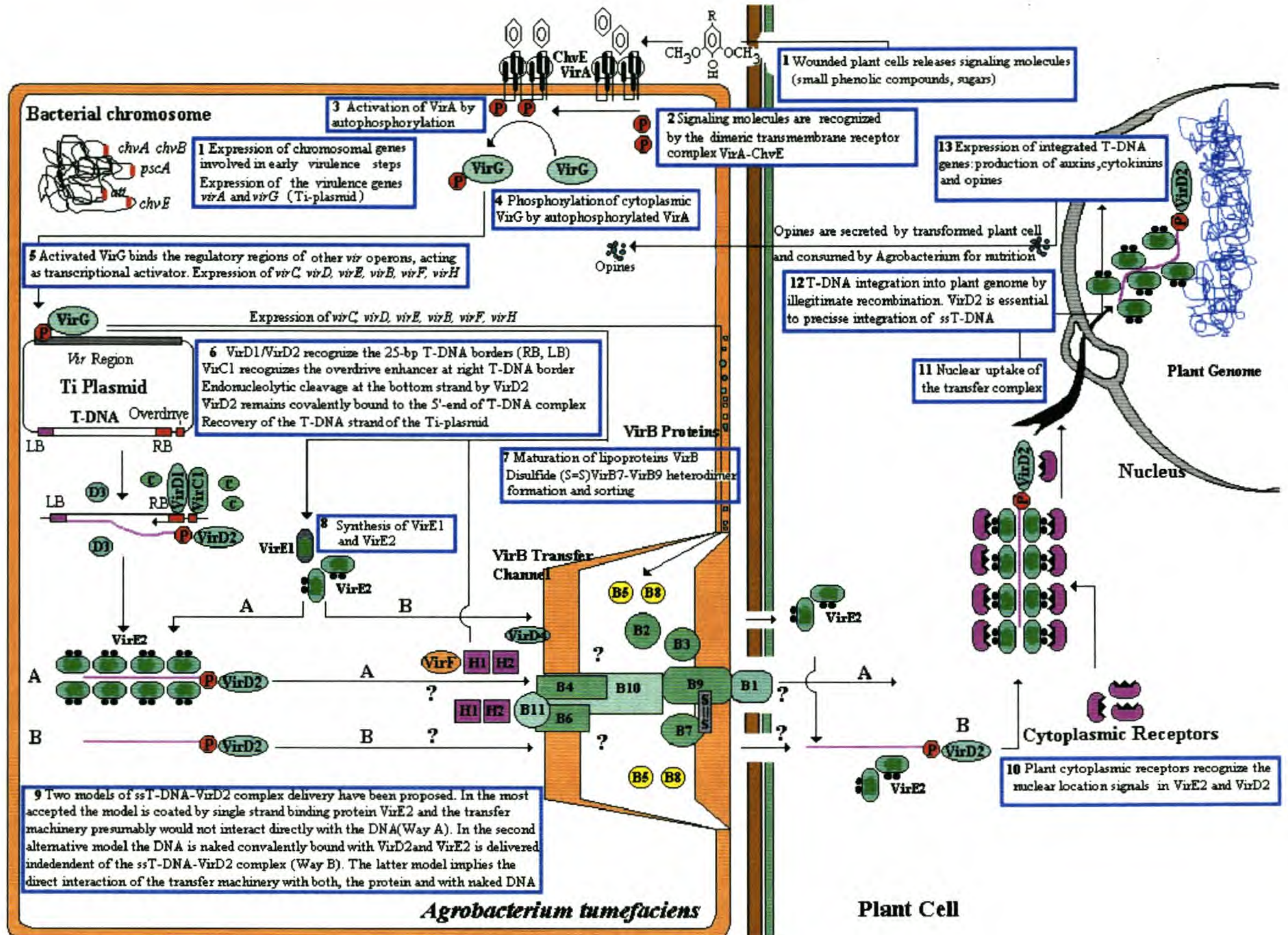

Fig. 3. The basic steps in transformation of plant cells by *Agrobacterium tumefaciens* (taken from De la Riva *et al*, 1998).

2.5.2.2. The infection process.

Agrobacterial chemotaxis. The most prominent structure in directional bacterial movement is the flagellum. In *E. coli,* a counter-clockwise (CCW) flagellar rotation causes the flagella to bundle and allows the cell to move in a straight path. A switch to clockwise rotation disentangles the bundle, causing the bacterium to tumble in a random fashion, thus allowing the bacteria to change direction. By altering the balance between runs

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(moving in a straight path) and tumbles, the bacteria are able to respond to extracellular stimuli (Zhu *et al*, 1997). Using specific transmembrane receptors, a five-member family of transmembrane methyl accepting chemotaxis proteins (MCPs), *E. coli* perceives spatial gradients as temporal changes in attractant or repellent concentrations. In effect, the cell compares its environment during the past second with the previous three-four seconds and responds accordingly (Spiro *et al*, 1997).

The signals initiated at these receptors are transduced through the cytoplasm by the products of the chemotaxis genes, *cheA*, *-B*, *-R*, *-W*, *-Y* and *-Z*. CheA (an auto-phosphorylating protein kinase) is autophosphorylated at a rate similar to which the ligand binds to the upstream chemoreceptors; the phosphoryl group is then transferred to the response regulators CheB and CheY (Shaw, 1991; Appleby and Bourret, 1998; Jasuja *et al*, 1999). CheY-P (phosphorylated CheY) interacts with the flagellar switch proteins and affects a reversal of flagellar rotation from CCW to CW, causing the bacteria to start tumbling and randomly change the direction of travel (Fig. 4). The autophosphorylation and phosphotransfer activities of the CheA kinase, as well as the dephosphorylation rate of CheY-P, control CheY-P levels *in vivo*. The dephosphorylation rate is controlled by the intrinsic autophosphatase activity of CheY and the CheZ activity (Zhu *et al*, 1997; Scharf *et al*, 1998).

Changes in the MCP methylation state are responsible for sensory adaptation. CheB-P, a methyl esterase, removes methyl groups from the MCPs, while CheR, a methylesterase, reversibly methylates these sites. When attractants interact with the MCPs, either directly, or indirectly via a periplasmic binding protein, the MCPs undergo a conformational change, and by interacting with CheA and W, suppress the phosphate flux. This results in a block of CheY-P accumulation, suppressing tumbles. Similarly, CheB-P will not be formed, and the MCP will acquire methyl groups resulting in an extended run (Appleby and Bourret, 1998). MCP methylation therefore provides the bacterium with a measure of previous receptor occupancy to compare with the current level. Methylated MCPs are less inhibitory to phosphate flux, allowing CheY-P to accumulate after an extended run. If attractant levels drop, MCP will signal non-occupancy, the phosphate flux increases, CheY-P is formed and a shortened run results (Jeziore-Sassoon *et al*, 1998).

The initial infection process of *Agrobacterium* in soil requires the proximity of plant roots towards which the bacterium moves and attaches (Chesnokova *et al*, 1997). Flagellar arrangement in *Agrobacterium* is unusual in the sense that two to four lateral

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filaments as well as a polar tuft of two flagella are typically found. The polar and lateral filaments, however, are not necessarily equivalent. This arrangement most probably facilitates the observed bacterial chemotaxis that leads to the infection process. The fact that cells are frequently attached in a polar fashion confirms this notion (Shaw, 1991; Chesnokova *et al*, 1997). *Agrobacterium* flagella rotate unidirectionally and in a clockwise fashion, driving the bacterium at approximately 60 $\mu m.s^{-1}$. *Agrobacterium* will, when exposed to an attractant gradient, move in the favourable direction due to a highly sensitive chemotaxis system, activated by a range of sugars and amino acids. Although the majority of chemotactic responses in *Agrobacterium* appear to be chromosomally encoded, chemotaxis also occurs in reaction to plant wounds (Ashby *et al*, 1988).

Wounded plant tissue exudes a range of compounds, some of which might play a role during chemotaxis. These include Ti plasmid-based *vir*-inducers, weak or non-inducers, attracting cured *Agrobacterium* strains; and non-inducing, non-attracting compounds (Shaw, 1991). *Vir* inducers are only considered to be chemo-attractants if *vir*A and *vir*G are present on the Ti plasmid. Strong *vir*-inducers such as acetosyringone, sinaptic acid and syringic acid are induced at a level 500-fold lower than the maximal *vir*-inducing concentration, suggesting a multifunctional role for the *vir*G/A system. At low *vir*-inducer concentrations, *vir*G/A induces chemotaxis, while at high *vir*-inducer concentrations, it triggers *vir*-induction (Shaw, 1991; Ashby *et al*, 1988). The constitutive level of *vir*G/A expression seems to be sufficient for chemotaxis and *vir*-induction does not seem to play a role in this process. In fact, when peak chemotaxis conditions exists, *vir*-induction is unlikely to occur. Moreover, *vir*-induction at high acetosyringone concentrations suppresses chemotaxis or motility, thus preventing exit from the wound sites (Shaw, 1991). In *Agrobacterium*, the *Vir*G/A signalling system is similar to other two component chemoreceptor-regulator systems. *Vir*A functions as an inner membrane chemoreceptor where the acetosyringone–binding action is situated within the second transmembrane spanning domain (Shaw, 1991). A putative chemotaxis operon has been identified in *A. tumefaciens* (Wright *et al*, 1998), which shares significant homology with that of chemotaxis operons found in the α-subgroup of proteobacteria. CheA and MCP-like mutants have proven that the cluster is involved in chemotaxis. It is, however, not clear which of the components interact with VirA or VirG (Wright *et al*, 1998).

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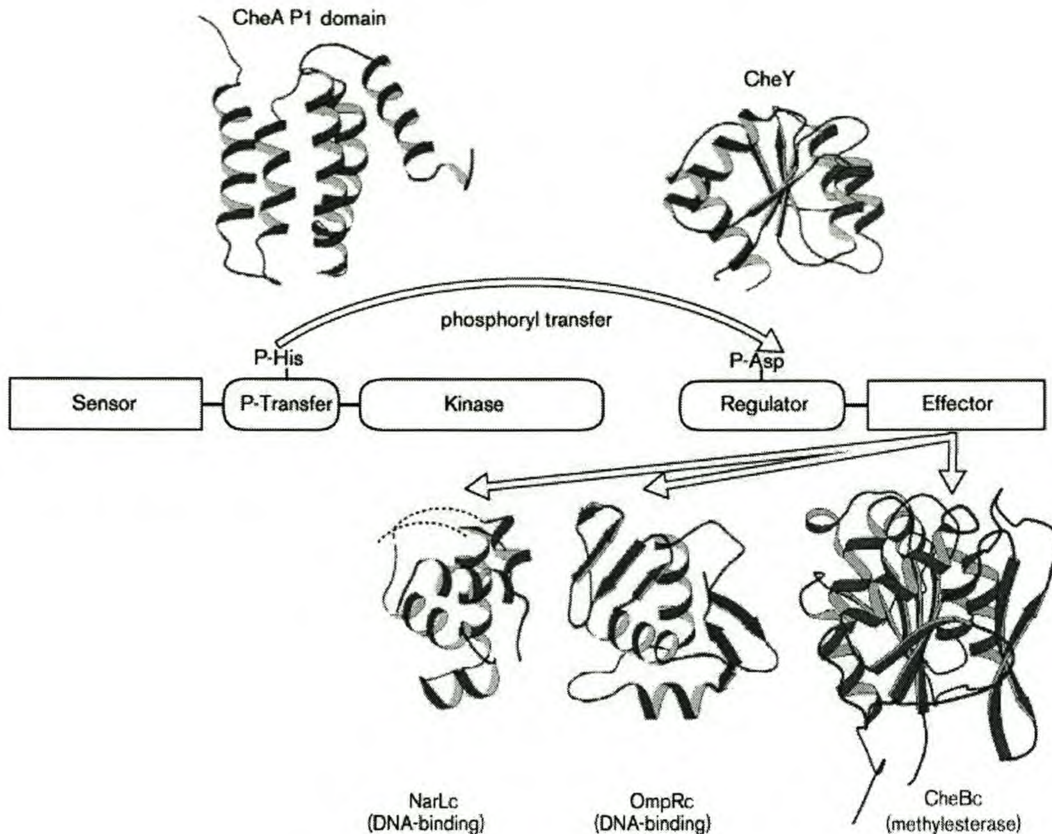

Fig. 4. Domain structure of two-component signal transduction proteins (taken from Goudreau and Stock, 1998).

Agrobacterial attachment. The next crucial step in the *Agrobacterium* infection process involves the attachment and colonisation of bacteria to wounded plant cells. Successful attachment is a prerequisite for agrobacterial colonisation; mutagenesis studies showed that non-attaching mutants lose their tumour-inducing capacity. Four chromosomal genes (*chv*A, *chv*B, *exo*C and *att*) code for the attachment of bacteria to plant cells, whereas *chv*B and *exo*C are also required for synthesis of a low molecular-weight, cyclic β-1,2-glucan (Cangelosi *et al*, 1989; Hooykaas and Schilperoot, 1992). These and other polysaccharides of the *Agrobacterium* cell surface seem to play an important role during the attachment process (De la Riva *et al*, 1998). The *exo*C (*psc*A) locus has been implicated in the production of an acidic, exopolysaccharide (EPS), directly correlated with the attachment of the wild-type bacterium to plant cells (Cangelosi *et al*, 1987; Thomashow *et al*, 1987). Moreover, if lipopolysaccharides (LPS) prepared from virulent strains were applied to bean leaves before bacteria were inoculated, the formation of tumours was inhibited (Cangelosi, 1989).

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Members of the *Rhizobiaciae* family of plant-associated bacteria, including *Agrobacterium*, also produce capsular polysaccharides (K-antigens). They are distinct from LPS, lacking a lipid anchor and having a highly ionic nature. Evidence suggests that the *att* locus, a 20 kb chromosomal fragment, may have a role in the production of K-antigens, as well as other genes implicated in attachment and virulence (Reuhs *et al*, 1997). Genes to the left of this region (*attA1-attH*) encode for proteins with homology to the ABC transporter systems in several bacteria, and mutational analyses of these genes suggest a signalling function (Matthysse *et al*, 1996; Reuhs *et al*, 1997; Matthysse and McMahan, 1998). Genes situated to the right off the *att* locus seem to encode proteins with a structural role in attachment. One such gene, *attR*, is thought to be required for the synthesis of an acidic polysaccharide which is correlated with the attachment of the C58 wild-type strain to carrot cells (Reuhs *et al*, 1997).

Following the initial attachment to plant cells, the bacteria synthesise cellulose fibrils, resulting in their tight binding to the plant cells (Matthysse, 1983b). The genes responsible for cellulose synthesis (*cel* genes) are located on the bacterial chromosome, in close proximity to the *att* genes. The fibrils definitely enhance the infection process, since mutants lacking the ability to synthesise cellulose show a significant reduction in virulence (Matthysse *et al*, 1995a, b).

Induction of the *Agrobacterium* virulence system. The generation and transfer of the T-DNA copy(s) is mediated by the 30-40 kb *vir* region of the Ti plasmid. This region is composed of at least six operons (*vir*A-*vir*G) and two non-essential operons (*vir*F and *vir*H) (Hooykaas and Schilperoort, 1992; De la Riva *et al*, 1998). The sizes of the individual operons differ: *virA, virG* and *virF* each contains a single gene; *virE*, *virC* and *virH* each contain two genes, whereas *virD* and *virB* comprise four and eleven genes, respectively (Zambryski, 1992; De la Riva *et al*, 1998). The operons are tightly regulated by the *virA-virG-chvE* signal transduction system. Central to the process where *Agrobacterium* senses environmental conditions favourable for T-DNA transfer, is the VirA protein, a dimeric transmembrane sensor protein that detects signal molecules originating from wounded plant cells (Engström *et al*, 1987; Peng *et al*, 1998).

The *vir*A gene is fully activated by several classes of signals typically originating from wounded plant cells, e.g. acidic pH, phenolic compounds such as acetosyringone and monosaccharides such as glucose, galactose and arabinose (Cangelosi *et al*, 1990;

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Hooykaas and Schilperoort, 1992). The VirA protein consists of an input domain, a transmitter domain and a receiver domain (Doty *et al*, 1996). The receiver domain includes a linker, a kinase and a carboxyl-terminal region that is homologous to the phosphorylated receiver domain of VirG (Doty *et al*, 1996; Peng *et al*, 1998). The linker domain is responsible for perceiving the presence of phenolic compounds and acidity, and apparently restricts the recognition of phenolic inducers by VirA (Peng *et al*, 1998).

ChvE, a sugar-binding protein encoded by the chromosomal *chvE* gene, is homologous to two periplasmic proteins involved in sugar recognition and uptake in *E. coli.* This protein has several roles. It functions in the uptake of specific sugars, the chemotaxis to these sugars, and in the VirA-VirG two-component signal transduction system of *Agrobacterium* (Kemner *et al*, 1997). Monosaccharide detection by VirA is an important amplification system and responds to low levels of phenolic compounds such as acetosyringone. The induction of this system is only possible through the interaction with ChvE, which interacts with the various inducing monosaccharides. The ChvE::monosaccharide complex in turn interacts with the periplasmic domain of VirA, exposing the amphiphatic helix to small phenolic compounds, suppressing the inhibitory effect of the periplasmic domain on the function of VirA, and thereby allowing autophosphorylation of VirA (Doty *et al*, 1996; Peng *et al*, 1998). Phosphorylated VirA transfers the phosphate group to VirG, which binds to the so called *vir* boxes upstream of each *vir* promoter. This interaction activates transcription from these promoters (Kalogeraki and Winans, 1998).

