Genetic Transformation of Broccoli and

Promoter Tagging in *Brassica* species

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GENETIC TRANSFORMATION OF BROCCOLI AND PROMOTER TAGGING IN *BRASSICA* SPECIES

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ABSTRACT

The genus *Brassica* includes many economically important vegetable, condiment and oilseed crops. Within this genus, *B. oleracea* includes many major vegetable crops, broccoli, cauliflower, Brussels sprout, kohlrabi, kale and cabbage. The key objective of this work was to develop an efficient *Agrobacterium*-mediated transformation system for broccoli (*Brassica oleracea* var. *italica*) and related crops to facilitate crop improvement through genetic engineering.

Five broccoli (*B. oleracea* var. *italica*) cultivars and one breeding line were evaluated for *in vitro* shoot regeneration. Hypocotyl and cotyledonary petiole explants taken from five-day-old seedlings were plated on media consisting of MS salts with B5 vitamins (MSB5) and conditions for obtaining direct shoot regeneration from explants were optimized by testing a range of growth regulator combinations. The optimal growth regulator combination varied with cultivar. The highest percentage of shoot formation (100%) and greatest average number of shoots per explant (28 for hypocotyls and 12 for cotyledonary petioles) were observed with the cultivar 'Cruiser'. Shoots rooted with up to 100% frequency after four weeks of culture on growth regulator free MSB5 medium. Shoot regeneration consistently occurred after two weeks of culture and plants with roots were obtained within six weeks.

Using the optimal shoot regeneration condition, *Agrobacterium*-mediated transformation methodology was developed. Cotyledonary petiole explants from the cultivars 'Cruiser' and 'Green Valiant' were co-cultivated with *Agrobacterium*

tumefaciens carrying a plasmid coding for β-glucuronidase (GUS), neomycin phosphotransferase (NPTII) and phosphinothricin acetyl transferase (PAT) which confers herbicide (phosphinothricin) tolerance under the independent regulation of the cauliflower mosaic virus (CaMV) 35S promoter. Plants resistant to 20 mg/L kanamycin were regenerated at a frequency of 1-10% from cotyledonary petiole explants and were shown to be transformed by GUS staining, NPTII and PAT assays, PCR analysis, Southern blotting and genetic segregation analysis. An efficient *Agrobacterium*-mediated transformation method was developed, with significant modifications subsequently made, to facilitate handling of large numbers of explants in a short period of time. Transformation of other *B. oleracea* varieties such as cauliflower, kale, kohlrabi, cabbage and Brussels sprout was also achieved with this method.

Agrobacterium-mediated transformation technology was employed to tag promoters in broccoli and *B. napus* by employing a *gus::npt*II promoterless fused gene construct. Seven promoter-tagged transgenic lines were produced by this method and these lines were analyzed for GUS expression in different tissues and at various developmental stages. Promoters tagged in this study included: constitutive, root predominant, phloem predominant and shoot specific promoters. Novel promoters, once isolated and characterized, could have important applications in crop genetic engineering strategies.

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their love, support, encouragement and inspiration:

Mother and Father

무한한 사랑과 희생, 그리고 교육에의 높은 식견으로
오늘의 이 학업을 마무리 지을 수 있도록 하여 주신 어머니/아버지께
이 논문을 헌정 합니다.

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Abbreviations	Descriptions
2,4-D	2,4-dichlorophenoxyacetic acid
als	acetolactate synthase gene
BA	6-benzylaminopurine
B5	Gamborg's salts and vitamins
bp	base pair
CaMV 35S	cauliflower mosaic virus 35S
CAT	chloramphenicol acetyl transferase
CTAB	cetyltrimethyl ammonium bromide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra acetic acid
g	gram
x G	centrifugal gravity
GA_3	gibberellic acid
GUS	β–glucuronidase
GUS::NPTII	bifunctional fusion protein of β–glucuronidase and neomycin phosphotransferase II
*gus*A (*gus,* syn. *uidA*)	β–glucuronidase gene
*gus::npt*II	β–glucuronidase and neomycin phosphotransferase II fused gene
Kb	kilo base pair

LB	T-DNA left border repeat
L-ppt	(L) form of phosphinothricin
IAA	indole-3-acetic acid
IBA	indole butyric acid
KIN	kinetin
MES	2-N-morpholinoethanesulfonic acid
μE	micro Einstein
μg	microgram
μL	microlitre
mg	milligram
mL	millilitre
mM	millimolar
MS	Murashige and Skoog's salts and vitamins
MTX	methotrexate
NAA	naphtalenacetic acid
NPTII	neomycin phosphotransferase II
*npt*II	neomycin phosphotransferase II gene
PAT	phosphinothricin acetyl transferase
pat	phosphinothricin acetyl transferase gene
PCR	polymerase chain reaction
PVP	polyvinylpyrolidine
RB	T-DNA right border repeat
Ri	root inducing plasmid
T-DNA	transferred DNA of Ti plasmid
Ti	tumor inducing plasmid of *Agrobacterium*
Tnos	transcriptional terminator of nopaline synthase gene
X-Gluc	5-bromo-4-chromo-3-indolyl-glucuronidase

INTRODUCTION

The genus *Brassica* includes several important oilseed and vegetable crop species, and is the subject of a growing number of studies on the principles of gene regulation and gene expression. Genetic studies have determined that these species are interrelated. Cytogenetic analyses have determined that *B. oleracea, B. rapa* (syn. *campestris*) and *B. nigra* are elementary diploid species, and that *B. napus, B. carinata* and *B. juncea* are amphidiploid species that have been derived from the diploid species through interspecific hybridization. *B. napus* and *B. rapa* are important oilseed crops in North America and Europe. *B. juncea* is grown as a condiment crop in North America and Europe but as an oilseed crop in Asia. *B. carinata* is grown regionally in parts of Africa and Asia as an oilseed and vegetable crop. *B. nigra* has been cultivated as a condiment in the U.S. in the past but it is now primarily recognized as a weed.

Brassica oleracea includes major vegetable crops such as broccoli, cauliflower, cabbage, kale, kohlrabi and Brussels sprout. These vegetable brassicas are amenable to *in vitro* manipulation, and shoot regeneration has been demonstrated for almost all from a number of vegetative explants as well as from reproductive or floral organs. In spite of a very well established regeneration system, a broadly-based and reliable transformation system is not available for *B. oleracea.*

Different types of explants from *B. oleracea* have been used in *Agrobacterium*-mediated transformation systems (Christey and Earle 1989; DeBlock *et al.* 1989; Eimert *et al.* 1992; Berthomieu *et al.* 1994). However, the results have sometimes been

difficult to reproduce. Moloney *et al.* (1989) have suggested that access to wound surfaces and subsequent shoot regeneration from those surfaces are the key factors in determining the efficiency of *Agrobacterium*-mediated transformation. The problem of low regeneration frequency, especially following *Agrobacterium* treatment, is exacerbated by inter-varietal differences in regeneration response. The majority of the reported explant regeneration and transformation studies have used the spring canola cultivar *B. napus* 'Westar'. Other commercial cultivars were less amenable in explant culture and transformation (Khehra and Mathias 1992).

