Genetic engineering for antibacterial activity in lettuce (*Lactuca sativa* L.)

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of M.Sc. (Plant Breeding and Biotechnology).

GENETIC ENGINEERING FOR ANTIBACTERIAL ACTIVITY IN LETTUCE (*Lactuca sativa* L.)

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Lettuce crop is affected by bacterial diseases (both in the field and in storage) with severe consequences which can be mitigated through improving disease resistance in lettuce. The numerous constraints associated with sexual crosses in lettuce render novel gene transfer via genetic manipulation a suitable method for lettuce improvement. This study was undertaken to transfer three chimaeric genes encoding antibacterial peptides into lettuce and to ascertain the effectiveness of these peptides in enhancing bacterial disease resistance.

Genetic manipulation of plants requires a good regeneration ability of genotypes in tissue culture, to ensure recovery of complete transformed plants. Twenty-two lettuce genotypes belonging to several morphological groups were thus screened for their regeneration response to defined tissue culture conditions and selection of amenable genotypes for genetic manipulation. Genotypic variation was observed in callus, shoot and root

production *in vitro*; two lettuce genotypes Bambino (a crisphead) and Cobham Green (a butterhead) with good tissue culture response were subsequently chosen for *Agrobacterium*-mediated transformation.

Two binary vectors carrying chimaeric genes (with T4 lysozyme, magainin II coding sequences) were obtained from Crop & Food Research, Lincoln; these vectors had pBINPLUS (a recently constructed binary plasmid) as the backbone. A third binary vector, similar in structure to the two others, with a chimaeric gene encoding Shiva-1 (a cecropin B analogue) was successfully constructed.

The three vectors, pBINPLYS, pBINMGN and pBINPLUSH (encoding T4 lysozyme, magainin II and Shiva-1 respectively) in *Agrobacterium* strain AGL1 were used for the transformation of Bambino and Cobham Green. Two transformation treatments: a delay of 5 days (after cocultivation) before explants were transferred to kanamycin selection medium and an immediate transfer of explants to selection medium were studied for their effect on transformation frequency. Successful regenerated transformed plants of Cobham Green were obtained in this study; however, attempts to transform Bambino proved futile and no regenerated transformed shoots of Bambino were obtained. The specific reasons for the failure to transform Bambino are unknown but crisphead lettuce genotypes are often recorded as being recalcitrant to *Agrobacterium*-mediated transformation. The delayed selection treatment gave higher transformation frequencies in Cobham Green than the immediate selection treatment. Polymerase Chain Reaction (PCR) with specific *npt* II oligonucleotide primers confirmed the presence of the selectable marker gene in all the 22 putative transgenic plants recovered. An inheritance study on the selfed progeny of

transformed lines, confirmed transmission of the kanamycin resistance and pointed to a single insertion of the *npt* II gene in most of the transformed lettuce lines.

The presence of the antibacterial genes was established in all the transformed plants, by PCR. The presence and effectiveness of the antibacterial peptides were determined by an *in vitro* assay and greenhouse evaluation of pathogen resistance in seedlings. Crude leaf extracts from selected transformed lines and control of untransformed Cobham Green were added to cultures of *Erwinia carotovora* subsp. *carotovora* and *Xanthomonas campestris* pv *vitians* to establish their effect on the growth of the pathogens. Growth of pathogens in cultures with crude extracts from transformed lines was inhibited while extracts from the control showed no such effect on the growth of the pathogens. In the greenhouse, selfed progeny of transformed lines and untransformed control were inoculated with the two lettuce pathogens to assess their response to disease development. Overall, delayed symptoms and reduced disease severity characterised the inoculated transformed lines, compared to the control seedlings. The potential of the antibacterial genes to effectively improve the resistance of bacterial diseases in lettuce was evident in these assays.

Keywords: lettuce, callus index, shoot index, standard genotype, pBINPLUS, pBINPLYS, pBINMGN, pBINPLUSH, *Agrobacterium*, genetic engineering, T4 lysozyme, magainin II, Shiva-1, cecropin B, polymerase chain reaction, antibacterial activity

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Abbreviations

χ^2	chi-square
µg	microgram
µl	microlitre
µM	micromolar
2n	diploid chromosome number
bp	basepair
CAT	chloramphenicol acetyltransferase
CI	callus index
CTAB	cethyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
EDTA	ethylene-diaminetetraacetic acid
g	gram
GUS	glucuronidase
IAA	indole acetic acid
IPTG	isopropylthiogalactoside
kb	kilobase
kPa	kilo Pascal
l	litre
LB	Luria broth
M	molar
mg	milligram
ml	millilitre
mM	millimolar
ng	nanogram
NOS	nopaline synthase
npt II	neomycin phosphotransferase II
NZ	New Zealand
OD	optimal density

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
rpm	revolutions per minute
SH	Schenk and Hilderbrandt
SI	shoot index
sp	species
subsp	subspecies
T-DNA	transfer deoxyribonucleic acid
TBE	tris-borate
TE	tris ethylene diaminetetraacetic acid
Ti	tumour inducing
TMC	tris magnesium chlorate
TMV	tobacco mosaic virus
USA	United States of America
V	volts
v/v	volume/volume
w/v	weight/volume
X-gal	5 bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YEB	yeast extract broth

CHAPTER 1.

Literature review

1.1 Importance and origin of lettuce

Lettuce (*Lactuca sativa* L.) is a cool season leafy vegetable grown in nearly all continents. It is consumed mainly as fresh, raw salad vegetable with the exception of stem lettuce which is cooked before eaten; few reports, however suggest it can be also used as a tobacco substitute (Ryder, 1986). Nutritionally, it rates low among other vegetable crops; 95% of the crop contains water with varying amounts of phosphorus, iron, sodium and potassium, depending on the morphological type. Cos and Leaf lettuces have higher levels of ascorbic acid, vitamin A and calcium (Pink and Keane, 1993).

Lettuce belongs to the family Compositae, the tribe Cichoreae and the genus *Lactuca*. The genus *Lactuca* was reported to have about three hundred species, though few are correctly documented (Ryder, 1979). Many of these names were later identified to be synonymous (De Vries, 1990). Earlier, Lindqvist (1960) recognised about a 100 species while Ferakova (1977) listed 70 species under the genus. Seven chromosome levels are observed in the genus; 2n = 10, 16, 18, 32, 34, 36, 48.

Lettuce is believed to have originated from the Mediterranean. Evidence from Egyptian tomb paintings indicates that it was cultivated before 4500 BC, from where it spread to Greece and Rome. The earlier forms grown in ancient Egypt are morphologically described as Cos lettuce (Lindqvist, 1960).

There is a general theory that cultivated lettuce originated from *Lactuca serriola*. The significant differences observed between them have been attributed to accumulation of mutations due to selection (De Candolle, 1959). The possibility of cultivated lettuce originating from hybrids between *Lactuca serriola* and other *Lactuca* species, has been propounded (Helm, 1954; cited by Lindqvist, 1960). Three different theories are advanced concerning the origin of cultivated lettuce: that it originated from wild forms of *Lactuca sativa*; through direct descent from *Lactuca serriola*, or through hybridization between different species, including *Lactuca serriola*.

Lactuca serriola and *Lactuca sativa* are found to be closely related and completely cross-compatible thus supporting the inferences drawn on the relationship between them (De Vries, 1990); a second wild species (in addition to *L. serriola*) is believed to have also contributed to the evolution of *Lactuca sativa* (Kesseli *et al.*, 1991).

1.2 Morphological types

Lettuce classification is based on leaf shape and heading characteristics, though recently they have also been classified according to cropping seasons (Curtis *et al.*, 1994). There are six broad morphological types (Michelmore and Eash, 1986). Crisphead cultivars, characterised by tightly folded and brittle textured leaves. The heads are usually large and heavy and are the most extensive and preferred type grown in most consumer countries, such as USA. The butterhead types, also known as cabbage lettuce, have relatively small heads which are loosely folded. They are characterised by their broad, oily, crumpled leaves and vary in size, colour and appearance. The leaf type, are of minor importance in

commercial lettuce production and have relatively short shelf life. They do not form heads and produce a rosette of leaves. Cos types have elongated leaves, which are coarse and tough and form loaf-shaped heads. The Latin types also have loose heads and elongated leaves while the stem types are characterised by thickened elongated stems.

1.3 Genetics and Breeding

The chromosome number of lettuce and three close wild relatives of cultivated lettuce, *Lactuca serriola*, *L. virosa* and *L. saligna;* have been determined as 2n=2x=18 (Michelmore and Eash, 1986; Ryder, 1986). These four species according to cytogenetic studies, form a useful breeding group that is reproductively isolated from the rest of the genus.

Zohary (1991) summarised the taxonomic and genetic information available on wild relatives of the crop; these included: *Lactuca aculeata*; *Lactuca altaica*; *Lactuca azerbaijanica*; *Lactuca dregeana*; *Lactuca georgica*; *Lactuca scarioloides*; *Lactuca serriola*. These seven wild relatives were described as morphologically close to cultivated lettuce. Based on morphological resemblance and interfertility among some, they were categorised as the primary gene pool of cultivated lettuce. *L. saligna* was classified under a secondary gene pool and *L. virosa* in a tertiary gene pool.

Lettuce has a relative short life cycle, fully self fertile and a higher rate of natural self pollination with moderate space requirement. These are useful features in breeding programmes. However, the structure of the lettuce flower obligates self-fertilization making it relatively difficult to obtain hybrids.

The common breeding objectives in lettuce include breeding for resistance to diseases, pests and postharvest disorders, uniformity in head formation and maturation, and adaptation to specific environments (Pink and Keane, 1993).

1.4 Diseases of lettuce

Several diseases caused by fungi, bacteria, viruses and nematodes have been identified to affect lettuce in the field and in storage. These include lettuce mosaic virus, big vein disease; lettuce drop by *Sclerotinia* species, anthracnose, downy mildew; root knot disease caused by *Meloidogyne* sp. Other abiotic diseases such as tipburn and russet spotting also affect lettuce (Campbell, 1985). Bacterial diseases also have major economic impact on lettuce production and these include, bacterial leaf spot and head rot, corky root rot and bacterial soft rots (Patterson *et al.*, 1986). Bacterial leaf spot and head rot are caused by *Xanthomonas campestris* pv *vitians* and is characterised by circular, translucent, water soaked lesions that become dark brown. Depending on severity of infection, the crop may be rendered unmarketable. Corky root rot is caused by a Gram negative bacterium *Rhizomonas suberifaciens* (Van-Bruggen *et al.*, 1989). Symptoms include brown banded lesions which occur on the taproots, giving the roots a corky appearance. Severely affected plants become stunted, chlorotic and wilted. Bacterial soft rot of lettuce is a postharvest disease caused mainly by *Erwinia carotovora* and *Pseudomonas marginalis* (Persley *et al*,. 1989; Patterson *et al.*, 1986). These two are secondary pathogens, wound invading and usually cause the disease in storage or transit. Possible avoidance methods include maintaining storage temperatures close to one degree Celsius and preventing injury to the crop during harvest.

The residual effects of chemicals in crops has rendered their use in controlling secondary pathogens particularly undesirable. The inherent ability of plants to withstand attack from pathogens therefore, is the preferred method of disease control (Stephens, 1990). Disease resistance can be obtained in cultivated crops through the introgression of genes; breeding for such resistance has been one of the most important objectives in vegetable breeding (Kalloo, 1988). Conventional breeding methods have limitations in achieving such objectives in lettuce. The gene pool of lettuce within which it is cross compatible, is limited and require transfer of genes from other sources for further improvement against diseases. The obligate self-fertilising nature of lettuce hinder such crosses, where genes are available, between itself and wild species.

1.5 Disease resistance in lettuce

Disease resistance and stress tolerance affect potential crop losses and are indirectly responsible for yield, hence the introduction of resistance genes through genetic manipulation remains an important breeding objective.

Disease resistance in plants is multifaceted involving structural and chemical components, some of which are preformed while others are inducibly produced in response to pathogen infection (Kleen, 1992; Moffat, 1992). Physical barriers such as cell wall, cuticle, root pericycle; biosynthesis of compounds such as phytoalexins, glucanases, and chitinases which are antagonistic to pathogens; and hypersensitive response, characterised by the death of plant cells in the vicinity of an invading pathogen, all act to defend plants against pathogenic diseases.

1.5.1 Breeding for disease resistance

The conventional procedures for the production of genetic variability involve crosses between plants with complementary traits, selection of superior genotypes and further development to give a new cultivar. This requires large numbers of plants in each program as well as careful examination of individual plants and their progeny. In species with narrow genetic base, obtaining such variability is limited. Useful genes may be available in wild or distantly related species, but their usage are accompanied with internal and external breeding barriers. In lettuce, difficulties in obtaining hybrids from crosses with wild *Lactuca* species have been reported (De Vries, 1990; Maisonneuve *et al.*, 1995).

Wild *Lactuca* species offer a valuable source of genes for genetic improvement, which could be transferred to the cultivated crop. Resistance genes for lettuce mosaic virus, turnip mosaic virus, powdery mildew, downy mildew, bidens mottle virus (which are economically important diseases of lettuce) have been identified (Robinson *et al.*, 1982). *Lactuca serriola* has been identified as source of resistance to downy mildew (Crute and Johnson, 1976). *L. virosa* is reported to be resistant to the leaf aphid and *L. saligna* resistant to the cabbage looper (Ryder and Whitaker, 1976; Eenink *et al.*, 1982). Cultivars of lettuce with resistance to brown blight, downy mildew, lettuce mosaic, tipburn have been released for cultivation (Ryder, 1986).

The difficulty in obtaining sexual crosses between various *Lactuca* species and the long period required to obtain new cultivars from introgression of genes from wild species, are drawbacks in improving lettuce crop. The introduction of genetic variability into plant

genomes and the use of cell culture methods have become imperative in lettuce improvement programmes.

1.6 Cell Culture

In vitro techniques offer substantial ways of improving crops, through genetic manipulation and rapid propagation of genetic materials. Successful manipulation of crops *in vitro* require their complete regeneration into mature plants and this is widely established for many crops species. The provision of appropriate *in vitro* conditions in an axenic environment enables an individual cell to grow and divide in a self regulating manner, regenerating into a whole organism. This capacity of a single cell to regenerate the phenotype of the complete and differentiated organism from which it is derived, is known as totipotency. It is a special characteristic of cells in young tissues and meristem and it requires the cells to acquire a meristematic characteristic, irrespective of its origin (Mantell *et al.*, 1985).

Totipotent cells, given their meristematic properties, are able to produce whole organisms given the right environmental conditions. In tissue culture, several factors influence regenerative response of explants; these include: the type and status of explant, the genotype of the donor plant from which the explant is taken, the composition of culture media and variations in culture environment.

Explants can be induced, under enabling conditions to produce callus, an essential tissue in most plant cell culture systems. Its importance in plant tissue culture is proven by the

ability to be maintained *in vitro* by routine subculture and manipulated to cause cell differentiation into organised tissues and whole plants.

Developmental pathways in differentiation resulting in the formation of recognisable tissues require cells with the morphogenetic potential and can occur with or without the proliferation of undifferentiated tissues. The two pathways discernible during morphogenesis, can both be controlled *in vitro*. Organogenesis *in vitro* describes a variety of complex developmental sequences which result from the experimental manipulation of plant parts in the formation of roots and shoots. The sequences may or may not include an intermediate callus phase, which has its own advantages and disadvantages when employed in culture (Hicks, 1980).

Callus can be induced to differentiate into whole plants by inclusion of appropriate growth regulators in growth medium and the right culture environment (Mantell *et al.*, 1985). Callus cells, under such induction, are often unstable in terms of chromosome constitution and result in plants with different genetic constitution (Sheridan, 1975; D'Amato, 1977). For example, differences in morphological characters observed among regenerated seedlings of alfalfa, were traced to genetic mutation during the callus phase (Johnson *et al.*, 1980). This suggests that, to maintain the genetic fidelity of clones for propagation purposes, the callus phase of the regenerative pathway should be avoided. However indirect organogenesis remains an important regenerative pathway for several crops (Hicks, 1980; Mantell *et al.*, 1985).

Development of morphogenetic tissues can also occur through the formation of bipolar adventive embryoids under the appropriate nutritional and hormonal conditions. The

formation of somatic embryoids from somatic tissues follows through pro-embryoid, globular and torpedo stages. Somatic embryogenesis parallels that of zygotic embryos and depending on the species it can occur indirectly from a callus phase or directly from explant tissues (Parrot *et al.*, 1992). Direct embryogenesis may occur from cells which are predetermined to become embryo producing before their transfer to culture or tissues related to their active role in nutrient transport (Sharp and Evans, 1982; Hepher *et al.*, 1988). Somatic embryogenesis is influenced by explant type and age, genotype and the growth hormones. For different crop species, the preferred explant type and age differs; however the growth substances used show a degree of similarity. The auxin, 2, 4, dichlorophenoxyacetic (2,4-D) has been the most widely used in stimulating embryogenic response (Bhaskaran and Smith, 1990; Parrot *et al.*, 1992). Other auxin types, such as naphthalene acetic acid (NAA), kinetin, benzyladenine have also been used.

Somatic embryogenesis is reported to be the preferred pathway of regeneration in cereal tissue culture (Bhaskaran and Smith, 1990). The choice of the morphogenetic pathway, in most studies, however seem subjective and may be dependent on the plant type or purpose of study.

1.6.1 Influence of explant on regeneration

The most suitable explants for tissue culture are those in which there is a large proportion of either meristematic tissue present or cells which retain an ability to express totipotency. Senescing tissues therefore would rarely give successful culture due to the diminishing regeneration potential of tissues with plant maturation or age of the plant.

The explant types, age and size used successfully in lettuce tissue cultures have varied; however, cotyledon explants are the most widely used with a greater degree of success. Other explant types used include young expanded leaves, protoplasts and apical segments. In lettuce, four day old cotyledons cut into 4 pieces and 5 day old whole cotyledons have been employed in successful plant regeneration (Michelmore *et al.*, 1987; Enomoto *et al.*, 1990). These studies were on genetic transformation and little emphasis was put on the regeneration potential of explants. Lettuce regeneration from explants of different age have shown that successful regeneration decreases with age (Torres *et al.*, 1993). The age of explants used in this experiment ranged from 24 to 120 hours after germination. No shoots were recovered from explants of 96 hours after germination and over; shoot regeneration was highest among 24-48 hours old explants. This result demonstrated a sharp difference with that of Michelmore *et al.*, (1987) and Enomoto *et al.*, (1990), cited above. Notwithstanding, lettuce cotyledons more than 120h after germination have been used in some regeneration experiments, producing callus, buds and shoots (Doerschug and Miller, 1967; Kadkade and Seibert, 1977). Growth regulators used in these regeneration studies varied and could account for the contrasting observations.

Other explant types have been used in successful regeneration to obtain lettuce explants. Alconero (1983) used young expanded leaves from 1-2 month old seedlings to obtain soft friable callus and later shoots. Koevary *et al.* (1978) used apical segments of expanded and non-expanded buds for regeneration of lettuce in tissue culture and successfully obtained shoots and roots. Lettuce regeneration from leaf protoplasts have been reported (Berry *et*

al., 1982; Engler and Grogan, 1982; Matsumoto, 1991), though plating efficiency ranged from as low as 0.1% to 34.4%.

Younger tissues are not invariably the best explants; callus may form more readily on explants from fully grown plants than from young seedlings or cotyledons (Wickham *et al.*, 1980). Webb and Torres (1984) investigated factors controlling organogenesis using whole lettuce cotyledons with age ranging from 3 to 10 days old. Root and shoot regeneration decreased as the age of explant increased with 10 day old explants performing poorly. The results, generally, substantiated the notion that regenerability of explants decreases with increase in age. However, 4-day old cotyledons were superior in shoot and root production compared to younger tissues. The meristematic condition or the availability of totipotent cells in an explant, could be the influencing factor and may be genotype dependent.

1.6.2 Genotypic influence on culture regeneration

Many genotype-dependent effects are caused by interaction between the plant genotype and the cultural environment. Plants of varying genotypes require specific media or environmental conditions that favour their regeneration, hence media and culture conditions often need to be varied from one genotype to another. The preferred method of micro-propagating new plants is best determined by experiment; either through the optimisation of the culture conditions for selected genotypes or by screening for those favoured by a given medium and environment.

George (1993) noted that the behaviour of plant tissues *in vitro*, in processes such as callus formation and morphogenesis seem to be under an over-riding genetic control, with other

factors exerting only a minor effect. This assertion is not fully supported by other regeneration studies in which factors, such as explant age and condition, media composition, environmental factors have had significant influence; though genotypic effects remained formidable (Trolinder and Xhixian, 1989; Paterson and Everett, 1985; Ranch *et al.*, 1985).

Regeneration in plant tissue culture is genetically controlled and can be improved through breeding (Balatero *et al.*, 1995; Sarrafi *et al.*, 1996). The variation in response to regeneration among genotypes may be due to genetic differences in production of endogenous hormone levels, which in combination with the exogenous plant growth regulators in the medium, elicits the regenerative response (Bhaskaran and Smith, 1990). Response to plant hormones is genotype dependent in *Phaseolus* species and controlled by a single dominant gene (Mok and Mok, 1977; Mok *et al.*, 1980). In some rice genotypes, results from diallel crosses suggested that low regeneration ability is dominant; with significant additive and dominant effects (Tsukahara *et al.*, 1995). The importance of genotype in regulating regeneration *in vitro* and the choice of suitable genotypes for manipulative studies is thus important and critical (Niederwieser and Staden, 1990).

In lettuce, there is evidence to the effect of genotype on regeneration. Regenerating lettuce protoplasts under same culture conditions, have showed significant cultivar differences in plating efficiencies (Matsumoto, 1991). Lettuce genotypes give different responses to tissue culture, in terms of callus, shoot or root production (Alconero, 1983; Xinrun and Conner, 1992; Curtis *et al.*, 1994). The assessment of genotype regeneration in lettuce prior to their genetic manipulation, is therefore justified.

1.7 Genetic transformation of plants

Rapid methods of creating genetic variability in plant species, their selection and testing has been necessitated by the indispensable need for new and improved crop cultivars. Introduction of genes from other sources into plants require somatic cell techniques and these have included: *Agrobacterium*-mediated transformation, viral vectors, polyethylene glycol mediated transformation, electroporation, micro-injection and biolistics (Kumar and Davey, 1991).

Vectors based on viral genomes, have ability to replicate in plant cells to achieve high copy numbers, which favours high transformation efficiency. However, they are limited by their inability to integrate into the host genome thus reducing transmission to sexual offspring (Potrykus, 1990). Precise insertion of foreign gene sequences is also required to avoid disturbing the expression mechanism of viral vectors (Futterer *et al.*, 1990). The inherent characteristics of a particular virus, such as genome structure, mode of expression, impose restrictions on its use as a vector (Walden, 1989). These and limited space available for foreign DNA cloning may be responsible for the limited use of vectors based on viral genomes.

DNA mediated gene transfer methods usually make use of isolated protoplasts to obtain transformed cells. Though stable transformed plants have been obtained with these methods in a number of species, the major limitation is with plant regeneration in protoplasts cultures (Steinbiss and Davidson, 1989; Weissinger, 1992; Maisonneuve *et al.*, 1995). The use of protoplasts is laborious and time consuming and unrelated genetic abnormalities can occur during plant regeneration as a result of long periods in cell culture

(Conner and Meredith, 1989). *Agrobacterium*-mediated transformation usually involves tissues which encounter little problem during regeneration but difficulties are encountered when used in some plant taxa, especially monocotyledonous species. However, it is the most widely used method of plant transformation (Grevelding *et al.*, 1993).

1.8 *Agrobacterium*-mediated transformation

Agrobacterium is a soil-borne organism which has long been identified to infect plants through wound sites. Two common species of *Agrobacterium* are known in this type of infection; *Agrobacterium tumefaciens* and *A. rhizogenes* causing crown gall and hairy root diseases respectively. A third species, *Agrobacterium rubi*, is mentioned but with little significance in the genetic engineering process (Grant *et al.*, 1991). The infections are characterised by a neoplastic growth-like proliferation of the wounded tissue by *A. tumefaciens*, and hairy root formation at wounded sites of the tissue by *A. rhizogenes*.

Susceptibility to *Agrobacterium* infection has been found in common crop plants such as potato, cotton, alfalfa, oilseed rape, sugar beet *etc.* Dicotyledonous plants are generally found to be receptive to the organism; in contrast many monocotyledonous plants remain recalcitrant to *Agrobacterium* infection. There are, however, reports of some monocotyledonous species transformed by *Agrobacterium* (De Cleene and de Ley, 1976; Conner and Dommisse, 1992). The reason for this contrast in susceptibility, between dicotyledonous and monocotyledonous plants is not clear, but could be due to the inherent difference in production of compounds which are identified to trigger the transformation process (Potrykus, 1990).

1.8.1 Ti plasmid

Tumour (or hairy root) induction and opine synthesis during infection are associated with the presence within the *Agrobacterium* of a plasmid of about 200-250 kb in size; the tumour inducing, Ti, plasmid found in *A. tumefaciens* (and the root inducing, Ri plasmid in *A. rhizogenes*). Four regions of homology exist in all strains of the Ti plasmid; these are the transferred DNA (T-DNA), the virulence region (*vir*), origin of replication and the conjugative transfer region (Armitage *et al.,* 1988). During tumour formation, a defined sequence of Ti plasmid, the T-DNA is transferred to the plant cell and integrated into the plant nuclear genome which can then be expressed. This transfer is mediated by the genes in the virulence region (Chilton *et al.,* 1977; Lemmers *et al.*, 1980; Thomashow *et al.*, 1980). The T-DNA region is flanked by 25 bp direct repeats and the endpoints of integrated T-DNA in the plant genome are found close to these sequences.

1.8.2 The transformation process

Agrobacterium transformation is preceded by the establishment of contact between the organism and the plant cell. The attachment to plant cells occur in a polar way by a single bacterium or in clusters, and this is mediated by the chromosomal virulence loci which are expressed in a constitutive fashion (Gheysen *et al.*, 1989). Primary cell walls of rapidly dividing cells are preferred by the bacterium; compounds produced in only actively growing and wounded plant cells, such as acetosyringone, have been identified to be responsible for inducing virulence functions in *Agrobacterium* (Bolton *et al.*, 1986).

Induction of virulence gene expression occurs during co-cultivation of *Agrobacterium* with wounded plant cells and during incubation of the organism in plant cell exudates. This

stimulates the expression of the inducible virulence genes, triggering *Agrobacterium* to initiate plant cell transformation. Wounding may also induce potentially competent cells in wound-adjacent cell layer to become competent for regeneration and transformation (Potrykus, 1990). The activation of virulence gene expression involve extracellular recognition by *Agrobacterium*, of a chemical attractant or nutritive source for the organism. The extracellular signal is then transmitted intracellularly, to activate the genes in the virulence region. Acetosyringone interacts with *vir* A gene products, which become responsible for the transmission of the signal resulting in the activation of the *vir* G gene product. The *vir* G protein then activates the rest of the *vir* genes B, C, D and E sequentially (Stachel *et al.*, 1985; Zambryski, 1992).

The oncogenic genes (*onc*) encoded by the Ti plasmid are not required for the transfer of the T-DNA and can be removed without affecting the ability of transfer. Consequently the virulence genes (*vir*) function in *trans* to the T-DNA; *Agrobacterium* carrying an attenuated or whole Ti plasmid, plus a binary vector with an inserted gene within the T-DNA borders of the Ti plasmid can be used to transfer foreign genes to plant cells (Zambryski *et al.*, 1983; Clare, 1990).

1.8.3 T-DNA transfer and integration

Virulence gene induction leads to the appearance of single-stranded nicks within the T-DNA border sequences and the excision of a single-stranded linear T-DNA molecule (Stachel and Zambryski, 1986; Albright *et al.*, 1987). The left and right borders of the T-DNA are conserved sequences that are of significance in the DNA transfer system. The

right border of a Ti plasmid is believed to be the startpoint of the T-DNA transfer with the left border acting as breakpoint (Wang *et al.*, 1984; Zambryski, 1992).

The nicking of the borders is caused by *vir* D specific products and the generated T-DNA traverses the bacterial cell membrane, the bacterial cell wall, the plant cell wall, the plant cell and nuclear membrane. This is mediated through the formation of a T-complex formed by the T-DNA and virulence specific proteins (Zambryski, 1992).