The regulation of the *vir*G gene is complex since transcription occurs from two tandem promoters that is situated 50 nucleotides apart (Zambryski, 1988; Chang and Winans 1996). These two promoters are designated P1 and P2 and induction of the P2 promoter appears to be independent of any Ti plasmid-encoded protein and usually occurs as a result of extracellular acidity. The P1 promoter in contrast, is induced by phosphorylated VirG as well as phosphate starvation. The induction of *vir*G by extracellular acidity, as well as phosphate starvation independent of the phosphorylation status of VirG, is speculated to establish a pool of VirG sufficient for the induction of the *vir* regulon. After phosphorylation this pool could potentially express *vir*G in a strong and positive autoregulated fashion (Chang and Winans, 1996).

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Generation of a T-DNA transfer complex. Upon activation of the *vir* genes, a single-stranded (ss) T-DNA copy, representing the bottom strand of the T-DNA, is generated (Hooykaas and Schilperoort, 1992). The T-DNA is flanked by a 24 bp repeat that is recognised by two *virD*-encoded proteins, VirD1 and VirD2. Together these proteins determine an endonuclease activity, capable of nicking the border repeats at a defined site. After endonucleotic cleavage and ss-T-DNA displacement by DNA replication, VirD2 remains covalently attached to the 5'-end via conserved tyrosine residues, Tyr-29 and Tyr-22, respectively, preventing exonucleolytic attack (De la Riva *et al*, 1998). VirD1 is dispensable for VirD2 mediated cleavage of the ss substrate *in vitro*, but is required for cleavage of supercoiled double-stranded substrate under the same conditions and for the generation of the free T-DNA complex *in vivo* (Christie, 1997). Although VirD1 is absolutely required for the nicking reaction *in vivo*, the precise function of this protein is still unknown (Relic *et al*, 1998). Only the N-terminal portion of VirD2 is required for the nicking reaction, whereas the C-terminal domain might be involved in the later stages of DNA transformation. VirD2 contains two nuclear localisation signals (NLS) at each end of the protein. The signal at the N-terminal domain is of the single-cluster type, whereas the signal located at the C-terminal belongs to the bipartite NLS group (Koukolikova-Nicola *et al*, 1993).

Deletion of the T-DNA right border almost completely abolishes T-DNA transfer, whereas deletions or mutations in the left border repeat lead to only a slightly lower frequency of transfer. This indicates a 5'–3' directional T-strand displacement from the right to left border (Fig. 5) (Hooykaas and Schilperoort, 1992) and termination takes place even if the left border is mutated or completely absent. The left border does sometimes initiate synthesis, but the efficiency is much lower compared to that of the right border (De la Riva *et al*, 1998). The 24 bp border sequences are highly conserved, implying that they all might capable of directing T-DNA transfer. Non-selective use of T-borders might, however, lead to non-productive T-DNA transfer events since genes not located between the T-DNA borders (i.e. non-oncogenes) would also be transferred.

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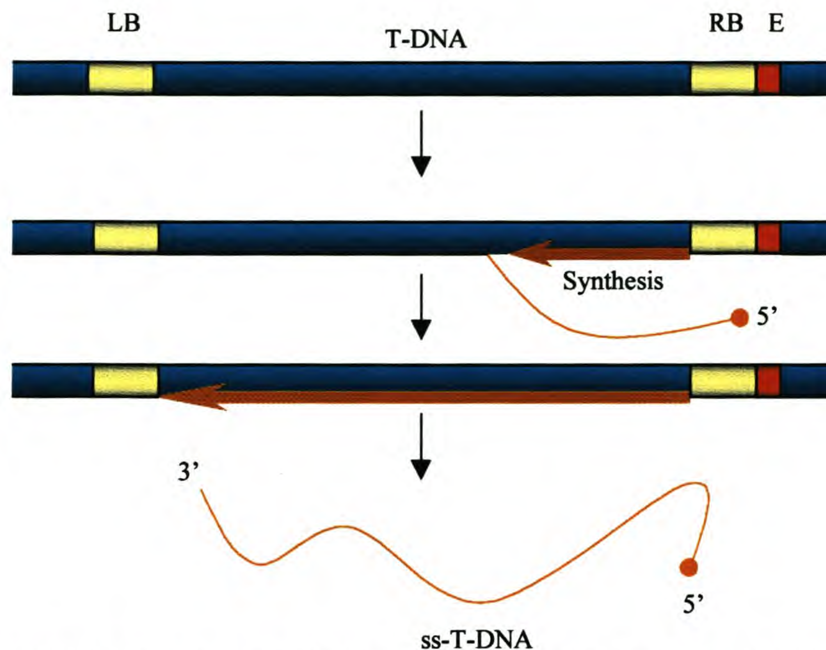

Fig. 5. A simplified schematic presentation of ss-T-strand synthesis, The red dot represents VirD2 covalently attached to the 5'-end of the T-DNA copy (adapted from Hooykaas and Schilerpoort, 1992).

A 25 bp DNA sequence (5'-TAARTYNCTGTRTNTGTTTGTTTG-3'), situated to the 3' end and adjacent to the right border sequences of the octopine Ti plasmid is essential for the efficient transfer of left (TL) and right (TR) T-DNA elements. This sequence is designated the overdrive element and acts similar to an enhancer element. It can stimulate transfer when placed upstream or downstream, as well as at a distance (up to 6 kb from the 24 bp border repeats) (Zambryski, 1988; Zambryski, 1992). VirD2 has been shown to interact specifically with both the T-DNA borders as well as the overdrive sequence. The *vir*C operon also enhances T-DNA processing through an interaction between the VirC1 protein and the overdrive sequence (Toro *et al*, 1989). Deletion of the *vir*C operon is followed by attenuation of virulence of *Agrobacterium* strains (De la Riva *et al*, 1998). It seems likely that the overdrive acts to efficiently direct the VirD2 endonuclease to the adjacent border sequences and that the *vir*C products may be important in this process (Toro *et al*, 1989). No sequences homologous to those of the octopine overdrive sequences are found adjacent to nopaline T-DNA borders, suggesting that if a different nopaline sequence has the same function, the nopaline-type enzymes that recognise the T-DNA border regions may have different requirements for binding (Zambryski, 1992).

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T-DNA transfer. In order to integrate stably into the plant genome, the ss-T-DNA-complex from *Agrobacterium* must translocate across membranes of diverse cell types, including the cytoplasmic and outer membranes of the *Agrobacterium* envelope, the plant plasma membrane, and the nuclear membrane (Christie, 1997). Several virulence proteins play a role; e.g. VirD4, at least 10 of the 11 *virB* operon encoded proteins, and VirE1 and E2, which are absolutely essential in the transfer process (Anderson *et al*, 1996; Fullner, 1998). According to the most accepted model of T-DNA transfer, the 69 kDa VirE2 protein, containing two NLSs, coats the ss-T-DNA-VirD2 complex inside the *Agrobacterium* cell (Zupan *et al,* 1996) and this complex is then transported via the transport machinery (De la Riva *et al*, 1998).

The association with the VirE2 protein results in conformational changes in the ss-DNA-complex, causing the formation of semi-rigid and hollow cylindrical filaments with a telephone-cord-like structure, 12.6 nm wide with a density of 58 kDa/nm. Since the outer diameter of these molecules exceeds the size-exclusion limit for nuclear diffusion channels (9 nm), it stands to reason that these molecules must be actively transported into the cell nucleus and therefore have to contain exposed and functional NLS sequences. The translocation of the 12.6 nm filament is also suspected to be unimpeded by binding of host cell receptors such as karyopherin α or its plant homologues that probably associate with multiple NLSs of VirE2 (Citovsky *et al*, 1997; Dombek and Ream, 1997). The NLSs from VirD2 and VirE2 are bipartite sequences that contain two interdependent basic domains, both necessary for full activity. The NLS-binding proteins recognise NLSs and direct NLS-containing proteins to nuclear pores, where transport into the nuclei occurs via ATP-dependent active transport through the nuclear pores (Dombek and Ream, 1997). Mutations in the NLS from VirD2 reduce, but do not totally abolish ss-T-DNA transfer and its integration, implying that the NLSs from VirE2 not only play an important role during nuclear targeting of the T-DNA complex, but can also assume the function of the VirD2 NLS (Citovsky *et al*, 1997).

Several recent studies have, however, prompted a re-evaluation of the T-complex model as originally proposed. An avirulent *Agrobacterium* strain lacking T-DNA, but containing a functional *vir*E2 gene, was able to functionally complement an avirulent *Agrobacterium* strain lacking a functional *vir*E2 gene, restoring virulence (Otten *et al*, 1984). This extracellular complementation implies that the T-DNA and VirE2 protein could be exported separately into the plant and that the T-strand does not have to be coated

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with VirE2 protein in the bacterium (Gelvin, 1998b). The accumulation of T-DNA strands in *vir*E2 mutants equals that of wild-type strains, indicating that VirE2 does not stabilise T-strands inside the bacterium. Transgenic tobacco plants producing VirE2 are susceptible to *Agrobacterium Vir*E2 mutants, indicating that VirE2 is only essential inside the plant cell (Dombek and Ream, 1997). Sundberg *et al* (1996) furthermore showed that VirE1 is essential for VirE2 export, but not for T-DNA export, again indicating that VirE2 is exported independently from the T-DNA strand. Collectively, these data suggest that the ss-T-DNA-VirD2 complex is exported separately from the VirE2 protein, and that assembly only takes place inside the plant cell (Gelvin, 1998b).

The apparatus for transporting the T-DNA complex as well as VirE2 across the *A. tumefaciens* envelope has been proposed to consist of most or all the 11 products of the ~9.5 kb *vir*B operon and the *vir*D4 gene (Fig. 6) (Fernandez *et al*, 1996a). Experimental data showed that the *virB* operon products associate with the *A. tumefaciens* envelope (Christie, 1997). Subcellular fragmentation studies have shown that most VirB proteins are found fractionating with both membranes, confirming the notion that these proteins assemble as a membrane-spanning protein channel (Fernandez *et al*, 1996a; Christie, 1997). Fusion analysis between *virB* and a periplasmically active alkaline phosphatase from *E. coli*, showed that each of the VirB proteins, except VirB11, possesses an exported and periplasmic domain typical of membrane-associated proteins (Fernandez *et al*, 1996a; Baron *et al*, 1997; Christie, 1997). In-frame deletions of all *virB* genes indicated that the loss of certain VirB proteins, such as VirB7 and VirB9, drastically reduces the steady-state levels of several others, such as VirB4, VirB5, VirB8, VirB10 and VirB11. This suggests that VirB7 and VirB9 play an important role in stabilising the VirB proteins during the assembly of the T-transport complex, whereas VirB2-6, VirB8 and VirB10-11, are essential core components of the T-complex transfer machinery. VirB7 interacts with VirB9, forming heterodimers and probably higher order multimeric complexes. VirB9 synthesis and stable accumulation is dependent on heterodimer conformation, indicating that VirB9 alone might be unstable and requires the association with VirB7 (Fig. 6) (Anderson *et al*, 1996).

VirB1 plays an auxiliary role in transport (Fernandez *et al*, 1996b; Baron *et al*, 1997; Fullner, 1998). The VirB1 protein is processed and a C-terminal truncation product, VirB1*, accumulates at the cell exterior and in the extracellular milieu. To date, VirB1* is the only *vir* gene product that has experimentally been shown to localise extracellularly. It

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is, however, possible that certain VirB proteins may be redistributed to the cell exterior or even transferred to another cell upon contact with recipient cells (Baron *et al*, 1997; Christie, 1997).

VirB4 and VirB11 are hydrophilic ATPases necessary for active DNA transfer. VirB4 possesses a transmembrane configuration, whereas VirB11 likely resides exclusively at the cytoplasmic face of the cytoplasmic membrane (Dang and Christie 1997). Both proteins contain consensus Walker A nucleotide-binding motifs, required for function. Both exhibit weak ATP hydrolysis activities, whereas purified VirB11 has been shown to autophosphorylate *in vitro* (Stephens *et al*, 1995; Christie, 1997). VirB11 belongs to a family of evolutionary related ATPases whose ATP-binding and/or hydrolysis activities are indispensable for assembly or function of a diverse array of DNA or protein transport systems in eubacteria. Members of this family of ATPases are generally hydrophobic proteins that lack obvious transmembrane domains and amino-terminal export signals. VirB11 lacks a continuous sequence of hydrophobic residues that typically form periplasmic domains. However, only one third of VirB11 constitutes its soluble fraction, whereas the rest of the protein remains associated with the cytoplasmic membrane (Rashkova *et al*, 1997). VirB4 has been shown to be tightly associated with the cytoplasmic domain (Dang and Christie, 1997) and two putative extracellular domains confer transmembrane topology to the protein, which presumably allows the ATP-dependent conformational change in the conjugation channels. VirB4 and VirB11 are thought to link energy via ATP hydrolysis to substrate translocation, and recent genetic analysis supports the notion that both VirB4 and VirB11 function as homo- or heteromultimers (Christie, 1997; Dang and Christie, 1997; Zhou and Christie, 1997).

Several steps in the assembly pathway of the T-complex transport machinery have been elucidated; VirB7 and VirB9 monomers are exported and processed into mature polypeptides and the monomers then assemble as covalently cross-linked dimers. The VirB7-VirB9 heterodimer is transported to and positioned at the outer membrane. The interaction of other Vir proteins with this heterodimer for assembly of the transfer channel with the contribution of the transglycosidase VirB1, is considered the next step in the process (Christie, 1997).

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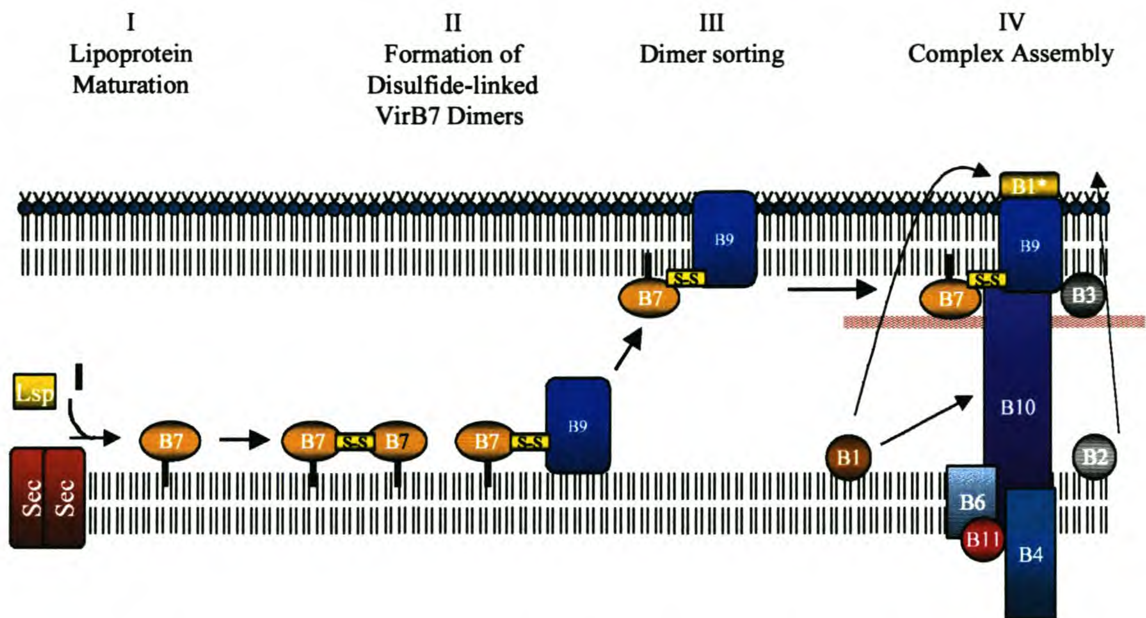

Fig. 6. A hypothetical model depicting early stages in the assembly of the T-complex transport apparatus. The fatty acid modification of VirB7 (B7) lipoprotein is depicted by the black rectangle, and Lsp indicates signal peptidase II. The dual role of VirB1 (B1) as a transglycosylase for local degradation of petidoglycan (red wavy lines) and as an extracellular factor (VirB1) [B1*] is clearly shown. Only the virB proteins for which there is experimental evidence of close proximity are shown in contact with one another. VirB5 (B5) and VirB8 (B8), however, are also likely components of this transport apparatus. Although the positioning of proteins is in accordance with predicted subcellular localisations, the model is not intended to portray spatial or stoichiometric relationships (adapted from Christie, 1997).

Two accessory *vir* operons, *virF* and *-H* enhance tumour formation. The protein VirF seems to play a role in the nuclear targeting of the ss-T-DNA-complex, whereas VirH enhances transfer efficiency, detoxifying certain plant components that can affect bacterial growth (De la Riva *et al*, 1998).

Integration of T-DNA into the plant genome. Although knowledge of the processes involved in the early events of T-DNA transfer has increased considerably, the fate of the T-DNA in the plant cell and host factors involved in T-DNA integration remains unclear (De la Riva *et al* 1998). The process of T-DNA integration is thought to occur via illegitimate recombination (IR). This process is normally not site-specific and could lead to small deletions of plant DNA at insertion sites. It was furthermore found that the 3' end of

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integrated T-DNA is less conserved compared to the 5' area (Bundock and Hooykaas, 1996; Rossi *et al*, 1996). According to this model, micro-homologies provide sufficient homology for a pre-annealing step between the T-DNA strand coupled with the VirD2 protein and plant DNA. The 3'-end or adjacent sequences of the T-DNA finds some homologies to the plant DNA and anneals and this annealing step results in a gap formed in the 3'-5' strand of plant DNA and the displaced plant DNA is subsequently cut at the 3' end position of the gap by endonucleases. The 3' overhanging ends of the T-strand, as well as the displaced plant DNA strand are subsequently digested away, either by endonucleases, or by 3'-5' exonucleases.