The first objective of this project was to establish an efficient shoot regeneration system in a wide range of broccoli (*Brassica oleracea* var. *italica*) cultivars using hypocotyl and cotyledonary petiole explants derived from *in vitro* grown seedlings. Five broccoli cultivars and one breeding line were evaluated for *in vitro* shoot regeneration response by screening both hypocotyl and cotyledonary petiole explants.

The second objective of this work was to develop an efficient *Agrobacterium*-mediated transformation method for broccoli (*Brassica oleracea* var. *italica*). Using optimal regeneration conditions, cotyledonary petioles were co-cultivated with *Agrobacterium tumefaciens* carrying a plasmid coding for β-glucuronidase (GUS), neomycin phosphotransferase (NPTII) and phosphinothricin acetyltransferase (PAT-which confers tolerance to the herbicide phosphinothricin) under the independent regulation of the cauliflower mosaic virus (CaMV) 35S promoter. Putative transgenic

lines were analyzed by PCR, Southern hybridization and progeny testing. The transformation method was also applied to other *B. oleracea* varieties such as cauliflower, kale, cabbage, kohlrabi and Brussels sprout.

The third objective of this work was to tag endogenous plant promoters from *Brassica napus* and *Brassica oleracea* var. *italica* using *Agrobacterium*-mediated transformation and a GUS::NPTII fused promoterless construct. Only transformation events in which the promoterless fusion gene integrated adjacent to a plant regulatory sequence were expected to produce viable green shoots under selection. Promoter tagged transgenic lines recovered from these experiments were analyzed for GUS expression in various tissues and cells at different developmental stages to determine the types of promoters tagged.

CHAPTER 1:

Literature Review

1.1 Taxonomy and origin of *Brassica* crops

The *Cruciferae* represents an agriculturally important family which contains approximately 3,000 species in 350 genera including species with highly diverse morphology and wide ranging utility (Weier *et al.* 1974). The genus *Brassica* has been reported to comprise 85 species, the majority of which are native to the Mediterranean region (Simmonds 1976). These species are herbaceous plants that are either annuals or biennials. Several of these species are now cultivated world-wide for human consumption, livestock feed or as a source of vegetable and industrial oil. This genus also includes several important vegetables (e.g. broccoli, cauliflower, kale, kohlrabi, Brussels sprout, turnip, swede, cabbage), condiments (brown mustard), oilseeds (rape or canola) and forages.

Genome relationships and evolution in *Brassica* and allied genera have attracted considerable interest over the past 60 years. Much of the information on *Brassica* species relationships has been derived from interspecific hybridization and cytogenetic studies (U 1935; Prakash and Hinata 1980; Quiros *et al.* 1986). The cultivated *Brassica* are represented by six interrelated species. Cytogenetic studies have determined that *B. oleracea*, *B. rapa* and *B. nigra* are the elementary diploid species, and that *B. napus*, *B.*

carinata and *B. juncea* are amphidiploid species derived from the diploid species by hybridization (Fig. 1). From analysis of chromosome morphology, Robbelen (1960) suggested that there were six basic chromosomes in the diploid species *Brassica rapa* (n=10), *B. nigra* (n=8) and *B. oleracea* (n=9). Evidence for genome duplication has been obtained from isozyme studies in two n=7 species, *Brassica adpressa* and *Diplotaxis erucoides* (Quiros *et al.* 1988). These and other results support the hypothesis that the genomes of diploid species in *Brassica* and allied genera have evolved in ascending chromosome number originating from a n=6 prototype (Prakash and Hinata 1980).

The origin of the amphidiploid species in *Brassica* was studied initially by interspecific hybridization and cytogenetic analysis. U (1935) proposed that among cultivated *Brassica* species, *B. napus*(n=19), *B. juncea*(n=18) and *B. carinata*(n=17) were amphidiploid species which evolved by interspecific hybridizations between the pairs of diploid species *B. oleracea* x *B. rapa*, *B. rapa* x *B. nigra* and *B. nigra* x *B. oleracea*, respectively. Many studies have confirmed U's hypothesis using a number of taxonomic criteria including flavonoid composition (Dass and Nybom 1967), seed protein serology (Vaughan 1977), isozymes (Coulthart and Denford 1982, Quiros *et al.* 1985, 1988), ribosomal DNA (Quiros *et al.* 1985) and RFLP/RAPD markers (Thormann *et al.* 1994). Additionally, studies from restriction pattern analysis of chloroplast DNA (Palmer *et al.* 1983) has provided information on the maternal contributors of various amphidiploids, specifically *B. juncea* has the cytoplasm of *B. rapa* and *B. carinata* has the cytoplasm of *B. nigra*, whereas the cytoplasm of *B. napus* has a more complex

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B. nigra
n=8
B
black mustard

B. carinata
n=17
BC
Abyssinian mustard

B. juncea
n=18
AB
brown mustard

B. oleracea
n=9
C
broccoli
cauliflower
kale
etc.

B. napus
n=19
AC
rapeseed (canola)
rutabaga (swede)

B. rapa (campestris)
n=10
A
turnip
Chinese cabbage
turnip rape (canola)
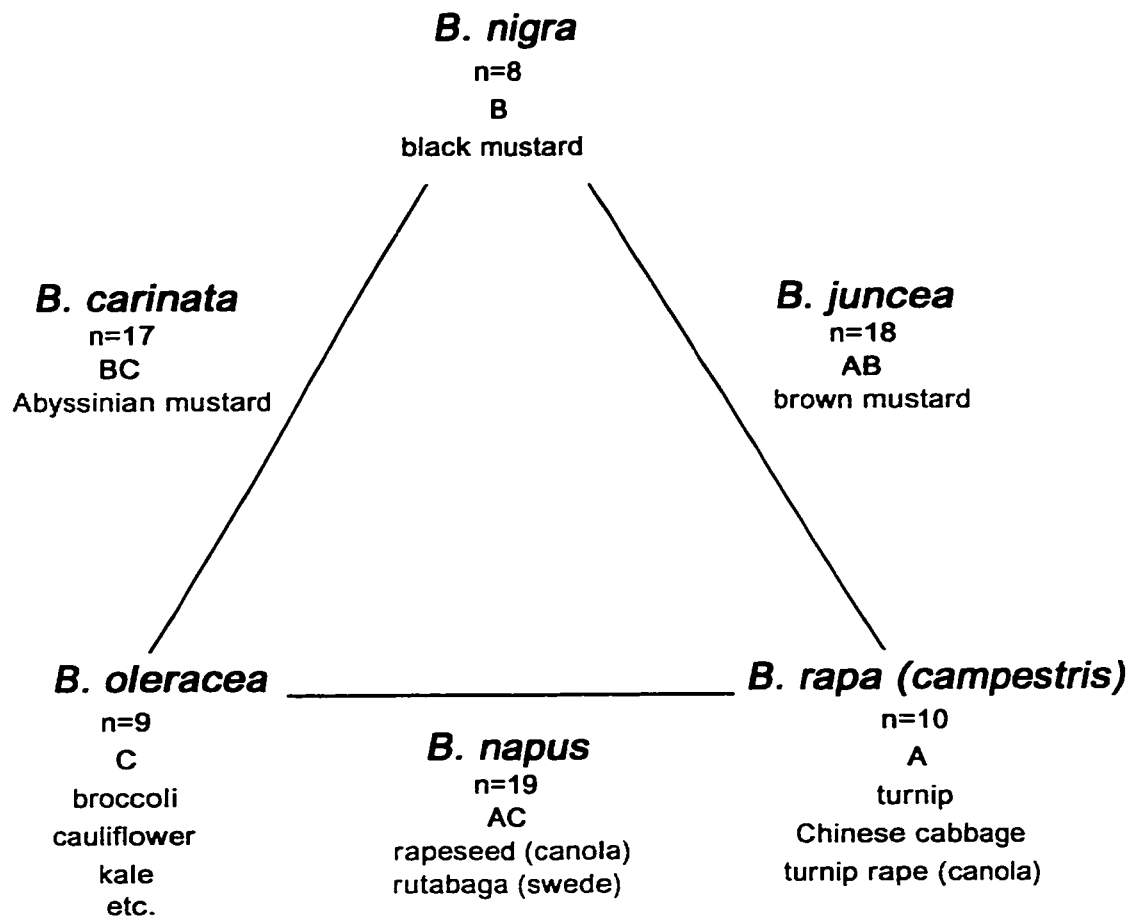
Figure 1 Genetic relationships in different *Brassica* species (modified from U 1935).