The final step in *Agrobacterium* transformation of plant cells is integration of the T-DNA strand into plant cell DNA. T-DNA insertions occur randomly into any chromosome but transcriptionally active regions are presumably more accessible and preferred (Ambros *et al.*, 1986; Herman *et al.*, 1990). The mechanism of T-DNA integration into plant cell DNA is thought to be analogous to recombination events involving integration of viral or transfected DNA in mammalian cell (Zambryski, 1992). The 5' end of the T-strand invades a nick in the plant DNA; partial pairing between both ends of the T-DNA and plant DNA occur due to short stretches of homology. The plant DNA further unwinds to form a gap which is followed by small deletion of plant target DNA. The T-DNA ends are then ligated to the plant DNA ends. A subsequent nick in the other plant DNA strand is followed by a gap repair and DNA synthesis occurs using the T-strand as template resulting in the final integration

1.8.4 *Agrobacterium* vectors

Agrobacterium vector systems are classified into cointegrate and binary vectors. Binary vectors are based on plasmids that can replicate in both *E. coli* and *Agrobacterium*, and

contain the T-DNA border sequences (Draper *et al.*, 1988). Binary and cointegrate vectors differ by the relative position of the transfer DNA with respect to the virulence genes (*vir*). The cointegrate vectors have the virulence genes physically linked to the transfer DNA on the same plasmid and acting in *cis*, while binary vectors have the *vir*, on separate plasmid, acting in *trans*.

Cointegrate vectors have high stability in *Agrobacterium*, however detailed knowledge of the Ti plasmid is required for its manipulation and there is relatively low rate of cointegrate formation (Grant *et al.*, 1991). Binary vectors are independent of the Ti plasmids in *Agrobacterium* and have high frequency of introduction into *Agrobacterium*. They are currently used to the virtual exclusion of cointegrate vectors because of their ease of construction (Draper *et al.*, 1988). The vector pBIN19 is one of the most widely used binary vectors in *Agrobacterium*-mediated plant transformation (Bevan, 1984; Draper *et al.*, 1988).

Desirable qualities of *Agrobacterium* binary vectors include: a smaller plasmid size with origins of replication for *Agrobacterium* and high copy number in *E. coli*; multiple cloning sites close to the right border, for DNA insertions between T-DNA borders; screenable or selectable marker gene located near the left border; and unique rare restriction sites.

The left junctions of T-DNA insertions are found to end at different sites within the 25 bp repeat suggesting that the left T-DNA border is not an exact boundary, though the right border appears to be precise (Zambryski *et al.*, 1982; Kwok *et al.*, 1985). Gene sequences positioned close to the right border, therefore have a high fidelity during integration. Positioning the gene of interest between the right border and the selectable marker genes

provides a greater probability that the gene of interest has been successfully integrated with the T-DNA (Van Engelen *et al.*, 1995). The presence of unique rare restriction sites facilitates subsequent Southern analyses of the integrated gene in the plant genome.

1.9 *Agrobacterium*-mediated transformation procedures

Plant transformations using *Agrobacterium* have followed a common trend, with modifications based on several factors. The basic requirement is the establishment of contact between wounded explant surfaces and the *Agrobacterium* vector, through co-cultivation *in vitro*. Co-cultivated explants are transferred to medium containing antibiotics, to suppress the growth of *Agrobacterium* and also select for cells that have been transformed. This is then followed by regeneration of transformed plants and subsequent confirmation of gene integration (Horsch *et al.*, 1988).

1.9.1 *Agrobacterium* strains

Different strains of *Agrobacterium* exist with different virulence systems. Strains can be classified as octopine, nopaline, agropine, mannopine, *etc.* depending on the opines produced. Octopine and the nopaline types are commonly used; *eg.* an octopine type plasmid is pTiAch5 and its T-DNA deleted derivative pAL4404. A supervirulent Ti plasmid, pTiBo542, is found in *Agrobacterium* strain A281 (Komari *et al.*, 1986).

The virulence systems of different *Agrobacterium* strains have common functions, leading to plant transformation. However, strain and cultivar specificity occur and some strains may give rise to more efficient transformation on some plant species than others. The

choice of *Agrobacterium* strain, plant genotype combination is thus important (De Kathen and Jacobsen, 1990).

1.9.2 Co-cultivation

Inoculation of explants with *Agrobacterium* cultures, subjects plant tissues to the selective agent used in the bacterial medium prior to the transformation process and could result in low transformation frequencies. Resuspension of *Agrobacterium* cells in a medium devoid of antibiotics before explant inoculation, has been widely used (An *et al.*, 1986). Different concentrations of *Agrobacterium* have been found to affect transformation frequency. High concentration of the bacteria result in submaximal rates of transformation due to stress on plant cells by such numbers. Very low concentrations may result in rare transformation, due to insufficient numbers of the bacterial cells (Michelmore *et al.*, 1987).

The use of stimulants such as acetosyringone; feeder or nurse cultures and preconditioning of explants by preculture have been employed in transformations. The use of acetosyringone mostly increases transformation rate (Sheikholeslam and Weeks, 1987; Rotino, *et al.*, 1992). However, it was reported to decrease transformation rate, in *Pisum sativum* (De Kathen and Jacobsen, 1990). The use of feeder or nurse cultures may have positive influence on transformation, by favouring regeneration but they are not absolute neccesity for adequate transformation frequency (Michelmore *et al.*, 1987; Horsch *et al.*, 1988; Curtis *et al.*, 1994).

The period of co-cultivation during transformation has varied for different studies. This is an important factor because it affects transformation results. Prolonged co-cultivation

period, to a certain extent, leads to high transformation frequency. A positive linear relationship was observed between co-cultivation period and transformation rate, however, after more than 5 days explant abortion resulted from bacterial overgrowth (De Kathen and Jacobsen, 1990). Two days of cocultivating explants with *Agrobacterium* appears to be the optimum for many plant species, including; tobacco (Horsch *et al.*, 1988); potato (Visser *et al.*, 1989; Filho *et al.*, 1994); *Phaseolus vulgaris* (Marrioti *et al.*, 1989); Chinese cabbage (Jun *et al.*, 1995); *Solanum gilo* (Blay and Oakes, 1996). This is an optimum period for cell transformation by *Agrobacterium* while preventing excessive overgrowth of bacteria on the explants. The growth of *Agrobacterium* cells therefore, presents a limiting factor on length of co-cultivation period.

1.9.3 Selection of transformants

Stable integration of foreign genes into plant genome occurs at a much lower frequency, during transformation; therefore a method for identifying transformed cells is necessary. Transfer of selectable marker genes alongside an agronomic gene, is a convenient way of identifying these transformed cells. Presence of selectable marker genes in cells, confer resistance to particular selective agents, which are toxic to wild type cells and thus allow preferential growth of those rare transformed cells. The selectable functions on most transformation vectors are antibiotic resistance which have been engineered to be expressed constitutively (Armitage *et al.*, 1988). Genes affording protection against specific herbicides have also been used succesfully as dominant selectable markers. The choice of a marker gene is very important since different plant species vary in sensitivity to

selective agents (Draper *et al.*, 1988). A number of selectable marker genes are available (Table 1).

Reporter genes are coding units whose products are easily assayed and therefore provide a rapid and definitive study of gene expression. They can be fused to a promoter of interest and their expression used to assay promoter function. Their quick transient assays are useful in optimisation of transformation procedures. Common examples include: chloramphenicol acetyltransferase (CAT), octopine and nopaline synthase, β-glucuronidase (GUS), β-galactosidase, luciferase genes.

The concentration of the active ingredient required to exert stringent selection pressure vary for type of antibiotic, plant genotype and regeneration conditions. Higher concentrations may affect regeneration of transformed plants while low concentrations may result in false positives. An optimum concentration is one that enables the quick identification of transformants while rigorously selecting against non-transformed tissues. In potato transformation, the following concentrations of antibiotics: 100 mg/l spectinomycin, 300 mg/l streptomycin, 50 mg/l kanamycin and 100 mg/l rifampicin, have been used in selection (Filho *et al.*, 1994).

The selection of transformed cells after cocultivation with *Agrobacterium* can be delayed while *Agrobacterium* overgrowth on explants is controlled with appropriate antibiotics. This will allow plant cells to develop further, before selection is applied. Such extended period may favour more cells in contact with *Agrobacterium* to be transformed. This method was used in the transformation of potato, and found to be effective (Conner *et al.*, 1991).

Table 1. Selectable marker genes and agents.

Marker gene	Selective agent used	Reference
bar	bialaphos phosphinothricin	De Block *et al.* (1987); Hartman (1994)
Bleomycin resistance	bleomycin	Hille *et al.* (1986)
Chloramphenicol acetyltransferase	chloramphenicol	De Block *et al.* (1985)
mutant acetolactate synthase	chlorsulfuron	Haughn *et al.* (1988)
5-enolpyruvylshikimate-3-phosphate synthase	glyphosate	Della-Cioppa *et al.* (1987); Fillati *et al.* (1987)
Hygromycin phosphotransferase	hygromycin B	Draper *et al.* (1988)
6' Gentamicin acetyltransferase	kanamycin, gentamicin	Gosselé *et al.* (1994)
Dihydrofolate reductase	methotrexate, trimethoprim	De Block *et al.* (1984); Draper *et al.* (1988)
Neomycin phosphotransferase II	neomycin, kanamycin, G418, paromomycin	Bevan *et al.* (1983); Torbert *et al.* (1995).
Paromomycin acetyltransferase (phosphotransferase)	paromomycin	Perez-Gonzalez *et al.* (1989)
Phenmedipham carbamate hydrolase	phenmedipham	Streber *et al.* (1994)
Phleomycin resistance	phleomycin	Perez *et al.* (1989)
Streptomycin phosphotransferase	streptomyicn	Jones *et al.* (1987); Maliga *et al.* (1988)
Dihydropteroate synthase	sulfonamides	Guerineau *et al.* (1990)
Lysopinedehydrogenase	toxic amino acid analogues eg. homo-arginine	Van Slogteren *et al.* (1982); Dahl and Tempe' (1983)

1.10 Confirmation of gene transfer

Putative transgenics, once obtained, are confirmed by detecting the presence and expression of the transferred gene in the plant genome. This step allows identification of plants that may have escaped through the selection process without the transferred gene. Common methods used to detect gene integration include molecular biology tools, such as Polymerase Chain Reaction (PCR) and Southern blot hybridization.

Southern blot hybridization allows DNA fragments corresponding to a particular probe to be identified directly from a digest of the genome, using restriction enzymes. The digested product is electrophoresed on agarose gel and the DNA bands are denatured to give single stranded DNA. The denatured fragments in gel are blotted onto a nitrocellulose paper and hybridised *in situ* with specific radioactive probe The target fragments are then identified by autoradiography. It is an ultimate proof of stable integration of T-DNA into genomic DNA (Southern, 1975). Southern blotting, in addition, gives information on the copy number of the transferred gene and whether multiples are tandemly linked.

PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences. Two specific oligonucleotide primers, with complementarity to sites on the target DNA, hybridise to the opposite strands and flank the region of interest. PCR is effective in screening putative transgenics plants for the presence of the transgene because of its specificity and the minute amount of template DNA required (Erlich *et al.*, 1988; Saiki *et al.*, 1988). It is a repetitive series of cycles involving template denaturing, primer annealing and the extension of the annealed primers by a DNA polymerase. The product at the end of each cycle serves as a template for the next, resulting in exponential increase in the amount

of the target DNA produced. Polymerase chain reaction is affected by several factors and requires optimization, especially in the temperatures used for the reaction steps.

Both Southern blot analysis and PCR have been used extensively, to confirm the integration of specific DNA segments in genomes.

1.11 Detection of expressed genes

The expression of genes can be ascertained through, detection of the gene product using enzyme assays; utilising specific antibodies to bind to the protein through immunodetection techniques (western analysis), or isolating specific RNA transcripts originating from the transferred gene (northern blot analysis) or PCR using reverse transcriptase. Other methods include *in vitro* screening of transformed progeny for the expressed gene, in the presence of appropriate selective agent (herbicides, pesticides, *etc.*); or the activity of isolated proteins against target agents.

Chloramphenicol acetyltransferase (CAT) inactivates chloramphenicol by acetylation at two sites within the antibiotic molecule (Scott *et al.*, 1988). The CAT assay protocols exploit the fact that non-acetylated and acetylated chloramphenicol display differential solubilities in chloroform and can be easily identified. Kanamycin phosphorylation by neomycin phosphotransferase II can occur using radioactively labelled phosphorous; which is detectable by autoradiography (McDonell *et al.*, 1987). A number of assays, fluorometric assay, *in situ* localization, and histochemical assay are also available for detecting β-glucuronidase (GUS) expression (Jefferson *et al.*, 1987; Scott *et al.*, 1988).

1.12 Lettuce transformation

The various genetic transformation methods are of potential use in lettuce improvement, though few have been used routinely.

Transgenic lettuce plants have been obtained through electroporation with plasmid DNA (Chupeau *et al.*, 1989). The yield of transformed plants in this study was limited by the number of transformed protoplasts and by the rate of regeneration from selected colonies. Similarly, the potential use of other DNA mediated transfers to plants is hampered by plating efficiencies and regeneration difficulties associated with the use of protoplasts (Weissinger, 1992). These have rendered the use of *Agrobacterium* vectors, the most effective method for lettuce transformation (Michelmore *et al.*, 1987).

Lettuce is not a natural host for *Agrobacterium* species but its susceptibility to artificial inoculation of *Agrobacterium* species has allowed gene transfer via *Agrobacterium* (Michelmore *et al.*, 1987). Several critical factors influence *Agrobacterium* transformation of lettuce. High bacterial concentrations result in submaximal rates of transformation while low concentrations may result in infrequent callus and rare regeneration. Two days of cocultivating explants with *Agrobacterium* has been found to be optimal for the lettuce cultivar Cobham Green and also with the cultivar Southbay (Michelmore *et al.*, 1987; Torres *et al.*, 1993). Prolonged co-cultivation periods result in overgrowth of bacterium and subsequent decrease in shoot regeneration (Enomoto *et al.*, 1990).

Kanamycin remains the most preferred antibiotic for selection in lettuce transformation. The intensity of selection has varied slightly among experiments. An optimal concentration of 50 mg/l was determined for selection in lettuce by Michelmore *et al.* (1987). Lower

concentrations allowed the growth of untransformed control while higher concentrations inhibited tissue growth. In another experiment involving a different lettuce genotype, 25 mg/l of kanamycin was sufficient to cause bleaching and subsequent death of untransformed tissues (Torres *et al.*, 1993). A higher concentration of 100 mg/l of kanamycin was recommended as more efficient in selecting transformed from non-transformed shoots than with a concentration of 50 mg/l (Curtis *et al.*, 1994). The optimal intensity of selection may be dependent on the genotype and its response to the selective agent used. The timing of selection has been determined largely by the length of the co-cultivation period. However, the imposition of selection can be independent of co-cultivation period (Conner *et al.*, 1991). The effect of varying the timing of selection while controlling bacteria overgrowth, has not been examined in lettuce transformation.

Genotypic variation in response to transformation is evident in studies involving lettuce. Several lettuce cultivars belonging to the various morphological categories, have been involved in the transformation studies mentioned. However, cultivars belonging to the crisphead category are found to be particularly recalcitrant to *Agrobacterium* transformation (Michelmore *et al.*, 1987). Some crispheads have been transformed, though; these include Southbay, a crisphead cultivar, which had previously failed in response to other transformation protocols (Torres *et al.*, 1993). A genotype independent transformation protocol for lettuce, recently reported, was used for thirteen cultivars belonging to various categories. Here, genotypic differences were also observed in their transformation response (Curtis *et al.*, 1994).

Genes that have been involved in lettuce transformation are mainly marker genes such as *npt II*, β-glucuronidase gene, chloramphenicol acetyltransferase gene (Michelmore *et al.*, 1987; Chupeau *et al.*, 1989; Torres *et al.*, 1993). Other genes that have been transferred include: a stress and salicyclic acid inducible protein (Enomoto *et al.*, 1990); a sweet protein, monellin, gene (Penarrubia *et al.*, 1992); rolAB genes from *A. rhizogenes* (Curtis *et al.*, 1996[a]); a pathogenesis related β-glucanase gene for inducing male sterility (Curtis *et al.*, 1996[b]); and a coat protein gene for protection against lettuce mosaic potyvirus (Dinant *et al.*, 1997).

The transfer of genes anticipated to confer disease resistance in lettuce has been limited though potential of such genes has been found in crops like potato and tobacco (Jaynes *et al.*, 1987; Anzai *et al.*, 1989; Destefano-Beltran *et al.*, 1990; Montanelli and Nascari, 1990).

1.13 Engineering disease resistance

There are potential ways by which disease resistance can be developed in lettuce using genetic engineering. A number of genes conferring resistance to viral diseases have been identified. The coat protein gene of viruses produce immunologically detectable virus coat protein when expressed in transgenic plants. The virus coat protein in plant cells interferes with the uncoating of an incoming virus and results in delayed and reduced disease symptoms (Wu *et al.*, 1990). Such resistance may be specific to the particular virus or effective against several other unrelated viruses (Anderson *et al.*, 1989). The 54-kDa gene of TMV encoding putative component of the viral replicase confers resistance to several

related TMV strains (Golemboski *et al.*, 1990). The use of virus satellite RNA genes and nucleocapsid protein gene have also yielded transgenic plants with resistance to viral diseases (Harrison *et al.*, 1987; Gielen *et al.*, 1991).

Pathogens that cause diseases by producing phytotoxins have the capacity to detoxify these compounds. Genes encoding the detoxification enzymes can thus be engineered into plants to protect them from the effects of these toxins (Anzai *et al.*, 1989; Fuente-Martinez *et al.*, 1992). However, such genes may be toxin specific and thus more genes will be required against the different toxins produced by pathogens. Pathogenesis related proteins (produced in plants) degrade cell walls of disease causing microbes and the genes responsible for these proteins encode enzymes, such as chitinases, α-amylases, peroxidases, β-fructosidases, which are secreted into the apoplastic space. The use of these genes can confer resistance to several pathogens, however their use will require a deeper understanding of the exact biochemical activities responsible for the control of pathogens, the nature of the regulatory feedback loops of elicitors, inducers, repressors and transacting factors at the molecular level (Harms, 1992).

The use of genes encoding antimicrobial proteins can divulge resistance to bacteria and fungal diseases in plants (Jaynes *et al.*, 1987; Destefano-Beltran *et al.*, 1990). Several of such genes conferring bacterial disease resistance have been identified and isolated. Their mode of action against bacteria may be lytic or bacteriostatic (Destefano-Beltran *et al.*, 1990). Humoral immunity in the Giant silk moth (*Hyalophora cecropia*) results in the production of at least three novel classes of bactericidal proteins: attacins, cecropins and lysozymes (Hultmark *et al.*, 1980). Other bactericidal peptides have been isolated, with

varying potency against bacteria pathogens. The lysozyme gene from the T4 bacteriophage is known for its antibacterial activity. The Andropin gene from *Drosophila melanogaster* (Samakovlis *et al.*, 1991) has a moderate antibacterial activity against Gram positive bacteria; however, little or no activity is observed against Gram negative bacteria. Tachyplesin, isolated from the horseshoe crab *Tachypleus tridentatus* (Nakamura *et al.*, 1988) and magainins from the African clawed frog, *Xenopus laevis* (Zasloff, 1987) were found to inhibit growth of some Gram positive and negative bacteria by forming complexes with bacterial lipopolysaccharides. Apidaecins, isolated from honey-bees are active against a range of Gram negative bacteria (Casteels *et al.*, 1989).

The cecropins are highly active against Gram positive and Gram negative bacteria with a lytic activity against bacteria cell wall (Boman, 1991). They are described as the most potent of the three classes of bactericidal peptides induced in *Hyalophora cecropia* (Jaynes *et al.*, 1987; Destefano-Beltran *et al.*, 1990). Apple seedlings transformed with Shiva-1 (Cecropin-B analogue) exhibited delayed symptoms and reduced disease severity after inoculation with *Pseudomonas solanacearum* (Norelli *et al.*, 1993). Contrastingly, the expression of cecropin peptide in transgenic tobacco did not reduce disease severity by *Pseudomonas syringae* pv *tabaci* (Hightower *et al.*, 1994). Cecropin B peptide was found to be degraded by endogenous proteinases in tobacco (Florack *et al.*, 1995); this could account for the contrasting observations in its effectiveness. The effect of these peptides and their expression may differ with respect to plant genotype. Antibacterial resistance has also been induced in potato plants using T4 lysozyme via *Agrobacterium* transformation

and its expression reduced maceration of tissues when inoculated with *Erwinia carotovora* (During *et al.*, 1993).

1.14 Aim and objectives

The overall aim of this thesis is to transform lettuce with three antibacterial genes (T4 lysozyme, Shiva-1 and magainin II) and to evaluate their effectiveness against disease-inducing pathogens.

The specific objectives of this study are to:

1. Define the genotypic response of lettuce to tissue culture regeneration and select amenable cultivars for genetic manipulation studies.

2. Construct a transformation vector with a chimaeric gene encoding an antibacterial protein.

3. Transfer the chimaeric genes encoding antibacterial peptides to lettuce cultivars via *Agrobacterium*-mediated transformation.

4. Confirm gene integration, through molecular techniques and gene expression by ascertaining protein activity against target pathogens.

CHAPTER 2

Genotypic variation in tissue culture response of lettuce (*Lactuca sativa* L.)

Abstract

Twenty-two lettuce genotypes belonging to different morphological groups were screened for their response to regeneration in tissue culture. A shoot regenerative medium, consisting of SH salts and vitamins, supplemented with 0.1 mg/l IAA, 0.5 mg/l kinetin, 0.05 mg/l zeatin, 30 g/l sucrose and 7 g/l bacteriological agar at pH 5.8 was used. Response in tissue culture measured by callus production, root and shoot regeneration were generally found to be statistically dependent on the genotype of lettuce used. Callus and Shoot indices were generated for all genotypes as indicators of performance. The use of Bronze Mignonette as a standard genotype enabled batches of genotypes to be compared in successive experiments. This approach detected minor variations among experiments and allowed the culture performance of all genotypes to be ranked. No correlation was observed between Callus index and Shoot index, and the variation in performance of genotypes was not statistically linked to their morphological groups. Genotypes that showed good shoot regeneration in tissue culture, included Bambino and Iceberg (crisphead types); Cobham Green and Sweet Butter, (butterhead type), Simpson Elite (leaf type), and Rosalita and Paris White (cos types).

Introduction

Lettuce (*Lactuca sativa* L.) is a cool season leafy vegetable which is grown worldwide (Michelmore and Eash, 1986; Patterson *et al.*, 1986). Though of relatively low nutritional value, lettuce is a high valued, economically important vegetable crop with diverse uses (Michelmore *et al.*, 1987; Toleman, 1987).

Various genetic manipulation and cell culture methods have been employed to improve lettuce; each requiring a sufficient level of regenerability to achieve the desired result. Somaclonal variation has been observed in cultured lettuce plants derived from callus (Sibi, 1976; Brown *et al.*, 1986). Direct transfer of foreign DNA into lettuce protoplasts was obtained through electroporation using plasmid DNA (Chupeau *et al.*, 1989); however low regeneration frequency was found to be partly responsible for the small number of transgenic plants recovered. Berry *et al.* (1982) observed that efficiency of plant regeneration from protoplasts of lettuce was dependent on several factors which account for the low regeneration rate. Somatic hybridization between cultivated lettuce and the wild *Lactuca* species was achieved using protoplast electrofusion (Matsumoto, 1991), though, genotypic differences were noted in the plating efficiency of the hybrid cells obtained. *Agrobacterium*-mediated transformation method as a means of introducing foreign genes into lettuce is known and has been successfully utilised (Michelmore *et al.*, 1987; Torres *et al.*, 1993; Curtis *et al.*, 1994; Dinant *et al.*, 1997).

The success of these genetic manipulation methods, as tools for crop improvement, depends on the response to regeneration in tissue culture of plant genotypes. In such experiments, the primary objective is to obtain mature plants with transferred genes or

desired traits. Therefore explants of genotypes chosen must have the regenerative capacity to undergo morphogenesis into complete plants. Variations in the response of genotypes to regeneration in tissue culture have been reported for most plant species including lettuce (Ranch *et al.*, 1985; Enomoto *et al.*, 1990; Xinrun and Conner, 1992; Curtis *et al.*, 1994). It is therefore important to determine the regenerative response of genotypes and explants to be used in such experiments. This can be accomplished through optimisation of regeneration medium for specific genotypes, or by screening for highly amenable genotypes on a particular tissue culture medium.

An improved culture medium for the regeneration of a wide range of lettuce genotypes has been recently defined (Xinrun and Conner, 1992). However, several lettuce cultivars identified to have high regeneration capacity on this medium, could not be successfully transformed with *Agrobacterium tumefaciens* (Conner *et al.*, 1992). It is therefore important to screen further lettuce cultivars to identify additional genotypes with high regeneration capacity for subsequent genetic manipulation experiments.

In this chapter the tissue culture response of an extensive range of lettuce genotypes belonging to different morphological groups was investigated with respect to callus, shoot and root production. The objective was to define the genotypic response to regeneration in tissue culture of lettuce and to select best genotypes for genetic transformation experiments.

Materials and Methods

Seeds of lettuce cultivars used (Table 1) were surface-sterilised with 70% ethanol for 30 seconds, transferred to a solution of 1% NaClO with 2 drops of Tween 20 detergent per litre for 30 minutes, then rinsed 2-3 times with sterile distilled water. Seeds of each genotype were sterilised separately and germinated on medium containing SH salts and vitamins (Schenk and Hilderbrandt, 1972); supplemented with 30 g/l sucrose, 7g/l of bacteriological agar.

Two days after germination, healthy cotyledons of uniform size were excised, longitudinally cut into two segments and placed on a regeneration medium consisting of the seed germination medium, supplemented with 0.1 mg/l (0.57 μM) indole acetic acid, 0.5 mg/l (2.32 μM) kinetin and 0.05 mg/l (0.23 μM) zeatin (Xinrun and Conner, 1992). Ten half-cotyledons were placed in each culture vessel and after 20 days the explants were transferred to fresh regeneration medium of same composition for a further 20 days.

The pH of all media was adjusted to 5.8 prior to addition of agar. Except for zeatin, which was filter sterilized and added after autoclaving, all the components were sterilized by autoclaving at 121°C, 103 kPa for 15 minutes. Molten media in 50 ml aliquots were then dispensed into ethylene oxide pre-sterilised plastic pottles of size 85 mm diameter x 35 mm height. All cultures were incubated at 23-24 ^{0}C with 16 hour photoperiod supplied by cool-white fluorescent lamps (70-85 μmol/m^2/sec).

Table 1. Lettuce cultivars and their sources

Morphological type	Cultivar name	Seed source
Crisphead	Bambino	Yates, NZ
	Iceberg	Burpee and Co. USA
	Mainspring	Crop and Food Research, NZ
	Mini- Green	Chas H. Lilly Co. USA
	Nevada	Chas H. Lilly Co. USA
	New York Head	Chas H. Lilly Co. USA
	Prize Head	Kings Seeds, NZ
	South Bay	University of Florida, USA
Butterhead	Bronze Mignonette	Watkins Seeds, NZ
	Cobham Green	UC Davis, USA
	Lollo Biondo	Kings Seeds, NZ
	Lollo Rosso	Kings Seeds, NZ
	Sangria	Kings Seeds, NZ
	Sweet Butter	Kings Seeds, NZ
Leaf	Black Seeded Simpson	Kings Seeds, NZ
	Oak Leaf	Kings Seeds, NZ
	Red Salad Bowl	Fothergills Seeds, Australia
	Royal Oak Leaf	Kings Seeds, NZ
	Simpson Elite	Kings Seeds, NZ
	Tango	Kings Seeds, NZ
Cos	Paris White	Kings Seeds, NZ
	Rosalita	Kings Seeds, NZ

The cultivars used in this study belonged to four morphological groups and were obtained from various sources (Table 1).

Regeneration was assessed through observations and measurements at the end of the culture periods. Explants showing regeneration and the type of response, such as callus or shoot, were noted. Observations made at the end of the second culture period included the kind of callus (such as friable or compact), position of callus growth (at the edges or over the surface of the explant), colour of the callus and the explant tissue, presence of roots and colour change in medium.

The measurements taken were, frequency of explants producing different amounts of callus (classified into Limited, Moderate or Extensive when 20%, 20-50% and >50%, respectively, of explant surface is covered with callus proliferation), frequency of explants with shoots and roots and the number of shoots per explant.

The study was conducted in a series of experiments, each including the genotype Bronze Mignonette as a standard. Each batch was analysed separately. An internal control of Bronze Mignonette cultivars, propagated in different environments and years, was undertaken under uniform culture conditions.