Micro-homologies between the 5'-nucleotides directly attached to the VirD2 protein result in an unstable annealing, bringing the electrophilic phosphotyrosine bond in proximity to the nucleophilic 3'-OH of the digested plant DNA. The upper strand of the plant DNA is nicked upstream of the annealing event. The phosphotyrosine bond can serve as substrate for the nucleophilic 3'-OH, resulting in the establishment of the new phosphodiester bond of the recombination product. Once ligation of the 5' end has been achieved to the 3'-OH of the plant DNA, the upper strand of the plant DNA is degraded and the T-DNA strand is copied by the plant repair machinery. The role of the VirD2 protein in providing the energy for the 5' integration could explain the conservation of the 5' area of the T-DNA, as well as the short deletion generated in the plant DNA (Tinland and Hohn, 1995; De la Riva *et al*, 1998).

2.5.3. *Agrobacterium*-mediated transformation systems

2.5.3.1. Disarming the pathogen and vector systems used

The unique ability of wild-type *Agrobacterium* species to genetically transform a range of dicotyledonous plants has been exploited in numerous ways, the most important being the use of *Agrobacterium* in plant transformation systems (Songstad *et al*, 1995). Genetic experiments showed that for the transfer of a T-DNA copy, no physical linkage is necessary between the T-DNA region and the rest of the Ti plasmid (Hooykaas and Schilperoort, 1992). The transfer mechanism providing the *vir-chv* genes as well as the 24 bp direct repeats which flank the T-region has made the construction of several transformation vectors possible. These include: i) *cis* systems in which new genes are introduced via homologous recombination into an artificial T-DNA already present on the

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Ti plasmid, and ii) binary systems in which new genes are cloned into plasmids containing an artificial T-DNA; these plasmids are subsequently introduced into an *Agrobacterium* strain harbouring a Ti plasmid with an intact *vir* region, but lacking the T-region (Deblaere *et al*, 1985; Klee *et al*, 1987). The most convenient way of manipulating the relatively big Ti plasmid is by removing and replacing segments through homologous recombination (Comai *et al*, 1983; Bevan, 1984; Hooykaas and Schilperoort, 1992). *Agrobacterium* strains (e.g. LBA4404) can be disarmed by removing the T-DNA (Hoekema *et al*, 1983). In an alternative strategy, a part of the T-DNA is left behind as homologous DNA for co-integrating vectors, as is the case with the split end vectors (SEV) system (Fraley *et al*,1985).

2.5.3.2. *Agrobacterium*-mediated transformation applications

The first record of transgenic tobacco expressing foreign genes appeared early in the previous decade, although many of the underlying processes were unknown at that stage (De la Riva *et al*, 1998). The natural hosts for *Agrobacterium* are mainly dicotyledonous plants (Hooykaas and Schilperoort, 1992) and transformation protocols for many agronomically important dicotyledonous plants have been developed. These include grapevine (Baribault *et al*, 1989; Scorza *et al*, 1996), tomato (Walden *et al*, 1997) and apple (Maximova *et al*, 1998). The system can be used in various transformation approaches, the simplest of which involves infiltration of *Agrobacterium* cells into *Arabidopsis* plants before flowering and direct selection for rare transformants in the resulting seedling populations. Unfortunately, small plant size, rapid generation time and high seed yields are prerequisites for this method, and these features are not shared by economically important plant species (Birch, 1997). Furthermore, some economically important plant species, e.g. grapevine, (Deng *et al*, 1995) have developed resistance towards *A. tumefaciens* infection and are therefore very difficult to transform with this system (Perl *et al*, 1996). In order to develop suitable methodologies for the transformation of economically important crops, including monocotyledonous plants, which were previously considered to be outside the *Agrobacterium* host range, it is important to take into consideration the critical aspects in the *Agrobacterium*-plant interaction. Cellular and tissue culture systems must be firmly established and suitable genetic material, e.g. appropriate transgenes and promoter constructs, binary vectors and reporter and marker genes, should be selected (Songstad *et al*, 1995). The

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Agrobacterium-plant interaction should be considered carefully; explant type, tissue necrosis and bacterial strain affect the attachment of the bacteria as well as DNA transfer (De la Riva *et al*, 1998). Taking these factors into account, several of the traditionally difficult crops to transform (e.g. cereals, legumes and woody plants) have since been added to the list of successfully transformed crops (Birch, 1997).

2.5.4. *Agrobacterium vitis*

2.5.4.1. Classification and unique characteristics

Agrobacterium vitis, previously referred to as *Agrobacterium* biovar 3, is related to the well known *A. tumefaciens, A. rubi* and *A. radiobacter.* DNA homology levels with the type strains of other *Agrobacterium* species, however, are less than 47%, whereas homology levels of approximately 90% exist between *A.vitis* strains. *A. vitis* strains have been isolated predominantly from grapevine and can be subdivided into octopine/cucomopine (o/c), nopaline (nop) and vitopine (vit) strains, based on the opine markers encoded by the Ti plasmid (Knauf *et al*, 1983; Ophell and Kerr, 1990; Paulus *et al*, 1991). These groups are associated with a particular chromosomal background and are characterised by the presence of specific bacterial insertion elements (Paulus *et al*, 1989).

The *A. vitis* o/c strains can further be divided into two groups, those containing Ti plasmids with a large TA region (OL), and those containing Ti plasmids with a small TA region (OS) (Otten *et al*, 1996). The o/c strains constitute approximately half of the isolated *A. vitis* strains and several of these Ti plasmids have been mapped. Some regions of these plasmids such as the virulence region, the T-DNAs and the octopine catabolism region are highly conserved, whereas others are strain-specific. Nopaline-type *A. vitis* strains are commonly found on grapevine and have a wide host range. Their plasmids also display significant homology to o/c-type Ti plasmids (Otten and De Ruffray, 1994). Vitopine strains are relatively rare on grapevine, and contain Ti plasmids with unique restriction patterns and three T-DNAs with low homology to other T-DNAs (Gérard *et al*, 1992).

Octopine/cucumopine-type strains. O/c *A. vitis* strains are divided into wide and limited host range strains (WHR & LHR), based on their ability to induce tumours on tomato and tobacco (WHR) or the induction of tumours exclusively on grapevine (LHR) (Paulus *et al*,

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1991). At least seven different T-DNA forms of o/c type strains have been identified by restriction analysis (Otten and Van Neunen, 1993). The Ti plasmids of *A. vitis* o/c strains usually carry two T regions, TA and TB, but some exceptions exist. *A. vitis* strain 2641 contains a single TA area and is the result of a deletion event in the parent strain 2608 where the TB area was lost (Fournier *et al*, 1994). The TA region of the WHR strains shows strong homology with the TL region of *A. tumefaciens* octopine strains, whereas the TA region of the LHR strains is a deleted version of the WHR TA area (Paulus *et al*, 1991). The TB region of both WHR and LHR strains are o/c Ti-specific and carry a set of auxin synthesis genes (TB-*iaaM* and TB-*iaaH*), as well as the cucumopine synthase (*cus*) gene (Fournier *et al*, 1993).

Though a variety of TA and TB structures exist in different o/c type strains, they were all derived from a common ancestor. The divergence was caused by the insertion of several bacterial insertion elements (IS) and deletions due to recombination between the insertions (Otten *et al*, 1993). Bacterial insertions are common in all bacteria and several families of IS elements have been identified in *Agrobacterium* species; these include IS*3*, IS*5*, IS*66* (the most common) and IS*630* (Mahillon and Chandler, 1998). *A. vitis* o/c strains harbour several unique inserted repeats (e.g. IS*867* and IS*868*), which may have been responsible for the formation of LHR plasmids by intraplasmid recombination (Paulus *et al*, 1991).

Nopaline-type strains. The T-DNA of the nopaline-type plasmid of *A.vitis*, pTiAB4, is a chimeric structure with recent evolutionary origins. On nucleotide level, the left area is 99.2% homologous to the corresponding area in the o/c Ti plasmids, whereas the right part is 97.1% homologous to the right part of an unusual nopaline T-DNA, recently identified in a biotype II strain isolated from wild berry. The isopentyl transferase (*ipt)* genes of the plasmids are also unique, and contain a 62 bp sequence derived from the coding sequence of an *ipt* gene of unknown origin, rendering the *ipt* gene potentially unstable (Otten and De Ruffray, 1994). The deduced *ipt*-protein is 20 amino acids longer at its N-terminal end, but is nevertheless biologically active. The nopaline plasmids also share homology with the o/c-type Ti plasmids in three other regions, and their arrangement and orientation are also similar. These facts suggest that these plasmids share a common ancestor (Otten *et al*, 1996).

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The smallest Ti plasmid found so far is that of the AB4 strain, with a size of only 157 kb. Significant areas of homology to that of the o/c-type Ti plasmids of *A. vitis* are found and although large parts of the *A. vitis* AB4 Ti plasmid hybridise to the *A. tumefaciens* nopaline-type Ti plasmid, no restriction site homology is detected. *A. vitis* nopaline-type Ti-plasmids therefore share a higher degree of homology with A. vitis o/c-type Ti plasmids than with their counterparts from *A. tumefaciens*. Although at least seven different o/c type T-DNAs are found, no variation in restriction maps of twenty *A. vitis* nopaline-type T-DNAs was observed (Otten and De Ruffray, 1994).

Vitopine-type strains. Octopine and nopaline-type plasmids are also found in *A. tumefaciens* and *A. rhizogenes*, whereas Ti plasmids encoding vitopine synthesis have been found exclusively in *A. vitis* strains (Canaday *et al*, 1992; Szegedi *et al*, 1996). The vitopine Ti plasmids are novel in several instances; i.e., vitopine oncogenes share no homology to that of *A. tumefaciens* (Gérard *et al*, 1992). Moreover, the Ti plasmids of the vitopine-type strains contain three separate T-DNAs (T1, T2 and T3) and were the first example of Ti plasmids of such a nature. The border sequences, delimiting the T-DNAs in these plasmids, also differ from previously described consensus sequences in the sense that one to three mismatches were observed (Canaday *et al*, 1992).

It has been shown that all three T-DNAs are present in tumour tissue, but that only the T2 and T3 borders function in a disarmed borderless *A. tumefaciens* vector. The absence of the overdrive sequence, found immediately adjacent to the right border in *A. tumefaciens* strains from all three T-DNAs, is surprising, since their transfer seems to be equally efficient as the T-DNA of *A. tumefaciens*. The possibility, however, still exists that an overdrive sequence might be located at a distance from the right borders. A distinctive GTGCATCC repeated motif was found adjacent to the T2 right border which might play a role in the efficient transfer of DNA, since similar motifs found in *A. rhizogenes* are necessary for DNA transfer. The vitopine-type *6b* and *ipt* genes show a significant increase in growth induction compared to that of their *A. tumefaciens* counterparts, implying a higher activity of their promoters or intrinsic differences in their encoded gene products (Canaday *et al*, 1992; Gérard *et al*, 1992; Szegedi *et al*, 1996).

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Tartrate utilisation. *A. vitis* species are some of the few *Agrobacterium* spp. capable of utilising tartrate. L(+)-tartrate, a substance abundant in grapevine, is the preferred carbon source of *A. vitis*, whereas *A. rhizogenes* is also capable of utilising L(+)-tartrate, but prefers glucose. L(+)-tartrate and D-malate are the most abundant organic acids in grapevine, and L(+)-tartrate concentrations in grapevine are much higher than those in other plant species. L(+)-tartrate degradation is thought to play a role in the host range specificity of *A. vitis* (Szegedi *et al*, 1992; Salomone *et al*, 1996).

Several tartrate degradation genes are carried by large conjugative plasmids. Three tartrate utilisation regions have been cloned: TAR-I is located on the 245 kb o/c type AB3 tartrate utilisation (pTrAB3) plasmid, TAR-II is part of the AB3 Ti plasmid; TAR-III is located on pTrAB4 of the nopaline *A. vitis* strain AB4 (Otten *et al*, 1995; Salomone *et al*, 1996). The TAR-III region from the tartrate pTrAB4 was sequenced and analysed by insertional mutagenesis and has been shown to contain five genes (*ttu*A-E), four of which (*ttu*A-D) are required for tartrate utilisation in the heterologous species *A. tumefaciens*. Based on functional studies and similarities with known genes, it is proposed that *ttu*A codes for a tartrate sensitive LysR-like protein which regulates the transcription of *ttu*B, C, D and E. The *ttu*B product mediates tartrate entry, while the *ttu*C encoded product catalyses the first step in tartrate degradation (Salomone and Otten, 1999). This yields an unidentified toxic intermediate that slows down growth unless further degraded by the *ttu*D product. The *ttu*E gene encodes a tartrate-inducible pyruvate kinase that is not required for tartrate utilisation, but may increase glycolysis. Restriction maps of TAR-I-III are shown in Fig. 7. In "small TA" o/c strains and nopaline strains, the type of TAR region correlates well with the chromosomal background. Most "small TA" o/c strains contain TAR-I and TAR-II, whereas several nopaline strains contain TAR-III. Both TAR-II and TAR-III are found in large TA o/c type strains (Salomone *et al*, 1996). Although vitopine strains utilise tartrate, they do not show homology to the TAR region maps. The tartrate utilisation system of the vitopine strain S4 is located on a large plasmid that was shown to be transferable to a cured *A. tumefaciens* recipient (Salomone *et al*, 1996). It is interesting to note that three rare *A. tumefaciens* strains isolated from grapevine carry a pTiAB3-like plasmid with its characteristic TAR region, and not a pTrAB3 plasmid. This suggests that the Ti plasmid and its TAR region can normally be transferred to avirulent *Agrobacterium* strains (Otten *et al*, 1995).

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Thus, although *A. vitis* dominates in grapevine crown galls, other *Agrobacterium* strains should be considered as potential recipients of Ti plasmids that can confer tartrate degradation. Although the utilisation of tartrate by most grapevine isolates suggests this to be the case, the correlation is not perfect and further studies are needed to clarify this issue (Crouzet and Otten, 1995).

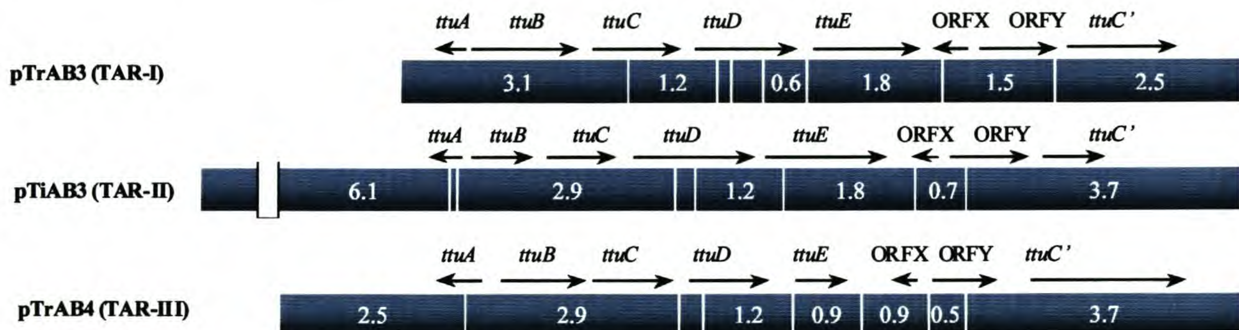

Fig. 7. *Pst*I maps of the tartrate utilisation areas TAR-I, -II and -III of *Agrobacterium vitis* strains AB3 (o/c) and AB4 (nop) (sizes in kb) (adapted from Salomone *et al*, 1996).

Polygalacturonase utilisation. Apart from the crown galls formed, *A. vitis* also sometimes cause sunken necrotic lesions on grapevine roots. These necrotic lesions are reminiscent of disease symptoms produced by pectolytic pathogens. The bacterium has been shown to produce a single extracellular polygalacturonase (PG), an enzyme that catalyses the hydrolytic cleavage of pectic polymers in plant cell walls. Tumorigenic, pTi-carrying strains as well as non-tumorigenic, pTi-lacking strains produce this enzyme, indicating that the enzyme is chromosomally encoded (Rodriguez-Palenzuela *et al*, 1991; Herlache *et al,* 1997). The enzyme is encoded by the pectic hydrolase gene (*pehA*) and is secreted either actively by a type II protein secretion system in *A. vitis*, or may simply leak out of older cultures (Herlache *et al*, 1997).