origin. Although previous studies have provided useful information on genomic relationships and the evolution of *Brassica* related genera, very little is known about the origins of the diploid species, and many questions remain concerning how the amphidiploid species evolved from their parental diploids (Prakash and Hinata 1980).

Each of the six cultivated *Brassica* species can be differentiated by chromosome number and by several morphological characters including leaf architecture, presence or absence of trichomes, petal color and size, silique position and length (Fernald 1950). Within each of these species there are diverse varieties with differences in vegetative, reproductive and root characters. For example, in *B. oleracea*, 14 varieties are distinguished by variations in internode length, stem swelling, axillary bud development, foliage curling, and inflorescence development (Prakash and Hinata 1980; Williams and Hill 1986) (Table 1.1).

B. oleracea represents an important source of several vegetables with practically every part of the plant utilized including the leaves (cabbage, kale), terminal (early cauliflower) and axillary buds (Brussels sprout), stems (kohlrabi), floral primordia (late cauliflower) and flower buds (broccoli). All morphotypes of *B. oleracea* are thought to have originated from a wild west European *B. oleracea*, which was domesticated during the first millennium B.C. (Prakash and Hinata 1980). The hypothesis of monophyletic origin was further supported by the RFLP analysis of nine cultivated morphotypes of *B. oleracea* (Song *et al.* 1990). On the other hand, a triple or even multiple origin for *B. oleracea* has been proposed (Mithen *et al.* 1987). Gates (1953) believed that cabbage,

Table 1.1 Classification of the major brassica morphotypes (Williams and Hill 1986)

SPECIES	VARIETY	COMMON NAME
B. nigra (n=8)		black mustard
B. oleracea (n=9)		
	acephala	kales
	alboglabra	Chinese kale
	botrytis	cauliflower, heading broccoli
	capitata	cabbage
	costata	Portuguese cabbage
	gemmifera	Brussels sprout
	gongylodes	kohlrabi
	italica	broccoli
	medullosa	marrow stem kale
	palmifolia	tree cabbage
	ramosa	thousand-head kale
	sabauda	savoy cabbage
	sabellica	collards
	selensia	borecole
B. rapa (syn. *B. campestris*) (n=10)		
	chinensis	pak choi
	narinosa	
	nipposinica	
	oleifera	turnip rape, toria
	parachinensis	choy sum
	pekinensis	Chinese cabbage, petsai
	perviridis	tendergreen, mustard spinach
	rapifera	turnip
	trilocularis	sarson
	utilis	broccoli raab
B. carinata (n=17)		Abyssinian mustard
B. juncea (n=18)		
	capitata	head mustard
	crispifolia	cut leaf mustard
	faciliflora	broccoli mustard
	lapitata	large petiole mustard
	multiceps	multishoot mustard
	oleifera	Indian mustard, raya
	rapifera	root mustard
	rugosa	leaf mustard
	spicea	mustard
	tsa-tsai	big stem mustard
B. napus (n=19)		
	oleifera	oil rape (canola)
	rapifera	swede, rutabaga

Brussels sprout and kale originated from *B. oleracea* in western Europe, while cauliflower and broccoli evolved from a related species in the eastern Mediterranean region. It has been suggested that various *B. oleracea* morphotypes evolved from a wild cabbage, which gradually gave rise to various forms, through mutations or chromosomal structure changes (Chiang and Grant 1975). Selective introgression from wild species, such as *B. incana* from the west and south coasts of Italy, Sicily and Yugoslavia; *B. montana* from Europe, northeast Spain and southern Italy; *B. rupestris* from Corsica and Sardinia substantially increased the variability and adaptability (Prakash and Hinata 1980). This was further confirmed by the occurrence of specific DNA markers found in certain kales, as well as in some accessions of *B. insularis* and *B. incana* (Hosaka *et al.* 1990). According to the hypothesis proposed by Song *et al.* (1990), the cultivated morphotypes of *B. oleracea* originated from a single progenitor that was similar to wild *B. oleracea* and *B. alboglabra*. The earliest cultivated *B. oleracea* was probably a leafy kale from which a variety of other kales originated. Ancient broccoli might have originated from a type of kale grown in Italy that had a specialized inflorescence. Cauliflower later evolved from broccoli. Ancient cabbage perhaps evolved from a leafy kale, and Portuguese cabbage may be an intermediate type from which common cabbage developed (Labana and Gupta. 1993).

1.2 *In vitro* plant regeneration from explants in brassica crops

1.2.1 *B. oleracea*

Regeneration of plants from leaf cuttings of *B. oleracea* was described many years ago (Isabell 1944; Ghosh 1965) and since that time various *Brassica oleracea* varieties have been examined in a range of tissue culture systems.

Broccoli (*Brassica oleracea* var. *italica*)

Plants have been regenerated from root segments, stem, leaf, hypocotyl, petiole, flower bud and peduncle explants (Table 1.2). In many cases MS basal medium has been used with IAA and kinetin combinations as growth regulators (Anderson and Carstens 1977; Johnson and Mitchell 1978). Hui and Zee (1980) used combinations of NAA (0.5mg/L), BA (0.5mg/L) and 2iP (2mg/L) as growth regulators with the medium supplemented with 500-1000mg/L of raw ginseng powder. With the addition of ginseng powder, regeneration frequency was increased from 25-30% to 55-60% in the case of cotyledon explants and from 10-25% to 25-40% in the case of hypocotyl explants.