Callus production in each experiment was analysed using Chi-square test of independence to ascertain the influence of genotype. To enable comparison among genotypes, a callus index (CI) was calculated using the formula $(n_1L + n_2M + n_3E)/(n_1 + n_2 + n_3)$; n_1, n_2 and n_3 being the numbers of explants producing Limited (L), Moderate (M) and Extensive (E) callus. The values 1, 2, and 3 were assigned to the L, M, E categories respectively. The frequency of explants with shoots, roots and the mean number of shoots per explant in each experiment were transformed with either $\text{Log}_{10}(x + 1)$ or $\sqrt{(x + 1)}$ to equate variances and analysed using Analysis of Variance. As an estimate of the tendency to produce shoots and

the intensity of shoot production for each genotype, a shoot index (SI), was generated for each genotype using the formula: $(\Sigma n_i X)/N_t$; n_i refers to the number of explants producing shoots in i^{th} replicate; X, the category of shoots produced depending on the number per explant (0=0, 1-2=1, 3-4=2, 5-6=3, 7-8=4 >8=5) and N_t, the total number of explants used for each genotype.

Results

Observations on callus produced by the lettuce genotypes revealed differences in qualitative characteristics such as colour of callus produced, softness and uniformity of development over explant surface (Figure 1). These were observed to be highly dependent on genotype. The type of callus produced by the genotypes were characterised into friable callus, identified by soft textured proliferation on explants and compact callus which was hard and crusty in nature. Generally, extensive callus production was associated with friable callus while compact callus, when observed was in limited amounts on explants.

The amount of callus produced by explants of genotypes varied markedly between genotypes (Table 2). Chi-square analysis of independence on callus production confirmed significant effect of genotype on the amount of callus produced. The proportion of explants producing shoots and roots showed marked variation between genotypes (Figure 2). The mean number of shoots produced per explant was also strongly influenced by genotype (Table 3).

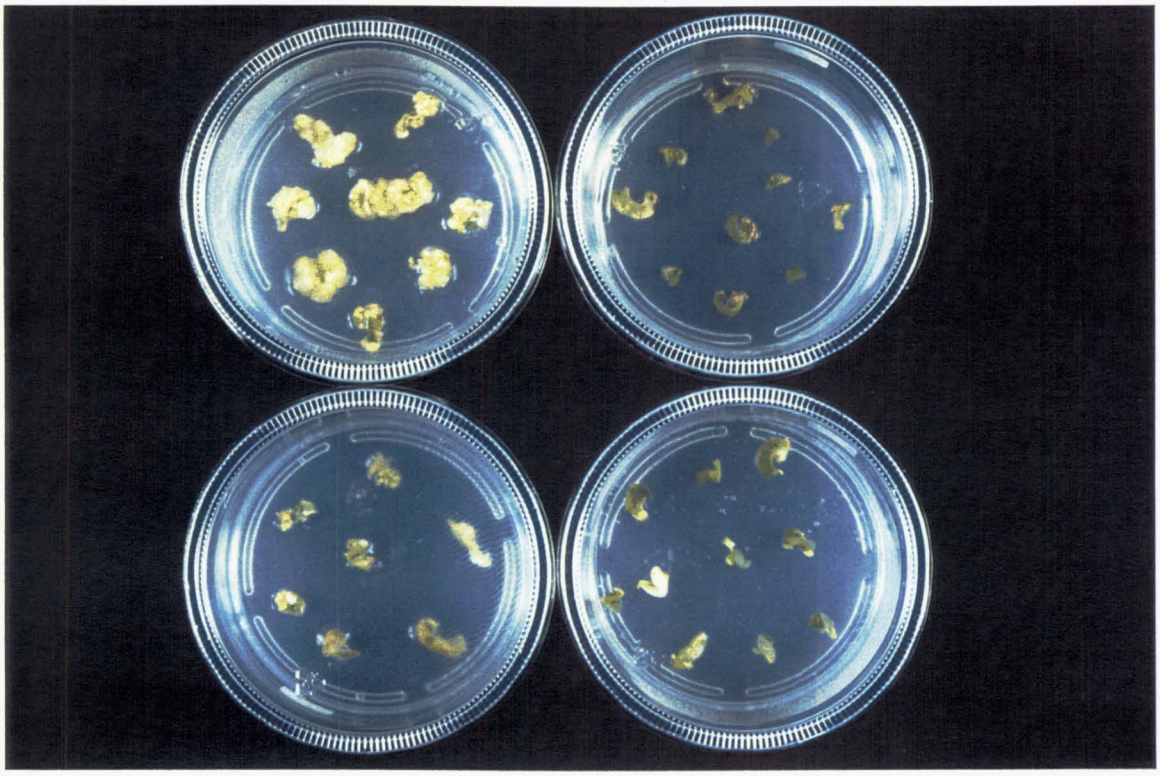

Figure 1. Callus production on explants of different lettuce cultivars in tissue culture. Differences in amount of callus and qualitative characteristics were observed among genotypes.

Table 2 Callus production on cotyledons of lettuce genotypes

Genotypes	Name code	Number of explants	Limited	Moderate	Extensive	Chi-square probability
Bambino	Bbn	60	65.0	35.0	0	
Red Salad Bowl	RSB	60	85.0	15.0	0	<0.001
South Bay	SBy	80	86.0	14.0	0	
Bronze Mignonette	BMg	70	70.0	28.5	1.5	
Black Seeded Simpson	BSS	50	32.0	42.0	26.0	
Lollo Biondo	LBd	50	34.0	48.0	18.0	
Lollo Rosso	LRs	30	36.7	40.0	23.3	
Mainspring	MSp	40	15.0	52.5	32.5	<0.01
Oak Leaf	OLf	40	20.0	30.0	50.0	
Royal Oak Leaf	ROL	50	6.0	38.0	56.0	
Tango	Tgo	40	52.5	45.0	2.5	
Bronze Mignonette	BMg	50	36.0	38.0	26.0	
Paris White	PWh	60	5.0	36.7	56.7	
Prize Head	PzH	60	13.0	47.0	40.0	
Rosalita	Rsl	70	13.0	26.0	61.0	
Sangria	Sgr	60	0.0	25.0	75.0	<0.01
Simpson Elite	SEl	80	67.5	31.0	1.5	
Sweet Butter	SBt	60	20.0	37.0	43	
Bronze Mignonette	BMg	50	28.0	62.0	10.0	
Cobham Green	CbG	100	5.0	35.0	60.0	
Iceberg	IcB	80	60.0	39.0	1.0	
Mini Green	MnG	80	74.0	25.0	1.0	<0.001
Nevada	Nvd	80	59.0	35.0	6.0	
New York Head	NYH	90	42.0	41.0	17.0	
Bronze Mignonette	BMg	100	44.0	49.0	7.0	

Percentages of total explants producing limited (+), moderate (++) or extensive (+++) callus, which were observed as 20%, 20-50%, and >50% of explant surface covered with callus proliferation. The total number of explants on which final measurement were in replicates of 10 explants (half cotyledons) each. The chi-square probability of independence showed highly significant effect of genotype in each experiment.

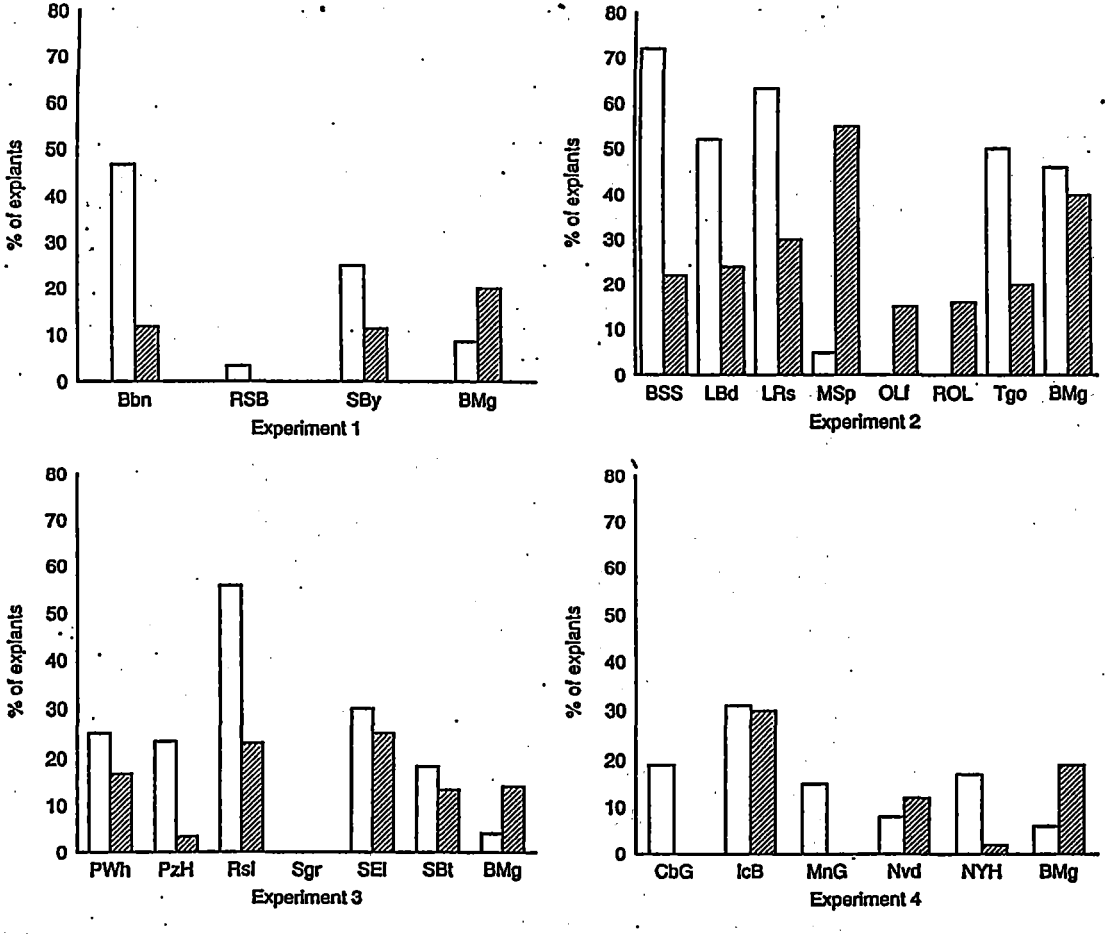

Figure 2. Explants producing shoots (open bars) or roots (hatched bars) for genotypes in each experiment. Probability values for effect of genotype on % of explants producing shoots or roots after Analysis of variance were as follows: (<0.001, <0.05); (<0.001, <0.05); (<0.001, <0.001) and (>0.05, <0.001) for experiments 1, 2, 3 and 4 respectively.

Table 3. ANOVA for "Number of Shoots per explant" by Genotype

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Mean Squares	2.27	0.39	0.25	0.92
Probability	<0.01	<0.001	<0.001	<0.05
F value	5.77	32.85	6.29	2.31

Genotypes	Means	Genotypes	Means	Genotypes	Means	Genotype	Means
Bronze Mignonette	1.0^{bc}	Bronze Mignonette	1.7^{ab}	Bronze Mignonette	0.6^{bc}	Bronze Mignonette	0.4^{b}
Bambino	1.8^{a}	Black S. Simpson	1.8^{a}	Prize Head	1.9^{ab}	Cobham Green	1.0^{a}
Red Salad Bowl	0.3^{c}	Lollo Biondo	1.3^{b}	Rosalita	1.4^{a}	Iceberg	0.9^{ab}
South Bay	1.2^{b}	Lollo Rosso	1.4^{ab}	Sangria	0.0^{c}	Mini-Green	1.4^{a}
		Mainspring	0.3^{c}	Simpson Elite	1.3^{a}	Nevada	0.8^{ab}
		Oak Leaf	0.0^{c}	Sweet Butter	0.7^{bc}	New York Head	0.8^{ab}
		Royal Oak Leaf	0.0^{c}	Paris White	1.3^{ab}		
		Tango	1.6^{ab}				

Analysis of Variance was used to test the statistical significance of genotype on the number of shoots produced per explant, after transformation of data by $\sqrt{(X + 1)}$ or $Log_{10} (X + 1)$ where appropriate. The mean number of shoots per explant was determined from the total number of shoots counted per the number of explants used in the experiment. These were separated by 95% Confidence Intervals of the means based on pooled standard deviations, using Minitab Statistical Package. Values with same letter in a column are not significantly different from each other at 0.05 probability level.

Callus index of the genotypes, as a measure of comparable callus production, in the various experiment also showed detectable differences among genotypes (Table 4).There was no significant linear correlation between Callus Index and Shoot Index as measures of callus and shoot production (Figure 3). Analyses to determine the relationship between morphological type and performance of genotypes, in callus, shoot or root production revealed non-significant statistical associations ($P>0.05$).

In the subsequent experiment involving five seed lots of Bronze Mignonette (produced in different years and environments) statistical analyses showed no significant effect of the various seed sources on callus production, shoot and root production (Table 5).

Table 4. Indices and relative ranking of genotypes for tisue culture performance

Genotype	Callus Index (CI)	Shoot Index (SI)	Adjusted CI	Adjusted SI
Bambino	1.40	0.53	1.07	5.89
Red Salad Bowl	1.10	0.03	0.85	0.33
South Bay	1.10	0.25	0.85	2.78
Bronze Mignonette	1.30	0.09	1.00	1.00
Black Seeded Simpson	1.94	0.80	1.02	1.48
Lollo Biondo	1.84	0.52	0.97	0.96
Lollo Rosso	1.87	0.63	0.98	1.16
Mainspring	2.18	0.05	1.15	0.09
Oak Leaf	2.30	0.00	1.21	0.00
Royal Oak Leaf	2.50	0.00	1.32	0.00
Tango	1.50	0.50	0.79	0.93
Bronze Mignonette	1.90	0.54	1.00	1.00
Prize Head	2.27	0.23	1.25	5.75
Sangria	2.75	0.0	1.51	0.0
Simpson Elite	1.34	0.31	0.74	7.75
Rosalita	2.48	0.56	1.36	14.00
Paris White	2.49	0.25	1.37	6.25
Sweet Butter	2.23	0.18	1.23	4.50
Bronze Mignonette	1.82	0.04	1.00	1.00
Cobham Green	2.55	0.19	1.56	3.17
Iceberg	1.41	0.31	0.86	5.16
Mini Green	1.27	0.19	0.78	3.17
Nevada	1.47	0.09	0.90	1.50
New York Head	1.75	0.18	1.07	3.00
Bronze Mignonette	1.63	0.06	1.00	1.00

Callus Index (CI), was calculated using the formular $(n_1L + n_2M + n_3E)/ (n_1 + n_2 + n_3)$; n_1, n_2 and n_3 being the numbers of explants producing Limited, Moderate and Extensive callus. Shoot Index, SI, was generated for each genotype using the formular: $(\Sigma\ n_iX)/N_i$; n_i refers to the number of explants producing shoots in i^{th} replicate and X, the category of shoots produced depending on the number per explant (0=0, 1-2=1, 3-4=2, 5-6=3, 7-8=4 >8=5). 'Adjusted CI' and SI', the ratio of CI or SI of each genotype to that of the standard (Bronze Mignonette) in each experiment.

Table 5. Callus production and indices of Bronze Mignonette types

Types[a]	% of explants producing callus				CI	SI
	Limited	Moderate	Extensive	χ^2 probability		
Y_1E_1	16.0	76.0	8.0		1.92	0.06
Y_1E_2	32.5	55.0	12.5		1.80	0.13
Y_1E_3	26.0	64.0	10.0	>0.05	1.84	0.10
Y_2E_3	17.5	75.0	7.5		1.90	0.15
Y_3E_3	17.5	82.5	0.0		1.83	0.05

[a]The different seed lots of Bronze Mignonette tested were produced in different years (Y) and in different environments (E). E_1, Crop & Food Research Ltd., Christchurch, New Zealand; E_2, private greenhouse, Christchurch; E_3, Watkins Seed company, New Zealand.

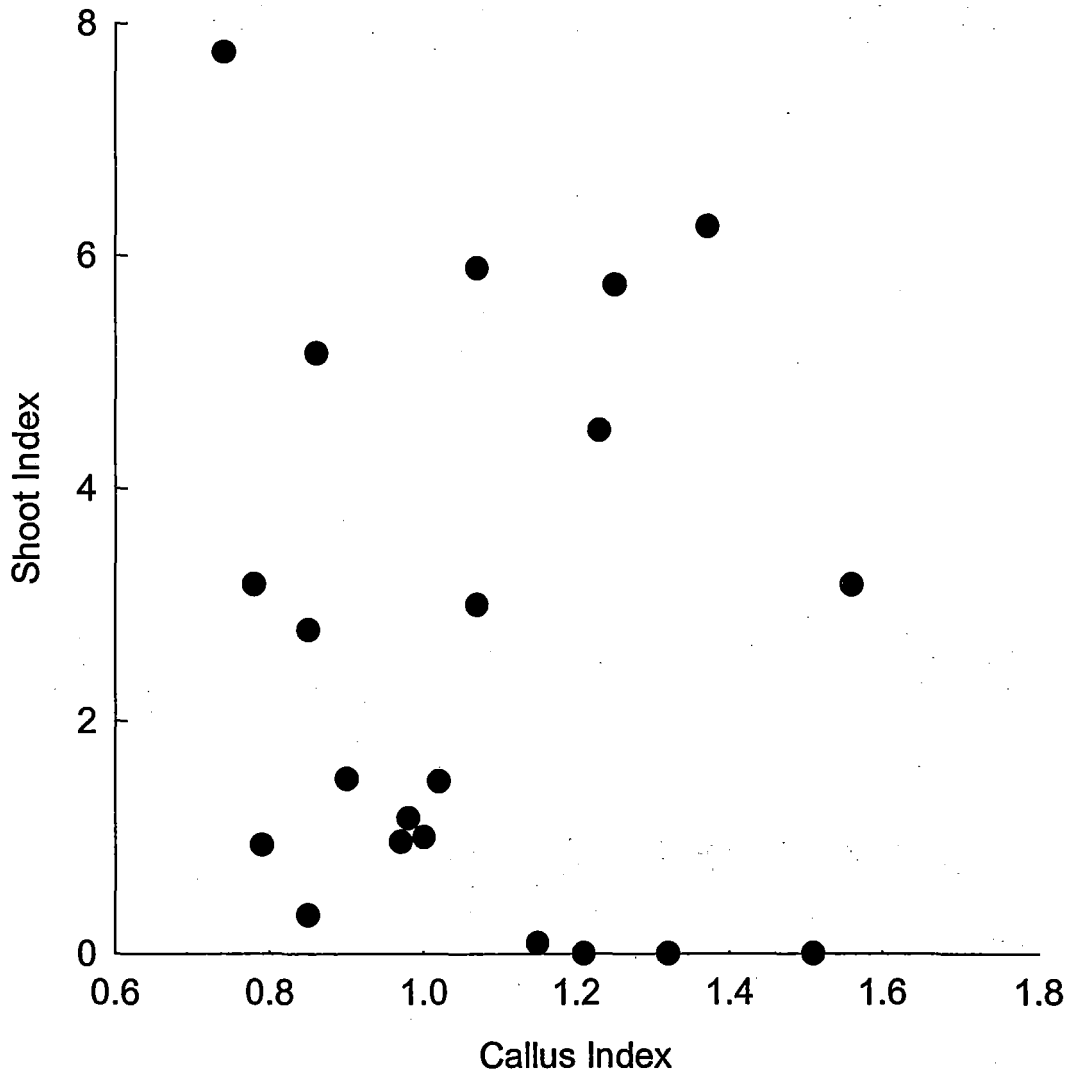

Figure 3. Scattergram between Callus Index and Shoot Index of all genotypes used in the study. Statistical analyses established little or no correlation between these two parameters (R^2 = 1.7%, P> 0.05).

Discussion

In general, culture conditions remained constant between experiments. Bronze Mignonette was chosen as a standard genotype in this study based on its intermediate performance, in an earlier regeneration study (Xinrun and Conner, 1992). It was therefore suitable in identifying superior as well as inferior performances in other cultivars.

Screening a large number of genotypes to ascertain their responses, in experiments, can be limited by space and time; which can introduce error variations. Tissue culture responses are affected by several factors, some of which cannot be measured or controlled (George, 1993). Therefore, grouping genotypes into batches with a standard to evaluate the performances of other genotypes in a given group is essential in such experiments. This is analogous to screening a large number of genotypes for their resistance to diseases, where grouping genotypes into batches, each including a standard as a check to evaluate their responses, is generally regarded as appropriate (Yang and Verma, 1992).

Bronze Mignonette as a standard revealed some differences among experiments in this study (Tables 2 and 3); thus detecting the effect of subtle variations in culture conditions, that are incidental to such experiments. Analysing the results separately for each group was therefore necessary to avoid inter-experimental variation.

The experiment involving different seed sources of Bronze Mignonette was performed to test the possible effect of different seed sources and environments under which seeds of genotypes are produced, thereby justifying the approach in this study of screening genotypes from different sources (Table 1). No obvious differences were observed in the

qualitative characters of the Bronze Mignonette from the different seed sources. Statistical analyses showed no effect of the different seed sources on the regeneration of the genotype. Lettuce is a highly inbreeding crop (Pink and Keane, 1993); breeding under different environmental conditions is likely to effect little or no change in the genetic constitution of genotypes.

Chi-square analyses of callus production showed that amount of callus produced was dependent on the genotype of lettuce. Explants within genotypes also produced different amounts of callus. The intra-genotypic variation observed in the replications is consistent with results obtained by Xinrun and Conner (1992) and Curtis *et al.* (1994). Clear genotypic differences in the culture response of lettuce cotyledons were obtained in both of these studies. Callus Index calculated as a measure of callus production enabled comparison among genotypes and was useful in detecting differences in the performance of the standard genotype over experiments. This measure enabled the ranking of all the genotypes based on the performance of the standard in each group (Table 4).

Shoot regeneration in culture indicated diverse response among genotypes. Some genotypes showed early shoot development eg. Bambino, a crisphead cultivar, produced shoots within fourteen days in the first culture medium. In this respect, it was distinct from the other genotypes, in which shoots were not noticed until the second culture period. Some genotypes, such as Bronze Mignonette, Red Salad Bowl, showed little tendency to produce shoots while Sangria produced no shoots in the regeneration medium. The medium used (SH salts and vitamins, 0.1 mg/l IAA, 0.5 mg/l kinetin, 0.05 mg/l zeatin, 30g/l sucrose and 7g/l agar) was designed for shoot regeneration (Xinrun and Conner,

1992) but some genotypes also produced roots. For Bronze Mignonette and Nevada the percentage of explants producing roots was higher than that for shoots while genotypes like Cobham Green, Mini-Green, Sangria and Red Salad Bowl did not produce roots. The tendency of various genotypes to produce shoots and/or roots further illustrates the strong genotypic variation of lettuce to organogenesis. Shoot and root production in culture is known to be strongly influenced by the ratio of cytokinin to auxin in the culture medium used (Skoog and Miller, 1957). The results of this study suggest that the genetic component is also critical to organogenic response in tissue culture.

In a similar tissue culture study with lettuce by Xinrun and Conner (1992), the genotype Bronze Mignonette gave a rather high percentage of explants producing shoots and is contrasted by the results obtained in this experiment. Culture conditions used were similar *i.e.* same media composition, temperature, light source and photoperiod. This gives an indication of the importance of subtle differences in environment on tissue culture response, which may be beyond experimental control. This is also evident in the variation between experiments detected during this study. Minor variations in lighting and temperature could be an important factor.

The rather weak correlation between Callus Index and Shoot Index (Figure 3) establishes that amount of callus produced has little or no influence on the tendency or rate of shoot regeneration in tissue culture.

Genotypic effects in tissue culture were observed in all measured characters in this study. It was clearly evident in the measure of explants producing shoots or roots. The primary objective of this study was to identify genotypes with high potential for adventitious shoot

regeneration. Bambino showed a high shoot regeneration capacity. Other genotypes with comparable regeneration capacity were, Iceberg, South Bay (crisphead), Cobham Green, Rosalita (cos) cultivars. Bronze Mignonette performed rather poorly, both in shoot and in callus production in contrast to a previous study. This emphasizes the higher shoot regeneration potential of the cultivars identified in this study compared to those identified earlier (Xinrun and Conner, 1992). The genotypes considered to have the highest potential for regeneration in tissue culture, and selected for genetic transformation experiment were Cobham Green and Bambino.

CHAPTER 3

Gene cloning into *Agrobacterium* vector system

Abstract

A chimaeric gene, including a Shiva-1 gene sequence, encoding an antibacterial protein (cecropin B analogue) was cloned into a binary vector plasmid (pBINPLUS) to be used in *Agrobacterium* mediated transformation. The gene insert was isolated from a pUC18 cloning vector, using restriction enzymes *Eco*RI and *Hin*dIII. A ligation reaction between the chimaeric gene insert (1.46 kb) and digested pBINPLUS vector (12.39 kb) resulted in a 13.85 kb plasmid, pBINPLUSH, which was used to transform *Escherichia coli* strain DH5α. The presence of the chimaeric gene insert was determined by screening *E. coli* cells, based on the lacZ' region, on IPTG, X-gal plates. Plasmid isolation and digestion confirmed the presence of the cloned insert within the T-DNA border sequences of the new binary vector. Competent cells of *Agrobacterium* strain, AGL1, were then transformed with pBINPLUSH and its presence assessed through selection of AGL1 cells on kanamycin containing LB plates. Digestion of the isolated plasmid, gave the expected DNA fragment sizes of 1.46 kb and 12.39 kb, confirming the presence of the correct plasmid vector.

Introduction

Agrobacterium-mediated gene transfer has revolutionised plant biotechnology via the introduction into crop plants of specific characteristics that are of interest to agriculture and industry (Angenon and Montagu, 1992). It is undoubtedly the most developed system for the transfer of foreign genes into plants, despite its limitations in monocotyledonous plants (Steinbiss and Davidson, 1989). *Agrobacterium tumefaciens*, referred to as nature's plant genetic engineer, has long been recognised as a natural vector for the transfer of DNA to plant cell genome (Lazo *et al.*, 1991).

The Ti plasmid of *Agrobacterium tumefaciens* provides the means of transferring specific segments of DNA, the T-DNA, into plants. This transfer is effected by the virulent genes found on the Ti plasmid which recognises the DNA sequences bordering the T-DNA. These properties of the Ti plasmid allow for the cloning of isolated DNA fragments for transfer to plants. The position of the *vir* genes that control this transfer does not necessarily, have to be on the same plasmid as the DNA to be transferred (Zambryski *et al.*, 1983; Conner and Meredith, 1989; Clare, 1990). This has enabled the use of binary vector systems, which carry cloned genes within T-DNA border sequences, for transfer into plant genome (Draper, *et al.*, 1988). Binary vectors exploit the observation that when the virulence region and the T-DNA region are on separate plasmids, the *vir* genes can act in trans to effect the transfer of T-DNA to plants. Genes producing desirable characteristics, therefore can be isolated, modified for transfer and expressed in plants. The oncogenic genes on the Ti plasmid can be physically replaced with desirable agronomic genes to obtain cointegrate vectors.

Gene cloning provides the opportunity of making specific and desirable genes, isolated from sources that would otherwise be inaccessible, available for introduction into plant genome and for their expression. A large number of vector constructs and regulatory sequences that allow for such gene transfer and expression in plants using *Agrobacterium* are available (Armitage *et al.*, 1988). The choice of vectors and *Agrobacterium* strain in a transformation experiment are essential, as they influence transformation frequency (Schmidt and Wilmitzer, 1988; Becker, 1990; Owens Y de Novoa and Conner, 1991). In this study, the *Agrobacterium* strain, AGL1, carrying the hypervirulent, attenuated tumor-inducing plasmid pTiBo542 (Lazo *et al.*, 1991) and a recently constructed binary cloning vector, pBINPLUS, found to yield high frequency of transgenic plants (Van Engelen, *et al.*, 1995) were used.

The overall aim of this research study was to transform lettuce genotypes with antimicrobial genes anticipated to confer resistance to bacteria diseases. Two vectors, similarly constructed and each coding for a different antibacterial protein (magainin II and T4 lysozyme) were obtained from Crop & Food Research, Lincoln, for this purpose. The aim of this chapter, therefore, was to construct and obtain in the same *Agrobacterium* strain, a third binary vector encoding antimicrobial cecropin B analogue (Shiva-1) similar to the two chimaeric genes available, for genetic transformation of lettuce.