PG production is required for root decay and contributes significantly to tumorgenicity in grapes since a *pehA*$^-$ mutant showed reduced tumorgenicity on grape cuttings. *A. vitis*, however, failed to produce decay in roots of any other non-host plants. This could either be attributed to grapevine-specific regulation of the enzyme, intrinsic changes in the activity of the enzyme, or to factors unrelated to PG production (Rodriguez-Palenzuela *et*

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al, 1991). Polygalacturonases are not absolutely required for tumorgenicity in grapes. Nevertheless, polygalacturonases can be conceived to contribute quantitatively to the tumorgenicity of *A. vitis* in the following ways: i) multiplication in wound sites, ii) bacterial attachment to host cells, iii) induction of *vir* genes, and iv) transfer of T-DNA through the host cell wall. A *pehA*$^-$ mutant showed a diminished ability to multiply on grape root seedlings and attached with a lower efficiently to grape roots than the wild-type strain. Attachment has been correlated with tumorgenicity and there is substantial evidence that a satiable receptor for *A. tumefaciens* binding is in the pectic fraction of the cell wall. It is therefore possible that PG cell wall degradation creates more binding sites, or in a yet undefined way, enhances binding. D-galacturonic acid, a potential product of polygalacturonase, is the monosaccharide with the strongest effect on *vir* induction at low concentrations. PG could also play a role in facilitating T-DNA transfer through the cell walls, although at present there are no data to support this notion (Rodriguez-Palenzuela *et al*, 1991).

2.5.4.2. Use in genetic transformation of its natural host, *Vitis vinifera*

The genetic transformation of grapevine has been attempted with strains of *A. tumefaciens* as well as *A. rhizogenes* (Baribault *et al*, 1989; Guellec *et al*, 1990) and although these systems are functional, grapevine has proven to be a particularly difficult species to transform and stable transformants are rarely reported (Martinelli and Mandolino, 1994). A major obstacle in the *A. tumefaciens*-mediated transformation of grapevine is due to the effect that the bacterium has on embryogenic grapevine callus. Co-cultivation of grapevine embryogenic calli with *A. tumefaciens* results in calli necrosis (browning) and eventual cell death, seriously hampering embryo regeneration and transformation efficiency (Perl *et al*, 1996).

As mentioned above, *A. vitis* harbours several unique characteristics rendering the bacterium an efficient grapevine pathogen. The ability to infect grapevine therefore implies that the bacteria have successfully managed to avoid triggering the grapevine HR and is capable of attaching and transferring a T-DNA copy to the grapevine host cells, genetically transforming recipient cells. Subsequently, it stands to reason that this compatible plant-pathogen interaction could be extended to grapevine embryogenic calli, resulting in less calli necrosis and a better transformation efficiency.

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CHAPTER THREE

RESEARCH RESULTS

Characterisation of an *Agrobacterium vitis* strain as a potential transformation agent for *Vitis vinifera*

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The data in this manuscript will form part of an article that will be submitted for publication in **Journal of Bacteriology**

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Characterisation of an *Agrobacterium vitis* strain as a potential transformation agent for *Vitis vinifera*

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Abstract

The main limitations of the *Agrobacterium tumefaciens* transformation system in general, can predominantly be attributed to the host range specificity of *A. tumefaciens*, as well as adverse effects on infected tissue due to plant defence mechanisms induced by plant-pathogen-interactions. Although usually a limitation, the host range specificity of certain *Agrobacterium* species can, however, be exploited to optimise the application of *Agrobacterium*-based transformation systems in a crop specific manner. In such an attempt to improve on the existing transformation systems for *Vitis vinifera*, we have selected and partially characterised an indigenous *A. vitis* strain for evaluation for this purpose. *A. vitis* strains have predominantly been isolated from grapevine and only a few of these strains have been fully characterised. The selected strain, characterised as an octopine type strain was assessed for infectivity on grapevine as well as for its effect on grapevine embryogenic callus. DNA-DNA hybridisation techniques were used to compare the T-DNA regions of characterised *A. vitis* strains with that of the selected indigenous strain. A partial restriction enzyme (RE) map of the T-DNA was constructed and the T-DNA as well as the flanking areas were cloned and partially sequenced. The sequencing results showed significant differences with other characterised octopine- as well as octopine/cucmopine(o/c)-type strains, most significantly the absence of the agrocinopine (*acs*) gene from the 5' area of the T-DNA. Three bacterial insertion elements (IS) were found flanking the T-DNA. Two of these elements were shown to belong to the IS*110* family of IS elements, which has not previously been identified in *A. vitis*. Moreover, these two elements seemed to correspond to the 5' and 3' ends of a *Pseudomonas* transposase gene, hinting at a possible evolutionary role.

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3.1. INTRODUCTION

The phytopathogens *Agrobacterium tumefaciens* and *A. vitis* are part of the Gram negative *Rhizobiaceae* family, a plant-associated group of bacteria that comprises *Rhizobium, Agrobacterium, Bradyrhizobium* and several other minor groups. They are predominantly soil-borne plant pathogens that can be present in infected soil for many years in association with plant rests (Ophel and Kerr, 1990; Burr *et al,* 1995). *Agrobacterium* spp. are responsible for the formation of crown galls and hairy root disease on dicotyledonous plants by transferring a discrete area of DNA (T-DNA) from a ±200 kb plasmid (Ti plasmid) to plant cells where it is incorporated into the plant genome (Zambryski, 1992). Transcription of this T-DNA fragment leads to phytohormone autonomy, resulting in cell proliferation and subsequently crown gall formation. T-DNA transfer is facilitated independently by the T-DNA border sequences and a large virulence (*vir*) region also located on the Ti plasmid operating in *trans*. It is therefore possible to exploit the DNA transfer mechanism of *Agrobacterium* by removing oncogenic areas from the T-DNA in a disarming strategy, and replacing it with the DNA to be transferred to susceptible host plants in a transformation event.

These disarmed *Agrobacterium* strains (mostly *A. tumefaciens* and *A. rhizogenes*) are frequently used for the stable transformation of both dicotyledonous and monocotyledonous plant species. (Hoekema *et al,* 1983, Klee *et al,* 1987, Gould *et al,* 1991). All plant species, however, are not susceptible to *Agrobacterium* infection and cultivars of *Vitis vinifera* are specifically recalcitrant to this bacterium. Successful grapevine transformation has been achieved using *A. tumefaciens, A. rhizogenes* and a combination of biolistic bombardment and *Agrobacterium* infection (Guellec et al, 1990; Martinelli and Mandolino, 1994; Scorza *et al*, 1996). Although successful, these transformation methods often result in low transformation frequencies and stable transformants are regenerated at relatively low frequency (Kikkert *et al*, 1996). Low transformation frequencies are partly caused by the long and difficult tissue culture phases of grapevine, as well as callus browning and necrosis induced by *A. tumefaciens* infection, seriously hampering regeneration of transgenic embryos (Perl *et al,* 1996). Tissue browning and necrosis have been linked to a severe oxidative burst associated with the active defence mechanism of the plant (Perl *et al*, 1996; Kazan *et al*, 1998).

Several *Agrobacterium* strains have, however, managed to circumvent this active defence response, and are able to infect and induce tumours on *V. vinifera* (Otten *et al,*

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1996). These strains have been re-classified as *A. vitis* strains, and are isolated mainly from grapevine tumours (Ophel and Kerr, 1990). Unlike their *A. tumefaciens* counterparts that are classified into several opine utilisation groups, *A. vitis* strains are categorised into either octopine/cucumopine (o/c), nopaline (nop) or novel vitopine (vit) groups (Szegedi *et al*, 1996). These groups are associated with a particular chromosomal background and are characterised by the presence of specific bacterial insertion elements (Paulus *et al*, 1989). The ancestral sequences of octopine type Ti-pasmids were shown to be highly conserved and their Ti plasmids form a homogeneous group (Knauf *et al*, 1983). Limited host range (LHR) o/c type strains have recently diverged from wide host strains (WHR) during a T-DNA deletion event resulting in the loss of a large TA-DNA area (Paulus *et al*, 1991). Also in contrast to *A. tumefaciens*, strains of *A. vitis* prefer tartrate (a substance abundant in grapevine) to glucose as carbon source (Otten *et al*, 1995) and also produce an acidic polygalacturonase that has been linked to the ability to infect grapevine (Herlache *et al*, 1997).

Here we report on the possibility of exploiting the ability of *A. vitis* to infect grapevine in an *A. vitis*-mediated transformation system for grapevine. An indigenous wild type *A. vitis* strain, with several unique characteristics, was selected and partially characterised. Strain selection was based on infectivity on grapevine cuttings and scored on lack of callus necrosis and subsequently partially characterised with regard to T-DNA structure. The T-DNA and flanking regions were partially characterised by mapping, cloning and sequencing. These cloned portions of the T-DNA and its flanking areas will be instrumental in an eventual disarming strategy rendering an *A. vitis* strain specific for transformation of *V.vinifera* cultivars.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains and culture conditions

All bacterial strains and plasmids used are listed in Table 1. *Agrobacterium* strains were routinely cultured at 28°C in Luria Bertani (LB) medium supplemented with 0.1% (w/v) glucose unless otherwise stated. *Escherichia coli* strains were grown at 37°C in LB media, or LB supplemented with 100 μg/ml ampicillin for the selection of transformants.

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3.2.2. DNA manipulations

Standard techniques for DNA cloning and mapping were performed according to Sambrook *et al,* (1989). Genomic DNA of *Agrobacterium* strains was prepared as described by Ausubel *et al,* (1994).

Table 1. Strains and plasmids used in this study

Strain or plasmid(s)	Relevant features or insert	Source or reference
E. coli strains		
DH5α	supE44 *lac*U169[*ϕ80lac*ZM15) *hsd*R17*rec*A1*gyr* A96*thi*-1*rel* A1]	Life Technologies (GIBCO/BRL)
A. tumefaciens strains		
EHA 105	Disarmed, succinomopine-type strain	Hood *et al,* 1993
LBA 4404		
A. vitis strains*		
2608	Octopine/cucumopine- type-strain	Fournier *et al,* 1994
Sis45	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
D1/1	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
M2/1$^{\Psi}$	Octopine type-strain	Phytobacterial culture collection, ARC, Pretoria, South Africa
F2/1	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
K2/1	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
H8/1	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
J8/1	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
H10/1	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
H11/1	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
R2/2	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
J4/2	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
J9/2	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
H14/2	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
F1/4	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
YI-1/6	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
9/2	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
4452	Nd	Phytobacterial culture collection, ARC, Pretoria, South Africa
Plasmids		
pBluescript (SK+)	cloning vector	Stratagene
pGEM-T Easy	pGEM5Zf(+) based PCR cloning vector	Promega
pGEM3Zf(+)	Cloning vector	Promega
Ab202	2.3 kb AB3 *Eco* RV TB fragment in pBlueskript (SK-)	Fournier *et al,* 1993
Ab36	*Hin*d III AB3 11 kb partial TB fragment in pPM1016	Fournier *et al,* 1993
pM2/1-1	2.6 kb M2/1 T-DNA PCR fragment in pBlueskript (SK+)	This study
pM2/1-2	3.5 kb M2/1 T-DNA *Eco* RV fragment in pBlueskript (SK+)	This study
pM2/1-3	3.0 kb M2/1 T-DNA *Eco* RV fragment in pBlueskript (SK+)	This study
pM2/1-4	2.2 kb M2/1 T-DNA *Eco* RI fragment in pGEM3Zf(+)	This study
pM2/1-5	0.65 kb M2/1 T-DNA *Eco* RI fragment in pGEM3Zf(+)	This study
pM2/1-6	2.2 kb M2/1 *Eco* RV T-DNA fragment in pGEM-T Easy	This study
pM2/1-7	1.05 kb M2/1 *Eco* RI T-DNA fragment in pGEM3Zf(+)	This study
pM2/1-8	5.2 kb M2/1 *Eco* RI T-DNA fragment in pGEM3Zf(+)	This study

Nd = Not determined

* Characterised as *A. vitis* by growth on Brisbane and Kerr medium (3DG), growth on NASA-medium (Staphorst, 1985), and 3-Keto lactose tests.

$^{\Psi}$ Confirmed as biovar 3 with a Biolog assay (Appendix C)

Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics and used according to the supplier's recommendations. Sequencing was done by the DNA

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Sequencing facility, Department of Genetics, University of Stellenbosch, using an ABI Prism 377 automated DNA sequencer from PE Biosystems.

PCR amplifications were done using Expand high fidelity DNA polymerase from Roche Diagnostics. All PCR primers used and their applications are listed in Table 2. PCR reactions were performed in 50 μl reaction mixtures typically consisting of 1x Expand high fidelity PCR buffer without $MgCl_2$, 200 μM dNTPs, 200 nM of each primer, 5 ng template DNA, and $MgCl_2$ added to the optimal concentration. Typical amplification conditions included an initial DNA denaturation step at 95°C for 2 minutes, followed by cycles of denaturation at 95°C for 10 seconds, primer annealing according to the specific primer melting temperatures, and elongation at 68°C, allowing 40 seconds per 1 kb amplified. Reactions proceeded for 30 cycles in a Biometra Trio-thermoblock cycler.

Table 2. Primer pairs and sequences used in this study

Primer	Sequence	Paired with	Template	Product
ipt 5'	GATCG(S)GTCCAATG(K)TGT	ipt 3'	pTi2608	420 bp of *ipt* gene
		T7 sequencing primer	Cloned M2/1 9.8 kb *Hind* III DNA fraction	7.2 kb 3' end of M2/1 9.8 kb *Hind* III fragment
ipt 3'	GATATCCATCGATC(K)CTT	T3 sequencing primer	Cloned M2/1 9.8 kb *Hind* III DNA fraction	2.6 kb 5' end of M2/1 9.8 kb *Hind* III fragment
IaaM 3'	ACCGTCCTTCAAGAACGCAC	T7 sequencing primer	Cloned M2/1 2.3 kb *Eco* RV DNA fraction	2.2 kb 5' end of M2/1 2.3 kb *Eco* RV fragment
		T3 sequencing primer	Cloned M2/1 9.5 kb *Pst* I DNA fragment	7 kb 5' end of M2/1 9.5 kb *Pst* I fragment
acs 5'	CGAGCTTCTCCAACGTATGTGTTTC	acs 3'	pTi2608	1000 bp of *acs* gene
acs 3'	CTGAGCGAGTTAGACGTCT			

Restriction sites included in primer sequences are underlined (*Eco* RV in ipt 3' and *Sac* I in acs 5') and degeneracies are indicated in parenthesis.

For Southern hybridisations, total DNA was digested, separated and blotted onto nylon membranes as described in Sambrook *et al,* (1989). Probe DNA was either labelled using the standard DIG random primed DNA labelling reaction, or PCR-labelled using the 10x

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DIG dNTP labelling mixture, both from Roche Diagnostics. All labelling reactions were done according to the manufacturer's specifications. Hybridisations were performed at 42°C for 16 hours using the standard DIG hybridisation buffer (from Roche Diagnositics) supplemented with 50% (w/v) formamide, unless otherwise stated. Signal detection proceeded according to the manufacturer's specifications.

3.2.3. Pathogenicity tests of *A. vitis* on *Vitis vinifera*

A. vitis strains were inoculated onto *V. vinifera* cv. Merlot material to verify the pathogenicity of the strains. Aging nodal cuttings were collected from the field and surface-sterilised in 75% ethanol for two minutes, soaked in 2.5% sodium hypochlorite for 20 minutes, and washed three times in sterile distilled water. The stem pieces were then trimmed aseptically and placed in half-strength B5 culture medium without the addition of any hormones (Gamborg *et al,* 1968). The stems were punctured ten times in an internodal section with a sterile needle. Bacteria were grown overnight, centrifuged and washed in 0.1 M phosphate buffered saline (pH 7.0). The cell concentrations were adjusted to OD_{600} = 0.3 in sterile distilled water and 1 μl (~10^5 bacterial cells) of each culture was inoculated onto wounded stems. Five to six explants were inoculated for each strain. The inoculated explants were incubated at 24°C in a growth chamber with a 16 hour photoperiod. Tumour formation was scored seven weeks after inoculations.

3.2.4. Grapevine callus infections.

Somatic embryos of *V. vinifera* cvs. Merlot, Sultana, and Chardonnay were inoculated with *A. vitis* strains M2/1 and H8/1 as well as with *A. tumefaciens* EHA105 and LBA4404 (controls), in an experiment to determine the extent of tissue necrosis induced by the various *Agrobacterium* strains. Somatic embryos were obtained from immature anther filaments based on a protocol described by Franks *et al,* (1998). *Agrobacterium* strains were grown overnight in 50 ml cultures to OD_{600} = 1.0, centrifuged and resuspended in 50 ml induction media (1 x AB salts (Chilton *et al*, 1974), 2 mM NaH_2PO_4, 40 mM MES, 0.5 % (w/v) glucose) supplemented with 100 μM acetosyringone and again incubated overnight. Cells were harvested and resuspended to OD_{600} = 0.3 in liquid culture media (1 x NN macro salts, 1 x MS micro salts, 1 x B5 vitiamins, 1 x Fe EDTA, 6% (w/v) sucrose, pH 6.2). Approximately 1-3 g of somatic embryos per 50 ml culture were incubated for

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seven minutes with slight agitation, after which the embryos were filtered from the solutions and briefly blotted on sterile filterpaper and placed on embryo maintenance medium (Franks *et al,* 1998). Co-cultivation proceeded for two days in the dark at 22°C. The embryos were washed extensively in sterile water containing 1000 mg/l cefotaxime after the co-cultivation period and replated on embryo-maintenance medium also containing 1000 mg/l cefotaxime. The cultures were incubated at 28°C in the dark and scored for necrosis with two-day intervals. An uninoculated control was subjected to all the above steps and media.

3.2.5. Opine characterisation of *A. vitis* strain M2/1

A freshly streaked culture of M2/1 was used to inoculate a plate of AB media (Chilton *et al*, 1974), supplemented with 0.15 g/l bromothymol blue, 5 g/l glucose and either NH_4Cl (positive control), octopine or nopaline at 0.01 g/l as a nitrogen source. The opine characterisation was done according to Hooykaas *et al*, (1980) where the inoculated plates were incubated for five days at 28°C until scoring for opine metabolism. According to this method, a yellow colour-change indicates nitrogen metabolism.