Cauliflower (*Brassica oleracea* var. *botrytis*)

Plants have been regenerated from root, stem, leaf, hypocotyl, curd and pedicel explants (Table 1.2). The most commonly used medium has been MS medium, but some modifications have also been employed (Pow 1969; Walkey and Woolfitt 1970). The cytokinins frequently used to induce shoots have been KIN and BA. In most cases, cytokinin/auxin combination has been employed to induce a high frequency of shoot

Table 1.2 Summary of regeneration of *Brassica oleracea* varieties

Variety	Common name	Explant	Reference
acephala	kale	hypocotyl	Dietert *et al.* 1982
		stem	Horak *et al.* 1975
botrytis	cauliflower	root	Grout and Crisp 1980
		hypocotyl	Dietert *et al.* 1982
			Singh and Chandra 1985a
		leaf	Bagga *et al.* 1982
			Pareek and Chandra 1978
		stem	Bagga *et al.* 1982
		flower part	Ilzuka *et al.* 1973
		pedicel	Gorecka 1985
		curd	Crisp and Walkey 1974
			Miszke and Skucinska 1976
			Pareek and Chandra 1978
			Pow 1969
			Singh and Chandra 1985b
			Walkey and Woolfitt 1970
capitata	cabbage	root	Lazzeri and Dunwell 1984b
			Lillo and Shahin 1986
		hypocotyl	Bajaj and Nietsch 1975
			Singh and Chandra 1985a
		leaf	Miszke and Skucinska 1976
		flower part	Ilzuka *et al.* 1973
		pedicel	Dunemann and Grunewaldt 1987
			Miszke and Skucinska 1976
		meristem	Walkey *et al.* 1980
gemmifera	Brussels sprout	root	Lazzeri and Dunwell 1984b
		hypocotyl	Dietert *et al.* 1982
			Ockendon 1984
		meristem	Clare and Collin 1974
		petiole	Clare and Collin 1974

Variety	Common name	Explant	Reference
gongylodes	kohlrabi	hypocotyl	Dietert *et al.* 1982
		pedicel	Becker-Zens and Grunewaldt 1984
			Dunemann and Grunewaldt 1987
italica	broccoli	root	Lazzeri and Dunwell 1984a,b
			Lazzeri and Dunwell 1986a,b
		hypocotyl	Dietert *et al.* 1982
			Hui and Zee 1980
			Lazzeri and Dunwell 1986a,b
			Robertson and Earle 1986
		cotyledon	Hui and Zee 1980
			Lazzeri and Dunwell 1986a
		leaf	Dunwell 1981
			Johnson and Mitchell 1978
			Lazzeri and Dunwell 1986a,b
		stem	Johnson and Mitchell 1978
		flower part	Ilzuka *et al.* 1973
			Pareek and Chandra 1978
		pedicel	Anderson and Carstens 1977
			Dunemann and Grunewaldt 1987
		peduncle	Christey 1989

regeneration. KIN+IAA (Pow 1969) and BA+IBA+GA (Margara 1969) combinations

have been used and a KIN+2,4-D combination has been employed for leaf vein culture

(Buiatti *et al.* 1974). The highest regeneration rates (up to 80%) have been obtained

from curd explants (Pow 1969; Walkey and Woolfitt 1970; Crisp and Walkey 1974;

Singh and Chandra 1985b).

Cabbage (*Brassica oleracea* var. *capitata*)

Regeneration has been reported from root segments, leaf explants, hypocotyl and

meristem tips in white cabbage and almost every organ in red cabbage (Bajaj and

Nietsch 1975). The most commonly used medium was MS with hormonal combinations

of IAA+KIN (Bajaj and Nietsch 1975) for hypocotyl explants, IAA+zeatin (Lillo and

Shahin 1986) for root segments and KIN alone for meristem tips (Walkey *et al.* 1980).

Brussels sprout (*Brassica oleracea* var. *gemmifera*)

Plants have been regenerated from root segments, hypocotyl and petioles (Clare

and Collin 1974; Lazzeri and Dunwell 1984b). MS medium has been mainly employed.

The major hormone combination has been IAA+KIN but IBA alone was shown to

induce shoots from petioles (Clare and Collin 1974).

Kohlrabi (*Brassica oleracea* var. *gongylodes*)

Becker-Zens and Grunewaldt (1984) have demonstrated up to 96% regeneration

from petals, petioles, mid ribs and leaf blades of several cultivars using Linsmaier and

Skoog (1965) medium with 1mg/L each of NAA and BAP. The results indicated a strong cultivar effect and also an explant-source effect.

Kale (*Brassica oleracea* var. *acephala*)

Horak *et al.* (1975) reported that 80% of stem pith explants from the cultivar 'Krasa' differentiated into roots and shoots.

1.2.2 Other *Brassica* species

In most cases, regeneration of *Brassica* species can be obtained from a wide variety of explants such as cotyledons, petioles, hypocotyls and stem segments. The best explants for efficient and reliable regeneration have been cotyledons and hypocotyls isolated from aseptically grown 4-6 day-old seedlings (Murata and Orton 1987; DeBlock *et al.* 1989; Sharma *et al.* 1990; Dale and Ball 1991; Babic 1994).

There are marked differences in the reported responsiveness of various explants to tissue culture. The most regenerative tissues of *B. napus* appear to be stem segments taken from shoot cultures (Pua *et al.* 1987), cotyledonary petioles (Moloney *et al.* 1989) and thin cell layers from floral internodes (Klimaszewska and Keller 1985; Charest *et al.* 1988). An extensive study on regeneration from cotyledonary petiole explants of *B. napus* has been done by Hachey *et al.* (1991). *B. rapa* has consistently proven to be one of the most recalcitrant members of the *Cruciferae* in tissue culture. This is evident from studies on shoot regeneration from callus (Dietert *et al.* 1982; Murata and Orton 1987), leaf discs (Dunwell 1981) and cotyledons (Jain *et al.* 1988; Narasimhulu and Chopra

1988), as well as from anther culture (Keller and Armstrong 1979). A high frequency of shoot regeneration from hypocotyl explants of *B. carinata* has been demonstrated by Yang *et al.* (1991) and Babic (1994). Most other parts of seedling explants of *B. carinata* such as root, cotyledonary petiole and cotyledonary disc were demonstrated as possible sources for shoot regeneration (Babic 1994). Efficient and reliable shoot and root regeneration from cotyledon explants were demonstrated for *B. juncea* (Sharma *et al.* 1990; Sharma and Bhojwani 1990; Sharma *et al.* 1991). Table 1.3 summarizes reports of regeneration for different *Brassica* species.