The objectives of this chapter were:

1. To clone a chimaeric gene construct encoding Shiva-1, into a binary vector with a high transformation frequency, pBINPLUS.

2. To transform and obtain in *Agrobacterium* strain, AGL1, the engineered transformation vector.

Materials and Methods

Bacteria strains and plasmids

Escherichia coli strain DH5α, harboured the plasmid vector pUC18 which carried a 1.46 kb chimaeric gene insert within the cloning site. The gene insert coded for Shiva-1 protein. *Agrobacterium tumefaciens* strain AGL1, with a resident hypervirulent but attenuated Ti plasmid, pTiBo542 was used (Lazo *et al.*, 1991). Both organisms were obtained from Crop & Food Research, Lincoln, New Zealand. A binary vector plasmid, pBINPLUS (12.39 kb in size) and possessing a multiple cloning site with a lacZ' region (Figure 1), was utilised for the gene cloning (Van Engelen *et al.*, 1995).

Bacterial growth and selection

The medium used for bacteria growth was Luria broth (5 g/l NaCl; 10 g/l Bactotryptone; 5 g/l Yeast extract). Aliquots of 40 ml were dispensed into 100 ml Erhlymeyer flasks and autoclaved at 103 kPa, 121°C for 15 minutes. The solid growth medium was obtained by adding 15 grams of bacteriological agar to one litre of liquid LB medium before autoclaving. Molten media were dispensed into Petri-dishes (90 mm diameter x 14 mm high). Blue-white selection plates were prepared by adding 100 mg/l kanamycin, 2% X-gal and 2% IPTG to LB solid medium. *E. coli* cells were streaked on the plates and allowed to incubate at 37°C for 24 hours.

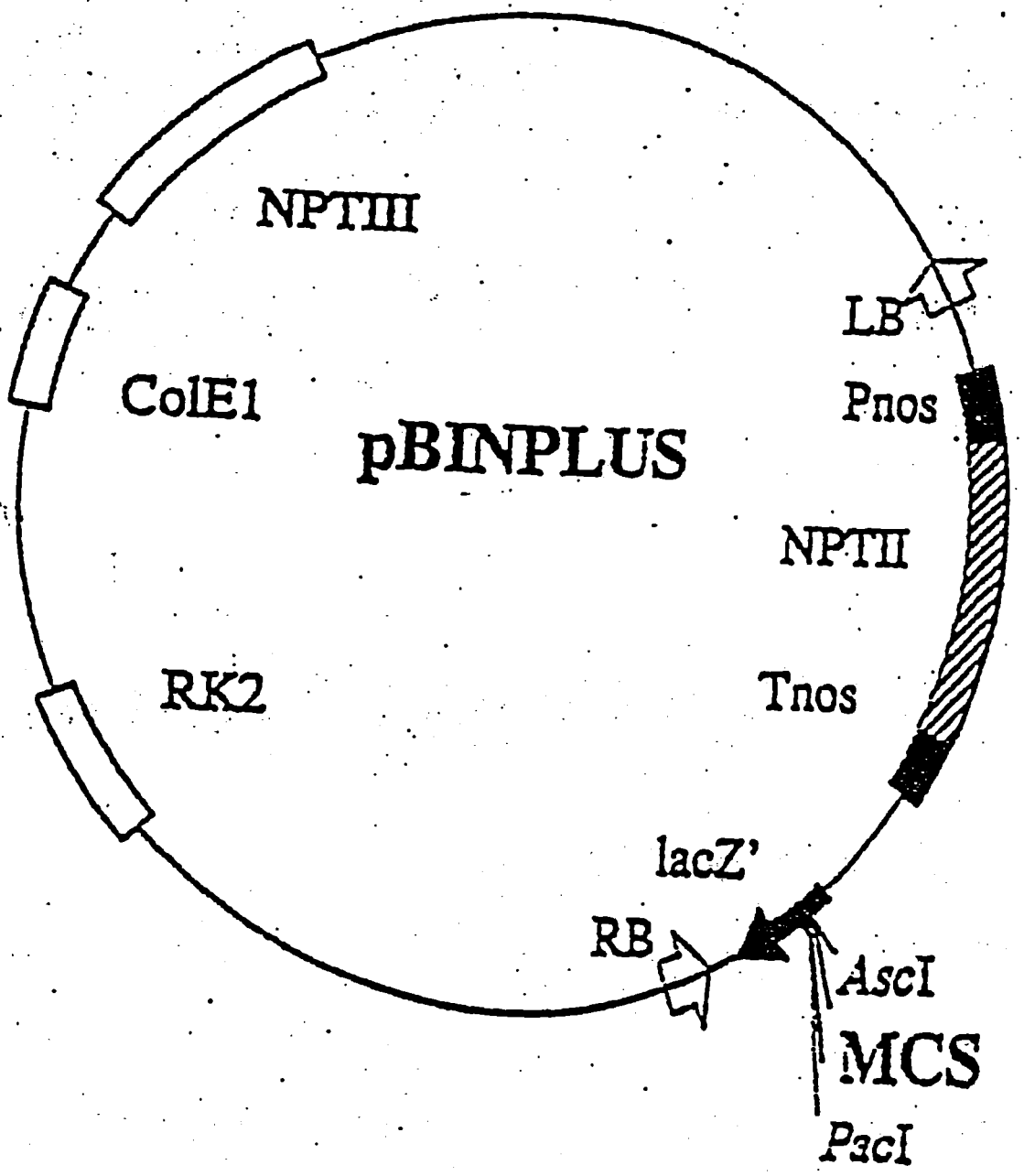

Figure 1. The plasmid map of pBINPLUS vector. The multiple cloning site (MCS) within the lacZ' region includes the pUC19 multiple cloning site and a few rare restriction sites [*Asc*I *Hind*III *Sph*I *Pst*I *Sal*I *Xba*I *Bam*HI *Sma*I *Kpn*I *Sac*I *Eco*RI *Pac*I].

Plasmid Isolation

Selected *E. coli* and *A. tumefaciens* colonies, carrying the appropriate plasmid vectors, were cultured overnight in 40 ml of Luria Broth (LB), supplemented with 100 mg/l of selection agent. These were incubated at 37°C on a gyratory shaker at 280 rpm. The DNA isolation method used was based on the alkaline lysis method described by Sambrook *et al.* (1989).

The overnight cultures were dispensed into tubes and centrifuged at 5,000 g at 4°C for 10 minutes. The pellet was resuspended in 5 ml of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) and incubated on ice for 5 minutes. Ten ml of freshly prepared solution II (0.2 N NaOH; 1% sodium dodecyl sulphate) was added to each tube and incubated at room temperature for 5 minutes. A 7.5 ml of solution III (3 M potassium acetate; 11.5% glacial acetic acid; pH 4.8) was then added to each tube and incubated on ice for five minutes.

The sample was centrifuged for 20 minutes at 12,000g at 4°C and the supernatant was passed through miracloth. A 5 ml volume of 50% PEG, 0.5M NaCl was added to the supernatant and incubated on ice for one hour. This was followed by centrifuging at 12,000 g, 4°C for 20 minutes. The supernatant was decanted carefully and the pellet (plasmid) was resuspended in 0.5 ml of sterile water. RNase was added to a concentration of 20 µg/ml and the sample was left at room temperature for 15 minutes and passed through a "Magic DNA clean up column" following manufacturer's (Promega) instruction. Plasmid DNA was eluted in 50 µl of water and stored at -20°C.

Plasmid Restriction

The restriction enzymes *Eco*RI and *Hin*dIII were used to digest pBINPLUS and pUC 18 with the gene insert. A total reaction volume of 20 µl was prepared for each sample as follows: 2.0 µl of each plasmid DNA (250 ng/µl), 0.2 µl of *Eco*RI (10 units/µl), 0.2 µl of *Hin*dIII (10 units/µl), 2.0 µl of 10X restriction buffer B [10mM Tris-HCl; 5 mM $MgCl_2$; 100 mM NaCl; 1 mM β-mercaptoethanol; pH 8.0] and 15.6 µl distilled water. The samples were incubated at 37°C for one and a half hours in Eppendorf tubes; the digested products were analysed on electrophoretic gel. The enzymes and reagents used were manufactured by Boehringer Mannheim.

Recovery of DNA insert

The digested sample of pUC 18 with insert, was run on a 1% electrophoretic gel prepared from 0.4 g 'low melting point' agarose in 40 ml of 1X TBE (89 mM Tris borate, 89 mM Boric acid, 2 mM EDTA), at 80 V for 45 minutes. Ethidium bromide (0.2 µg/ml) was used for staining DNA fragments in gel. The separated DNA fragments were viewed under UV light (320 nm) on a transilluminator with a photographing device and the sizes of the fragments were confirmed by comparing to the molecular marker bands of 1 kb ladder (Boehringer Mannheim). The gene insert band was cut out of the gel using a sterile scalpel.

The weight of the agarose piece was determined and 0.04 volume of 25X Agarase buffer (750 mM BisTris, 250 mM EDTA) added. The tube was incubated for 15 minutes in a bath at 65°C. This was followed by the cooling of the molten agarose to 45°C and 1 unit of

DNAse-free Agarase was added per 100 mg of agarose. The solution was carefully mixed with a pipette tip and incubated at 45°C for one hour for the Agarase to cleave the agarose.

A tenth volume of 3 M sodium acetate at pH 5.5 was added to mixture; this was incubated on ice for 15 minutes. It was then centrifuged at 4°C for 15 minutes in a microcentrifuge to precipitate the oligosaccharides. The supernatant was removed and 3X volume of ice cold ethanol added, followed by centrifugation at 13,000 rpm in a microcentrifuge for 20 minutes. The supernatant was then carefully discarded using a micro pipette and the DNA pellet dried.

Ligation

A ligation reaction between the *Eco*RI and *Hin*dIII digested product of the pBINPLUS vector and the isolated gene insert was set up in a total reaction volume of 20 µl including: 2.0 µl of ligation buffer; 1.0 µl aliquot of T4-DNA ligase; 2.0 µl of digested pBINPLUS DNA (200 ng/µl); 2.0 µl chimaeric gene insert DNA (250 ng/µl) and 13 µl of water was added. The sample was incubated at 14°C for 36 hours to enable ligation between the ends. The ligated product was run on a gel to confirm ligation and also the band size.

Preparation of competent *E. coli* DH5α cells

A starter culture of DH5α in 5 ml of LB was grown overnight at 37°C. One ml of the overnight culture was diluted into 100 ml of LB supplemented with 10 mM $MgCl_2$. This was incubated at 37°C with shaking until about an OD_{500} of 0.5 was obtained and then centrifuged at 5,000 rpm, 4°C for 5 minutes. The pellet was resuspended in 50 ml of ice cold TMC (10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$, 50 mM $CaCl_2$). The resuspension

was left on ice for 15 minutes and centrifuged at 5,000 rpm for 5 minutes at 4°C. The pellet was resuspended in 8 ml of TMC with 15% glycerol and aliquots of 500 µl was used in transformation or stored in -80°C freezer for later use.

Transformation of competent DH5α

Competent *Escherichia coli* cells (200 µl) were added to plasmid DNA in an Eppendorf tube and stored on ice for 45 minutes. The mixture was heat shocked at 42°C for exactly 2 minutes, then put back on ice. This was diluted into one ml of LB medium and cultured with shaking at 37°C for 100 minutes. 200 µl of the sample was plated onto each of three Petri-dishes containing solid LB medium with 100 mg/l kanamycin, 2% X-gal and 2% IPTG. Non-transformed DH5α cells were plated onto the same medium as a control. The plates were incubated overnight at 37°C.

Preparation of competent AGL1 cells

This was based on the protocol of Hofgen and Wilmitzer (1988). An overnight culture of *Agrobacterium tumefaciens* strain AGL1 was diluted in 200 ml of YEB (1.0g/l yeast extract, 5.0g/l beef extract, 5.0g/l peptone, 5.0g/l sucrose, 0.5g/l $MgSO_4.7H_2O$; pH 7.0) medium and maintained at room temperature for 3-4 hours. The growing cells were centrifuged at 3000 g for 20 minutes at 4°C. The pellet was washed in 10 ml pre-cooled TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and resuspended in 20 ml fresh YEB medium. 500 µl aliquots were stored in 15% glycerol at -80°C for use in transformation.

Transformation of AGL1 cells

Competent cells were thawed slowly on ice prior to transformation. About 200 µl of the competent cells were mixed with 1.0 µg of the plasmid DNA and the sample incubated on ice for 5 minutes. The mixture was frozen in liquid nitrogen for 5 minutes, thawed at 37°C for 5 minutes and then put back on ice. One ml of LB was added to the mixture immediately and incubated for 4-5 hours at 28°C. The samples were plated on LB selection plates containing 100 mg/l kanamycin. Non-transformed AGL1 cells were plated onto same medium as control. Plasmid DNA was isolated from selected cells and confirmed through restriction digestion.

Results

Plasmid vector construction

The *Eco*RI and *Hin*dIII digestion of pUC18 carrying the chimaeric gene insert resulted in two DNA fragment bands of sizes 1.46 kb (chimaeric gene insert) and 2.60 kb (plasmid DNA vector) observed on electrophoretic gel. In the digestion of pBINPLUS with same restriction enzymes one single DNA fragment band, of size 12.39 kb was observed on the gel.

The recovery of the plasmid insert from the low melting point gel yielded about 10 µg DNA which was diluted to give a concentration of 0.5 µg/µl. The ligation reaction between the digested pBINPLUS and the chimaeric gene insert resulted in a single DNA fragment band of size 13.85 kb on the electrophoretic gel (Figure 2).

Selection and screening of *Escherichia coli* strain DH5α, transformed with the constructed plasmid vector, on kanamycin and IPTG/X-gal plates resulted in blue and white cell colonies. 95% of the colonies observed were white. No cell colonies were obtained with the control of non-transformed *E. coli* cells on this medium. Plasmid isolation from the six selected white colonies of *E. coli* on these plates yielded the plasmid DNA of the correct sizes for the pBINPLUS backbone and the Shiva-1 chimaeric gene following restriction digestion using *Eco*RI and *Hin*dIII (Figure 3).

Transformation of AGL1

The obtained plasmid vector was used to transform *Agrobacterium* strain AGL1. Cells of *Agrobacterium* strain AGL1, transformed with the constructed plasmid vector were obtained through selection on kanamycin containing LB medium. No cell colonies were obtained on the control plates of non-transformed AGL1 cells. Isolated plasmid from transformed AGL1 cells gave the expected DNA fragment bands of 1.46 kb for the chimaeric gene insert and 12.39 kb for the cloning vector following restriction with *Eco*RI and *Hin*dIII.

The DNA band sizes, 1.46 kb, for the gene insert and 12.39 kb, for pBINPLUS vector obtained, thus confirmed the transformation of AGL1 with the plasmid vector, pBINPLUS with gene insert. Transformed AGL1 cells were cultured overnight and stored in glycerol at -80°C.

The plasmid vector, pBINPLUS carrying the chimaeric gene insert, encoding Shiva-1 protein, will be known and referred to as pBINPLUSH.

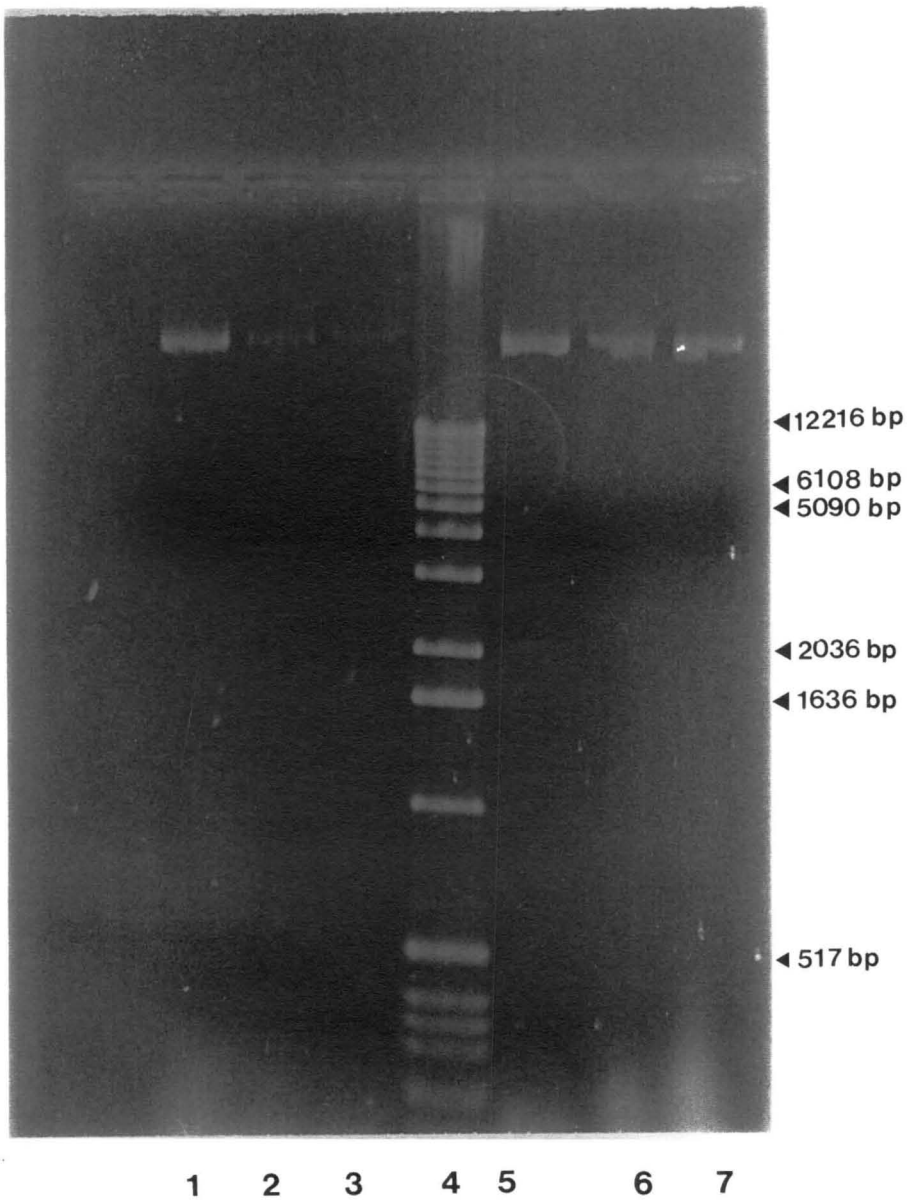

Figure 2. Electrophoretic gel assessment of pBINPLUSH (13.8 kb) isolated from six selected colonies of *Escherichia coli* (lanes 1-3, 5-7). Lane 4 contains 1 kb DNA ladder.

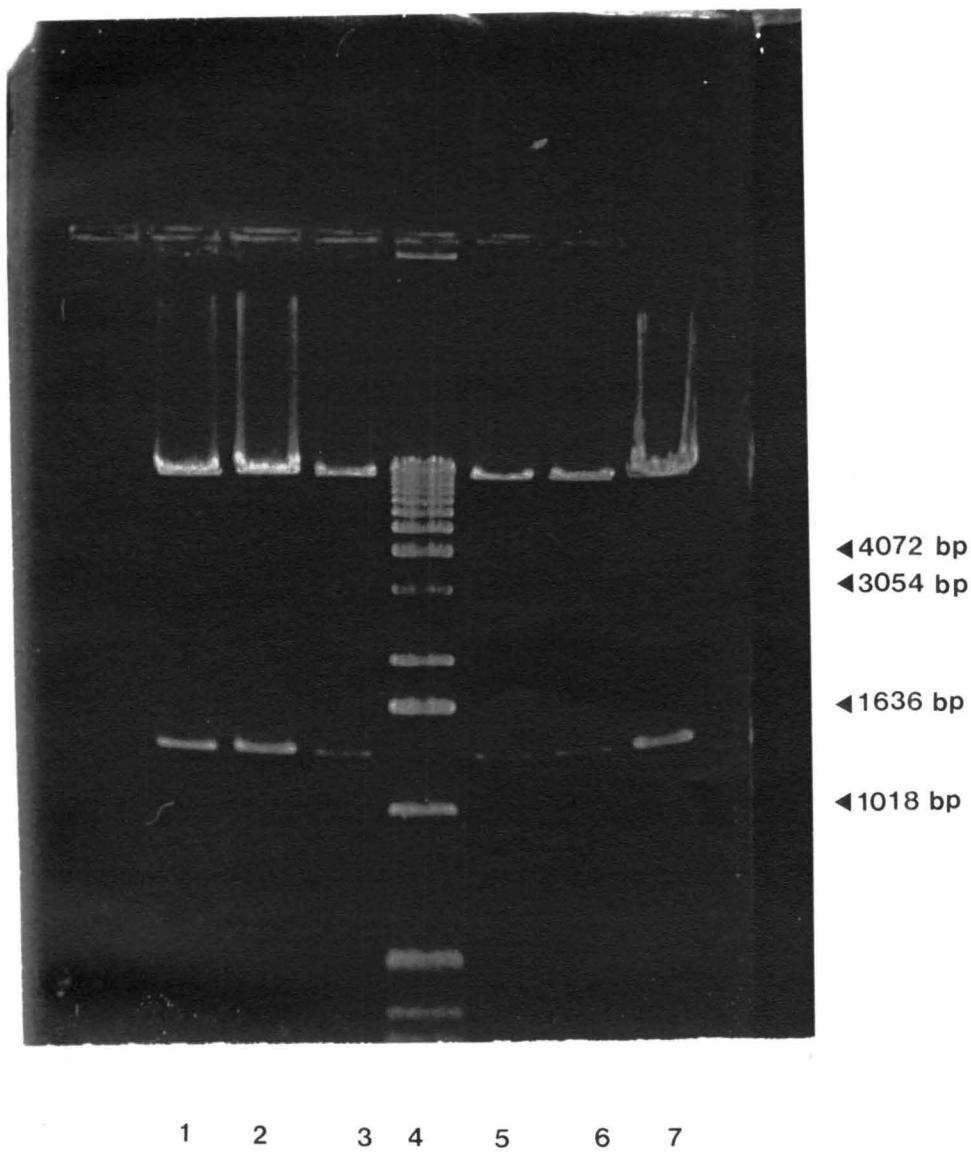

Figure 3. *Eco*RI and *Hin*dIII digestion of pBINPLUSH, isolated from selected colonies of transformed DH5α (Lanes 1-3, 5-7). Lane 4 contains 1 kb DNA ladder. The approximate sizes of the fragment bands obtained were 12.3 kb representing the pBINPLUS vector DNA and 1.4 kb insert containing the Shiva-1 gene insert.

Discussion

Escherichia coli is a preferred organism for most gene cloning experiments due to attributes that allow for quick assessment of cloned genes. The greatest variety of cloning vectors exist for use with *E. coli* as the host organism (Brown, 1990). *E. coli* cells are quick growing and allow for rapid propagation of the recombinant DNA molecule introduced. The organism also enables the use of IPTG and X-gal plates for easy screening of cells with or without cloned inserts.

The *Agrobacterium* strain AGL1, used in this study, carried the hypervirulent, attenuated tumour-inducing plasmid pTiBo542 (Lazo *et al.*, 1991). The pBINPLUS cloning vector, unlike most vectors, carried the selectable marker gene (*npt* II) close to the left T-DNA border. This feature is expected to reduce the possibility of obtaining transgenic plants with the selectable marker but without the gene of interest (Van Engelen *et al.*, 1995). It also has a higher copy number in *E. coli*, rare restriction sites around the multiple cloning site that allow for easier analysis of T-DNA insertions in plant genomes and a lacZ' gene to facilitate the detection of DNA insertion events.

The multiple cloning sites of pUC 18 includes restriction sites for *Eco*RI and *Hin*dIII enzymes flanking the gene of interest (Shiva-1). The use of *Eco*RI and *Hin*dIII enzymes for DNA digestion generated ends that were not compatible, eliminating the possibility for the cloning vector to religate without the insert. It also permitted the correct orientation of the insert within the vector.

The plasmid vector pUC 18 with chimaeric gene insert had a total size of 4.0 kb. The *Eco*RI /*Hin*dIII digestion resulted in two distinguished fragments on the LMP agarose gel. The 1.46 kb DNA insert chimaeric gene comprised of a nopaline synthase (NOS) terminator region at the 3' end (0.39 kb), Shiva-1 coding sequence (0.12 kb) fused to a pre-PRS protein from tobacco (75 bp) and AMV leader sequence (38 bp) with a 35S promoter from Cauliflower Mosaic Virus (0.85 kb) at the 5' end. The use of LMP gel in separation of DNA fragments made it easier for the recovery of the DNA gene insert after the digestion. Agarase was used in cleaving the agarose, releasing the DNA trapped in it.

The single DNA band observed on gel after the digestion of pBINPLUS with the two restriction enzymes (*Eco*RI and *Hin*dIII) is because the smaller fragment (48 bp), migrated beyond the electrophoretic gel. The use of *Eco*RI and *Hin*dIII for the digestion of both pUC18 and pBINPLUS vectors allowed ligation of ends in a particular orientation during the cloning of the chimaeric gene insert (1.46 kb) into pBINPLUS. The lacZ' region in the pBINPLUS vector allowed the screening of *E. coli* cells for disruption of the region or otherwise, as a result the cloning. The multiple cloning sites of the vector are within this region, cloning of the insert resulted in disruption of the lacZ' region and the cell colonies with such disruptions appeared white on the plates containing IPTG inducer and X-gal substrate. Selection with kanamycin was carried out on the same plates. The blue colonies that were observed indicate cells transformed with vectors with undisrupted lacZ' region. This could be due to pBINPLUS vectors that were not digested by the two restriction enzymes since the ends generated by *Eco*RI and *Hin*dIII digestion were not expected to allow religation without insert.

Truncations can occur during gene insertions into plasmid vectors; their isolation and digestion with enzymes provide the means of establishing the correct vector size. Transformation of *E. coli* cells with the ligated plasmid vector, pBINPLUSH, was successful as indicated by the proportion of white colonies observed on the IPTG and X-gal plates. Moreover, the six white colonies selected randomly from the plates, when further analysed gave the expected DNA fragments (Figure 3). The resulting DNA fragment bands from the digestion of isolated plasmid from *E. coli*, 1.46 kb for the chimaeric gene insert and 12.39 kb for the cloning vector, confirmed the successful construction of pBINPLUSH.

In the transformation of AGL1 cells with the pBINPLUSH vector, a small number of cell colony growths were obtained on the kanamycin selection plates as compared to the transformation of *E. coli* strain DH5α. Plasmid isolation from selected colonies were of the correct size (13.85 kb) and digestion with restriction enzymes *Eco*RI and *Hin*dIII yielded the expected DNA fragments, 1.46 kb for the chimaeric insert and 12.39 kb for the pBINPLUS cloning vector. The results from the electrophoretic gel analyses carried out during this experiment confirm that *Agrobacterium* cells with the Shiva-1 chimaeric gene and a selectable *npt* II marker gene, cloned between left and right T-DNA border sequences have been obtained. The left and right border sequences are recognised by the virulence genes present in the resident *Agrobacterium* disarmed Ti plasmid. The transformed *Agrobacterium* cells were inoculated in LB medium with 100 mg/l kanamycin and stored in glycerol at a temperature of -80°C.

A successful cloning of Shiva-1 chimaeric gene insert into pBINPLUS vector and a subsequent transformation of *Agrobacterium* strain AGL1 was achieved in this study. Two similarly constructed *Agrobacterium* transformation vectors with T4 lysozyme and magainin II genes were obtained from Crop & Food Research, Lincoln. These three vectors were used in the transformation of lettuce cotyledons in the next chapter.

CHAPTER 4

Agrobacterium-mediated transformation of lettuce (*Lactuca sativa* L.)

Abstract

Agrobacterium-mediated transformation was attempted for two lettuce cultivars with high regenerative potential in tissue culture: Bambino (crisphead) and Cobham Green (butterhead). Binary vectors based on pBINPLUS, with either of three chimaeric genes encoding T4-Lysozyme, magainin II and Shiva-1 proteins, were transferred to *Agrobacterium* strain AGL1. All three vectors have a neomycin phosphotransferase II (*npt* II) selectable marker gene, conferring resistance to kanamycin, situated close to the left T-DNA border.

Following co-cultivation of lettuce cotyledons with *Agrobacterium*, putative transgenic shoots were observed for Cobham Green, but not for Bambino. A treatment involving a delay of 5 days on timentin containing medium, before explants were transferred to kanamycin supplemented medium, proved more effective in producing vigorous growing transformed shoots for Cobham Green.