3.2.6. Partial characterisation, mapping and cloning of *A. vitis* M2/1 T-DNA region

Based on known T-DNAs from characterised o/c strains of *A. vitis*, partial mapping studies were done using DNA-DNA hybridisation. In an attempt to characterise the 5' end of the M2/1 T-DNA, the 1000 bp amplification product of the 3' end of the agrocinopine synthase (*acs*) gene (Table 2), located on the 5' end of all characterised o/c strains (Paulus and Otten, 1992), was used as a probe. To locate the TB area of M2/1, a 2.3 kb *Eco* RV fragment situated on the 3' flanking area of *A. vitis* AB3 (pAb202) was used as a probe. A hybridising ~9.5 kb M2/1 *Hin*d III fragment was used as a reference point to isolate a pool of ~9.5 kb *Hin*d III fragments from a total DNA digest. These fragments were cloned into pBlueskript (SK+) and screened by colony blots using the 2.3 kb *Eco* RV fragment from pAb202 as a probe. A positive clone was obtained and partially sequenced. A 1.45 kb *Eco* RI fragment (from pAb36) from the AB3 TB region was used as an additional probe for the TB area of M2/1 (Fig. 1).

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To map the T-DNA of M2/1, a 420 bp amplification product from the 5' area of the isopentyl transferase gene (*ipt*) gene of *A. vitis* strain 2608 was used as a probe to identify a 9.8 kb T-DNA *Hin*d III fragment. A 9.8 kb Hin*d* III fraction, referenced by the hybridising band, was isolated from a total DNA digest and ligated to pBlueskript (SK+). The transformation mix of this ligation was directly amplified as a "library" of recombinant plasmids and used to extract DNA. This "library" of plasmid DNA was subsequently used as template DNA in a PCR-based plasmid walking technique (Fig. 2). Briefly, PCR amplified DNA segments, harbouring DNA inserts that hybridised to the probe originally used in the Southern Blot, could easily be obtained by pairing plasmid specific T7 and T3 sequencing primers with the forward (ipt 5') and reverse (ipt 3') primers of the 420 bp probe, respectively. This strategy yielded PCR-amplified products of 2.6 and 7.2 kb of M2/1 T-DNA.

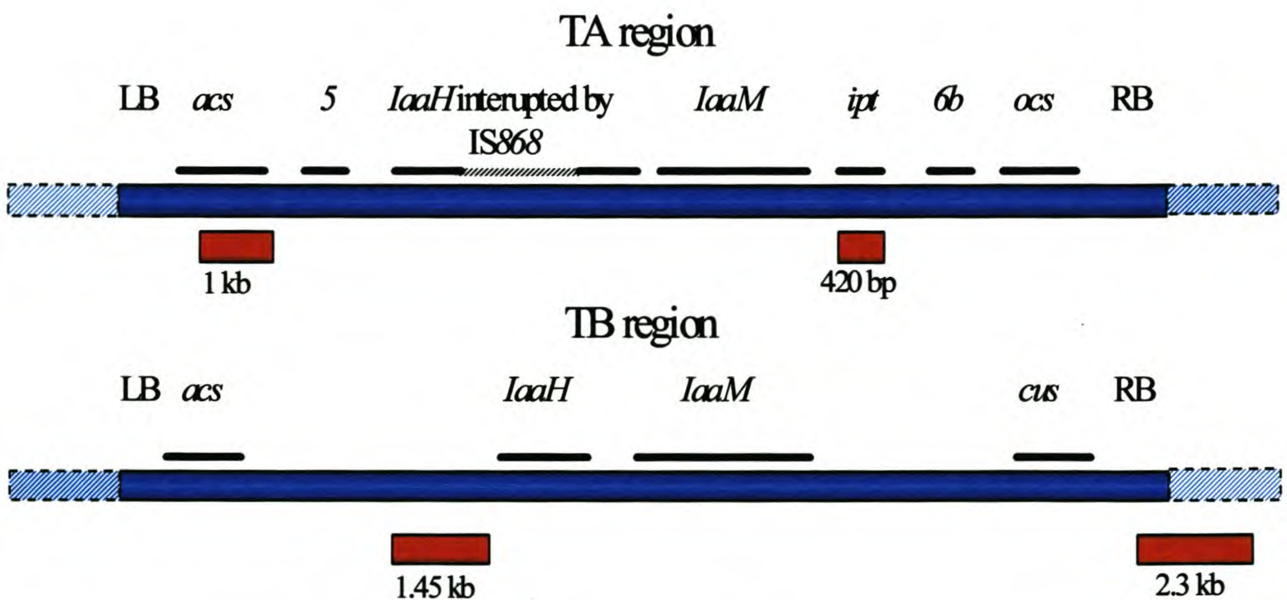

Fig. 1. Schematic representation of the probes used originating from the *A. vitis* strain 2608 TA and TB regions. Probes used are indicated with thick red bars. *Acs* = agrocinopine synthase, *IaaH* = indoleacetamide hydrolase, *IaaM* = tryptophan monooxygenase, *ipt* = isopentyl transferase, *ocs* = octopine synthase, *cus* = cucumopine synthase

The 2.6 kb M2/1 T-DNA fragment was cloned into the *Eco* RV and *Sac* I sites of pBluescript (SK^+) to yield pM2/1-1. The 7.2 kb PCR fragment was digested with *Eco* RV, yielding fragments of 3.5 and 3.0 kb which were coned into pBlueskript to generate

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pM2/1-2 and pM2/1-3, respectively. An *Eco* RI digestion of the original 7.2 kb PCR fragment yielded two fragments, 2.2 and 0.65 kb in size which were cloned into pGME3Zf(+), yielding pM2/1-4 and pM2/1-5, respectively. The 2.6 kb fragment of plasmid pM2/1-1 was digested with *Hpa* I and a 0.6 kb 5' fragment (corresponding to the 3' end of the *IaaM* gene) was isolated and used as a probe to identify a 2.4 kb *Eco* RV fragment overlapping and flanking the 5' end of the 9.8 kb *Hin*d III fragment.

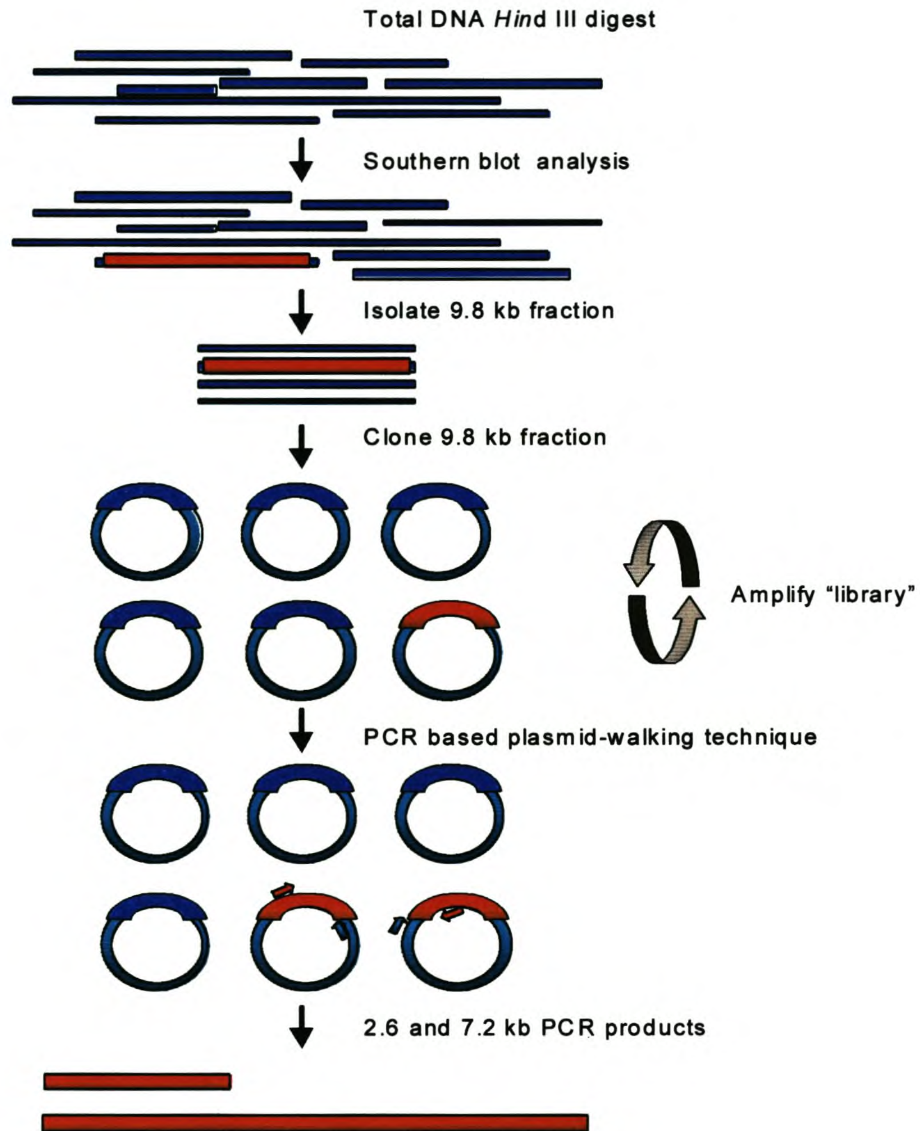

Fig. 2. Schematic representation of the plasmid walking technique employed to clone the T-DNA of *A. vitis* strain M2/1. Depicted is the cloning of a 9.8 kb *Hin*d III T-DNA fragment fragment.

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As in the previous cloning, the 2.4 kb fraction from a total DNA digest was isolated and cloned into pBluescript (SK^+). The "library" of recombinant plasmids was used as template material in a PCR reaction with the IaaM 3' primer paired with the T7 sequencing primer, yielding a 2.2 kb fragment. This fragment was subsequently cloned into pGEM-T Easy vector, yielding pM2/1-6. Using this 2.2 kb fragment, harbouring the 3' end of the *IaaH* gene, from pM2/1-6 as a probe, a ~9.5 kb *Pst* I fragment was identified that overlapped with the 5' end of the 9.8 kb *Hin*d III fragment. This hybridising fragment included the 2.4 kb *Eco* RV fragment already isolated and extended an additional ~6 kb past the 5' end of the 2.4 kb *Eco* RV clone. The ~9.5 kb fraction was isolated from a M2/1 total DNA *Pst* I digest and cloned into pGEM3Zf(+). The DNA from this plasmid pool was amplified and used as template in a PCR reaction with the IaaM 3' primer paired with the T7 plasmid specific sequencing primer, yielding a ~7 kb fragment. The fragment was digested with *Eco* RI and two resulting fragments, 1.05 and ~5.2 kb in size, were cloned into pGEM3Zf(+) to yield pM2/1-7 and pM2/1-8 respectively.

Approximately 600 bp of either end of all eight clones (pM2/1-1 to pM2/1-8) were sequenced employing universal primers for forward and reverse reactions. The obtained sequences were aligned against existing sequences in the Genbank database, using the advanced BLAST system (Altschull *et al*, 1997).

3.3. RESULTS

3.3.1. Grapevine cane infections

In our efforts to identify a useful *A. vitis* strain with possible application as a transformation agent of *V. vinifera*, several uncharacterised strains of *A. vitis* isolated from tumours on *V. vinifera* were screened for desirable attributes such as infectivity and lack of necrotic induction in grapevine somatic embryos. Wounded, nodal cuttings of *V. vinifera* cv. Merlot, inoculated with 17 *A. vitis* strains, were scored for tumour development after seven weeks of incubation. The plant material used was in the early stages of secondary growth (wood formation) and was kept at 24ºC. Several strains caused small tumours at the site of infection, whereas two strains (M2/1 and H8/1) caused large tumours (Fig. 3). The tumour development capacity of the strains is summarised in Table 3.

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Table 3. Evaluation of tumour-inducing ability of indigenous *A. vitis* strains on Merlot cuttings.

A. vitis strain	Tumour-inducing ability Merlot cuttings
Sis45	++
D1/1	-
M2/1	+++
F2/1	-
K2/1	-
H8/1	+++
J8/1	-
H10/1	-
H11/1	-
R2/2	-
J4/2	-
J9/2	+
H14/2	-
F1/4	-
YI-1/6	+
9/2	-
4452	++

No tumour development was scored as negative (-), whereas small and large tumours were scored positively (+ and +++, respectively).

3.3.2. Grapevine callus infections

Somatic embryogenic cultures of *V. vinifera* cvs. Merlot, Chardonnay and Sultana were infected with *A. tumefaciens* EHA105 and LBA4404 as control experiments, and *A. vitis* strains H8/1 and M2/1 based on their strong infectivity on grapevine cutivars. Uninoculated somatic embryo cultures subjected to media alone were used as negative controls. The embryogenic cultures used were all in the early stages of embryogenesis, represented by mainly globular and heart-shaped embryos. The infected embryos were evaluated on the level of browning/necrosis compared to the uninoculated control as well as on recovering ability (Table 4). In all cultivars tested, the *A. vitis* strains caused significantly less tissue browning/necrosis of the embryo cultures than *A. tumefaciens* EHA105 and LBA4404 (results of LBA4404 not shown). Of the two *A. vitis* strains tested, M2/1 induced the least browning/necrosis (Fig. 4) and allowed a markedly faster recovery of the embryos post-infection. In fact, the Merlot embryos infected with M2/1 compared well in appearance with that of the uninfected controls after seven days. Based on the infectivity studies and the observed effect on grapevine somatic embryos, strain M2/1 was selected for further genetic characterisation.

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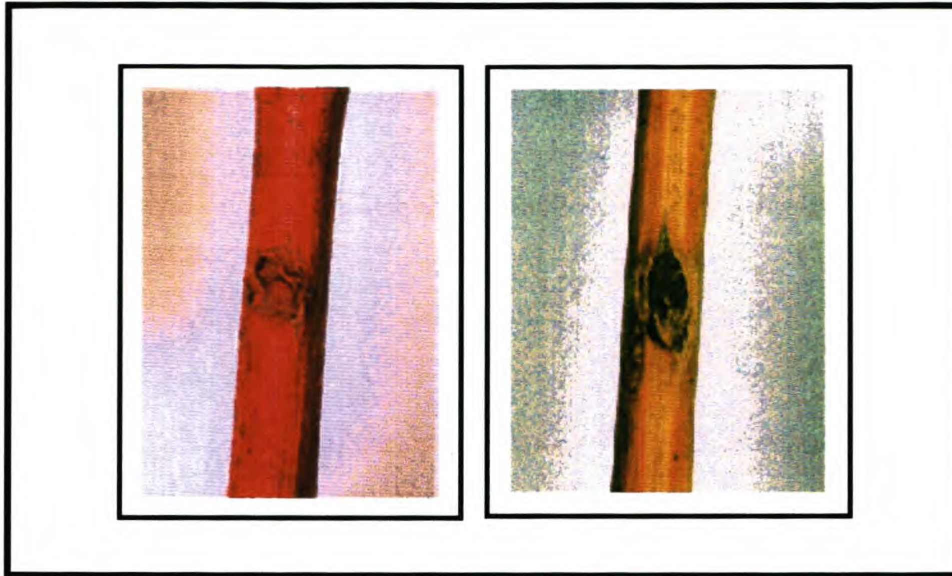

Fig. 3. Tumours induced by *A. vitis* strains by M2/1 (right) and H8/1 (left) on Merlot cuttings after a seven week incubation period at 24°C, respectively. Cuttings were surface-sterilised and punctured with a sterile needle before inoculated with ~10^5 bacterial cells.

Table 4. Effect of *A.vitis* and *A. tumefaciens* strains on embryogenic grapevine cultures

Grapevine cultivar			Callus necrosis effect	
	Agrobacterium strain	M2/1 (A. vitis)	H8/1 (A. vitis)	EHA105 (A. tumefaciens)
Merlot		+	++	+++
Chardonnay		+	+	+++
Sultana		+	Nd	++
			Callus regeneration	
	Agrobacterium strain	M2/1 (A. vitis)	H8/1 (A. vitis)	EHA105 (A. tumefaciens)
Merlot		+++	++	+
Chardonnay		+++	++	+
Sultana		++	Nd	

Severe effects are indicated by +++, Nd indicates not determined

3.3.3. Genetic characterisation of *A. vitis* M2/1

A. vitis strain M2/1 was identified as an octopine [N^2-(1-D-carboxyethyl)-L-arginine] type (o/c) strain by substituting octopine as sole N-source in a plate assay where the production of acid is indicated by bromothymol blue as indicator (Cho *et al,* 1996, Desaux *et al,* 1988) (Fig. 5). Preliminary PCR experiments indicated that the strain could belong to the WHR type, based on the fact that a 420 bp fragment of the 5' end of the *ipt* gene

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could be amplified from this strain, indicating a complete copy of the *ipt* gene. This could be an indication of the wide host range status, since that specific portion of the *ipt* gene is deleted in LHR strains of the o/c type. These results must, however, be verified by host range tests.