The most commonly used basal medium for regeneration has been Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Cytokinins are required for shoot regeneration and BA has been mainly employed at a concentration of 1-10mg/L (Charest *et al.* 1988). In some cases the medium has been supplemented with kinetin for *B. carinata* (Yang *et al.* 1991) and zeatin for *B. juncea* (Jain *et al.* 1988a,b). In most cases, the addition of cytokinin alone has been sufficient to induce shoots but low concentrations (0.01-0.1mg/L) of auxins (IAA, NAA, 2,4-D) have improved shoot regeneration (Murata and Orton 1987; Yang *et al.* 1991; Babic 1994). Each study involved a different variety and specific culture method and thus it is difficult to compare the relative efficiencies of regeneration.

Table 1.3 Summary of regeneration systems for *Brassica* species

Variety	Explant	Reference
B. napus	cotyledon	Narasimhulu *et al.* 1988a,b
		Murata and Orton 1987
	petiole	Moloney *et al.* 1989
		Dale and Ball 1991
	hypocotyl	DeBlock *et al.* 1989
	leaf	Dunwell 1981
		Stringam 1979
	root	Graves *et al.* 1991
B. rapa	cotyledon	Murata and Orton 1987
		Deng *et al.* 1991
		Jain *et al.* 1988a
		Narasimhulu and Chopra 1988
	petiole	Dale and Ball 1991
	hypocotyl	Singh *et al.* 1981
		Faciotti *et al.* 1989
	leaf	Dunwell 1981
	root	Lazzeri and Dunwell 1984b
B. juncea	cotyledon	Murata and Orton 1987
		Jain *et al.* 1988b
		George and Rao 1983
		Chi *et al.* 1990
	petiole	Sharma *et al.* 1991
	hypocotyl	Kirti and Chopra 1989
		Chi *et al.* 1990
B. carinata	cotyledon	Jain *et al.* 1988b
		Narashimhulu *et al.* 1989
		Jaiswal *et al.* 1987
		Narashimhulu and Chopra 1988
	cotyledonary petiole	Babic 1994
	hypocotyl	Yang *et al.* 1991
		Bajaj and Mohapatra 1987
		Jaiswal *et al.* 1987
	root	Jaiswal *et al.* 1987

1.3 Development of genetic transformation techniques

1.3.1 *Agrobacterium*-mediated transformation

Agrobacterium is a Gram-negative facultative aerobic soil bacterium (Holt 1981). Virulent strains of *Agrobacterium tumefaciens* elicit crown gall tumors in infected plant tissues. These pathogenic responses result from the expression of genetic information (i.e. components of the Ti plasmid) transferred from the bacterium into host cells (Gheysen *et al.* 1985; Hooykaas and Schilperroort 1984). The resultant transformed plant cells produce novel sugar and amino acid conjugates, termed opines, which can be used by the inciting bacteria in two ways: as a source of carbon and nitrogen and as an inducer of Ti plasmid transfer between bacteria. Different *Agrobacterium* strains carry different types of Ti plasmids, which can be used to categorize the bacteria. In addition to opine synthesis, the T-DNA genes encode the biosynthesis of the plant growth regulators, auxin and cytokinin (Buchmann *et al.* 1985; Thomashow *et al.* 1986). The expression of these genes in the transformed cells results in the unregulated accumulation of the hormones and is responsible for the non-self-limiting growths that are recognized as tumors. Thus, the DNA inserted into plant cells by *Agrobacterium* species results in the synthesis of opines for the bacteria and causes multiplication of plant cells. Numerous recent reviews have focused on the utilization of *Agrobacterium*-based vectors for plant genetic engineering and on the expression and functions of the transferred genetic information from *Agrobacterium* (Hoekema *et al.* 1983; Bevan 1984; An 1985; Klee *et al.* 1987; Schell 1987; Zambryski 1988; Potrykus 1991).

1.3.2 Other transformation techniques

Several genetic transformation methods other than *Agrobacterium*-mediated method are available for plants. These include direct gene transfer methods such as biolistic particle bombardment (Klein *et al.* 1987; Sanford 1988; Klein *et al.* 1989; Finer *et al.* 1992; Hunold *et al.* 1995), microinjection (Neuhaus *et al.* 1987; Pena *et al.* 1987), sonication (Xu *et al.* 1990; Joersbo and Brunstedt 1990,1992), electroporation of protoplasts (Shillito *et al.* 1983; Bradshaw *et al.* 1987) and intact tissues (Dekeyser *et al.* 1990; D'Halluin *et al.* 1992; Klöti *et al.* 1993), organ electrophoresis (Griesbach and Hammond 1993) as well as transformation mediated through lasers (Weber *et al.* 1988; Guo *et al.* 1995), silicon carbide whiskers (Appel *et al.* 1988; Kaeppler *et al.* 1990,1992; Frame *et al.* 1994; Wang *et al.* 1995) and liposomes (Deshayes *et al.* 1985; Rosenberg *et al.* 1988).

1.4 *Agrobacterium*-mediated genetic transformation of brassica crops

1.4.1 *Brassica oleracea*

There is abundant tissue culture information available for various tissues and organs of *B. oleracea* (Table 1.2) but only a few reports are available on *Agrobacterium*-mediated transformation of *B. oleracea*. Various explants such as peduncle, leaf, hypocotyl and cotyledonary petioles have served as recipient cell systems and *both A. tumefaciens* and *A. rhizogenes* have been used to introduce plasmid DNA (Table 1.4).

Broccoli (*Brassica oleracea* var. *italica*)

Transgenic plants have been recovered from peduncle explants of the cultivar 'Green Comet' (Christey 1989; Christey and Earle 1989; Toriyama *et al.* 1991; Metz *et al.* 1995) and leaf tissues of the cultivar 'Early De Cico' (Hosoki *et al.* 1991). Peduncle and leaf tissue explants have produced a lower frequency of recovery of transgenic plants than cotyledonary petioles. Christey and Earle (1989) obtained only one transgenic plant from their study while Toriyama *et al.* (1991) obtained seven from the cultivar 'Green Comet'. Hosoki *et al.* (1991) produced 13 transgenic lines from secondary hairy roots using *A. rhizogenes*-mediated transformation but three out of the 13 lines had an abnormal appearance as is often found in Ri plasmid-transformed plants (Tepfer 1984). Christey (1989) has used *A. tumefaciens* LBA4404 harboring the plasmid pBI121 which carries *gus* and *npt*II genes.