Polymerase Chain Reaction (PCR) on DNA isolated from the putative transgenic plants confirmed integration of transferred genes into lettuce genome. Specific oligonucleotide primers, for the *npt* II gene, were used to amplify the gene. Segregation analysis on the selfed progeny, with regards to kanamycin sensitivity, showed monogenic inheritance in seven of eight lines analysed.

Introduction

Lettuce crop is afflicted by several diseases; the prevalent ones, by fungal pathogens. Bacteria diseases also attack lettuce, both in the field and in storage (Patterson *et al.*, 1986). Examples are leaf rot caused by *Xanthomonas campestris* pv. *vitians* and leaf soft rot, by *Erwinia carotovora* subsp. *carotovora*. These diseases reduce the market value of the crop and result in substantial losses. Chemical control of such diseases are often associated with residue problems in plant tissues and the environment, thus making inherent resistance to diseases in plants much desirable.

However, the lack of disease resistance genes in germplasm and /or their presence in distantly related species, limits gene introgression through sexual crosses and traditional breeding. Genes for resistance against certain lettuce fungal diseases, particularly *Bremia lactucae,* have been identified in wild *Lactuca* species (Crute and Norwood, 1981) however, little has been reported of resistance genes for bacteria diseases in the gene pool of lettuce. In addition, the limited number of seeds produced per cross, difficulty in producing hybrids as a result of the self pollinating nature, the incorporation of undesirable traits, and the long periods required to obtain the final desired variety are some of the problems encountered in lettuce breeding (Robinson *et al.*, 1982). Due to the constantly evolving nature of disease pathogens, breeding for disease resistance require more efficient methods which allow introduction of novel genes by genetic engineering.

Cloning of genes and their introduction into plant genomes through genetic manipulation methods offer the prospects of transcending barriers encountered in sexual crosses. The identification and isolation of gene sequences encoding antibacterial peptides has enhanced the effort against bacterial diseases. Genes encoding a number of such peptides are available for introduction into plants via genetic manipulation methods (Jaynes *et al.*, 1987; Casteels *et al.*, 1989; Zasloff, 1987; Nakamura *et al.*, 1988; Samakovlis *et al.*, 1991). The different peptides have varying potency against bacteria pathogens and may also be dependent on the plant species into which they are introduced. In this study, cloned chimaeric genes containing sequences for T4 lysozyme, Shiva-1 (a cecropin B analogue) and magainin II proteins were used.

The different methods of genetic manipulation in plants have varying successes in their use in crop improvement, however, *Agrobacterium*-mediated transformation is a well developed system for genetic manipulation of plants. It is widely utilised and has a greater degree of success in obtaining regenerated transformed plants (Conner and Meredith, 1989; Angenon and Montagu, 1992; Zambryski, 1992). *Agrobacterium*-mediated transformation has been successfully used and remains an effective method for gene transfer to several lettuce cultivars (Michelmore *et al.*, 1987; Torres *et al.*, 1993; Curtis *et al.*, 1996[ab]; Dinant *et al.*, 1997). The success of obtaining transformed cells, expressing the transferred gene, is influenced by a number of factors. Extended co-cultivation of explants and *Agrobacterium* results in increased frequency of transformed plants but this is limited by the growth of *Agrobacterium* on explants (De Kathen and Jacobsen, 1990). The overgrowth of *Agrobacterium* on explants, however, can be

controlled with antibotics while delaying the imposition of selection (Conner *et al.*, 1991); the effect of delaying selection on transformation frequency, was compared in this study.

The objectives of this experiment were to:

1. Transform two lettuce cultivars, Cobham Green (butterhead type) and Bambino (crisphead type) with three chimaeric genes encoding antibacterial peptides.

2. Determine the effect of delaying selection, after co-cultivation of explants and *Agrobacterium*, on transformation frequency.

3. Confirm the integration and inheritance of the transferred genes in the lettuce genome.

Materials and Method

Bacterial strain and vectors

Agrobacterium tumefaciens strain AGL1, with a hypervirulent, tumour-inducing plasmid, pTiBo542, from which the T-region DNA sequences have been precisely deleted (Lazo *et al.*, 1991) was transformed with three binary vectors: pBINPLUSH, pBINPLYS and pBINPMGN. The plasmid vectors pBINPLUSH, pBINPLYS and pBINPMGN carried, within the polylinker site of pBINPLUS (Van Engelen *et al.*, 1995) chimaeric gene constructs encoding Shiva-1, T4-Lysozyme and magainin II proteins respectively (Figure 1). The Shiva-1 and magainin II chimaeric genes were under control of a 0.85 kb CaMV 35S promoter and a 0.39 kb nopaline synthase (NOS)

terminator. The T4 lysozyme gene was under control of a 0.40 kb CaMV 35S promoter and a 0.22 kb CaMV 35S polyadenylation terminator sequence. A neomycin phosphotransferase gene (nos-*npt* II-nos) was situated close to the left border of the T-DNA region, in each binary vector. A bacterial expressed *npt* III gene present in pBINPLUS, outside the T-DNA region, provided a means of selecting for the presence of the binary vectors in the bacteria.

Cell colonies of *Agrobacterium* vectors were selected on solid Luria Broth (LB) medium, containing 100 mg/l of kanamycin, grown at 28°C, for 72 hours. Single colonies were cultured overnight in 40 ml of LB liquid medium containing 100 mg/l kanamycin, at 280 revolutions per minute and temperature of 28°C. Log phase growing cells were pelleted by centrifuging at 5,000 rpm, the pellet was then diluted in 400 ml of SH medium of salts and vitamins.

Genotypes and explants

Lettuce genotypes, Cobham Green (butterhead) and Bambino (crisphead) were used in this experiment. Seeds were surface-sterilised, washed and germinated as described in chapter 2. Two-day old uniform cotyledons (excised from germinated seedlings) were longitudinally cut into half segments and used as explants for *Agrobacterium* co-cultivation.

Media

The seed germination medium consisted of SH salts and vitamins, 30 g/l sucrose, 7 g/l bacteriological agar. Co-cultivation of explants and *Agrobacterium* vectors was done on

shoot regeneration medium, made up of SH salts and vitamins, 30 g/l sucrose, 7g/l bacteriological agar, 0.1 mg/l IAA, 0.5 mg/l kinetin and 0.05 mg/l zeatin. The selection medium for shoot regeneration included 50 mg/l of kanamycin and 200 mg/l of timentin. The selective rooting medium included the seed germination medium supplemented with 50 mg/l kanamycin and 100 mg/l timentin.

All media preparations were sterilised by autoclaving at 121°C, 103 kPa for 15 minutes; except where zeatin, kanamycin or timentin were included. These were filter sterilised and added after autoclaving.

Transformation procedures

Half cotyledon explants were inoculated with the 1:10 dilution of *Agrobacterium* culture by fully immersing explants in a Petri-dish containing the inoculum for 10 minutes. Explants were blotted dry with clean sterilised Whatman filter paper and were cocultivated for two days on shoot regeneration medium followed by transfer to selective shoot regeneration medium. In an alternative treatment, explants were transferred to regeneration medium containing only timentin at 200 mg/l for five days, after co-cultivation, before transfer to the selective regeneration medium. In both treatments, explants were transferred to fresh selective medium every two weeks. Data were taken on the number of explants producing shoots on selection medium and the number of putative transgenic shoots produced with each treatment. Putative transformed shoots were excised and placed on fresh shoot selective medium for three weeks and then transferred to selective rooting medium.

After rooting, the putative transformed shoots were transferred to kanamycin free medium containing SH salts and vitamins, for further growth. The plants were then topped to remove all leaves and the gelled medium washed from their roots in tepid water. They were planted into pots, 36 cm^2 x 7 cm deep and subsequently transferred to pots of 78 cm^2 x 9 cm deep, containing soil mix (Conner *et al.*, 1994).

Plant DNA isolation

Genomic DNA was isolated from leaf tissues obtained from the individually grown putative transgenic plants. The CTAB method for DNA isolation (Doyle and Doyle, 1990) was employed. Fresh leaf tissues were ground in 10 ml of preheated (60°C) CTAB isolation buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0)]. Samples were incubated in a waterbath at 60°C for 30 minutes, with gentle swirling of tubes. An equal volume of chloroform-isoamylacohol (24:1; v/v) was added to the samples in tubes and mixed gently by inverting tubes. Samples were centrifuged at 8,000 rpm for 10 minutes; the aqueous phase was transferred to fresh centrifuge tubes and two-third volume of cold isopropanol added. These were then centrifuged at 6,000 rpm for 5 minutes. The supernatant was discarded and the DNA pellet resuspended in 25 ml of wash buffer [76% (v/v) ethanol, 10 mM ammonium acetate] for an hour. The samples were then centrifuged again at 6,000 rpm for 5 minutes to re-pellet the DNA. The pellet was dissolved in 400 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). An equal volume of phenol-chloroform-isoamylacohol (24:1:1, v/v) was then added to the tubes and gently mixed. These were centrifuged briefly at 10,000 rpm in a microcentrifuge. The aqueous

phase was removed and this step repeated. Two-third volume of cold iso-propanol was then added to the aqueous phase and centrifuged at 14,000 rpm to precipitate the DNA. DNA pellet was dissolved in 100 µl of TE. RNase A was added to a final concentration of 10 µg/ml and incubated at 37°C for 30 minutes. The samples were diluted with sterile distilled water, 7.5 M ammonium acetate was added to a final concentration of 2.5 M and twice volume of cold ethanol (100%) added. Samples were then centrifuged at 14,000 rpm in a refrigerated microcentrifuge. The DNA pellets were air dried and resuspended in TE.

Polymerase Chain Reaction for *npt* II gene

The PCR mixtures were prepared using Expand™ High Fidelity PCR System, manufactured by Boehringer Mannheim, including a thermostable DNA polymerase mixture containing Taq DNA polymerase and Pwo DNA polymerase (isolated from the thermophilic *Thermus aquaticus* and *Pyrococcus woesei*, respectively). Two oligonucleotide *npt* II primers: 5'-ATGACTGGGCACAACAGACAATCGCTGCT-3' (primer A); 5'-GCTATCAGGACATAGCGTTGGCTACCCG-3' (Primer B) were used in the amplification.

A total reaction volume of 25 µl for each sample was prepared in 500 µl PCR tubes, as follows: 0.3 µl of 3.5 units/µl DNA polymerase mixture, 1.25 µl each of 20 µM *npt* II primer A and primer B, 2.5 µl of PCR buffer containing magnesium (10X), 2.0 µl of 2.5 mM PCR nucleotide mix, 2.0 µl of 50 µg/ml of genomic DNA and 15.7 µl of sterile distilled water.

Samples were placed in automated PCR thermocycler for 40 cycles of 94°C for 1 minute, 60 °C for 1 minute, 72 °C for 2 minutes and a post-treatment of 72 °C for 5 minutes. A control with DNA from a non-transgenic Cobham Green and a blank of no DNA were included.

Gel electrophoresis

PCR products were run on an electrophoretic gel for visual assessment. A 2% agarose gel was prepared using 1.0 g of agarose in 50 ml of 1X TBE (0.089 M Tris borate, 0.089 M Boric acid, 0.002 M EDTA). These were put in 100 ml flask and heated gently for agarose to dissolve. The molten agarose was cooled in a gel tray with an inserted comb, to create wells. 15.0 µl of each PCR product was mixed with 3.5 µl of loading dye and loaded into the wells. A 5.0 µl of 2.0 µg/µl DNA molecular marker III (Boehringer Mannheim), mixed with dye was loaded alongside samples. An electric field of 80 volts was applied to the gel, in a tank filled with TBE, and allowed to run for 60 minutes. Gel was then immersed in 50 ml of 0.5 µg/ml ethidium bromide solution for 30 minutes, to stain the DNA bands. The DNA bands in gel were viewed under long wave ultraviolet light and a photograph taken.

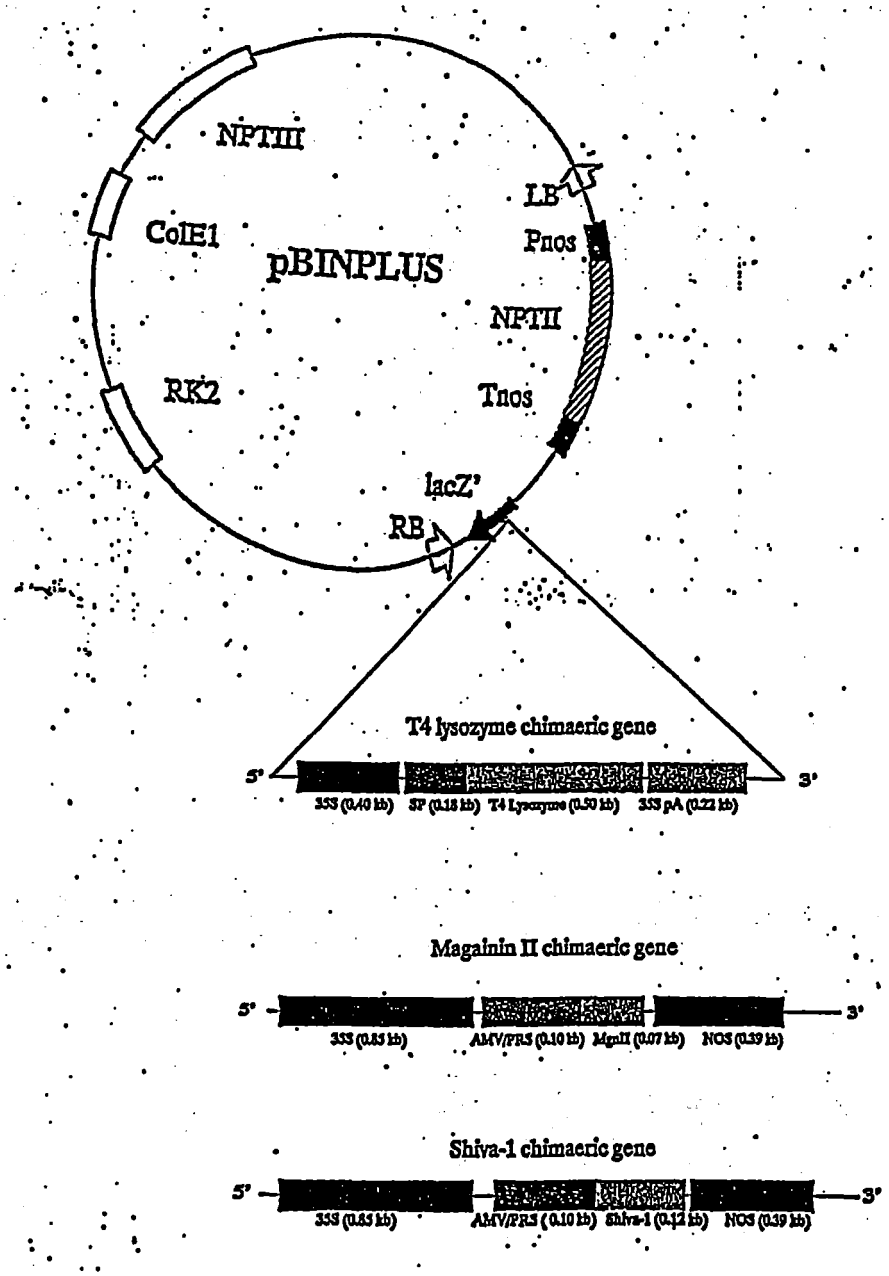

Figure 1. The three chimaeric genes, used in this transformation were cloned into the multiple cloning site within the lacZ' region of pBINPLUS. The T4 lysozyme gene was fused to barley α-amylase signal peptide (SP) under cauliflower mosaic virus (35S) promoter and a polyadenylation signal. The Shiva-1 and magainin II genes were fused to 5' leader of AMV and a signal peptide from the tobacco PR-S gene (pre-pRS) under expression control of a 0.89 kb 35S promoter and a nopaline synthase (NOS) terminating sequence.

Inheritance and expression of *npt* II gene

Seeds obtained from natural self pollination of the transformed plants were surfaced-sterilised and sown on seed germination medium as described above. Three days after germination, seedlings were transferred to a medium of SH salts and vitamins, 30 g/l sucrose, 7 g/l bacteriological agar, with 50 mg/l kanamycin. Non-transgenic Cobham Green seedlings, were included as control. The number of seedlings showing resistance or susceptibility to kanamycin were recorded and tested with chi-square for goodness of fit to the expected 3:1 segregation ratio.

Results

There were no regenerated transformed shoots obtained with the cultivar Bambino. All of the 198 explants of Bambino inoculated with the three *Agrobacterium* vectors became necrotic and appeared brownish at the cut edges, after their transfer to kanamycin selection medium. These were later bleached completely on the selection medium. Bambino explants, in the delayed selection treatment, showed callus growth during the delayed period; their subsequent transfer to selection medium gave no transformed shoots. Regenerated transformed shoots were obtained with Cobham Green, in both of the transformation procedures. Inoculated explants showed restricted growth compared to explant regeneration on kanamycin-free medium. The control of uninoculated explants were completely bleached on the kanamycin containing medium (Figure 2).

The proportion of explants observed with regenerated shoots on selection medium was greater for the delayed selection treatment than the immediate selection. Although, this effect was relatively minor, it was consistent across all three vectors, used (Table 1). More importantly, the total number of shoots excised during selection and the number of putative transgenic shoots finally recovered (after the subsequent transfers to kanamycin selection medium) was greater for the delayed selection than the immediate selection treatment. Putative transgenic shoots recovered from the delayed selection treatment exhibited vigorous growth in culture.

The overall transformation frequency, calculated as percentage of explants producing shoots on selection medium, was 31.3% for pBINPLYS, 13.2% for pBINMGN and 27.3% for pBINPLUSH.

The PCR with the specific oligonucleotide *npt* II primers resulted in a 600 bp DNA fragment in all the transgenic genomic samples used. No DNA fragment band was observed with the control genomic DNA (Figure 3). Some unexpected DNA bands were, however, obtained from the amplification. The 600 bp fragment corresponded to the expected fragment size of the amplified *npt* II gene.

Table 1. Outcome of *Agrobacterium*-mediated transformation of Cobham Green cultivar.

Selection treatments[1]	Vectors	Total number of explants inoculated	Number of explants selected[2]	Number of shoots excised on selection medium	Number of putative transgenic shoots
	T4 lysozyme	30	9	22	2
Immediate	Magainin II	16	1	2	2
selection	Shiva-1	30	4	4	0
	Total	76	14	28	4
	T4 lysozyme	34	11	26	8
Delayed	Magainin II	21	4	8	1
selection	Shiva-1	36	6	14	9
	Total	91	21	48	18

1. Inoculated explants were either transferred to kanamycin selection medium immediately after co-cultivation (immediate selection) or transferred to medium containing timentin for 5 days, before subsequent transfer to selection medium (delayed selection).
2. The number of explants observed with regenerated shoots on selection medium.

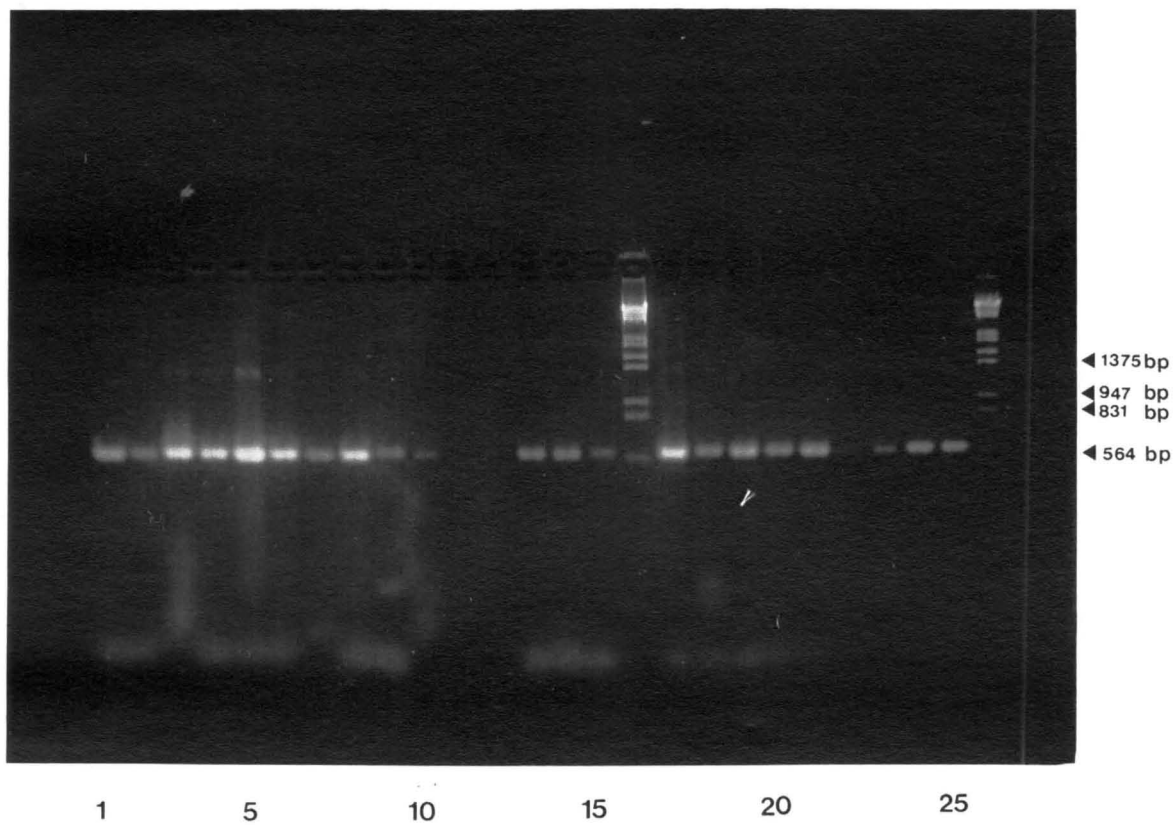

Figure 3. Electrophoretic gel assessment of PCR amplification using *npt* II oligonucleotide primers. The 600 bp fragment (arrowed) is the result of the amplified *npt* II gene and was observed in all the putative transformed lines. The lanes 11 and 12 contained the blank and control samples, respectively. Additional fragment bands were also observed in some of the lines (Lanes 3, 5 and 18). Lanes 16 and 26 contained DNA molecular marker III (Boehringer Mannheim).

In the inheritance study, seedlings from the transformed lines segregated for kanamycin resistance (Figure 4). Non-transformed Cobham Green seedlings, transferred to kanamycin containing medium (as control) all showed kanamycin sensitivity and with bleached leaves. A chi-square goodness of fit test revealed that all the lines, except for one, did not significantly differ from the 3:1 segregation ratio expected for a single integration of the T-DNA (Table 2). More sensitive seedlings than expected were observed in transgenic line 17. The segregation in three of the lines (Line 13, 14 and 19) though not statistically different from the expected ratio was found to be marginal or with a rather small probability of conformity.

Seeds obtained from natural selfing of transformed lines and untransformed Cobham Green were grown in a containment greenhouse to observe any morphological changes in transformed lines. No abnormalities or changes in morphological characteristics were found in the transformed lines compared to the control. However, two lines transformed with T4 lysozyme were characterised by poor seed germination and lack of vigour in seedling growth (Figure 5).

Table 2. Segregation of *npt* II gene in selfed progeny of transformed plants.

Transgenic line	Kan^R seedlings	Kan^S seedlings	χ^2 statistic[a]	Probability[b]
12	40	13	0.006	90%<p<95%
13	44	9	1.817	10%<p<20%
14	33	18	2.882	5%<p<10%
17	33	20	4.580	2%<p<5%
19	32	18	3.226	5%<p<10%
20	39	11	0.240	50%<p<70%
21	39	11	0.240	50%<p<70%
22	41	11	0.410	50%<p<70%

[a] Chi-square goodness of fit to 3:1 segregation ratio; kanamycin resistant (Kan^R): kanamycin sensitive (Kan^S).

[b] The probability, p, that the observed ratio conformed to the expected 3:1 ratio.

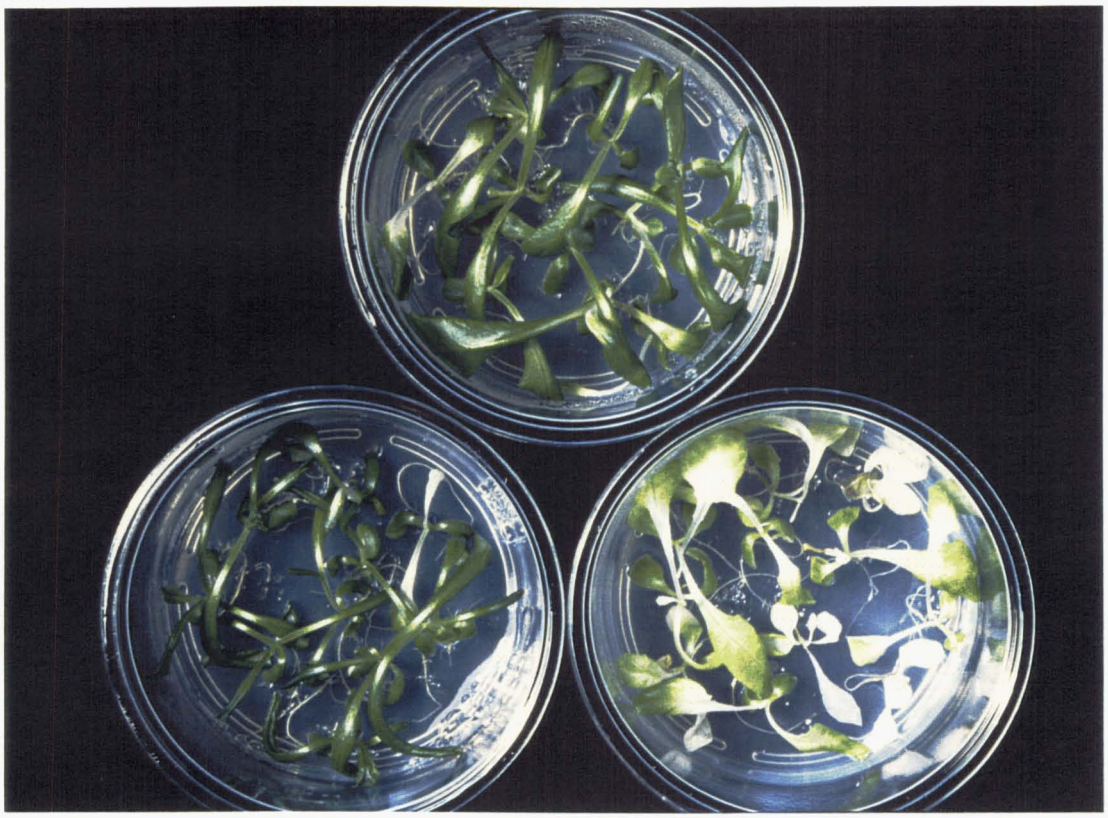

Figure 4. Segregation of progeny seedlings of transformed plants on 50 mg/l kanamycin containing medium. All the control seedlings showed sensitivity to kanamycin (right).

Figure 5. Picture of transformed and control Cobham Green seedlings in soil. Poor seed germination and lack of vigour were observed in some T4 lysozyme transformed lines (LYS).

Discussion

The three binary vectors used for *Agrobacterium*-mediated transformation of lettuce were similar in structure, except for differences in the size and DNA sequence of the chimaeric genes. The promoter for the lysozyme gene was a 0.40 kb CaMV 35S sequence with a 0.22 kb CaMV 35S polyadenylation terminating sequence; a 0.85 kb CaMV 35S promoter and 0.39 kb NOS terminator sequence in pBINPMGN and pBINPLUSH. The sizes of the binary vectors were 13.69, 13.80 and 13.85 kilobasepairs for pBINPLYS, pBINPMGN and pBINPLUSH respectively.

The choice of explant type, age and size, as well as the media used, were based on the earlier tissue culture experiments (chapter 2), where the selected genotypes showed good response in similar conditions. Bambino and Cobham Green are both highly amenable to tissue culture and have the genetic potential for regeneration compared to other lettuce cultivars (Ampomah-Dwamena *et al.*, 1997). The resuspension of the *Agrobacterium* cells, in the liquid medium of SH salts and vitamins, prior to explant inoculation was appropriate to avoid the lettuce explants from being subjected to a higher (100 mg/l) concentration of kanamycin from the bacterial culture medium.