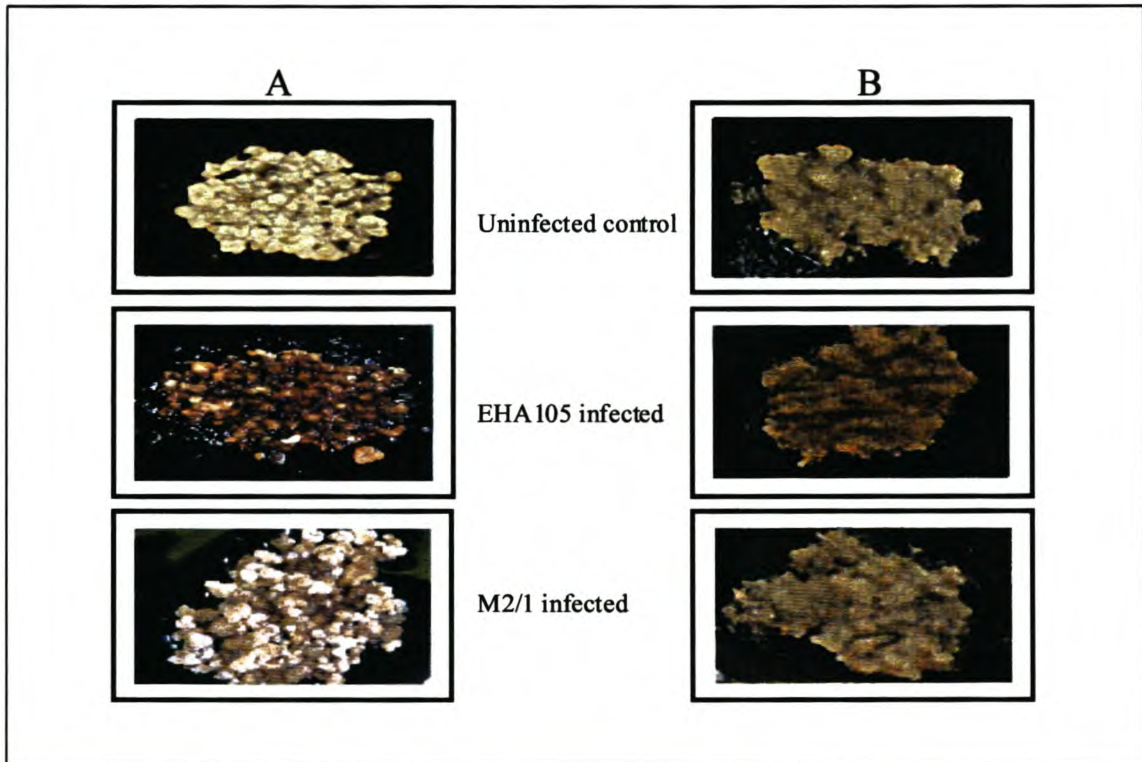

Fig. 4. The effect of *A. tumefaciens* EHA 105 and *A. vitis* M2/1 on embryogenic cultures of A) Merlot, and B) Sultana. The top frames represent the uninfected control culture, the middle frames embryos subjected to EHA105 and the bottom frames represent embryos subjected to M2/1.

In an attempt to partially characterise and map the T-DNA areas of M2/1, a hybridisation procedure was used employing various probes containing known genes or T-DNA specific areas from the T-DNA of characterised o/c strains (Fig. 1 and 2 depict the probes and strategy used). Hybridisation experiments with the 1.45 kb *Eco* RI fragment from pAb36 yielded no results (Appendix B), whereas the 2.3 kb fragment from pAb202 identified a ~9.5 kb *Hin*d III fragment. After isolation and cloning of this fragment, the first 600 bp on either side was sequenced (Appendix A) to determine the position of the fragment on the TB area relative to that of characterised o/c strains. Sequencing results, however, indicated no homology to any known TB areas. These results indicated that

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either the TB area of M2/1 is unique and shared no homology with known TB areas, or is absent from pTiM2/1.

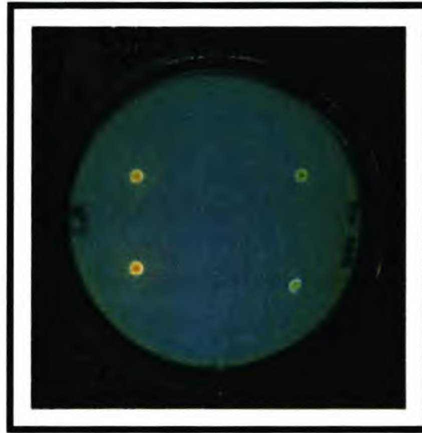

Fig. 5. Opine utilisation of M2/1. M2/1 colonies are on the left showing a surrounding yellow zone, whereas *A. tumefaciens* EHA105, a succinomopine type strain is shown on the right as a negative control.

In an attempt to characterise the 5' end of the T-DNA, a 1000 bp PCR fragment from the 3' end of the *acs* gene was used as a probe in a Southern hybridisation. No area of homology could be identified on pTiM2/1, indicating the absence of the *acs* gene on the 5' end of the T-DNA. A large T-DNA region from M2/1 was identified using the 420 bp *ipt* PCR fragment as a probe. This region, along with the rest of the M2/1 T-DNA, was cloned using a PCR-based plasmid walking technique and eight clones, pM2/1-1 to pM2/1-8 were obtained. These clones were partially sequenced (Appendix A) and the sequences aligned against existing sequences in the Genbank database. Four T-DNA-associated genes were identified; the indoleacetamide hydrolase (*laaH*) gene, tryptophan monooxygenase (*laaM*), *ipt* and octopine synthase (*ocs*) gene. These genes share homology levels of 99%, 95%, 97% and 98% respectively on nucleotide level with the corresponding genes from the *A. tumefaciens* octopine type pTi15955 (Gielen *et al*, 1984), as well as a conserved gene order (Fig. 6). Homology to corresponding genes from *A. vitis* o/c type pTiTm4 and octopine type pTi CG474 varies between 85% and 90% on nucleotide level.

The LB flanking region is 98% homologous to the pTiA6 sequence AF034854 over its full length, while a stretch of 496 bp from the RB flanking region is 94% homologous to the

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pTi15955 sequence X00493. Two families of bacterial insertion (IS) elements were identified on the LB and RB flanking regions from the sequencing results. An IS element homologous to the IS*110* family (Mahillon and Chandler, 1998) was identified flanking the 5' area of the T-DNA, showing 63% homology on amino acid level to the 5' end of a putative transposase from *Pseudomonas syringae* pv. *phaseolicola* (Jackson *et al*, 1999). An IS*3*-like element flanking the 3' end of the T-DNA show 94% homology on nucleotide level to the 529 bp IS*51*-like fragment of *A. tumefaciens* pTiAch5 (Yamada *et al*, 1985). The third IS element identified on the T-DNA is located approximately 350 bp to the right of the IS*3*-like element and belongs to the IS*110* family (Mahillon and Chandler, 1998). The element showed 61% homology on amino acid level to the 3' end of the putative transposase from *P. syringae* pv. *phaseolicola* (Jackson et *al*, 1999).

The data from the hybridisation experiments in correlation with data from the partial sequences were used to construct a restriction enzyme (RE) map of the M2/1 T-DNA (Fig. 6).

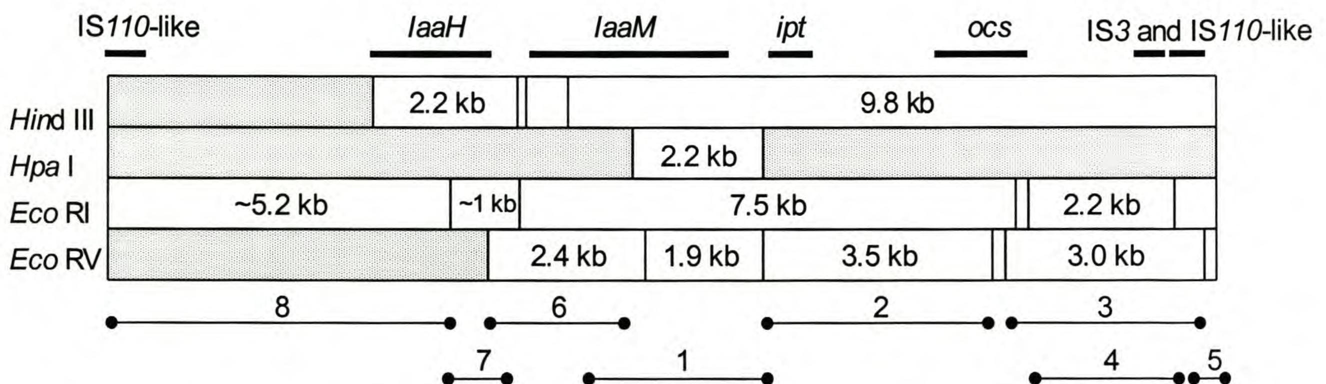

Fig. 6. Restriction maps of the M2/1 TA DNA area. Shaded blocks represent RE fragments of unknown length. Lines with dotted ends numbered 1-8 represent clones pM2/1-1 to 8. Thick black lines indicate conserved genes. The position and order of the identified genes relative to the obtained clones are indicated.

3.4. DISCUSSION

Although the existing *A. tumefaciens* transformation system has successfully been used to transform grapevine, several problems exist which significantly decrease the transformation efficiency of this system. A severely limiting factor in the transformation of grapevine results from the necrotic effect on embryogenic grapevine calli caused by

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A. tumefaciens. This necrosis can be attributed to the active defence system of grapevine culminating in the hypersensitive response (HR). This response has been associated with *A. tumefaciens* infection of grapevine embryogenic callus, leading to an oxidative burst that results in subsequent callus browning and necrosis (Perl *et al,* 1996).

Several *Agrobacterium* strains have been isolated from crown gall tumours on grapevine (Szegedi *et al,* 1989), implying that these *Agrobacterium* strains, designated *A. vitis*, have evolved certain characteristics to allow infection of grapevine without eliciting the defence response of the plant. In this case the host-pathogen interaction on grapevine is favourable for disease development. These strains therefore are ideal for a disarming strategy to obtain a grapevine-specific transformation agent. In this hypothesis, it is also assumed that apart from excellent colonisation and infection of tissue, the favourable host-pathogen interaction will cause limited tissue damage.

In order to test this hypothesis, we tested the infectivity of 17 indigenous *A. vitis* strains on Merlot cuttings in the early stages of secondary growth or wood formation. Relatively few strains (only six) induced tumours, and most of these tumours were small and slow to develop, given the incubation period of seven weeks. The relatively inefficient tumour formation might be attributed to the developmental stage of the plant material, (not actively growing) as well as a test system perhaps not conducive of optimal infection. Two strains however, H8/1 and M2/1, were seemingly unaffected by the conditions and were able to consistently produce large tumours within the incubation period. These two strains also produced large tumours on *in vitro* infected plantlets (results not shown).

The extent of browning induced on somatic embryos of grapevine by the *A. tumefaciens* strain as well as both the *A. vitis* strains varied between the different grapevine cultivars. Furthermore, the observed browning effect of *A. vitis* varied between the two strains, H8/1 and M2/1; H8/1 induced a slightly more severe effect on Merlot, as well as Chardonnay than M2/1. These results are indicative of the important role of plant and bacterial genotype in plant-bacterial interactions, emphasising the high degree of specialisation found in certain plant pathogens in order to infect susceptible host plants successfully. *A. vitis* strain M2/1 induced the least amount of necrosis on all grapevine cultivars tested and was therefore selected for further genetic analysis.

The M2/1 strain was shown to be of the octopine type by a simple plate assay indicating the production of a keto-acid as a result of opine utilisation (Hooykaas, 1980).

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The results of the plate assays were later confirmed by partial sequence analysis of the cloned T-DNA. PCR analysis indicated that the strain could belong to the WHR type, although this indication needs to be verified by host range tests. This initial finding was confirmed by the partial sequencing as well, indicating that a large portion of the T-region from M2/1 is intact.

All characterised o/c *A. vitis* strains to date, save one, were shown to have two T-DNA regions, a TA and TB region. The one exception occurred due to intraplasmid recombination resulting in the loss of the TB region (Fournier *et al*, 1994). Hybridisation experiments with the 2.3 kb *Eco* RV fragment from pAb202 identified a 9.5 kb fragment which was subsequently cloned and partially sequenced. According to characterised strains, the 5' end of this 9.5 kb should either overlap the cucumopine synthase (*cus*) gene and/or IS*869* and IS867 (Fournier *et al*, 1993). Sequence analysis, however, revealed no sequence homology to any known TB genes, indicating the absence of either the *cus* gene, or the IS*869* and IS867 elements. The 1.45 kb *Eco* RI fragment from pAb36, also did not hybridise to any sequences of the M2/1 Ti plasmid. Furthermore, hybridisation experiments with the 2.6 kb fragment from pM2/1-1 and the 2.2 kb fragment from pM2/1-6, which were shown to contain the 3' end of the M2/1 *IaaM*- and the 3' end of the *IaaH* gene (Fig. 6), respectively revealed a single copy of each gene (Appendix B), both of which are usually present in characterised TB areas of o/c type strains (Fournier *et al*, 1993). These results seem to confirm the notion that the TB area of M2/1 is either deleted, or is not homologous to any characterised TB area of o/c type strains.

The T-DNA of M2/1 was identified on a 9.8 kb *Hin*d III fragment and overlapping ~9.5 kb *Pst* I fragment. The complete T-area, as well as the flanking regions, was cloned using a PCR-based plasmid walking technique. Eight clones, pM2/1-1 to 8, were obtained and partially sequenced. Partial sequence analysis revealed four conserved T-DNA genes, *IaaH, IaaM, ipt* and *ocs.* The order of these genes also remained conserved. It is highly likely that other conserved T-DNA genes such as gene *5* and *6b* might also be conserved on the T-DNA, although this could not be confirmed with the limited sequence analyses performed to date. The identified genes and flanking areas show a high level of homology to that of the octopine type *A. tumefaciens* pTi15955. Furthermore, the 500 bp sequence to the right of the T-DNA, contains an IS element of the IS*51* family, characteristic for *A. tumefaciens* octopine strains (Yamada *et al*, 1985). This sequence is lacking in *A. vitis* o/c type strains. The sequence to the left of the *ipt* gene is also characteristic of TL-DNA

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of octopine type *A. tumefaciens* strains and not of the TA-DNA of *A. vitis* o/c type strains. These findings indicate a closer resemblance to the Ti plasmids of *A. tumefaciens* octopine-type strains than to the octopine- or o/c-type Ti plasmids of *A. vitis.* M2/1, have, however been classified as a biotype 3 strain (Appendix C) and have been found to efficiently induce tumours on grapevine. It is therefore possible that M2/1 does contain an octopine type *A. tumefaciens* Ti plasmid within an *A. vitis* chromosomal background. If this is indeed the case, it is imperative that the possible existence of a TR area should be investigated.

Data from the hybridisation experiments were used in correlation with the sequencing results to construct a partial RE map of the T-DNA (Fig. 6). In comparison with RE maps of characterised o/c type strains (Otten and DeRuffray, 1994), it became evident that the partial map of the M2/1 T-DNA is unique in several instances. The IS*3*-like element flanking the 3' end of the T-DNA compares well with the 529 bp IS*51* fragment from pTiAch5 and fits in with the proposed model from Yamada *et al*, (1985) that *Agrobacterium* T-DNA *iaa* genes may have been acquired from other bacterial species as part of a transposon flanked by IS*51* (IS*51* forms part of the IS*3* family). The two IS*110*-like elements flanking the T-DNA seems to be the 5' and 3' ends of the same element and it appears that a T-DNA insertion event could have disrupted this element (Fig 6 shows the relative positions of the IS*110* fragments on the T-DNA of M2/1). Another possibility is that M2/1 acquired its T-DNA from another *Agrobacterium* strain through a double homologous recombination step involving the IS*110*-like element. Elements of the IS*110* family have, to our knowledge, never been reported in *A. vitis*. This study demonstrated, however, that they might have a possible evolutionary role in the development of *A. vitis* and should be investigated further.

What is clearly shown in this study, is that pTiM2/1, although conserved in ancestral sequences, differs significantly from characterised *A. vitis* octopine and o/c type Ti plasmids. Whether the T-DNA and/or Ti plasmid does in fact originate from an octopine type *A. tumefaciens* should be investigated further. M2/1 does, however, exhibit several characteristics that could be exploited in a grapevine transformation system, including efficient DNA transfer ability and reduced necrosis on grapevine embryogenic callus. We hope to exploit these characteristics in the further development of an efficient *Agrobacterium*-mediated grapevine transformation system using M2/1.