Table 1.4   Reports of *Agrobacterium*-mediated transformation of *B. oleracea*

Variety	*Agrobacterium*	Explant	Selection	Frequency(%)	Reference
italica	*tumefaciens*	pd	km	1 line	Christey and Earle 1989
(broccoli)	*tumefaciens*	pd	hm	7 lines	Toriyama *et al.* 1991
	rhizogenes	l	aut	13 lines	Hosoki *et al.* 1991
	tumefaciens	pd	km	5.1	Metz *et al.*. 1995
	tumefaciens	h	km	9.8	Metz *et al.*. 1995
	tumefaciens	cp	km	1.8	Metz *et al.*. 1995
botrytis	*tumefaciens*	l	km		Srivastava *et al.* 1988
(cauliflower)	*tumefaciens*	h	km	8-30	DeBlock *et al.* 1989
	rhizogenes	h	aut		David and Tempe 1988
	tumefaciens	seed	km	2.3	Eimert *et al.* 1992
	tumefaciens	pp	km		Eimert and Siegemund 1992
acephala	*rhizogenes*	l	aut		Hosoki *et al.* 1989
(kale)	*rhizogenes*	cp	km	14 lines	Christey and Sinclair 1992
capitata	*rhizogenes*	lp	hm	6	Berthomieu and Jouanin 1992
(cabbage)			km	0.3	Berthomieu and Jouanin 1992
	tumefaciens	s	km	4	Berthomieu *et al.* 1994
	tumefaciens	h	km	9.4	Metz *et al.*. 1995
	tumefaciens	cp	km	10.3	Metz *et al.*. 1995
alboglabra	*tumefaciens*	pd	hm	3 lines	Toriyama *et al.* 1991
(Chinese kale)					

pd: peduncle, h: hypocotyl, l: leaf, cp: cotyledonary petiole, s: nipped adult stem,
pp: protoplast, seed: direct injection into seed. lp: leaf petiole
km: kanamycin, hm: hygromycin, aut: hormone autonomous growth

Cauliflower (*Brassica oleracea* var. *botrytis*)

Transgenic plants have been recovered by *Agrobacterium*-mediated transformation of leaf explants of the cultivar 'Synthetica' (Srivastava *et al.* 1988); hypocotyls of the cultivars 'Walcheria' and 'Anderson' (DeBlock *et al.* 1989); and hypocotyl derived protoplasts of the line '3/10' (Eimert and Siegemund 1992). Transformation by direct injection of *Agrobacterium* into seed of the line '3/10' has also been reported (Eimert *et al.* 1992). David and Tempe (1988) have demonstrated *A. rhizogenes*-mediated transformation. All experiments were done using kanamycin selection for the recovery of transgenic plants except in the secondary hairy root regeneration study carried out by David and Tempe (1988). In most cases, transformation frequencies were not reported, except for direct seed injection (2.3%, Eimert *et al.* 1992) and hypocotyl transformation (8-30%, DeBlock *et al.* 1989). Transformation using hypocotyl explants (DeBlock *et al.* 1989) seems to be the most promising for cauliflower, but reported frequencies (8-30%) are based on kanamycin selection without further confirmation, and these may include some escapes. The unusual approach of direct injection of *Agrobacterium* into sterilized seeds used by Eimert *et al.* (1992) may omit the regeneration step but the transformation process in which each seed is inoculated using a small sharp pin after dipping it into a suspension of *Agrobacterium* is more tedious and laborious. The injection of 1476 seeds resulted in the production of 54 green plants (3.7%). Of the plants tested, 80% expressed the *npt*II gene resulting in a 2.3% transformation frequency.

Kale (*Brassica oleracea* var. *acephala*)

Hosoki *et al.* (1989) have demonstrated *A. rhizogenes*-mediated transformation using leaf tissue through secondary hairy root formation based on the principle of Ri-plasmid transfer but the frequency of transformation was not reported. Christey and Sinclair (1992) have also used *A. rhizogenes* for transformation of four cultivars, 'Kapeti', 'Medium Stem', 'Midas' and 'Rawara' using 7 to 12 day-old cotyledonary petioles and 7 to 24 day-old leaf tissues as explants with a feeder cell layer of tobacco suspension cells. They transferred *als*, *gus* and *npt*II genes using the *A. rhizogenes* strain A4T harboring the plasmid pKIWI110. They obtained 14 transgenic lines in total but transformation frequency was not reported. These studies have shown that *A. rhizogenes* can be used to produce transgenic plants in kale.

Cabbage (*Brassica oleracea* var. *capitata*)

Ten-day-old leaf petioles from *in vitro* grown seedlings and one-month-old internodes and leaf petioles from greenhouse plants were used for transformation of rapid cycling cabbage by *A. rhizogenes* (Berthomieu and Jouanin 1992). Six transgenic plants were recovered from 100 root explants using hygromycin selection, and one transgenic plant from 300 root explants using kanamycin selection. All transgenic roots were obtained from greenhouse grown materials. Unlike most other *A. rhizogenes*-mediated *Brassica* transformants, these transgenic lines had no hairy root phenotype. Transformation of 20 day-old rapid cycling cabbage with *A. tumefaciens* using nipped stems from *in vitro* grown seedlings was reported by Berthomieu *et al.* (1994). The

transformation frequency was about 4% but most transgenic plants had multiple inserts and were chimeric.

Chinese kale (*Brassica oleracea* var. *alboglabra*)

Toriyama *et al.* (1991) obtained a total of three transgenic plants from the cultivar 'Kairan' using peduncle explants based on hygromycin selection.

1.4.2 Other *Brassica* species

Most transformation studies have been carried out with *B. napus* because of its importance as an oilseed. *Agrobacterium*-mediated transformation frequencies have been reported to range from 1-55% for *B. napus*, but are more commonly in the range of 3-5% with both hypocotyl and cotyledonary petiole explants (Radke *et al.* 1988; Moloney *et al.* 1989). Other *Brassica*s such as *B. juncea*, *B. nigra*, *B. rapa* and *B. carinata* have also been transformed by *Agrobacterium* but the efficiency of transformation was generally not as good as that of *B. napus* (Table 1.5). However, *B. carinata* appears to be an exception as Babic (1994) obtained high transformation frequencies (in the order of 50%). *B. rapa* is considered to be recalcitrant in terms of amenability to *Agrobacterium*-mediated genetic transformation.

Agrobacterium-mediated transformation of brassicas appears to be cultivar dependent. For example, in *B. napus* the cultivar 'Westar' is more responsive to *Agrobacterium* co-cultivation and has been most commonly used in transformation

Table 1.5   Reports of *Agrobacterium*-mediated transformation of *Brassica* species excluding *B. oleracea*

Species	Cultivar	Explant	Selection	Frequency(%)	Reference
B. napus	Westar	h	km	30-41	DeBlock *et al.* 1989
	Westar	h	km	0.4-2.5	Radke *et al.* 1988
	Westar	cp	km	55	Moloney *et al.* 1989
	Westar	s	km	7	Fry *et al.* 1987
	Westar	s	mtx	11-14	Pua *et al.* 1987
	Westar	cp	hm	0-10	Mehra-Palta *et al.* 1991
	Westar	h	hm	5.5-6.6	Mehra-Palta *et al.* 1991
	Topaz	me	km	0.5	Swanson and Erickson 1989
	Giant	cp	km	12 line	Christey and Sinclair 1992
B. rapa	Emma,Tobin	h	km	1-9	Radke *et al.* 1992
	Pusa Kalyani	h	km	7-13	Mukhopadhyay *et al.* 1992
B. juncea	Indian mustard	h	km	9 line	Barfield and Pua 1991
	RLM198	h	hm	11-36	Deepak *et al.* 1993
	Indian mustard	h	km	10 line	Pua and Lee 1995
B. nigra	IC257	h	km	60 line	Gupta *et al.* 1993
B. carinata	#171	s	km	1.5	Narasimhulu *et al.* 1992
	#1163	cp	km	7.5-47	Babic 1994

h: hypocotyl, s: stem, l: leaf, me: microspore-derived embryo, cp: cotyledonary petiole
km: kanamycin, hm: hygromycin, mtx: metotrexate based on its low response in tissue culture.

studies (Table 1.5). In most cases, *in vitro* grown materials or young seedlings have been employed as a source of explants for transformation because they can be easily obtained in a short time (4-6 days) compared to explants from mature plants. In particular, hypocotyls and cotyledonary petioles from *in vitro* grown seedlings have been the most preferred explant source for *Agrobacterium*-mediated transformation (DeBlock *et al.* 1989; Moloney *et al.* 1989).