The concentration of kanamycin used in the plant selection medium (50 mg/l) was chosen to avoid extensive inhibition of callus formation while providing rigorous selection for kanamycin resistance (Michelmore *et al.*, 1987). Observed callus production on selection medium was not as prolific as found in explants regenerated on kanamycin-free medium. This was apparently due to the effect of the antibiotics, as relatively few cells were expected to proliferate on the selection medium.

Bambino, a crisphead lettuce, could not be successfully transformed in this experiment. Crisphead cultivars have been identified to be recalcitrant to *Agrobacterium*-mediated transformation, suggesting genotypic variation in response to transformation (Michelmore *et al.*, 1987). Notwithstanding, there are a few reports on successful *Agrobacterium*-mediated transformation of crisphead lettuce. South Bay cultivar, a crisphead, has been transformed using *Agrobacterium*-mediated method with acetosyringone as a stimulant (Torres *et al.*, 1993). Some genotypes categorised as either 'crisp' or 'head' cultivars were among those transformed using *Agrobacterium*-mediated method by Curtis *et al.* (1994). Here, clear genotypic differences were also observed for gene transfer and integration, with some of the crisp or head cultivars being recalcitrant. Genotypic variation in response to *Agrobacterium*-mediated transformation procedures have been reported in other crops. The potato cultivars 'Aracy' and 'Baronesa' could not be transformed, using a procedure that was successful with the cultivar 'Mantiqueira' (Filho *et al.*, 1994). Snyder and Belknap (1993) also observed that the efficiency of potato transformation with *Agrobacterium*, was genotype dependent. However, a transformation procedure for potato, using leaf discs, had earlier been reported as genotype-independent (De Block, 1988).

The proportion of explants with indication of producing shoots on selection medium coupled with the number of transformed shoots finally obtained, clearly showed that the delayed selection treatment was more effective than the immediate selection treatment. The delay in imposing selection can be compared to extended co-cultivation period, as examined by Torres *et al.* (1993); where prolonged co-cultivation of 4-5 days had an

inhibitory effect on shoot regeneration. Longer periods of co-cultivation results in overgrowth of the *Agrobacterium* cells on the explant but the transfer of inoculated explants to medium containing timentin (in this study) controlled the growth of the bacterium while delaying the imposition of selection. The survival of cells in selection medium depends on the stable integration and expression of the marker gene and this could be favoured by the further delay in applying selection. The mechanism of T-DNA transfer involves induction of *vir* gene expression, with sequential events leading to integration and expression of the transferred gene in the host plant (Stachel and Zambryski, 1986). The success thus may be influenced by duration of cocultivation. *Agrobacterium* cells are known to persist in tissues after their inoculation, well after treating with antibiotics (Matzk *et al.*, 1996); a delay in imposing selection therefore could lead to an increased number of cells being transformed and able to express the gene. Delaying selection on inoculated explants allows development of cells and favours quick regeneration. In this study, more callus growth was observed on explants on which selection was delayed than on the immediate selection medium. The regeneration ability of transformed cells could be enhanced by delaying selection.

The initial development of shoots on selection medium was more frequent than the number of putative transformants finally recovered (Table 1); subsequent transfers to fresh kanamycin selection medium identified escape plants by their bleached appearance when in contact with kanamycin. Escape plants may occur as a result of low level expression by intercellular *Agrobacterium* still present in the explants protecting cells in the vicinity by degrading the selective agent. Some parts of the explants may be in poor

contact with the selective agent in the medium (due to possible protection by callus) and may result in regeneration of non-transformed cells. Transient expression in plant cells may also allow non-transformed cells to survive and regenerate on selection medium. The frequent transfer of recovered shoots to fresh selection medium therefore reduced the number of false positives that could have survived. While the number of escape plants, eliminated in the subsequent transfers, was observed to be greater in the delayed selection treatment, the percentage escapes was higher for the immediate selection treatment (Table 1). More putative transgenics were therefore recovered with the delayed selection than with the immediate selection treatment.

In the amplification of the integrated *npt* II gene, the primers under the optimised conditions used were expected to give a 600 bp fragment. The presence of this fragment in all the treatment samples and its absence in the control genomic DNA, confirmed integration of the *npt* II gene in the genome of all the putative transgenic plants. The additional fragments observed could be a result of re-arrangement in the transferred gene, deletions or tandem arrangements as found to occur in some T-DNA insertions (Hepburn *et al.*, 1983; Kwok *et al.*, 1985). Additional fragments can also result in PCR amplification products when low annealing temperatures are employed; the annealing temperature of 60°C used in this study, was the expected optimum for the primers used.

The results obtained in the screening of progeny from the transformed plant on kanamycin-supplemented medium, showed that the *npt* II gene was inherited in a Mendelian manner, in segregation ratios expected for insertion of a single dominant gene (Umbeck *et al.*, 1989; Maiti *et al.*, 1989; Enomoto *et al.*, 1990). The prior

germination of the seeds before their transfer to kanamycin containing medium, prevented the inhibition of seed germination by the selective agent and allowed normal uniform seedlings from all the lines to be used in the assays.

The greater than expected number of sensitive seedlings observed in line 17 may be attributable to downregulation in gene expression (Schmidt and Willmitzer, 1988). Gene silencing via mechanisms such as methylation of cytosines in DNA, production of antisense transcripts and ectopic gene pairing (Meins Jr and Kunz, 1994) in the *npt* II gene may be responsible for the greater number of sensitive seedlings observed in line 17. Gene silencing is observed more frequently as soon as two or more sequences with a certain degree of homology are present in the same genome (Matzke *et al.*, 1994). This may occur between multiple gene copies that are closely linked or arranged in tandem arrays and between homologous genes at allelic or non-allelic locations. The ratio observed in this line conformed to a 1:1 ratio which can be interpreted as a homology-induced silencing of the marker gene in the homozygous state.

In lines 14 and 19, the marginal probabilities of the expected ratio gives indication of an unusual trend. In both lines, there were relatively more sensitive seedlings than would be expected (Table 2). Though inheritance studies established a single locus for kanamycin resistance, there is still the possibility of multiple T-DNA copies in tandem repeats at the same insertion site. Michelmore *et al.* (1987) detected multiple insertions of the *npt* II gene in lettuce, using Southern hybridization, though segregation analyses were consistent with a singly inserted dominant gene. The transmission of kanamycin

resistance observed in the inheritance further confirmed integration of the transferred gene.

In this study, successful transformation of Cobham Green with binary T-DNA vectors in *Agrobacterium* has been achieved. The presence of the *npt* II gene gives a high probability that the other linked genes have been also integrated. This is inferred from the position of the *npt* II gene in the T-DNA, which was close to the left T-DNA border (Zambryski, 1992; Van Engelen *et al.*, 1995). The delay in imposition of selection after co-cultivation of explants, was found to be very effective in producing transgenic shoots despite increased number of escapes.

CHAPTER 5

Integration and expression of antibacterial genes

Abstract

Polymerase chain reaction technique, using specific oligonucleotide primers, was used to confirm integration of the chimaeric genes encoding antibacterial peptides (T4 lysozyme, magainin II and Shiva-1) in the genome of the transformed lettuce plants. All the *npt* II transformed plants also carried the linked antibacterial gene. Expression of the antibacterial genes was ascertained by the activity of their products on bacterial pathogens. Crude leaf extracts from transformed and untransformed lines were used in an *in vitro* assay of peptide activity against two lettuce pathogens: E*rwinia carotovora* subsp. c*arotovora* and *Xanthomonas campestris* pv *vitians*. Leaf extracts from selected T4 lysozyme, magainin II and Shiva-1 transformed lines had an inhibitory effect on the growth of the pathogens compared to extracts from untransformed control plants. Seedlings of transformed lines, inoculated with the pathogens in the greenhouse, exhibited delayed disease symptoms and reduced disease severity compared to the control seedlings.

Introduction

The final phase of a plant transformation process involves the stable integration and subsequent expression of the transferred gene. Selection of transformed cells expressing the phenotype of a selectable marker gene and confirmation of its integration, give a high probability that the gene of interest (present in the T-DNA) has been fully integrated. However, a problem often associated with transformation is the rearrangement of the T-DNA during its insertion into the plant genome, therefore an unselected gene of interest may not be present in the transformant as an intact gene (Hepburn *et al.*, 1983; Kwok *et al.*, 1985; Lassner, 1989). Besides, the expression of a transferred gene is influenced by several factors. The successful integration and expression of a gene of interest, therefore, must be confirmed in selected transformed plants.

Two common bacteria pathogens, *Erwinia carotovora* subsp. *carotovora* and *Xanthomonas campestris* pv *vitians* (which cause soft rot and leaf rot, respectively, in lettuce) can result in severe crop losses (Campbell, 1985). The most desirable and effective strategy of preventing such economic losses has been the incorporation of disease resistance genes into commercially acceptable cultivars (Kleen, 1992). Such improvement through sexual crosses has been limited to genes that exist in one relatively small group of related species and associated with genetic recombination between genomes. Genetic engineering, however, overcomes these drawbacks and greatly expands the potential of obtaining disease resistance in plants, via several approaches (Jaynes *et al.*, 1987; Harms, 1992). Introduction of genes encoding antimicrobial peptides, identified from various sources, into

plant genome may significantly enhance a crop plant's natural resistance to bacterial pathogens (Boman *et al.*, 1991; Montanelli and Nascari, 1991; Düring *et al.*, 1993).

In this chapter, the objectives were to ascertain the successful integration of the antibacterial genes in the genome of the transformed lines obtained and to examine antimicrobial activity of the expressed gene products against two pathogens of lettuce (*Erwinia carotovora* subsp. *carotovora* and *Xanthomonas campestris* pv *vitians*).

Materials and Methods

Confirmation of gene integration

DNA isolated from transformed lines (as described in chapter 4) was used in this experiment. Oligonucleotide primers, specifically designed for Cauliflower mosaic virus (CAMV) 35S promoter, CAMV 35S3' terminator and NOS3' terminator sequences were used in the amplifications. The primer sequences were as follows:

5' CAGTCTCAGAAGACCAAAGGG 3' (35S promoter)

5' TTATCTGGGAACTACTCACAC 3' (35S terminator)

5' GCGGGACTCTAATCATAAAAA 3' (NOS terminator)

PCR mixtures were prepared in a reaction volume of 25 µl for each sample. This included: deoxynucleotide triphosphates (dNTPs), PCR buffer with $MgCl_2$, Taq DNA polymerase, (Boehringer Mannheim); and the designated primers for each gene sequence (Table 1).

Table 1. PCR reagents and concentrations used.

Reagent	Stock concentration	Volume taken	Final Concentration
PCR buffer (with $MgCl_2$)	10X (15 mM)	2.5 µl	1X (1.5 mM)
dNTPs	2.5 mM	2.0 µl	200 µM
Primers 1,2	20 µM	1.25 µl	1 µM
Taq DNA polymerase	5 units/µl	0.2 µl	1.0 U
DNA template	50 µg/ml	2 µl	4.0 µg/ml
Sterile distilled H_2O	--	15.7 µl	--

For the T4 lysozyme gene (with primers from the 35S promoter and 35S terminator), a cycle of 95°C for 1 minute, 58°C for 1 minute, 72°C for 2 minutes and post-treatment of 72°C for 5 minutes, was employed in the amplification. The reaction cycle 95°C (1 minute), 56°C (1 minute), 72°C (2 minutes) final extension of 72°C (5 minutes) was used for the amplification of both Shiva-1 and magainin II genes (using primers from the 35S promoter and NOS terminator).

A negative control of DNA from untransformed Cobham Green, as well as a blank sample containing PCR reagents but no DNA were included in all the samples. PCR products were electrophoretically run on a 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) for visual assessment under ultraviolet light.

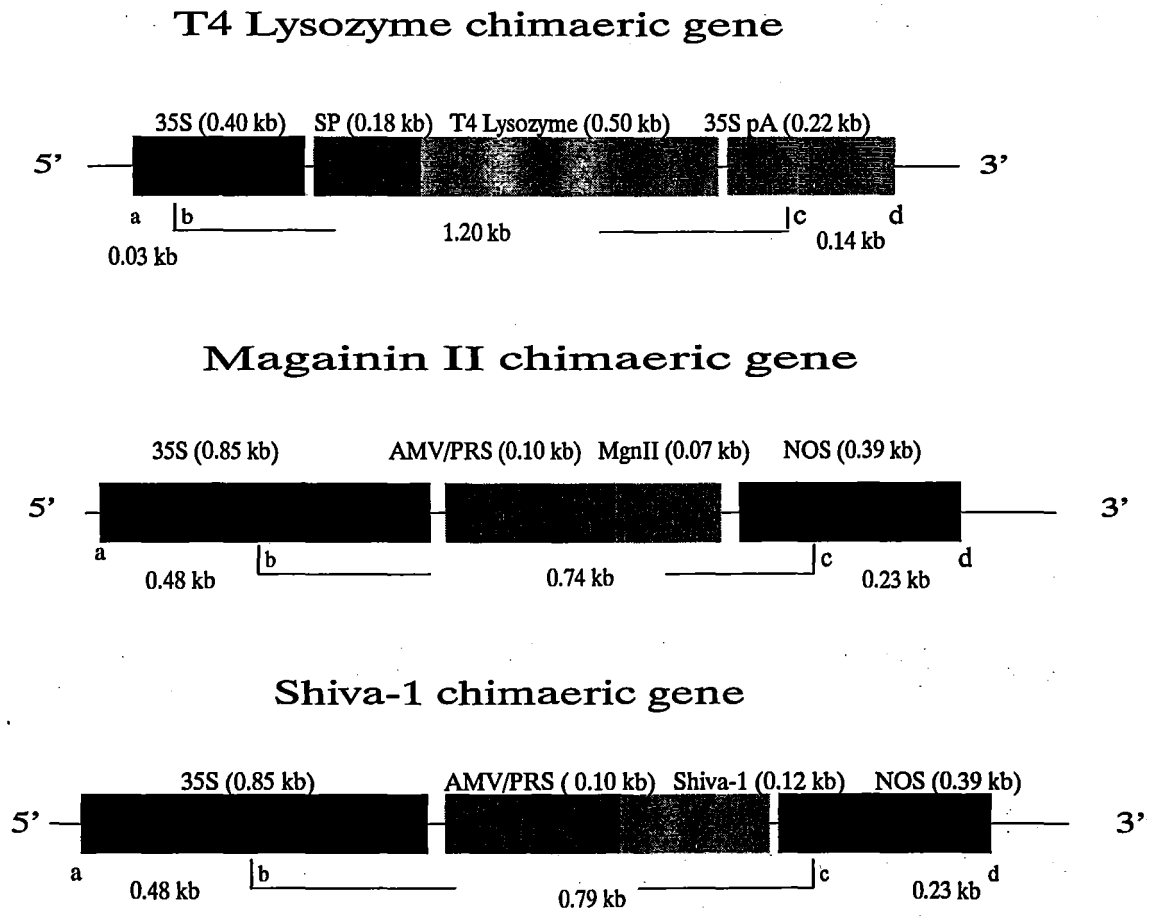

Figure 1. Maps of the three chimaeric genes transferred into Cobham Green showing the expected size of the amplified fragment. The marked positions designate the primer annealing sites (b, c) and their location from the ends of the gene (a, d). For explanations to abbreviations in diagram, refer to Figure 1 of chapter 4.

***In vitro* antibacterial assay**

Selfed progeny seedlings of selected transgenic lines and untransformed Cobham Green were grown in tissue culture medium [SH salts and vitamins, 30 g/l succrose, 7 g/l bacteriological agar, pH 5.8] with no added antibiotics. Fresh leaves (0.5 g) were excised from 30-40 seedlings of each line, after 4 weeks. These were freeze dried with liquid nitrogen (30 ml per sample) in a mortar and ground to fine debris. Samples were transferred into centrifuge tubes and 200 µl of phosphate buffered saline, PBS (0.14M NaCl; 0.003M KCl; 0.008M Na_2HPO_4; 0.002M KH_2PO_4; pH 7.4) added. The mixtures were vortexed briefly and put on a shaker for 5 minutes. Samples were centrifuged at 6,000 rpm for 6 minutes to pellet the debris and the supernatant (crude leaf extract) transferred to fresh Eppendorf tubes.

Single cell colonies of *Erwinia carotovora* and *Xanthomonas campestris* were cultured overnight, in LB medium at 28°C, 280 rpm. Overnight cultures of pathogens were diluted to a standard concentration (determined by plating an aliquot) and used in all the treatments. 150 µl of the diluted pathogen culture was put in fresh sterile 1ml Eppendorf tubes and 100 µl of crude leaf extracts added. Two replications of each treatment were included. The samples were incubated at 28°C, at 240 rpm. Control samples included bacterial culture with crude leaf extracts from non-transformed Cobham Green, and PBS only. After 36 hours of incubation, 10 µl from each sample was plated on LB solid medium; further aliquots were plated after an additional 24 hours. The number of bacterial colonies on each plate were counted and the mean calculated for each treatment sample.

The mean number of colonies for the control samples were determined from 10^{-8} dilutions of the bacterial culture.

Evaluation of pathogen resistance in seedlings

Seeds from selected transgenic lines and untransformed Cobham Green were sown in 12-plug cavity trays with individual cells of 3 cm^2 x 5 cm deep, containing formulated soil mix (Conner *et al.*, 1994). After germination, seedlings were thinned to one per cell and left to grow in containment greenhouse. Five-week old seedlings were wounded in the leaves by perforating with sterile needles and inoculated with two bacterial pathogens. Overnight cultures of *Erwinia carotovora* subsp. *carotovora* (ICMP 1475) and *Xanthomonas campestris* pv *vitians* (ICMP 6461) were diluted with a solution of SH salts to obtain a concentration of 2 x 10^4 colony forming units per ml. Each seedling of 4-6 leaves was inoculated with 100 µl of the bacterial dilution. Inoculated seedlings were transferred to humidity chamber (RH 70-80%) after 5 days. Disease development and severity of symptoms were compared with control seedlings.

Results

PCR for antibacterial genes

The PCR amplification of the antibacterial genes resulted in fragments of size 1.20 kb for the T4 lysozyme transformed lines (Figure 2); 0.74 kb for the magainin II transformed lines (Figure 3) and a 0.79 kb fragment from the Shiva-1 transformed lines (Figure 4). These were the expected fragment sizes from amplification of the genes with the specific

primers (Figure 1). No observed amplified DNA fragment was observed with the control of untransformed Cobham Green and the blank samples included in the amplification procedures.

Antibacterial activity in leaf extracts

In the *in vitro* assay, the growth of *Erwinia carotovora* subsp. *carotovora* and *Xanthomonas campestris* pv *vitians*, was restricted in the cultures with crude extracts from transformed lines. The number of colony forming units determined after incubation with extracts was significantly lower in transformed lines. There was uninhibited cell growth observed in the control as colony formation was not significantly different from the PBS added culture. Colony forming units of *Erwinia carotovora* counted in the treatments (after 36 hours of incubation) was less than the initial concentration used, except in the controls (Table 2). There was a marked reduction in colony growth after 60 hours of incubation with no colonies observed in cultures involving the T4 lysozyme and magainin II transformed lines (Figure 5).

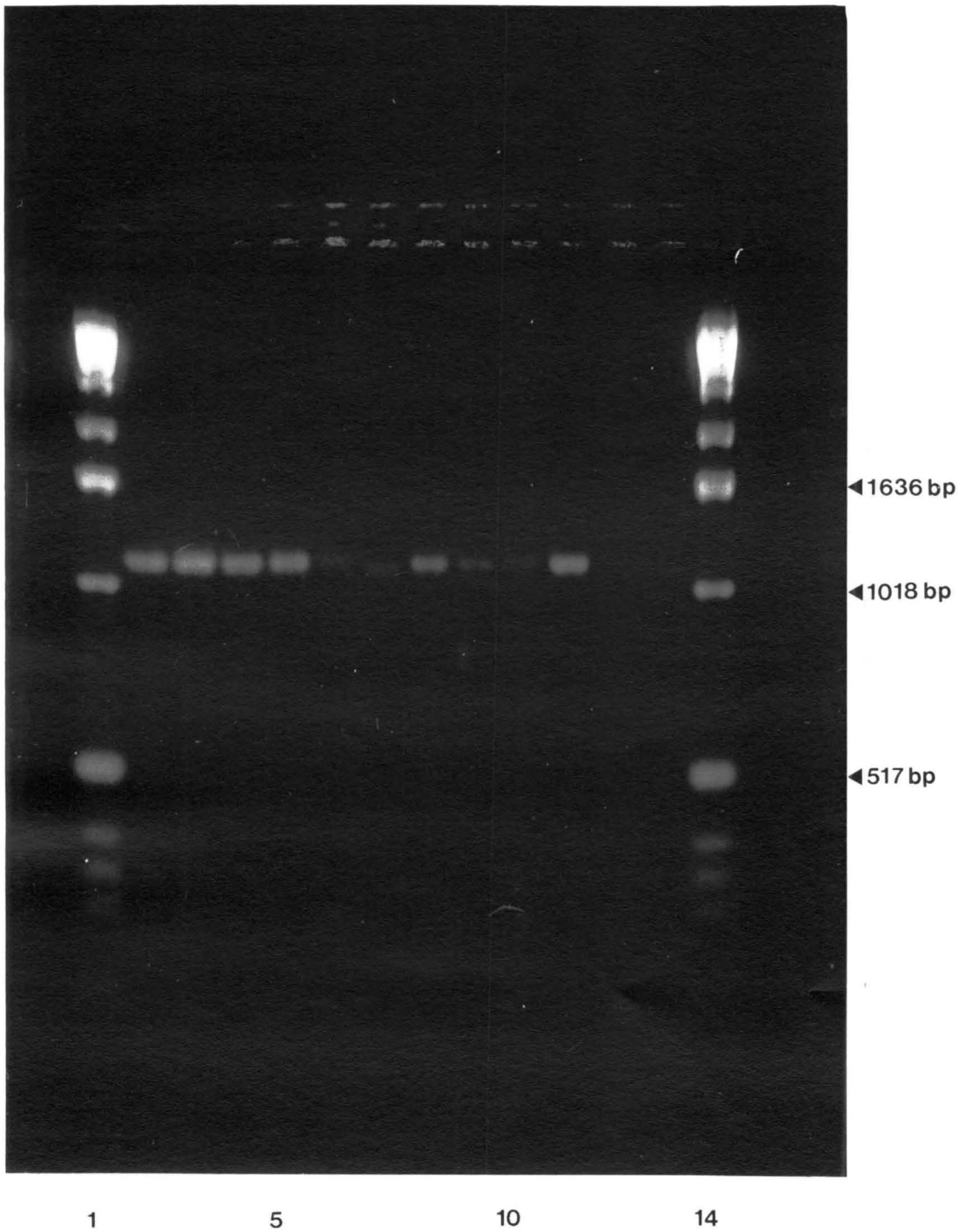

Figure 2. PCR amplification of the T4 lysozyme gene in transformed lines. Lanes 2-11 contained amplified DNA fragment (1.20 kb) from transformed lines; lanes 12 and 13 carried blank and control DNA samples respectively; lanes 1 and 14, 1 kb DNA molecular ladder.

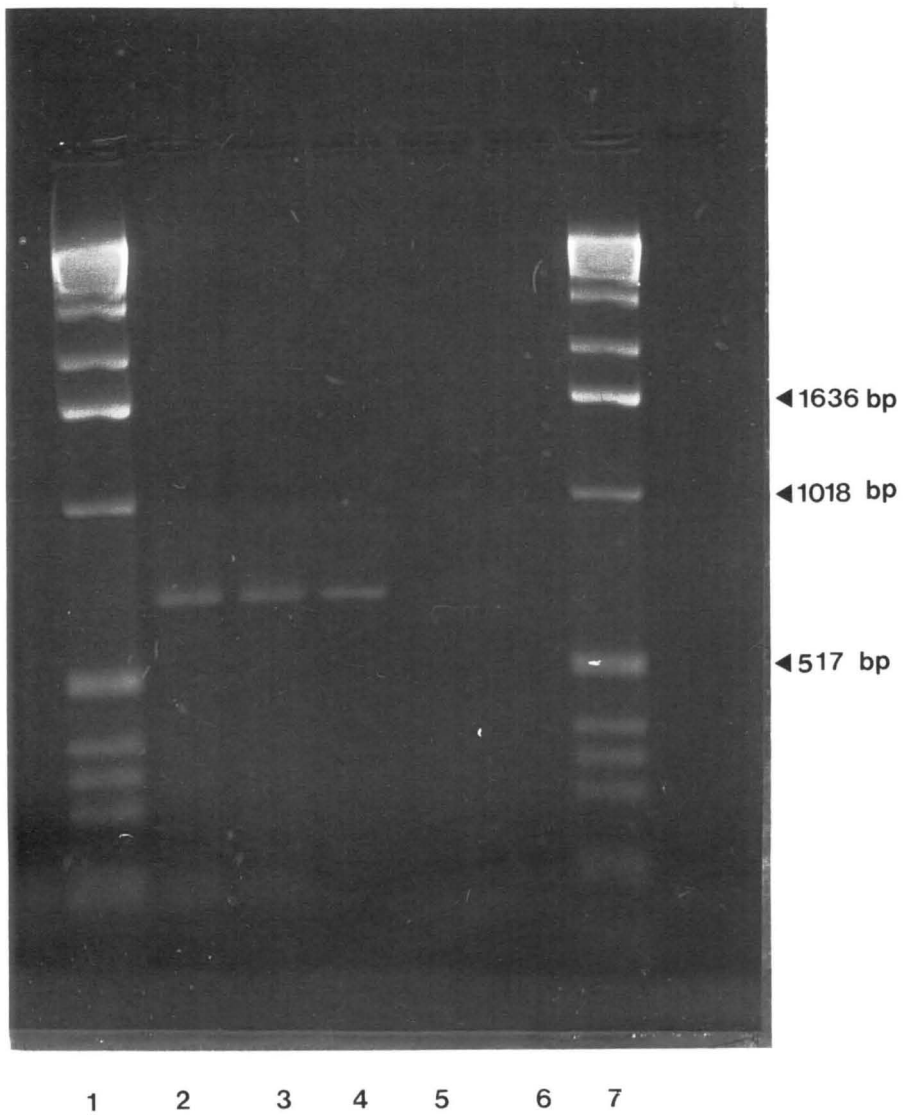

Figure 3. PCR amplification of the magainin II gene resulted in 0.74 kb band (lanes 2-4). No DNA band was observed with the blank and control samples (lanes 5, 6). Lanes 1 and 7 carried 1 kb DNA ladder.

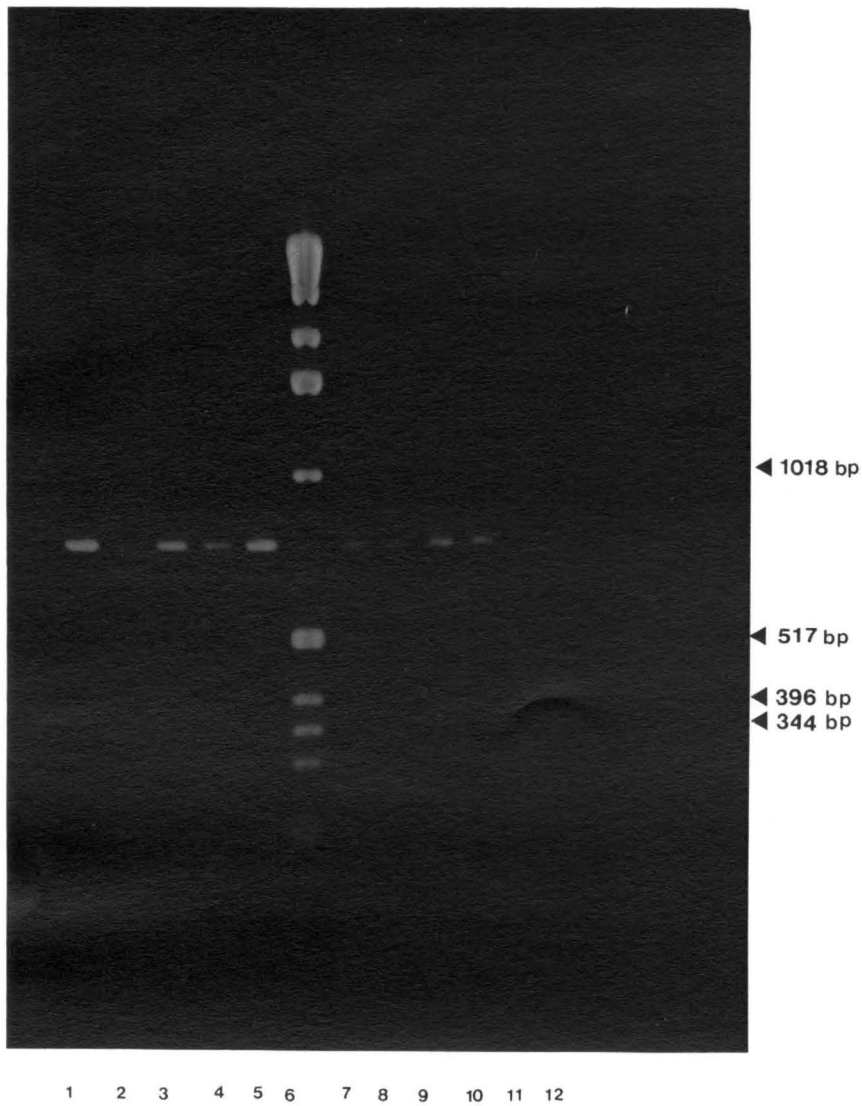

Figure 4. Amplification of the Shiva-1 chimaeric gene. A resultant 0.79 kb DNA fragment obtained from the samples (Lanes 1-5, 7-10). Lane 6 contained 1 kb DNA ladder; lanes 11 and 12, blank and control samples respectively.