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Appendix A:

A. vitis M2/1 T-DNA partial sequence data:

1. LB border flanking area: (5' pM2/1-8)

ATGGAGCCGTTCTTTCCCGCAAAGTCAGCCGGTCGAAGTTGGCCGAGACTATAAAAGCGATTTCTC
CGAAGACAATAGCAATGGAAGCCTGCGCCAGCGCTCATCATTGGGGAAGACAGTTCAGGCAAGCAG
GACGAGAAGTCCTTCTCATCAATCCACGCTTCGTGAAGCCATTTGTACGCGGCTCCAAGAACGATG
CGGTCGACGCAGCGGCGATATATGAAGCTGCGACACGGCCAACCATGCGGTTCGTACCGGTTAAGT
CTACCGCGCAGCAAGATCTTCAGTCGCTACATAGAATTAGAGAGCGTCTCATTATCCAGCGCACAT
CGCTTTTCAATCATGCGCGTGGCCTTCTCGCCCGAATATGGGATCGTACTGCCACAAGGACCGTGG
AACTTCGCAGCCCAGGCACCCTCGGCGGTCGAGAATGCACCTCAGTCGGATCTTGGGCGTGAGCTA
TTCCTGGAACTTCTTGATCAACTTACCGATGTGAATGGCCGCGTATTCAGGATCGATAGGAAAGAT
CAAGTGAGATTTGCCGCGAACGAAAMCGATTGGCCGTCGTCTTTTCCGAGTTGGCANG

2. *IaaH* gene and flanking regions (3' pM2/1-8, 5' & 3' pM2/1-7, 5' pM2/1-6)

TTAAAAGTTTGTNCAAAATTCTCCATCGCAAGTTATTATTCGATTTATAACATAAGGCACTTGTTG
TTCATAGACGCAATACAAGGATTGATTGTCATCAATCTGAAAATTGTAAAAACGAACATGGTAGAA
AGTTTAATTGGGTAAACCGGCAAAATATCGGAATCCAATGGCTTCTCAAACAGGAATGCTCAAGCC
AGGTAGGCCTGCGTTGCTGCTTGGGTCCACATTTCGCACGTAGATCTTGAATGTGTCCAGCATCGT
GCCATTGTGGATAACTGAGGAATCCTGACCTATGGGTCTGGCCACCAAGGGTGCTGTTGGGAAGAG
AATAGCATCTAATCTATTCAGTTTGAAGTAGTTGCGATAGGTGGCTTGAAGTCTTGGTCTGAAGGA
GTGGCGGGCCAGTTCATATTCAGCTTTGGAAATTTGATGTCCATCAATTTGCGCATTGGCAATGTT
GGCACATCAGGGctacgAATTCCTTTGATGACGTCAGAAAAAGAAACAGTTTTTACAAGTCGTCGA
GATACTGTTTTAGAGCGTGTGGAAATTCATAGAGTGCAACTGGGAAGCTGGCCCCTTTATTCAGTT
CGTCAAGGTGGGGAATGTTAGCTTCAACAAAAGTTACGCCTTTGTTTGCTAGCAGGCGAATCGTTG
TTTCAGCTGCTAGGGCCACATCAGCATCAAGGTCATCATAAAAGTAGGTTGTAGGGAGGCCGATCC
TTAGCCCCTTCAGCGGCACGGGTGGTATTCTCTCCGGTGTGCCGGAAATTATCCGGTCGAGGATTA
CAACATCGGCTACGCACTGCGCTATGATTCCGGGAGTGTCCCGGGTAGGGCTAACCGGTATTATCC
GATCTCCCGGATATCTACCAAGCGTCGGTCGAAATCCTACTACGCCACACAGGGCTGCGGGTAGGC
GAACAGATGCACCGGTATCGGTGCCTATGCCGCCTAACATCAATCGGCTKGCTACCGCAGCAGCCA
CACCACCGCTTGAGCCCCCTGGTATCAGATCTGGATTCCACGGGTTTCGCACCGCCCCGGTGGCAT
AGTTGTTGCTTGTAATTCCAAACGATAACTCATGCATATTTCCCGAGGCACCCGGCAGTGCTCCAG
CTGAAAAAAGTCTTTCTGCGACGCGGGATGGTATCTTTGGCAAGGTGWTTTATCAGCGCCCGGCGT
AACGGCGCT

3. Partial *IaaM* (5' pM2/1-6, 3' pM2/1)

NCAATGTTACCCNCNNAGTCCAACCTTTGGTTAGCTTNCAAAGGCTGCCAATGTCGATTTTCCCCG
AGATTTCAACTGGTGAAANGNANCAGTTCTCCCGcTTATATCAATATATTGCAAAGAAATTTGGGG
CGGAAACTTGAATCGAACCTTGGGCGCGGAGGCCACCAgtgAGTGNTCTCGTNCCATCGACTTGGC
ACCATTTGGCATGNGAATTTCTCCGAAGCACAACTAATCCAAGCCNTGTTTTTGCtgAGCGGTAAA
GCAGATGGCACCGATTGATCTTAGTCATTTCGTGGCCATTTCAATCTCTAAGCAACTGCCGGCTTT
CGAACCCTGCCAATGCCGCTGTACGAGAATGGCACGATGAGAAATGCGTTACCGGGTTTACCATAA
CCCTTGAAGGGGCCGTGCCATTTGAAACATGGTAGCTTATGGTCGAAACCTGATGCTGAAGGGTTC
GGCAGGTTCCCGTTTCCAACAATCGACTTGCTCTACGACTACAGACCGTTTTTTGACCAATGCGTT
CCGATAGTGGACGGATCGGCTTCTTTCCGGAGGATGTTCCTAAGCCGATTAAGTGGCGGTCATTGG
CGCTGGCATTTCCGGACTCGTGGTGGCAAACGAATGCTGCTTCATGCTGGGTAGACGATGTTACA

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ATATATGAAGCAAGTGATCGcaTGTTGGAGGCAAGCTTTGGTCACATGCTTTCAGGGACGCTCC
TAGTGTCGATTGGCCGAAATGGGGGCGATGCGATTTCCTCCTGCTGCATTCTGCTTGTTTTTCTTC
CTCGAGCGTTACGGCCTGTCTTCGATGAGGCCGTTCCCAAATCCCGGCACAGTCGACACTTACTTG
GTCTACCAAGGCGTCCAATACATGTGGAAAGCCGGGCAGCTGCCACCGAAGCTGTTCCATCGCGTT
TACAACGGTTGGCGTGCGTTCTTGAAGGACGGTTTCTATGAGCGAGATATTGTGTTGGCTTCGCCT
GTCGCTATTACTCAGGCCTTGAAATCAGGAGACATAGGTGGGCTCATGACTCCTGGCAAATTTGGC
TGAACCGTTTCGGGAGGGAGTCCTTCTCTTCAGGGATAGAGAGGATCTTTCTGGGCACACATCCTC
CTGGTGGTGAAACATGGAGTTTTCCTCATGATTGGGACCTATTCAAGCTAATGGAATAGGATCTG
GCGGGTTTGGTCCASTTTTTGAAAGCGGGTTTATTGAGATCCTCCGCTTGGTCATCAACGGATATG
AAGAAAATCAGCGGATGTGCCCTGAANGAATCTTAAAACTTCCACGTCGGATCGSATCTGAAGTGG
TTAACGGNGTGTCTGKGAACCCCCNCATATGCCATGTTCAAGTCAGGGCGATTCAGAAGGGAAAGR
MCNAAAATTAAGGATTAAGGNTTAAAAASCGGGATWTTTGAMCYTTATKAAAANGGGGGGGCAMM
ATCTGGACTCSCAAAWATTCCMACTCAAGCANTTGNCYGGCCMTGCCGAANACCAATATTTTTTA
ANGGCNCCCAKTGGAACCCAAGCCGGGTGWWTAACMCCCCCCTTTTGAMMGGGATYNKYCAAAAA
AYYYTTTYCYGKAAGNMCNNT

4. *ipt* gene and flanking regions (5' pM2/1-1, 3' pM2/1-2)

GCCNTTCSGTTAAGTGACAAATTGGCTTTCAAAGAAGACAAGCCAATGCCCCNMCATTTTGTTGAA
AAAACAAAGTKCCNTTTTGGGAATCCGGTAAAAGCCAGCTTGCACTTCAAATAATGAATTTCAAGG
AGNCAATATACCCGCNTCTGATACCNCATTTCTTTTAATATAAAAATCAGTTTTGTATTCAATATA
CTGCAAAAAACTTATGGACCTGCATCTAATTTTCGGTCCAACTTGCACAGGAAAGACGACGACCGC
GATAGCTCTTGCCCAGCAGACAGGGCTTCCAGTCCTTTCGCTTGATCGGGTCCAATGCTGTCCTCA
ACTATCAACCGGAAGCGGACGACCAACAGTGGAAGAACTGAAAGGAACGACGCGTCTCTACCTTGA
TGATCGGCCTCTGGTGGAGGGTATCATCGCAGCCAAGCAAGCTCATCATAGGCTGATCGAGGAGGT
GTATAATCATGAGGCCAACGGCGGGCTTATTCTTGAGGGAGGATCCACCTCGTTGCTCAACTGCAT
GGCGCGAAACAGCTATTGGAGTGCAGATTTTCGTTGGCATATTATTCGCCACAAGTTACCCGACCA
AGAGACCTTCATGAAGGCGGCCAAGGCCAGAGTTAAGCAGATGTTGCACCCCGCTGCAGGCCATTC
TATTATTCAAGAGTTGGTTTATCTTTGGAATGAACCTCGGCTGAGGCCCATTCTGAAAGAGATCGA
TGGATATCGATATGCCATGTTGTTTGCTAGCCAGAACCAGATCACGGCAGATATGCTATTGCAGCT
TGACGCAAATATGGAAGGTAAGTTGATTAATGGGATCGCTCAGGAGTATTTCATCCATGCGCGCCA
ACAGGAACAGAAATTCCCCCAAGTTAACGCAGCCGCTTTCGACGGATTCGAAGGTCATCCGTTCGG
AATGTATTAGGTTACGCCAGCCCTGCGTCGCACCTGTCTTCATCTGGATAAGATGTTCGTAATTGT
TTTTGGCTTTGTCCTGTTGTGGCAGGGCGGCAAAACTTCCGACAATCCATCGTGTCTTCAAACTTT
ATGCTGGTGAACAAGTCTTAGTTTCCACGAAAGTATTATGTTAAATTTTAAAATTTCGATGTATAA
TGTGGCTATAATTGNAAAAATAAACTATCGTAAGTGTGCGTGTTATGTATAATTTGNCTAAATGTT
TAATATATATCATAGAACGCAATAAATATTAAATATAGCGCTTTTATGAAATATAAATACATCATT
ACAAGTTGGTTATATTTCGGGTACCTTTTNCATTATTTTGCGCAACAAGTCACGGATATTCGKGAA
AACCACAAAAACTGCGAAANTTGCGGCAGGGCCTTCAGTTTTCCTATTAATATTTAAGTTTGACAN
CCAGTTGCTATTMATTGCGGGCCAASCTCAGCTTGTTTT

5. *ocs* gene 3' and flanking region (3' pM2/1-2, 5' pM2/1-3 and 5' pM2/1-4)

CCTTGCTCGACGGTATCTTTTGCAGCGAGAATTCCAGCAGGGTGAGCAACAGGGTTGGTATTTGAA
AAAAATATCGACGCGGGATTTTGATACCACTGAAGCCGATTTGGAAAGAGAATCTCGAAGCCCCCC
CTAACCTCTTCGCTCAAAGCCCGAGTTGACGCAACTTCGAACGTTCTCTTCACACTTAGCATTAGC
ACCTGTGCATTGACACGGCGGCATGCATAGGGAGATGTCGTTGCTTCGATGACGGCGATAGGTGCA
AAAGCTGGAGTTAAAGTCTGCTTGCATGCCAGAGACGTTGCGCTACCGGGCAATGCTACGAGGACC
GAGCTGCTCAGATTGAAGTTCGCCAACTCGCAAAGAATTCCTTGCTGGCCCATGGTCGGGACCGTA
AGAAAAATGAACGCCGCGCCTGAAATCGCTGTTTCAAGATCATCCTCCAATTGCGGCTGAAAGTCG
CCTCCATAGTCCGGCCCTACTAGCTCCAAGGAGCCAAGGGACCTCACAGAGTTGAAGCTGTTCCTG

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TTGGAGATTGGCGCCCAGATTGAGGACACCTGGCCGAGCCTCCGGGCGAGATCACCTGCAAGAG
TAAGAGCCACGTTTCCCGCACCCAAAATTGCCACTTTAGCCATTTGGTAGATTGCAAATATAATGG
TTTGGCGATTATCCTTGAGGCCACACCTTTAAATAGATCAATGAATGGGCAAGTATTGACTTGCGA
TAATCCTTTAACTTTCTGCCTACCATCAATGTGGATGAGTTGTCGGTAAAAAGGATCCCTGAAAGC
GACGTTGGATGTTAACATCTACAAATTGCCTTTTCTTATCGACCATGTACGTAAGCGCTTACGTTT
TTGGTGGACCCTTGAGGAAACTGGTAGCTGTTGTGGCCTGTGGTCTCAAGATGGATCATTAATTT
CCACCTTCACCTACgATGGGGGGCATCGCACCCGTGAAGTAATATTTGTACCGGCTTAAAAACCG
AAATTTTGGCCTGGTAAgAACCTTCAAATTGGCGAGCCTTTTTTTAAATTTTTAAAAACCTANTTT
CNGGGGNCCCTTAAACNTTTTTNGGGGGGGTGGAATKGAAATGCCTTTAACCTNGGGGCAAGGGGA
AAWATTATTNANCCCGGGTT

6. RB area (3' pM2/1-3 and pM2/1-4, 5' & 3' pM2/1-5)

NCCTCTAGGACGCCTTTTTGGAGACTTTTTTTGNTCAAGCAATTCGGCCNGTTCTGATGAATGGTT
CGCAATCCCTTCCCNAACACTTATACAAGCAATTTNNTCNCCTGGGCNACACGGAAAAANTGCGTA
CCATTNCCCCCGGCGGGTTTTGACCNATCACCATGTCTTTTGGGGAGAGNCTTGAGTAGCCNCCNC
NCGNCNGGAANGCGAGAAAtgATcCTgCAGatcGGATCGCGTTAAGGTTTTTGCNCATAACGaCAA
CCGGGAATCAgCTTTGCGCATGCCGTACTTATGtCgCACTTGGAAGGcTTTNGTTACGTATGACTT
TCGTGATCGACGTCTTCGCCGTTCGCATTGTCGAATGGCGGCCAATGgCGGACAGCACATGCAGCT
TTTGTCCTCGATGCCCTCGAGAGGCTTCATCGCATGATGGCGTCCCGCTCATGGCGGCCTAGTGCA
TCACTCGGATCGCGGTGTGTTCAATACGTGTCCTTTCGCTATTCCGAGCGGTTGCAGAAGCAGGTA
TCGAGAGCCATCTATCGGAAGCGTCGGCGACAGCTACGACAACGCCCTCGCAGAAACGCGATCAAC
GGTCTTTACAAGGCCGAGGTCATTCATCGGCGTGGACCATGTTGAGGAGCTTCGAAGCGGTCGAGT
TCGCTACCTTGGAATGGATAGACTGGTTATCAACCACGGCGGCTTTTGAAGCCCATCGGCAATATA
CCGCCAGCCGAAGCSACGAGGATCAGTATTACGCCATGCTGGACGAAGCAGCCATGGCTGCGCATC
ATTTAACGAAATGGCCTCCGGCAAACCCGGTGCGTGTGTGGATGCCCCCGGNATTTGGCAAGAAGA
TTTTTTTGACGGTAGTCCGCGAACGAGGAATTCTATCGGTCGTGTGTCAGGCCCTCTTGATGTGGC
CTGATGAtAGCCACGGGCCGGGAtGCAGTTCGAAGAACAGAGGCCACATCGGTTCANGGAGAGCTT
TGTGCTCGAGCGCCGGGACTGGTTTACCCCATTCTGAACATTGAAGTCCTTTCCGATGGTTCCATC
AGTCGATCTTTCTCACCGGGGcTTTTGGCCCCAGGCATTCACCGCCTTGCTCTTTCGAAAAAAAAC
AGCGGATCACGCCATTCTGTAATTCTGCTCCCTCGTCATCATCGCCCAAATGATGCGGGCCATCTT
GTTTGCGAGTGCAACAGCCGCGACCATTCGAGGCTTTCGTCCCAGAACGCCTCCCAGCCAATTGTC
CGGCAACACGCCCTTACGCACGATCCAGCGGATCAGGCTCATGGCTCCGACGATGAGCAATTTACG
GATATCCGTTTGCCCCATCTTGCTCACGCCTCCCAGCCGGGTCTTTCCTCCGGTTGAATGCTGGCG
GGGTACAAGGCCGAGCCAGGCAGCAAAATTCCGGCCACTTCTAAATGCGCGAAGATCTGGAGCAAA
CGCGAGGATCGCTCCGGCAGTCACGGGTCCGACGCCGGGAACGGTGCAAAGTCGACGCATATCTTT
ATTCGCCTTGGTTGCTTCTC

7. Sequences from "TB fragment"

CTMMSCTTCGCCGCCCACGTTTCTCTTTCTTCTCMTATTMAATTGTCMAAAAACAGACCGTATCAA
ACAGTCACAAAATCATTCCCCCAAAGCCTAAACCCCGGGAAACAAACAAGCTATCAGCCCATCCGC
TTGATTTCTTTAGAACGAAAGTCGTCGTCGCCAGCAGCGCCGCCGCCCTCGTTCAGTGAGCGGACT
TATAAATCCACCCCACCAAACAAGTCAACACAGCAATATCAAAAAAATCAAGAAATGTGACAGATT
ACTGATTTTAAAAGGGAATTTGAGATTAATCGTACAGATTAACGGATTTTAACGACTTTCGCCGTG
CAAACGATAAGTGGGGTTAGCCTTCCCCTATATAGGGTTTCCATTTCGCAATTAAAGGACGCGAGC
GACCTTACGGATATCAAGTCGGAAGACGGGCTGAAACGGGCGACCAGGGCATGGGCATTGCCAGGA
TAGGTGAAGCGCACATAGGTCACGGCACCGGAATTGCTCCAGGTCGACGCTCGAMCACAAGACANG
CCTGCTGTCGGAGATGCCCGAGCANGCGCGCGATCTNCTTCGACGGNGCCTCGGCACTGATCCGAT
GTTCGGGAGTGCTCCCCGGACATGGGCAAGCAGCCANGGGCCTGGCGCAATTTCGGTAAATCGGGT
TTARNCCGNTTTCGGNCNGGCGTNAAMTKATKANACCTTSNTTKMMMMAAAATTNKCGCCCCCCCG