1.4.3 Selectable markers and reporter genes used in brassica transformation

Most transformation methods require the use of a selective agent for the efficient recovery of transformants. With *Brassica* spp., several selectable markers have been employed with variable success (Table 1.4 and 1.5). Aminoglycoside antibiotics such as kanamycin and G418 have been widely used (Fry *et al.* 1987; Radke *et al.* 1988; Charest *et al.* 1988). Neomycin phosphotransferase II (NPTII) when expressed in transformed cells confers resistance to 15-100mg/L kanamycin. For *Brassica* species, kanamycin has been shown to be a very effective antibiotic for selecting transformed cells. However, Barfield and Pua (1991) reported a high level of non-transformed 'escapes' using this gene as a selectable marker in *B. juncea*. An alternate selection marker using hygromycin was also successful in *Brassica* species (Barfield and Pua 1991; Mehra-Palta *et al.* 1991; Radke *et al.* 1992; Deepak *et al.* 1993). Though hygromycin has not yet been as widely used as kanamycin in *Brassica* transformation studies, hygromycin selection has been used for the recovery of transformants in *B. napus* (Radke *et al.* 1988; DeBlock *et al.* 1989), *B. rapa* (Radke *et al.* 1992; Mukhopadhyay *et al.* 1992), *B.*

juncea (Deepak *et al.* 1993), *B. nigra* (Gupta *et al.* 1993) and *B. oleracea* (David and Tempe 1988; DeBlock *et al.* 1989).

Chloramphenicol acetyltransferase (CAT) has found only limited utility as a selectable marker in *N. tabacum* (DeBlock *et al.* 1984). Generally, it has been used more effectively as a reporter gene in studies on the regulation of promoters in transformed tissues that have been recovered with an efficient selectable marker such as the *npt*II gene (Herrera-Estrella *et al.* 1984; Hauptmann *et al.* 1987; Thornburg *et al.* 1987; Timo *et al.* 1985). In several *Brassica* spp., endogenous CAT-like activity has been detected (Balazs and Bonneville 1987). This poses problems for the use of CAT either for selection or as a reporter in *Brassica* species. Charest *et al.* (1989) found evidence for inhibition of bacterial CAT activity in extracts of *B. napus*. These extracts inhibited CAT activity of transgenic tobacco to a level equivalent to the background CAT-like activity normally found in *Brassica* extracts. This finding limits the possibilities for the use of CAT in gene expression studies in *Brassica*. Some workers found that the *Brassica* CAT-like activity was quite heat-labile, whereas the CAT activity produced by bacterial genes was not. It was suggested that a 10-min pretreatment at 65°C eliminates most of the endogenous CAT activity (Balazs and Bonneville 1987).

DeBlock *et al.* (1989) used the *bar* gene which confers resistance to the herbicide phosphinothricin (at 5-20mg/L) as a selectable marker in transformation of *B. napus* and *B. oleracea*. An advantage of this marker gene is that it facilitates the direct production of herbicide resistant transgenic plants.

While the genes from bacterial origin have been used most widely as selectable markers, one mammalian gene has also been used in plants; mutant mouse DHFR specifying resistance to MTX (methotrexate) was used in *Petunia hybrida* (Eichholtz *et al.* 1987) and *B. napus* (Pua *et al.* 1987). Transformed callus and plants of *B. napus* grew on 0.005-0.01mg/L of methotrexate which is very toxic to untransformed *Brassica* species.

GUS from the *uid*A (*gus*) gene of *E. coli* (Jefferson 1987) has been widely used as a screenable marker and reporter protein in brassica transformation studies (Kridle *et al.* 1989; Facciotti *et al.* 1989). GUS expression can be histologically and biochemically assayed in transgenic plants. In order to avoid expression from bacterial cells and false positive detection, an intron-GUS fused reporter gene system has been developed as it expresses only in plant cells (Vancanneyt *et al.* 1990; Deepak *et al.* 1993).

A recent report by Chalfie *et al.* (1994) indicated that green fluorescent protein (GFP) has the characteristics required to function as a sensitive reporter for *in vivo* gene expression studies. The green bioluminescence of the jellyfish *Aequorea victoria* is due to a protein-protein energy transfer from a blue fluorescent protein, aequorin, to GFP in the presence of Ca^{2+} (Morise *et al.* 1974). The GFP chromophore appears to be derived from the primary amino acid sequence of the protein (Cody *et al.* 1993). Fluorescence of purified GFP requires no exogenous cofactors, only excitation with near UV or blue light, and is easily visible under normal room fluorescent lighting (Morise *et al.* 1974; Prasher 1995). GFP is stable and is only denatured under extreme conditions (Ward and Bokman 1982). GFP maintains its fluorescence when expressed in heterologous systems

such as *E. coli* (Chalfie *et al.* 1994; Kain *et al.* 1995), *Mycobacterium smegmatis* (Kremer *et al.* 1995), *Dictyostelium* (Fey *et al.* 1995; Hodgkinson 1995), *Caenorhabditis elegans* (Chalfie *et al.* 1994), *Drosophila melanogaster* (Wang and Hazelrigg 1994; Brand 1995), and mammalian cells (Kain *et al.* 1995; Pines 1995). In plants, Niedz *et al.* (1995) have demonstrated the GFP expression in sweet orange (*Citrus sinensis*) protoplasts. Haseloff and Amos (1995) have also demonstrate the GFP expression under the control of the CaMV 35S promoter in *A. thaliana* with mild toxicity and inhibition of regeneration. They have suggested that the *gfp* gene was efficiently mis-spliced when expressed in *A. thaliana* because of intron-like sequences within GFP coding sequences resulting in low fluorescence in transformed plants. They have characterized this improper mRNA processing, deleted the 84 nucleotide intron from within the GFP coding sequences, and constructed a modified version of GFP which has been successfully used to produce green fluorescence in transgenic *Arabidopsis* plants. The modified *gfp* gene has proved very useful for the visualization of cellular and sub-cellular details in *A. thaliana* (Dr. J. Haseloff, personal communication). Although there is no report yet of the application of the GFP in *Brassica* species, as GFP expression has been demonstrated in plant cells, the GFP may be useful as a sensitive reporter for *in vivo* gene expression studies in *Brassica* species.