Table 2. Colony growth (cfu/ml) of *Erwinia carotovora* subsp. *carotovora* (ICMP 1475) in cultures supplemented with crude leaf extract[a].

Lines	T_0	T_1	T_2
Non-transformed	8.3×10^3	1.6×10^7	1.1×10^{10}
8 (magainin II)	8.3×10^3	4.1×10^3	0
12 (T4 lysozyme)	8.3×10^3	13.5×10^3	0
13 (Shiva-1)	8.3×10^3	4.8×10^3	8×10^2

[a] Crude leaf extracts were isolated from the transformed lines as well as untransformed Cobham Green (control). T_0 refers to the initial concentration of bacteria before incubation; T_1; 36 hours T_2; 60 hours of bacterial incubation with extracts

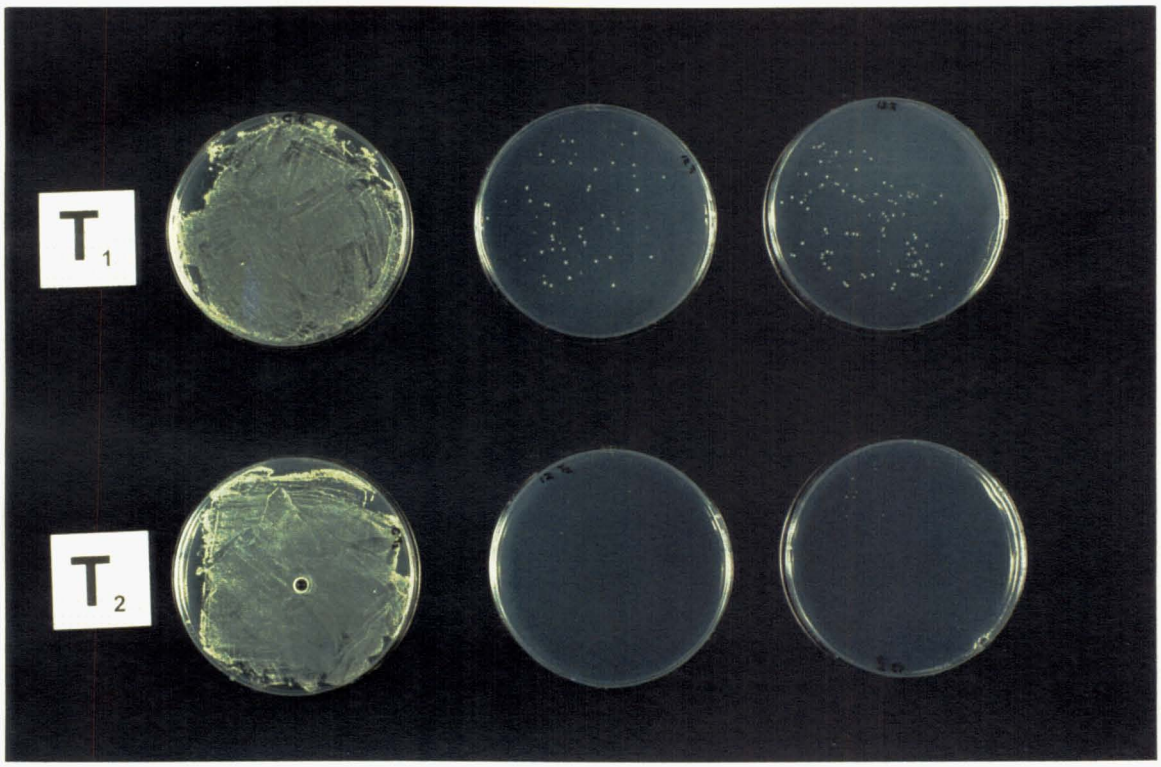

Figure 5. Colony growth of *Erwinia carotovora* subsp. *carotovora* after treatment with crude leaf extracts from untransformed Cobham Green (extreme left) and lines transformed with antibacterial genes after T_1 (36 hours) and T_2 (60 hours) of incubation.

Response to pathogen inoculation

In the greenhouse assay of pathogen testing, differences in susceptibility to *Erwinia carotovora* subsp. *carotovora* and *Xanthomonas campestris* pv *vitians* were observed between control seedlings and those of transformed lines. Overall, delayed symptoms and reduced disease severity were demonstrated in seedlings of transformed lines as compared to the control (Figure 6).

Figure 6. Seedlings of Shiva-1 transformed (S) and control of untransformed (C) Cobham Green, four weeks after their inoculation with *Xanthomonas campestris* pv *vitians*.

Discussion

The molecular evidence obtained from the PCR amplification of the antibacterial gene sequences, confirmed their integration in the transformed lines. This was provided by the presence of the expected amplified DNA bands in the transformed lines and their absence in the control. The presence of the intact antibacterial gene in all the lines found to possess the *npt* II gene could be attributed to the physical position of the antibacterial gene relative to the marker gene, on the T-DNA. The marker gene was proximate to the left T-DNA border; since T-DNA integration is more precise on the right border than the left border, there is a higher likelihood of cotransfer of the gene of interest (Zambryski *et al.*, 1982; Deroles and Gardner, 1988; Van Engelen *et al.*, 1995).

The lines used in the *in vitro* pathogen assay were selected from the transformed lines for each antibacterial gene with good seed production. The activity of the crude leaf extracts (from the transformed lines) on the *in vitro* growth of the pathogens, as compared to the control, established the presence and effectiveness of the expressed antibacterial genes. Crude leaf extracts from lines transformed with antibacterial genes have been used to demonstrate the control of bacterial growth *in vitro* by antibacterial peptides (Montanelli and Nascari, 1991). Selfed progeny seedlings from selected transformants were however, used in this study; these were grown in culture without kanamycin or any antibiotic included in the medium. The likelihood of any residual effect of such antibiotics (in the seedlings) on the pathogens, was eliminated. The potential of the antibacterial genes and the activity of the peptides in enhancing disease resistance (in lettuce) is thus emphasised by the results obtained in this assay.

The action of synthetic antimicrobial peptides on bacterial pathogens of potato has been demonstrated recently (Wilson and Conner, 1995). In that study, cecropin peptides were found to control bacterial growth more effectively than Magainin peptides. Complete inhibition of cecropins against *Erwinia* isolates was determined at a concentration of 37.5 μg/ml of active ingredient. Equal weight of fresh leaves was taken from each of the lines used for leaf extraction (in this study) but the concentration of the peptides in the leaf extracts, was undetermined. This would depend on the level of expression of the gene and the actual proportion of the (30-40) segregated seedlings carrying the expressed gene, which may differ among lines (During *et al.*, 1993). T4 lysozyme, magainin II and Shiva-1 are reported to display antibiotic activity against numerous Gram-negative and Gram-positive bacteria (Zasloff, 1987; Destefano-Beltran *et al.*, 1990; During *et al.*, 1993).

Symptoms of bacteria soft rot caused by *Erwinia carotovora* (soft, wet and slimy rot of leaves) and leaf rot by *Xanthomonas campestris* (characterised by water-soaked spots and yellowing of leaves) were observed in inoculated seedlings of the transformed as well as control plants. However, the extent of disease development was reduced in the transgenic lines compared to the control. A proportion of the seedlings in the transgenic lines were expected to be lacking the antibacterial genes due to segregation of the transgenes; this made assessment very difficult. A uniform population of transformed seedlings, such as a true breeding homozygous line, would be more suitable for disease assessment.

In this chapter, integration of the antibacterial genes (gene of interest) their expression and effectiveness against bacterial pathogens as well as their potential to induce disease resistance in lettuce plants were established.

CHAPTER 6

General Discussion

6.1 Background

Plant diseases can be disruptive to agricultural production, often with disastrous consequences resulting in substantial economic and social impact; for instance the infamous Irish potato famine of 1845-1860 (Jaynes *et al.*, 1987). The natural ability of plants to resist attack by pathogens has been widely and successfully exploited as a disease control strategy and is a prime objective in most plant breeding programmes. Traditional breeding through sexual crosses has been beneficial in this direction, however, the goal of any breeder basically is to provide new genetic material with minimal rearrangement to the genome of the preferred genotype. The specific targeting of single or a few genes that control a character and the small changes that result in the host genome after gene transfers, have made genetic engineering a preferred technique for crop improvement. Improving genetic disease resistance has thus taken the turn of searching for broad principles that may be used to protect a wide range of plant species against disease causing agents (Moffat, 1992). Disease resistance (of all the strategies to control plant pathogens) is the least expensive, of low risk to humans and the environment, and a dependable form of control (Stephens, 1990). Enhancing the self defence mechanism of plants, through genetic engineering, may reduce the use of chemicals in disease control and result in a positive effect on the environment and humans. Useful application of genetic engineering in crop improvement has involved enhanced quality, resistance to pathogens, insects and controlled reaction towards herbicides.

The goal of this research was to transform lettuce with genes encoding antibacterial peptides, to ascertain their effectiveness against bacterial pathogens and their potential to confer disease resistance in lettuce plants

6.2 Summary of findings

The ultimate objective of any plant genetic manipulation procedure is to obtain regenerated transformed plants. Cells undergo stress during manipulation which may affect their regeneration; good regeneration ability is thus a prime requisite of genotypes chosen for genetic manipulation. Though tissue culture performances can be continuously improved through non-genetic factors, such as optimising culture conditions, extensive testing of culture conditions may be required to effectively overcome genotypic dependency of tissue culture response (even for a few genotypes). Screening of plant genotypes to ascertain their response to regeneration conditions is a viable alternative. This has been carried out in several crops and genetic factors are found to be fundamental in the determination of *in vitro* responses (Henry *et al.*, 1994).

The observations made during the regeneration of genotypes (in this study) confirmed genotypic influence on cell growth characteristics of lettuce in tissue culture (Xinrun and Conner, 1992; Curtis *et al.*, 1994). The ranking of callus production into the three categories, limited, moderate and extensive (chapter 2) was appropriate, as a measure of the different amounts of callus produced on explants of the same genotype. The subsequent derivation of callus index (CI) as a single measure of callus proliferation enabled comparisons and detected differences among the genotypes. Good callus producing

genotypes were easily identified, nevertheless, shoot regeneration *in vitro* was of primary importance in determining response of genotypes to tissue culture. This was defined by the number of explants producing shoots and number of shoots per explant, with significant differences observed among the genotypes. The Shoot index (SI) also, gave a single measure of the tendency to produce shoots and the intensity of shoot production on explants. CI and SI, both provided a summarised description of the genotypes response to tissue culture.

The absence of significant correlation between CI and SI indicated that amount of callus produced had little influence on shoot production. Callus growth and shoot regeneration may be independently controlled by different genes as was reported in rice by Tsukahara *et al.* (1995). Further, while significant genotypic effects were evident in the measured characteristics, these were found to be independent of the morphological groups to which the genotypes are classified. The genes controlling regeneration in lettuce may also be independent of those responsible for determining the morphological characteristics.

The method utilised in the regeneration study was convenient for screening large number of genotypes in batches while taking into account the subtle changes that may accompany such procedure. The standard genotype (Bronze Mignonette) included in all the experimental batches, came from a single packet of seed; the differences observed in its performance (in the batches) were attributed to uncontrolled environmental factors such as fluctuations in temperature, photoperiod *etc.* during the experimental period.

The cloning of the chimaeric gene encoding Shiva-1 into pBINPLUS vector was to provide a third binary vector to be used in the transformation of lettuce cultivars. Two binary

vectors with chimaeric genes encoding T4 lysozyme and magainin II, cloned into the multiple cloning sites of pBINPLUS, were available. The third binary vector was constructed similarly to the other two, to avoid any variation in structure that could result in differences during transformation. A distinguished feature of these vectors was the position of the selectable marker gene, which was close to the left T-DNA border of pBINPLUS. The left junction of integrated T-DNA varies over at least 70 bp with much less variation on the right (Zambryski *et al.*, 1982). The mechanism of T-DNA integration and subsequent stabilisation is thus precise at the right border and imprecise on the left border. The position of the selectable marker gene to the left T-DNA border, in the binary vector, provide a greater chance that genes close to the right border are present in cells where the marker gene is integrated.

In the transformation of the two lettuce cultivars (Bambino, Cobham Green), the recalcitrance of Bambino to transformation confirmed genotypic variation in response to *Agrobacterium* transformation of lettuce as observed by Michelmore *et al.* (1987). Crisphead cultivars were particularly noted for this response. The dead cells that characterised the explants of Bambino after their transfer to kanamycin selection medium suggested a hypersensitive response by the wounded cells to the selective agent. Where the explants were left to stay longer on kanamycin-free medium, regenerated shoots were obtained but these were found to be kanamycin sensitive after their transfer to selection medium. The rapid response, therefore, could not be attributed to the presence of *Agrobacterium* cells in the tissues. Bambino has a good shoot regeneration capacity in

tissue culture and transformed cells would be expected to regenerate without total inhibition by the selective agent.

The cultivar Cobham Green was responsive to transformation by both the delayed and immediate selection treatments. Delaying selection on inoculated explants resulted in more transformed shoots and was established as an effective method for increased transformation frequency in plants. All putative lines obtained after selection were confirmed as positive transformants using the molecular technique, PCR. The rigorous selection procedure implemented which involved several transfers to kanamycin containing medium contributed to this, by eliminating false positives.

The polymerase chain reaction (PCR) is a powerful technique for amplifying specific sequences from genomic DNA and can be used to examine the integrity of foreign DNA in transgenic plants. The amplified fragments are easily visualised on ethidium bromide stained agarose gels. Results from PCR are, however, easily influenced by imperfect amplification conditions and contaminated reagents. When using the *npt* II primers for PCR reactions unexpected DNA bands were observed in the amplified product of some transformed lettuce lines. Such bands have been observed in other studies and attributed to imperfect amplification conditions or a possible result of T-DNA re-arrangements and deletions during insertions (Lassner, *et al.*, 1989; Christey and Sinclair, 1992). The presence of the expected DNA band (600 basepairs) in the amplified product of all the transformed lettuce lines and the absence of this band from the untransformed plant provided molecular evidence of integration of the *npt* II gene. Transmission of the

kanamycin resistance to the progeny of transformed plants (as observed in the inheritance study) further confirmed integration of the *npt* II gene.

Segregation of T-DNA in the progeny of transformed plants is dependent on the number of loci into which the T-DNA is inserted during the initial transformation event (Herle-Bors *et al.*, 1988). In addition, factors that influence gene expression may have an effect on the results obtained from inheritance analysis. The *npt* II gene was inherited as a dominant character in a Mendelian manner and was consistent with results from other studies on the *npt* II gene (Maiti *et al.*, 1989; Umbeck *et al.*, 1989). The 3:1 segregation ratio observed in the progeny of transformed lines implies a single insertion of the T-DNA in the plant genome; nevertheless, multiple but linked insertions of a gene could give similar results (Michelmore *et al.*, 1987).

Differences in the frequency of transformed explants and the number of transformed shoots obtained with the binary vectors, were evident. The T4 lysozyme gene gave a higher frequency in both assessments. The binary vectors differed slightly in size; pBINPLYS had a smaller size compared to pBINMGN and pBINPLUSH. The promoter and terminator sequences of the T4 lysozyme differed from the magainin II and Shiva-1 genes, however the *npt* II gene, whose phenotype was used for the selection of transformed explants had the same promoter and terminator sequences in all the vectors. Transformation frequency can be influenced by several factors and may include the size of binary vector (An *et al.*, 1986; Rotino *et al.*, 1992).

The *in vitro* assay of peptide action (using the leaf extracts) confirmed the potency of the expressed proteins resulting from the inserted genes. This provides evidence for the

potential control of diseases caused by *Erwinia carotovora* and *Xanthomonas campestris* in the transgenic lettuce. The activity of peptides against pathogens is known to be stoichiometrical rather than catalytical (Boman, 1991). Their effectiveness against disease incidence will depend on the level of gene expression, accumulation and concentration of the peptide produced in the plant genome which may differ among transformed lines even with same promoter sequences (During *et al.*, 1993). The seedlings used in the greenhouse assay were segregated progeny and therefore a proportion of them were expected to be susceptible to the pathogens, as the control. Severity of symptoms and disease incidence was greater in the control seedlings than was observed in the transgenic lines. The elimination of non-transgenic seedlings among the progeny could have been achieved by their prior growth on kanamycin-supplemented medium. However, this was not done to prevent the residual effect of kanamycin (in the cells) on the pathogens. Nevertheless, such selection of seedlings followed by selfing will result in a population more suitable for assessing disease resistance.

6.3 Contributions of this thesis

The analyses of callus proliferation and regeneration using a standard genotype accounted for effect of subtle variations, typical of tissue culture experiments. Such variations, if unchecked may introduce error and result in wrong assessment. The calculated callus and shoot indices and the adjusted values provided for concise measurement and comparisons of tissue culture response of genotypes. These can be altered to suit specific experimental circumstances. The indices also provided an overall illustration of relationship between callus, shoot production and morphological categories.

The binary transformation vector pBINPLUSH is a new construction based on an efficient plasmid cloning vector. The delayed selection method of transformation, employed by Conner *et al.* (1991) was demonstrated to have positive effect on transformation frequency; this can be easily used in other crops. A few useful genes have been used to transform lettuce as at the time of this research (Curtis *et al.*, 1996[a]; Curtis *et al.*, 1996[b]; Dinant *et al.*, 1997). This work reports the first successful transfer and expression of antibacterial genes in lettuce and therefore establishes a potential for obtaining bacterial disease resistance in lettuce. The antibacterial peptides encoded by the gene have little or no harmful effect on eukaryotic cells, according to Boman (1991). Thus they can be a useful part of the primary defense against bacterial infections in lettuce.

Generally, the aim and objectives of this thesis have been achieved. The difficulties encountered, such as failure to transform Bambino cultivar, insufficient assessment of disease resistance in transgenics, present new challenges to be tackled in further research. An efficient transformation protocol for crisphead cultivars need further investigation to enable transfer of useful genes into the most preferred lettuce types. A detailed examination of gene expression of transferred genes in lettuce, effect of expressed products on host cells and on disease incidence is very important and needs attention.

A significant contribution to obtaining bacterial disease resistance in lettuce has been made in this research study and further development (as suggested) could ultimately result in an improved lettuce cultivar.

References

Albright, L.M.; Yanofsky, M.F.; Leroux, B.; Ma, D.Q.; Nester, E.W. (1987). Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single stranded T-DNA. *Journal of Bacteriology* 169(3): 1046-1055.

Alconero, R. (1983). Regeneration of plants from cell suspensions of *Lactuca saligna, Lactuca sativa, and Lactuca serrola. Hortscience* 18(3): 305-307.

Ambros, P.F.; Matzke, A.J.M.; Matzke, M.A. (1986). Localization of *Agrobacterium rhizogenes* T-DNA in plant chromosomes by in situ hybridization. *Embo Journal* 5: 2073-2077.

Ampomah-Dwamena, C.; Conner, A.J.; Fautrier, A.G. (1997): Response of lettuce genotypes to in vitro regeneration. *Abstract, 12th biennial meeting of IAPTC (New Zealand branch) 10-14 February 1997, Hanmer Springs, New Zealand.* p. 58.

An, G.; Watson, B.D.; Chiang, C.C. (1986). Transformation of tobacco, tomato, potato, and Arabidopsis thaliana using a binary Ti vector system. *Plant Physiology* 81: 301-305.

Anderson, E.J.; Stark, D.M.; Nelson, R.S.; Powell, P.A.; Tumer, N.E.; Beachy, R.N. (1989). Transgenic plants that express the coat protein genes of tobacco mosaic virus or alfalfa mosaic virus interfere with disease development of some non-related viruses. *Phytopathology* 79: 1284-1290.

Angenon, G.; Van Montagu, M. (1992). Transgenic plants: *Agrobacterium*-mediated transformation and its application in plant molecular biology research and biotechnology. In: *Biotechnology and crop improvement in Asia*. Moss, J.P. (Editors). pp. 181-203. ICRISAT, Patancheru, India.

Anzai, H.; Yoneyama, K.; Yamaguchi, I. (1989). Transgenic tobacco resistant to a bacteria disease by the detoxification of a pathogenic toxin. *Molecular-and General Genetics* 219(3): 492-494.

Armitage, P.; Walden, R.; Draper, J. (1988). Vectors for the transformation of plant cells using *Agrobacterium*. In: *Plant genetic transformation and gene expression. A laboratory manual.* Draper, J.; Scott, R.; Armitage, P. and Walden, R. (Editors). pp. 1-67. Blackwell Scientific Publications, Oxford.

Balatero, C.H.; Darvey, N.L.; Luckett, D.J.; Mather, K.; Jinks, J.L. (1995). Genetic analysis of anther culture response in 6X triticale. *Theoretical Applied Genetics* 90(2): 279-282.

Becker, D. (1990). Binary Vectors which allow the exchange of plant selectable markers and reporter genes. *Nucleic Acids Research* 18(1): 203.

Berry, S.F.; Lu, D.Y.; Pental, D.; Cocking, E.C. (1982). Regeneration of plants from protoplasts of *Lactuca sativa* L. *Zeitschrift fur pflanzenphysiologie* 108: 31-38.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* 12: 8711-8721.

Bevan, M.W.; Flavell, R.B.; Chilton, M.-D. (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* 304: 184-187.

Bhaskaran, S.; Smith, R.H. (1990). Regeneration in cereal tissue culture: a review. *Crop Science* 30(6): 1328-1337.

Blay, E.; Oakes, J.V. (1996). *Agrobacterium tumefaciens*-mediated transformation of Solanum gilo Raddi as influenced by explant type. *Plant Cell Reports* 15: 582-585.

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

Boman, H.G. (1991). Antibacterial peptides: Key components needed in immunity. *Cell* 65: 205-207.

Boman, H.G.; Faye, I.; Gudmundsson, G.H.; Lee, J.-Y.; Lidholm, D.-A. (1991). Cell-free immunity in Cecropia. *European Journal of Biochemistry* 201: 23-31.

Brown, C.; Lucas, J.A.; Crute, I.R.; Walkey, D.G.A.; Power, J.B. (1986). An assessment of genetic variability in somacloned lettuce plants (*Lactuca sativa*) and their offspring. *Annals of Applied Biology* 109: 391-407.

Brown, T.A. (1990). *Gene cloning: an introduction.* Chapman and Hall, London.

Campbell, R.N. (1985). Common names for plant diseases: Lettuce (*Lactuca sativa*). *Plant Disease* 69: 662.

Casteels, P.; Ampe, C.; Jacobs, F.; Vaeck, M.; Tempst, P. (1989). Apidaecins: antibacterial peptides from honeybees. *Embo Journal* 8(8): 2387-2391.

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

Christey, M.C.; Sinclair, B.K. (1992). Regeneration of transgenic kale (*Brassica oleracea* var. *acephala*), rape (*B. napus*) and turnip (*B. campestris* var. *rapifera*) plants via *Agrobacterium* rhizogenes mediated transformation. *Plant science* 87: 161-169.

Chupeau, M.-C.; Bellini, C.; Guerche, P.; Maisonneuve, B.; Vastra, G.; Chupeau, Y. (1989). Transgenic plants of Lettuce (*Lactuca sativa*) obtained through electroporation of protoplasts. *Bio/Technology* 7: 503-508.

Clare, G. (1990). *Agrobacterium* in plant disease, biological disease control and plant genetic engineering. *Science Progress* 74: 1-13.

Conner, A.J.; Christey, M.C.; Xinrun, Z. (1992). *Agrobacterium*-mediated transformation of lettuce. *Proceedings of the New Zealand Genetical Society* 25-28 August. p 28.

Conner, A.J.; Dommisse, E.M. (1992). Monocotyledonous plants as hosts for *Agrobacterium*. *International Journal of Plant Science* 153 (4): 550-555.

Conner, A.J.; Meredith, C.P. (1989). Genetic Manipulation of Plant Cells. In: *The Biochemistry of Plants: A comprehensive Treatise.* Vol. 15. Marcus, A. (Editors). pp. 653-688. Academic Press, Orlando.

Conner, A.J.; Williams, M.K.; Abernethy, D.J.; Fletcher, P.J.; Genet, R.A. (1994). Field performance of transgenic potatoes. *New Zealand Journal of Crop and Horticultural Science* 22: 361-371.

Conner, A.J.; Williams, M.K.; Gardner, R.C.; Deroles, S.C.; Shaw, M.L.; Lancaster, J.E. (1991). *Agrobacterium*-mediated transformation of New Zealand potato cultivars. *The New Zealand Journal of Crop and Horticultural Science* 19: 1-8.

Crute, I.R.; Johnson, A.G. (1976). The genetic relationship between races of *Bremia Lactucae* and cultivars of *Lactuca sativa*. *Annals of Applied Biology* 83: 125-137.

Crute, I.R.; Norwood, J.M. (1981). The identification and characteristics of field resistance to lettuce downy mildew (*Bremia Lactucae* Regel). *Euphytica* 30: 707-717.

Curtis, I.S.; He, C.; Power, J.B.; Mariotti, D.; De laat, A.; Davey, M.R. (1996)[a]. The effects of *Agrobacterium* rhizogenes rolAB genes in lettuce. *Plant science* 115: 123-135.

Curtis, I.S.; He, C.; Scott, R.; Power, J.B.; Davey, M.R. (1996)[b]. Genomic male sterility in lettuce, a baseline for the production of F1 hybrids. *Plant science* 113: 113-119.

Curtis, I.S.; Power, J.B.; Blackhall, N.W.; de Laat, A.M.M.; Davey, M.R. (1994). Genotype-independent transformation of lettuce using *Agrobacterium tumefaciens*. *Journal of Experimental Botany* 45: 1441-1449.

D'Amato, F. (1977). Cytogenetics of differentiation in tissue and cell cultures. In: *Plant Cell, Tissue and Organ Culture*. Reinert, J. and Bajaj, Y.P.S. (Editors). pp. 343-357. Springer-Velag, Berlin.

During, K.; Porsch, P.; Fladung, M.; Lorz, H. (1993). Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. *The Plant Journal* 3:4: 587-598.

Dahl, G.A.; Tempe, J. (1983). Studies on the use of toxic precursor analogs of opines to select transformed plant cells. *Theoretical Applied Genetics* 66: 233-239.

De Block, M. (1988). Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*. *Theoretical Applied Genetics* 76: 767-774.

De Block, M.; Botterman, J.; Vandewiele, M.; Dockx, J.; Thoen, C.; Gossele, V.; Movva, N.R.; Thompson, C.; Van Montagu, M.; Leemans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *Embo Journal* 6(9): 2513-2518.

De Block, M.; Herrera-Estrella, L.; Van Montagu, M.; Schell, J.; Zambryski, P. (1984). Expression of foreign genes in regenerated plants and in their progeny. *Embo Journal* 3(8): 1681-1689.

De Block, M.; Schell, J.; Van Montagu, M. (1985). Chloroplast transformation by *Agrobacterium tumefaciens*. *Embo Journal* 4(6): 1367-1372.

De Candolle, A. (1959). *Origin of cultivated plants*. Hafner publishing Co., New York.

De Cleene, M.; Ley, J.D. (1976). The host range of crown gall. *Botanical Reviews* 42: 389-466.

De Kathen, A.; Jacobsen, H.-J. (1990). *Agrobacterium tumefaciens*-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors. *Plant Cell Reports* 9: 276-279.

De Vries, I.M. (1990). Crossing experiments of lettuce cultivars and species (*Lactuca* sect. *Lactuca*, Compositae). *Plant Systematics and Evolution* 171: 233-248.