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CNAAATGGGTKSCNAANAATNTTNARCCACNATKNTGNCCRMCGGAAACNTNNARCCNSTTNCC
AAACCCCCNTTTTTGGGGSTKGGGCCTTTTTGNNCYTCCNGGANYTTTNAANCCCTNTTTNWNGGC
CCAGGGGWTTSAMTTTNSNCCCCNWKNTMNTTGAATTTTNNNAAANGGMNAAMTGGGWTNTTNNGG
NTKGGGAAACAAAAATKSCCGGGNTTTTTTGGGGGGGTNGWNNAAACCC

>M2-1TB1T7
CAAWTCGACTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTTCGCAGCTGA
AGGCGTTGAATGCGCGCATCGGCGGGCTGAGCGTGGTTGCCGATAGCGCGGTCACGATTGCCGGTC
TTGCCGATACATTCGAGGCAGTAATGCCGCTTGCCGTGCTTGTCGAATGCGATACGGGCGGCAAGC
GCTGCGGCGTGCAGACGCCGGAAGCGGCATTGGCGCTTGCGGAAAAGATTGTTGCGGCCCCGGGCC
TCAGATTCAAGGGCATTTTGACCTATCCGGCACCGGATGGGGCCGAGACGGTGGAAAACTTCCTTC
GCAAAACCATGGCGTTGCTTGCGGCAAGGGGCATTGACTGCCGGTCCGTTCCAGCGGCGGTTCGSC
CGATTTTTATTCCGCCATCTCGTTCCTTCCGCGACCGAACATCGGGCAGGCACCTATGTCTATAAT
GATCGCGCCATGCTGCGCGCGGGACATTGCACGACCGGCGATCTCGCCATGCATGTTCTCGCCACC
GTCGTTTCGAGGCCTACNGNCGATCNGGCNGTGCTGGACAACGGATCCAANGCCTTGACCTCGGAT
CTTCTGGGTTTCANCGATTATTGGCTGATCCAAAGGTTGTGAAGGCGCAAAAATCGCTCSTTTCNG
NAAAAAACCCCSTCSNTGATTTGTCCGGTKGNCTTTCNGGCGGCYTKAAATCGGTGATGTNGGAAA
AGGNNNNGCCCAAACCMMACCCTSCGTNGNYARSNAATNTTTTTNSAAMMGGAAGGGGTTTTCCMT
TCNAAAACGGGGNYNKNAAMMMCCCCNTTNANAAAGGGYAAMAANNGSCNNCGGWWCCCYNTTGGT
AAAANTWCCCCGGNTAATTTAAATNGGNAWYNGGAAASYYGNNSAAAAWTCNNAWWNCCCGCNMMC
NANAAAATTNGGGAAAWWTKNNSYTTTTTTMMCGGGGGNKSNTTSCCMMWYAA

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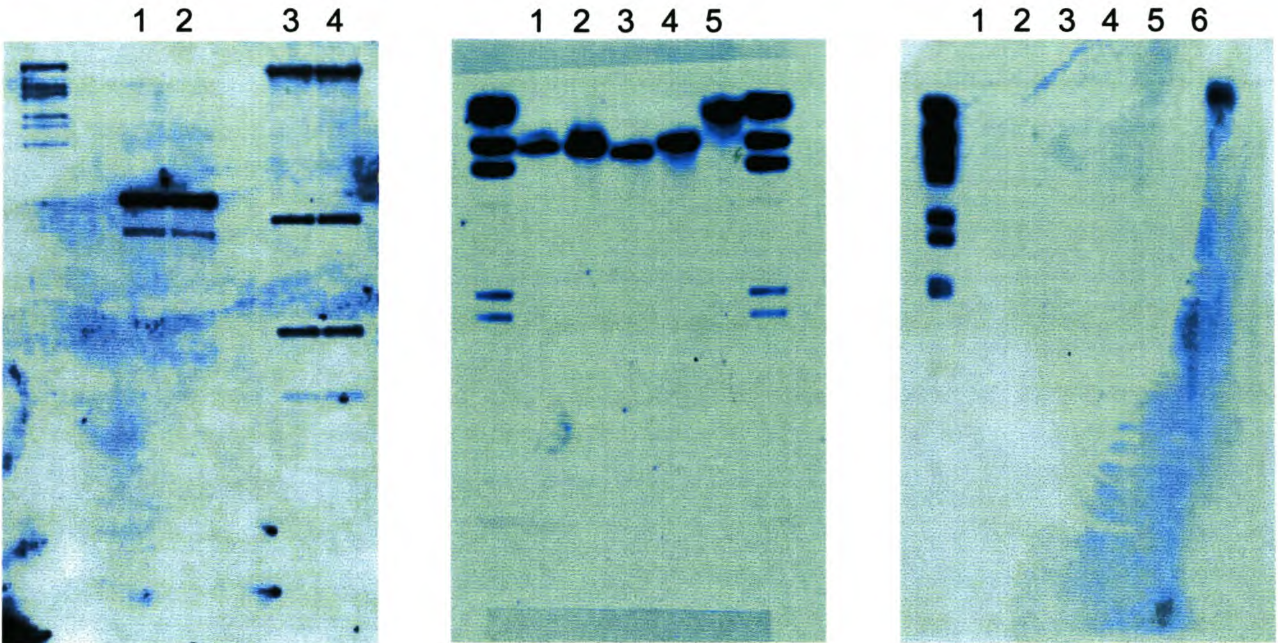
Appendix B

Hybridisation results indicating the absence of a TB area in M2/1



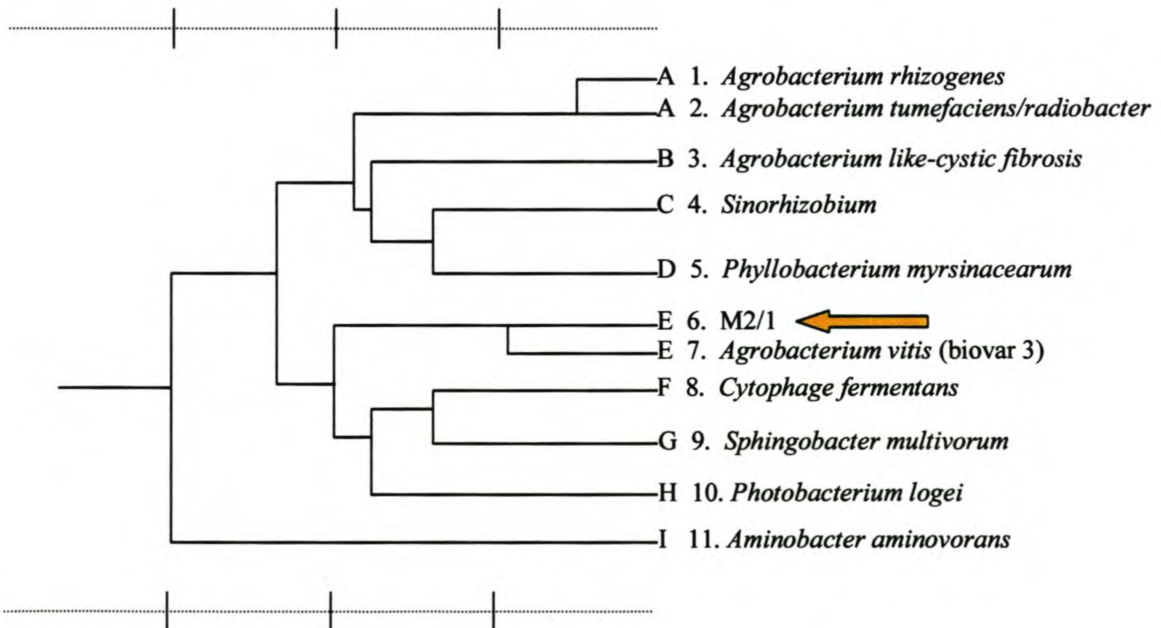
Lane 1&2: M2/1 *Eco*RV
Lane 3&3: 2608 *Eco*RV digests

Lane 1-5: M2/1 *Hpa*I/*Xho*I, *Xho*I, *Hpa*I/*Pst*I, *Pst*I, *Hpa*I digests

Lane 1-5: M2/1 *Eco*RV, *Eco*RI, *Hind*III, *Pst*I, *Hpa*I digests. Lane 6: AB3 *Bam*HI digest

Appendix C:

Results form Biolog assay (represented by a dendogram)



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CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSION

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4.1. GENERAL DISCUSSION AND CONCLUSION

The development of an efficient DNA transfer system is an essential component in all plant transformation strategies. Although established as the DNA transfer method of choice for many plant species due to the low copy number of introduced transgenes, as well as the relatively high frequency of stable transformants obtained (Birch, 1997; Gelvin, 1998), several plant species have proven to be recalcitrant to *Agrobacterium tumefaciens* infection. This could be attributed among others to the host range limitations of *Agrobacterium tumefaciens*, and/or the induction of an active plant defence response upon infection (Songstad *et al*, 1995; Hutcheson, 1998). In order to circumvent these limitations intrinsic to *Agrobacterium*, several alternative DNA transfer strategies have been developed. These mainly include direct DNA transfer methods such as protoplast transformation and microprojectile bombardment (Negrutiu *et al*, 1987; Cao *et al*, 1992). These methods are useful due to their applicability to a wide range of plant species and target materials, but they typically do not produce high frequencies of stable transformants. Furthermore, the transformants that are obtained frequently show high copy numbers of the transgenes, leading to several problems such as gene disruption or transgene silencing (Birch, 1997). The practical implications of these limitations have refocused attention to manipulating the *A. tumefaciens*-mediated transformation system in order to extend its host range and avoid some of the detrimental effects caused by the active defence mechanism of plants. This research has resulted in the transformation of several monocotyledonous plants such as rice (Hiei *et al*, 1994), previously thought to fall outside the host range of *A. tumefaciens*. Although advances have been made to combat the effect of the active defence response on explant tissue, (i.e. the addition of anti-oxidants in culture media) (Perl *et al*, 1996), the necrotic effect of this response still seriously hampers transformation efficiency.

Although a serious limitation to the *A. tumefaciens* transformation system, the host range limitations of other *Agrobacterium* spp. could be exploited to transform plants that have proven recalcitrant to *A. tumefaciens* infection. One such sp., *A. vitis*, has been found to preferentially infect grapevine, a crop that has been especially difficult to transform with the *A. tumefaciens* system (Otten *et al*, 1996). It should therefore be possible to genetically alter a suitable *A. vitis* strain to facilitate specific DNA transfer, thereby exploiting the ability of *A. vitis* to bypass the active defence response of grapevine. As discussed in chapter one, the purpose of this study was to investigate the possibility of

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utilising an indigenous pathogenic *A. vitis* strain as an alternative to *A. tumefaciens* in order to facilitate the consistent, efficient transfer of foreign DNA to grapevine cells without tissue damage.

In order to select an *A. vitis* strain with suitable characteristics to be used as a transformation agent, we tested the infectivity of seventeen indigenous *A. vitis* strains on *Vitis vinifera* cv. Merlot. Infectivitity, as measured by tumour formation, varied significantly among the seventeen strains with many strains failing to induce tumours. Two *A. vitis* strains, M2/1 and H8/1, induced large tumours on nodal cuttings of Merlot as well as on *in vitro* grapevine plantlets (results not shown). These strains were subsequently used in a test on embryogenic grapevine cultivars to estimate the induction of tissue necrosis by these strains. *A. vitis* as a pathogen of *V. vinifera* cultivars suggests a compatible host-pathogen interaction, a fact that will presumably be reflected in limited or no tissue necrosis after co-cultivation with the strains. This notion was proven accurate by significantly less tissue damage and excellent recovery of somatic embryos infected with *A. vitis* strains in comparison with the cultures infected by *A. tumefaciens* EHA105. These reactions were calibrated against a uninfected controls, which clearly confirmed that the *A. vitis* strains tested induced a lesser defence strategy than *A. tumefaciens.* However, variation was observed between the amount of necrosis induced by M2/1 and H8/1 on Merlot callus. This is surprising since both strains induced tumours of approximately the same size on Merlot cuttings during the seven-week incubation period. The variation observed could be indicative of a genotypic effect of *Agrobacterium* and/or grapevine affecting the interaction between plant and pathogen. If the observed variation is indeed caused by a cultivar-specific genotypic effect, it could potentially limit the applicability of a disarmed *A. vitis* strain in a grapevine transformation system. It is premature, however, to question the usefulness of a wild type strain before it has been disarmed and tested, since the oncogenes present on the wild type Ti plasmid might contribute to the observed host range limitations. A true reflection of the strain's ability to function as an universal grapevine transformation tool would only be obtained once the oncogenes are removed and the foreign DNA transfer ability of the strain has been assessed. The wild type *A. vitis* strain, M2/1, effecting minimal callus necrosis on several grapevine cultivars was thus selected for further characterisation.

The strain was shown to belong to the octopine group of strains. Hybridisation experiments as well as sequence analyses indicated that the M2/1 Ti plasmid deviates

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significantly from the characterised octopine and o/c type *A. vitis* strains. Most significantly, the results indicate that the M2/1 T-DNA do not carry any sequences homologous to known TB areas. It was, however, confirmed that pTiM2/1 carried at least four of the conserved o/c type T-DNA genes. These included; two genes encoding for the production of indoleacetic acid; one coding for indoleacetamide hydrolase (*laaH*), and one coding for tryptophan monooxygenase (*laaM*); the isopentyl transferase (*ipt*); and octopine synthase (*ocs*) genes. The deduced amino-acid alignments confirmed the intact open reading frames of the encoding genes. Huss *et al*, (1990) showed that tumour formation can be induced by the *A. vitis* o/c type *laaH*, *laaM* and *ipt* genes, either seperately or in a synergistic action. Theoretically, if all three genes identified are actively expressed, tumour formation mediated by the genes located on the T-DNA of M2/1 should be possible, thus rendering the presence of a TB area obsolete. These results strongly suggest the absence of the TB area.

Strong homology to the TL sequences of pTi15955 was observed for both conserved T-DNA genes, as well as flanking areas. Hybridisation results, in correlation with sequencing, data were used to construct a restriction enzyme (RE) map of the TA-DNA area of pTiM2/1. The map differed considerably from all other characterised o/c type strains (Otten and De Ruffray, 1994). Major differences originated from intergenic areas on the T-DNA; the most significant difference, however, was found on the 5' end of the T-DNA. Hybridisation results indicated that the agrocinopine synthase (*acs*) found on the 5' end of most characterised *A. vitis* o/c type strains, was absent from the T-DNA found in M2/1. The strain was, however, identified as a biovar 3-type strain and is capable of inducing tumours effectively on grapevine. It is, however, possible that M2/1 carries a Ti plasmid that strongly resembles that of octopine *A. tumefaciens* strains. If this is indeed the case, it would be imperative to investigate the presence of a TR area, characteristic of octopine *A. tumefaciens* strains (Hooykaas and Schilperoort, 1992). With the limited sequence analyses performed in this study, it was impossible to determine the precise cause of the absence of the *acs* gene, but it might be attributed to a deletion, or mutation of the 5' end of the T-DNA. This is an important aspect that needs further investigation.

Sequence analyses of the flanking regions of the T-DNA revealed two bacterial insertion elements (IS) of two different families, IS*3* and IS*110* (Mahillon and Chandler, 1998). IS elements are commonly found in *A. vitis* Ti plasmid- as well as chromosomal DNA and have been associated with rearrangements and deletions of the T-DNA (Paulus

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et al, 1991). The IS*3*-like element found flanking the 3' end of the T-DNA correlates well with the existing hypothesis that the *Agrobacterium* T-DNA *iaa* genes may have been acquired from another bacterial species as part of a transposon flanked by IS*51* (Yamada *et al*, 1985). The IS*110*-like elements, flanking the 5' end of the T-DNA as well as 3' end of the IS*3*-like element, however, appear to be part of a single element disrupted by the intervening T-DNA. This apparent disruption could imply that the T-DNA insertion event happened inside this gene, or, that M2/1 acquired the T-DNA segment from another *Agrobacterium* strain or species through a double homologous recombination event involving the IS*110*-like element. The presence of IS*110*-like elements in *A. vitis* has not previously been reported, and this study hints at a possible evolutionary role for this element in *A. vitis*. This observation, however, is not tested at this point and needs further investigation before evolutionary assumptions are made.

A. tumefaciens-based plant transformation protocols were central to the study of plants during the last generation. Apart from the obvious economic importance of a gene transfer system for plant species, the genetically transformed products of these transformants enabled scientists to study various physiological and genetic features of processes key to plant growth and metabolism. It is fitting then, that a process so important to plant science and biotechnology in general, becomes optimised for its various applications. To this end, this study has attempted to provide the fundamental background to develop a specific transformation system for grapevine, exploiting some of the unique characteristics of *A. vitis*. We have selected a unique *A. vitis* octopine type strain and was able to clone the T-DNA as well as the T-DNA flanking regions using a PCR-based chromosome walking technique. The flanking regions will be used in a previously proven disarmament strategy based on double homologous recombination to remove the oncogenic region. The resulting disarmed M2/1 strain will be used in grapevine transformation experiments, determining the effectiveness and application range of this strain as a transformation tool.

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