Della-Cioppa, G.; Bauer, S.C.; Taylor, M.L.; Rochester, D.E.; Klein, B.K.; Shah, D.M.; Fraley, R.T.; Kishore, G.M. (1987). Targeting a herbicide-resistant enzyme from *Escherichia coli* to chloroplasts of higher plants. *Bio/Technology* 5: 579-584.

Deroles, S.C.; Gardner, R.C. (1988). Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium* transformation. *Plant Molecular Biology* 11: 365-377.

Destefano-Beltran, L.; Nagpala, P.G.; Cetiner, M.S.; Dodds, J.H.; Jaynes, J.M. (1990). Enhancing bacterial and fungal disease resistance in plants: Application to potato. In: *The Cellular and Molecular Biology of Potatoes*. Vayda, M.E. and Park, W.D. (Editors). pp. 205-221. CAB International, Wallingford.

Dinant, S.; Maisonneuve, B.; Albouy, J.; Chupeau, Y.; Chupeau, M.-C.; Bellec, Y.; Gaudefroy, F.; Kusiak, C.; Souche, S.; Robaglia, C.; Lot, H. (1997). Coat protein gene-mediated protection in *Lactuca sativa* against lettuce mosaic potyvirus strains. *Molecular Breeding* 3: 75-86.

Doerschug, R.M.; Miller, C.O. (1967). Chemical control of adventitious organ formation in *Lactuca sativa* explants. *American Journal of Botany* 54(4): 410-413.

Doyle, J.J.; Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Draper, J.; Scott, R.; Armitage, P.; Walden, R. (1988). *Plant genetic transformation and gene expression. A laboratory manual.* Blackwell scientific publications, Oxford.

Eenink, A.H.; Groenwold, R.; Dieleman, F.L. (1982). Resistance of lettuce (*Lactuca*) to the leaf aphid *Nasonovia ribis-nigris* 1. Transfer of resistance from *L. virosa* to *L. sativa* by interspecific crosses and selection of resistant breeding lines. *Euphytica* 31: 291-300.

Engler, D.E.; Grogan, R.G. (1982). Isolation, culture and regeneration of lettuce leaf mesophyll protoplasts. *Plant Science Letters* 28: 223-229.

Enomoto, S.; Itoh, H.; Ohshima, M.; Ohashi, Y. (1990). Induced expression of a chimeric gene construct in transgenic lettuce plants using tobacco pathogenesis-related protein gene promoter region. *Plant Cell Reports* 9: 6-9.

Erlich, H.A.; Gelfand, D.H.; Saiki, R.K. (1988). Specific DNA amplification. *Nature* 331: 461-462.

Ferakova (1977): The genus *Lactuca* L. in Europe. *Univerzita Komenskeho, Bratislava.* p 68.

Filho, E.S.F.; Figueiredo, L.F.A.; Monte-Neshich, D.C. (1994). Transformation of potato (*Solanum tuberosum*) cv. Mantiqueira using *Agrobacterium tumefaciens* and evaluation of herbicide resistance. *Plant Cell Reports* 13: 666-670.

Fillatti, J.J.; Kiser, J.; Rose, R.; Comai, L. (1987). Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. *Bio/Technology* 5: 727-730.

Florack, D.; Allefs, S.; Bollen, R.; Bosch, D.; Visser, B.; Stiekema, W. (1995). Expression of giant silkmoth cecropin B genes in tobacco. *Transgenic Research* 4(2): 132-141.

Fuente-Martinez, J.M.; Mosqueda-Cano, G.; Alvarez-Morales, A.; Herrera-Estrella, L. (1992). Expression of a bacterial phaseolotoxin-resistant ornithyl transcarbamylase in transgenic

tobacco confers resistance to *Pseudomonas syringae* pv *phaseolicola*. *Bio/Technology* 10: 905-909.

Futterer, J.; Bonneville, J.M.; Hohn, T. (1990). Cauliflower mosaic virus as a gene expression vector for plants. *Physiologia Plantarum* 79: 154-157.

George, E.F. (1993). *Plant propagation by tissue culture. Part 1. The technology*. Exegetics Ltd., Edington, England.

Gheysen, G.; Herman, L.; Breyne, P.; Montagu, V.; Depicker, A. (1989). *Agrobacterium tumefaciens* as a tool for the genetic transformation of plants. In: *Genetic transformation and expression*. Butler, L.O.; Harwood, C. and Moseley, B.E.B. (Editors). pp. 151-174. Intercept, Andover.

Gielen, J.J.L.; De Haan, P.; Kool, A.J.; Peters, D.; Van Grinsven, M.Q.J.M.; Goldbach, R.W. (1991). Engineered resistance to tomato spotted wilt virus, a negative stranded RNA virus. *Bio/Technology* 9: 1363-1367.

Golemboski, D.B.; Lomonossoff, G.P.; Zaitlin, M. (1990). Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. *Proceedings of National Academy of Science*, USA 87: 6311-6315.

Gossele, V.; Van Aarssen, R.; Cornelissen, M. (1994). A 6' gentamicin acetyltransferase gene allows effective selection of tobacco transformants using kanamycin as a substrate. *Plant Molecular Biology* 26: 2009-2012.

Grant, J.E.; Dommisse, E.M.; Christey, M.C.; Conner, A.J. (1991). Gene transfer to plants using *Agrobacterium*. In: *Advanced methods in Plant Breeding and Biotechnology*. Murray, D.R. (Editors). pp. 50-73. CAB International, Wallingford.

Grevelding, C.; Fantes, V.; Kemper, E.; Schell, J.; Masterton, R. (1993). Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. *Plant Molecular Biology* 23: 847-860.

Guerineau, F.; Brooks, L.; Meadows, J.; Lucy, A.; Robinson, C.; Mullineaux, P. (1990). Sulfonamide resistance gene for plant transformation. *Plant Molecular Biology* 15: 127-136.

Harms, C.T. (1992). Engineering genetic disease resistance into crops: biotechnological approaches to crop protection. *Crop Protection* 11: 291-306.

Harrison, B.D.; Mayo, M.A.; Baulcombe, D.C. (1987). Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. *Nature* 328: 799-802.

Hartmann, C.L.; Lee, L.; Day, P.R.; Tumer, N.E. (1994). Herbicide resistant turfgrass (*Agrostis palustris* Huds.) by biolistic transformation. *Bio/Technology* 12: 919-923.

Haughn, G.W.; Smith, J.; Mazur, B.; Sommerville, C. (1988). Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulphonylurea herbicides. *Molecular and General Genetics* 211: 266-271.

Heberle-Bors, E.; Charvat, B.; Thompson, D.; Schernthaner, J.P.; Barta, A.; Matzke, A.J.M.; Matzke, M.A. (1988). Genetic analysis of T-DNA into the tobacco genome. *Plant Cell Reports* 7: 571-574.

Helm, J. (1954). *Lactuca sativa* in morphologisch-systematischer Sicht. *Die Kulturpflanze II.*

Henry, Y.; Vain, P.; De-Buyser, J. (1994). Genetic analysis of in vitro plant tissue culture responses and regeneration capacities. *Euphytica* 79: 45-58.

Hepburn, A.G.; Clarke, L.E.; Blundy, K.S.; White, J. (1983). Nopaline Ti-plasmid pTiT37, T-DNA insertions into a flax genome. *Journal of Molecular and Applied Genetics* 2: 211-224.

Hepher, A.; Boulter, M.E.; Harris, N.; Nelson, R.S. (1988). Development of a superficial meristem during somatic embryogenesis from immature cotyledons of soybean (*Glycine max* L.). *Annals of Botany* 62: 513-519.

Herman, L.; Jacobs, A.; Van Montagu, M.; Depicker, A. (1990). Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. *Molecular-and General Genetics* 224(2): 248-256.

Hicks, G.S. (1980). Patterns of organ development in plant tissue culture and the problem of organ determination. *Botanical Reviews* 46: 1-23.

Hightower, R.; Baden, C.; Penzes, E.; Dunsmuir, P. (1994). The expression of cecropin peptide in transgenic tobacco does not confer resistance to *Pseudomonas syringae* pv *tabaci*. *Plant Cell Reports* 13: 295-299.

Hille, J.; Verheggen, F.; Roelvink, P.; Franssen, H.; Van Kammen, A.; Zabel, P. (1986). Bleomycin resistance: a new dominant selectable marker for plant cell transformation. *Plant Molecular Biology* 7: 171-176.

Hofgen, R.; Willmitzer, L. (1988). Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Research* 16(20): 9877.

Horsch, R.B.; Fry, J.; Hoffmann, N.; Neidermeyer, J.; Rogers, S.G.; Fraley, R.T. (1988). Leaf disc transformation. *Plant Molecular Biology Manual* : A5:1-9.

Hultmark, D.; Steiner, H.; Rasmuson, T.; Boman, H.G. (1980). Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *European Journal of Biochemistry* 106: 7-16.

Jaynes, J.M.; Xanthopoulos, K.G.; Destefano-Beltran, L.; Dodds, J.H. (1987). Increasing Bacterial Disease Resistance in Plants Utilizing Antibacterial Genes from Insects. *BioEssays* 6:6: 263-270.

Jefferson, R.A; Kavanagh, T.A.; Bevan, M.W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *European Molecular Biology Organisation Journal* 6: 3901-3907.

Johnson, L.B.; Stuteville, D.L.; Skinner, D.Z. (1980). Phenotypic variants in progeny of selfed alfalfa regenerated from calli. *Plant Physiology* 65 (suppl): 36.

Jones, J.D.G.; Svab, Z.; Harper, E.C.; Hurwitz, C.D.; Maliga, P. (1987). A dominant nuclear streptomycin resistance marker for plant cell transformation. *Molecular-and General Genetics* 210: 86-91.

Jun, S.I.; Kwon, S.Y.; Paek, K.Y.; Paek, K.-H. (1995). *Agrobacterium*-mediated transformation and regeneration of fertile transgenic plants of chinese cabbage (*Brassica campestris* ssp. *pekinensis* cv. 'spring flavor). *Plant Cell Reports* 14: 620-625.

Kadkade, P.; Seibert, M. (1977). Phytochrome-regulated organogenesis in lettuce tissue culture. *Nature* 270: 49-50.

Kalloo, D. (1988). *Vegetable Breeding*. Vol. 1. CRC Press, Boca Raton, Florida.

Kesseli, R.; Ochoa, O.; Michelmore, R. (1991). Variation at RFLP loci in *Lactuca* spp. and origin of cultivated lettuce (*L. sativa*). *Genome* 34: 430-436.

Kleen, N.T. (1992). The molecular biology of disease resistance. *Plant Molecular Biology* 19: 109-122.

Koevary, K.; Rappaport, L.; Morris, L.L. (1978). Tissue culture propagation of head lettuce. *Hortscience* 13(1): 39-41.

Komari, T; Halperin, W; Nester, E.W. (1986). Physical and functional map of supervirulent *Agrobacterium tumefaciens* tumor-inducing plasmid pTiBo542. *Journal of Bacteriology* 166: 88-94.

Kumar, V.; Davey, M.R. (1991). Genetic improvement of legumes using somatic cell and molecular techniques. *Euphytica* 55(2): 157-159.

Kwok, W.W.; Nester, E.W.; Gordon, M.P. (1985). Unusual plasmid DNA organization in an octopine crown gall tumor. *Nucleic Acids Research* 13 (2): 459-471.

Lassner, M.W.; Peterson, P.; Yoder, J.I. (1989). Simultaneous amplification of multiple DNA fragments by Polymerase Chain Reaction in the analysis of transgenic plants and their progeny. *Plant Molecular Biology Reporter* 7(2): 116-128.

Lazo, G.R.; Stein, P.A.; Ludwig, R.A. (1991). A DNA transformation-competent Arabidopsis genomic library in *Agrobacterium*. *Bio/Technology* 9: 963-967.

Lemmers, M.; De Beuckeleer, M.; Holsters, M.; Zambryski, P.; Depicker, A.; Hernalsteens, J.P.; Van Montagu, M.; Schell, J. (1980). Internal organization, boundaries and integration of

Ti plasmid DNA in nopaline crown gall tumours. *Journal of Molecular Biology* 144: 353-376.

Lindqvist, K. (1960). On the origin of lettuce. *Hereditas* 46: 319-349.

Maisonneuve, B.; Chupeau, M.C.; Bellec, Y.; Chupeau, Y. (1995). Sexual and somatic hybridization in the genus *Lactuca*. *Euphytica* 85: 281-285.

Maiti, I.B.; Wagner, G.J.; Yeargan, R.; Hunt, A.G. (1989). Inheritance and expression of the mouse metallothionein gene in tobacco. *Plant Physiology* 91: 1020-1024.

Maliga, P.; Svab, Z.; Harper, E.C.; Jones, J.D.G. (1988). Improved expression of streptomycin resistance in plants due to deletion in the streptomycin phosphotransferase coding sequence. *Molecular-and General Genetics* 214: 456-459.

Mantell, S.H.; Matthews, J.A.; McKee, R.A. (1985). *Principles of plant biotechnology. An introduction to genetic engineering in plants*. Blackwell Scientific Publications, Oxford.

Mariotti, D.; Fontana, G.S.; Santini, L. (1989). Genetic transformation of grain legumes: *Phaseolus vulgaris* L. and *P. coccineus* L. *Journal of Genetics and Breeding* 43: 77-82.

Matsumoto, E. (1991). Interspecific somatic hybridization between lettuce (*Lactuca sativa*) and wild species *L. virosa*. *Plant Cell Reports* 9: 531-534.

Matzk, A.; Mantell, S.; Schiemann, J. (1996). Localization of persisting *Agrobacteria* in transgenic tobacco plants. *Molecular Plant-Microbe Interactions* 9(5): 373-381.

Matzke, M.; Matzke, J.M.; Scheid, O.M. (1994). Inactivation of repeated genes - DNA-DNA interaction? In: *Homologous Recombination and Gene Silencing in Plants*. Paszkowski, J. (Editor). pp. 271-307. Kluwer Academic Publishers, Dordrecht.

McDonell, R.E.; Clark, R.D.; Smith, W.A.; Hinchee, M.A.A. (1987). Simplified method for the detection of neomycin phosphotransferase activity in transformed tissue. *Plant Molecular Biology Reporter* 5: 380-386.

Meins Jr., F.; Kunz, C. (1994). Silencing of chitinase expression in transgenic plants: An autoregulatory model. In: *Homologous Recombination and Gene Silencing in Plants*. Paszkowski, J. (Editor). pp. 335-348. Kluwer Academic Publishers, Dordrecht.

Michelmore, R.; Marsh, E.; Seely, S.; Landry, B. (1987). Transformation of lettuce (*Lactuca sativa*) mediated by *Agrobacterium tumefaciens*. *Plant Cell Reports* 6: 439-442.

Michelmore, R.W.; Eash, J.A. (1986). Lettuce. In: *Handbook of Plant Cell culture*. Vol. 4. Evans, D.A.; Sharp, W.R. and Ammirato, P.V. (Editors). pp. 512-551. Macmillian, New York.

Moffat, A.S. (1992). Improving plant disease resistance. *Science* 257: 482-483.

Mok, M.C.; Mok, D.W. (1977). Genotypic responses to auxin in tissue cultures of *Phaseolus*. *Physiologia Plantarum* 40: 261-264.

Mok, M.C.; Mok, D.W.S.; Armstrong, D.J.; Rabakoarihanta, A.; Kim, S.G. (1980). Cytokinin autonomy in tissue cultures of *Phaseolus*: A genotype-specific and heritable trait. *Genetics* 94: 675-686.

Montanelli, C.; Nascari, G. (1990). Introduction of an antibacterial gene in potato using a binary vector in *Agrobacterium* rhizogenes. *Rivista di agricoltura subtropicale e tropicale* 84(3): 329-346.

Montanelli, C.; Nascari, G. (1991). Introduction of an antibacterial gene in potato (Solanum tuberosum L.) using a binary vector in *Agrobacterium* rhizogenes. *Journal of Genetics and Breeding* 45: 307-316.

Nakamura, T.; Furunaka, H.; Miyata, T.; Tokunaga, F.; Muta, T.; Iwanaga, S. (1988). Tachyplesin, a class of antimicrobial peptide from the hemocytes of the Horseshoe Crab (*Tachypleus tridentatus*). *Journal of Biological Chemistry* 263(32): 16709-16713.

Niederwieser, J.G.; Staden, J.V. (1990). The relationship between genotype, tissue age and endogenous cytokinnin levels on adventitious bud formation on leaves of *Lachenalia*. *Plant cell, Tissue and Organ Culture* 22: 223-228.

Norelli, J.; Aldwinckle, H.; Destefano-Beltran, L.; Jaynes, J. (1993). Increasing the fire blight resistance of apple by transformation with genes encoding antibacterial proteins. *Acta-Horticulturae* 338: 385-386.

Owens Y de Novoa, C.; Conner, A.J. (1991). Responses of Rubus genotypes to strains of *Agrobacterium*. *Journal of Genetics and Breeding* 45: 359-368.

Parrot, W.A.; Bailey, M.A.; Durham, R.E.; Matthews, H.V. (1992). Tissue culture and regeneration in legumes. In: *Biotechnology and crop improvement in Asia*. Moss, J.P. (Editors). pp. 115-148. ICRISAT, Patancheru, India.

Paterson, K.E.; Everett, N.P. (1985). Regeneration of *Helianthus annuus* inbred plants from callus. *Plant science* 42: 125-132.

Patterson, C.L.; Grogan, R.G.; Campbell, R.N. (1986). Economically important diseases of Lettuce. *Plant Disease* 70(10): 982-987.

Penarrubia, L.; Kim, R.; Giovannoni, J.; Kim, S.H.; Fischer, R.L. (1992). Production of the sweet protein monellin in transgenic plants. *Bio/Technology* 10(5): 561-564.

Perez, P.; Tirbay, G.; Kallerhoff, J.; Perret, J. (1989). Phleomycin resistance as a dominant selectable marker for plant cell transformation. *Plant Molecular Biology* 13: 365-373.

Perez-Gonzalez, J.A.; Lopez-Cabrera, M.; Pardo, J.M.; Jimenez, A. (1989). Biochemical characterization of two cloned resistance determinants encoding a paromomycin acetyltransferase and a paromomycin phosphotransferase from *Streptomyces rimosus forma paromomycinus*. *Journal of Bacteriology* 171(1): 329-334.

Persley, D.M.; O'Brien, R.; Syme, J.R. (1989). *Vegetable Crops:A disease management guide.* Queensland Department of Primary Industries, Brisbane.

Pink, D.A.C.; Keane, E.M. (1993). Lettuce, *Lactuca sativa* L. In: *Genetic improvement of vegetable crops*. Kalloo, G. and Bergh, B.O. (Editors). pp. 543-571. Pergamon Press, New York.

Potrykus, I. (1990). Gene transfer to plants: assessment and perspectives. *Physiologia Plantarum* 79: 125-134.

Ranch, J.P.; Oglesby, L.; Zielinski, A.C. (1985). Plant regeneration from embryo-derived tissue cultures of soybeans. *In Vitro Cellular & Developmental Biology* 21 (11): 653-658.

Robinson, R.W.; McCreight, J.D.; Ryder, E.J. (1982). The genes of lettuce and closely related species. *Plant breeding reviews* 1: 267-293.

Rotino, G.L.; Perrone, D.; Ajmone-Marsan, P.; Lupotto, E. (1992). Transformation of *Solanum integrifolium Poir* via *Agrobacterium tumefaciens*: Plant regeneration and progeny analysis. *Plant Cell Reports* 11: 11-15.

Ryder, E.J. (1979). *Leafy salad vegetables*. AVI Publishing Co, Westport, CT.

Ryder, E.J. (1986). Lettuce Breeding. In: *Breeding vegetable crops*. Bassett, M.J. (Editors). pp. 433-474. AVI Publishing Company, Inc., Westport, Connecticut.

Ryder, E.J.; Whitaker, T.W. (1976). Lettuce. *Lactuca sativa* (Compositae). In: *Evolution of crop plants*. Simmonds, N.W. (Editors). pp. 39-41. Longman, London.

Saiki, R.K.; Gelfand, D.H.; Stoffel, S.; Scharf, S.J.; Higuchi, R.; Horn, G.T.; Mullis, K.B.; Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.

Samakovlis, C.; Kylsten, P.; Kimbrell, D.A.; Engstrom, A.; Hultmark, D. (1991). The andropin gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster*. *Embo Journal* 10(1): 163-169.

Sambrook, J.; Fritsch, E.F.; Maniatis, T. (1989). *Molecular cloning. A laboratory manual*. Cold Spring Harbor laboratory press, New York.

Sarrafi, A.; Roustan, J.P.; Fallot, J.; Alibert, G. (1996). Genetic analysis of organogenesis inthe cotyledons of zygotic embryos of sunflower (*Helianthus annuus* L.). *Theoretical Applied Genetics* 92(2): 225-229.

Schenk, R.U.; Hilderbrandt, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* 50: 199-204.

Schmidt, R.; Willmitzer, L. (1988). High efficiency *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. *Plant Cell Reports* 7: 583-586.

Scott, R.; Draper, J.; Jefferson, R.; Dury, G.; Jacob, L. (1988). Analysis of gene organization and expression in plants. In: *Plant genetic transformation and gene expression. A laboratory manual.* Draper, J.; Scott, R.; Armitage, P. and Walden, R. (Editors). pp. 263-339. Blackwell Scientific Publications, Oxford.

Sharp, W.R.; Evans, D.A. (1982): Application of somatic embryogenesis to crop improvement. In: *Proceedings of 5th International Congress of plant tissue and cell culture,* Tokyo, Japan. pp 759-762.

Sheikholeslam, S.N.; Weeks, D.P. (1987). Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens. Plant Molecular Biology* 8: 291-298.

Sheridan, W.F. (1975). Plant regeneration and chromosome stability in tissue cultures. In: *Genetic manipulations with plant material.* Vol. 3. Ledoux, L. (Editors). pp. 263-295. Plenum Publishing Co., New York.

Sibi, M. (1976). La notion de programme genetique chez les vegetaux superieurs. *Annales de l'amelioration des plantes* 26: 523-547.

Skoog, F.; Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symposia of the Society of Experimental Biology* 11: 118-130.

Snyder, G.W.; Belknap, W.R. (1993). A modified method for routine *Agrobacterium*-mediated transformation of in vitro grown potato microtubers. *Plant Cell Reports* 12: 324-327.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* 98: 503-517.

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

Stachel, S.E.; Zambryski, P.C. (1986). *Agrobacterium tumefaciens* and the Susceptible Plant Cell: A novel adaptation of extracellular recognition and DNA conjugation. *Cell* 47: 155-157.

Steinbiss, H.H.; Davidson, A. (1989). Genetic manipulation of plants: from tools to agronomical applications. *Science Progress* 73: 147-168.

Stephens, C.T. (1990). Minimizing Pesticide Use in a Vegetable Management System. *Hortscience* 25(2): 164-168.

Streber, W.R.; Kutschka, U.; Thomas, F.; Pohlenz, H.-D. (1994). Expression of a bacterial gene in transgenic plants confers resistance to the herbicide phenmedipham. *Plant Molecular Biology* 25: 977-987.

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

Toleman, E. (1987). Techniques for better lettuce. *The New Zealand Gardener* 43(7): 22-23.

Torbert, K.A.; Rines, H.W.; Somers, D.A. (1995). Use of a paromomycin as a selective agent for oat transformation. *Plant Cell Reports* 14: 635-640.

Torres, A.C.; Cantliffe, D.J.; Laughner, B.; Bieniek, M.; Nagata, R.; Ashraf, M.; Ferl, R.J. (1993). Stable transformation of lettuce cultivar South Bay from cotyledon explants. *Plant cell, Tissue and Organ Culture* 34: 279-285.

Trolinder, N.L.; Xhixian, C. (1989). Genotype specificity of the somatic embryogenesis response in cotton. *Plant Cell Reports* 8: 133-136.

Tsukahara, M.; Hirosawa, T.; Nagai, E.; Kato, H.; Ikeda, R.; Maruyama, K. (1995). Genetic analysis of plant regeneration ability in cell suspension cultures of rice (*Oryza sativa*). *Breeding Science* 45: 425-428.

Umbeck, P.; Swain, W.; Yang, N.-S. (1989). Inheritance and expression of genes for kanamycin and chloramphenicol resistance in transgenic cotton plants. *Crop Science* 29: 196-201.

Van Engelen, F.A.; Molthoff, J.W.; Conner, A.J.; Nap, J.-P.; Pereira, A.; Stiekema, W.J. (1995). pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Research* 4: 288-290.

Van Slogteren, G.M.S.; Hooykaas, P.J.J.; Planque, K.; De Groot, B. (1982). The lysopinedehydrogenase gene used as a marker for the selection of octopine crown gall cells. *Plant Molecular Biology* 1: 133-142.

Van-Bruggen, A.H.C.; Brown, P.R.; Jochimsen, K.N. (1989). Corky root of lettuce caused by strains of a gram-negative bacterium from muck soils of Florida, New York and Wisconsin. *Applied Environmental Microbiology* 55(10): 2635-2640.

Visser, R.G.F.; Jacobsen, E.; Witholt, B.; Feenstra, W.J. (1989). Efficient transformation of potato (*Solanum tuberosum* L.) using a binary vector in *Agrobacterium rhizogenes*. *Theoretical Applied Genetics* 78: 594-600.

Walden, R. (1989). *Genetic transformation in plants*. Prentice Hall Inc., New Jersey.

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

Webb, D.T.; Torres, L.D. (1984). Interactions of growth regulators, explant age, and culture environment controlling organogenesis from lettuce cotyledons in vitro. *Canadian Journal of Botany* 62: 586-590.

Weissinger, A.K. (1992). Physical methods of gene transfer. In: *Biotechnology and crop improvement in Asia*. Moss, J.P. (Editors). pp. 213-233. ICRISAT, Patancheru, India.

Wickham, K.; Rodreguez, E.; Arditti, J. (1980). Comparative phytochemistry of *Parthenium hysterophorus* L. (*Compositae*) tissue cultures. *Botany Gazzette* 141: 435-439.

Wilson, C.R.; Conner, A.J. (1995). Activity of antimirobial peptides against the causal agents of Common scab, Black leg and Tuber soft rot diseases of potato. *New Zealand Natural Sciences* 22: 43-50.

Wu, X.; Beachy, R.N.; Wilson, T.M.; Shaw, J.G. (1990). Inhibition of uncoating of tobacco mosaic virus particles in protoplasts from transgenic tobacco plants that express the viral coat protein gene. *Virology* 179: 893-895.

Xinrun, Z.; Conner, A.J. (1992). Genotypic effects on tissue culture response of lettuce cotyledons. *Journal of Genetics and Breeding* 46: 287-290.

Yang, J.; Verma, P.R. (1992). Screening genotypes for resistance to pre-emergence damping-off and post emergence seedling root rot of oilseed rape and canola caused by *Rhizoctonia solani* AG-2-1. *Crop Protection* 11: 443-448.

Zambryski, P.; Depicker, A.; Kruger, K.; Goodman, H.M. (1982). Tumor induction by *Agrobacterium tumefaciens*:Analysis of the boundaries of T-DNA. *Journal of Molecular and Applied Genetics* 1: 361-370.

Zambryski, P.; Joos, H.; Genetello, C.; Leemans, J.; Van Montagu, M.; Schell, J. (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *Embo Journal* 2 (12): 2143-2150.

Zambryski, P.C. (1992). Chronicles from the *Agrobacterium*-plant DNA transfer story. *Annual Review of Plant Physiology and Plant Molecular Biology* 43: 465-490.

Zasloff, M. (1987). Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proceedings of National Academy of Science*, USA 84: 5449-5453.

Zohary, D. (1991). The wild genetic resources of cultivated lettuce (*Lactuca sativa* L.). *Euphytica* 53: 31-35.

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Appendix

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Ampomah-Dwamena, C; Conner, AJ and Fautrier, AG (1997). Agrobacterium-mediated transformation of lettuce with antibacterial genes. Abstract, NZ IAPTC Conference, Hanmer Springs, 10-14 February, 1997.

Ampomah-Dwamena, C; Conner, AJ and Fautrier, AG (1997). Response of lettuce genotypes to *in vitro* regeneration. Abstract, NZ IAPTC Conference, Hanmer Springs, 10-14 February, 1997.

Ampomah-Dwamena, C; Conner, A.J. and Fautrier, A.G. (1997). Genotypic response of lettuce cotyledons to regeneration *in vitro*. *Scientia Horticulturae* (in press)