1.4.4 Promoters used in brassica transformation

Appropriate control of genes that are introduced into plants is a critical aspect of plant genetic engineering studies. For this purpose, foreign genes need to be

conntrolled by effective promoters. The most commonly used promoters in transformation vectors are the cauliflower mosaic virus 35S (CaMV 35S) promoter and Ti-plasmid's nopaline synthase (NOS) promoter. The NOS promoter is known to be a weaker promoter than CaMV 35S in brassicas (Pua *et al.* 1987; Charest *et al.* 1988). In spite of its weaker expression in brassicas, NOS promoter based constructs have also been used (Fry *et al.* 1987; Charest *et al.* 1988; Narasimhulu *et al.* 1992). Other types of promoters have been used for specific regulation of trans-genes. These include: the oleosin promoter (Moloney *et al.* 1993; Plant *et al.* 1993; Van Rooijen and Moloney 1995), the napin promoter (Radke *et al.* 1988; Moloney *et al.* 1993; Plant *et al.* 1993) and anther/pollen specific promoters (Arnoldo *et al.* 1992; Fabijanski 1992; Leemans 1992; Hong 1996).

1.5 Improvement of brassicas through genetic transformation

Genetic modification/engineering of crop plants via genetic transformation is the essential link between molecular biology and crop improvement. It will play an increasingly important role in providing mankind with crop plants that are agronomically superior with new features.

Many transgenic *Brassica*s have been produced through the use of *Agrobacterium*-mediated gene transfer methods and *B. napus* is dominant amongst them.

1.5.1 Herbicide tolerance

Herbicide tolerance has been the main target for transformation in many crops including oilseed *Brassicas*. DeBlock *et al.* (1989) have produced glufosinate resistant transgenic lines from *B. napus* and *B. oleracea* var. *botrytis* using the *bar* gene which confers resistance to the non-selectable herbicide, phosphinothricin.

The *pat* gene whose function is similar to *bar* gene but which has been artificially modified to be suitably expressed in plant cells has also been introduced into *B. napus* (Oelck *et al.* 1991,1993), *B. rapa* (Swartz *et al.* 1993), *B. carinata* (Babic 1994) and *B. juncea* (W. Keller, personal communication).

Miki *et al.* (1990) have produced chlorsulfuron tolerant transgenic *B. napus* by inserting a mutant *Arabidopsis* acetolactate synthase (*als*) gene. Christey and Sinclair (1992) have introduced the *als* gene into *B. oleracea* var. *acephala.*

Freyssinet *et al.* (1992) have produced transgenic *B. napus* with tolerance to bromoxynil, a contact herbicide that acts by blocking photosynthesis in dicots.

Glyphosate tolerant *B. napus* has been obtained by introducing bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) genes (Mitchell 1993).

1.5.2 Insect resistance

The toxins produced by *Bacillus thuringiensis* strains have been shown to be toxic to insects belonging to the groups *Lepidoptera* and *Coleoptera* species. The *Bt* toxin genes that have been introduced into *B. oleracea* var. *italica* (Metz *et al.* 1995), *B. napus* (Cardineau *et al.* 1993) and *B. rapa* (Mehra-Palta *et al.* 1991) have been shown to confer resistance to lepidopteran insects. Puttick *et al.* (1993) have introduced the proteinase inhibitor II gene into *B. napus*.

1.5.3 Disease resistance

Plants respond to fungal attack by activating a number of defensive mechanisms such as production of phytoalexins or lytic enzymes such as chitinase. Chitinase catalyzes the hydrolysis of chitin, the major cell wall component of most fungi. Broglie *et al.* (1991) have introduced a CaMV 35S regulated endochitinase gene from bean into *B. napus*, and these transgenic plants have shown resistance to the root-rot disease caused by the fungal pathogen, *Rhizoctonia solani*.

1.5.4 Modification of carbon metabolism

Pyruvate kinase is considered to represent a key regulatory enzyme for carbon metabolism in plants. This gene from potato has been isolated by researchers at Queen's University (Dennis and Blakeley 1993). Transgenic *B. napus* lines produced with this potato pyruvate kinase gene with the aim of increasing seed oil content have been evaluated in the field since 1992 (W. Keller, personal communication).

1.5.5 Seed oil modification

Calgene has recently announced a number of fatty acid modifications in *Brassica* oilseed species through genetic engineering. Through the use of a seed specific antisense gene construct, the stearyl-ACP desaturase activity in developing embryos of *B. rapa* and *B. napus* was reduced, resulting in stearate levels ranging from the normal 2% or less to as high as 40% (Knutzon *et al.* 1992).

Voelker *et al.* (1992) have redirected the synthesis of fatty acids in *B. napus* into lauric acid, which is used for production of soaps, detergents, lubricants and other industrial products, through the introduction of the 12:0-ACP thioesterase enzyme gene from the California bay tree under the control of the napin promoter to express this gene specifically in seeds. Thioesterase is thought to play an important role in the production of medium chain fatty acids. It has been shown that these transgenic plants produced lauric acid as the major fatty acid (Thompson *et al.* 1993).

Murphy *et al.* (1994) have cloned the Δ_{12} oleate hydroxylase gene from castorbean. This gene is responsible for the production of the ricinoleic acid that is used

in cosmetics, pharmaceuticals, lubricant, plasticisers, coatings and surfactants. The transfer of this gene to oilseed rape is being attempted by Murphy group.

1.5.6 Seed meal quality improvement

Lysine and methionine are the two most common limiting amino acids in livestock feed and therefore genetic engineering strategies have been developed to enhance content of these amino acids in seeds (Krebbers *et al.* 1991). Altenbach *et al.* (1993) have expressed the methionine-rich 2S albumin seed storage protein of Brazil nut (*Bertholletia excelsa*) in *B. napus* under the control of phaseolin gene regulatory sequences. Progeny from these transformed plants expressed 1.7 to 4% of the total protein as the heterologous methionine-rich protein, containing 18.8% methionine. It accumulated at the same time as the normal 11S *B. napus* protein and disappeared rapidly on germination. Mehra-Palta *et al.* (1993) were able to obtain transgenic *B. napus* plants that expressed the phaseolin gene, which encodes for a high level of methionine accumulation. Phaseolin comprised up to 2% of the *B. napus* seed meal (Dr. L. Sernyk, personal communication).

1.5.7 Male sterility

Researchers from Plant Genetic Systems have applied the technique of expressing a toxic molecule to inhibit pollen production and creating male sterility in transgenic *B. napus* plants and restoring fertility through the use of a specific inhibitor of the toxic gene (Mariani *et al.* 1990;1992; Leemans 1992; Reynaerts *et al.* 1993). Two

nuclear, dominant genes that interfere with the vital functioning of cells, RNase T1 from *Aspergillus oryzae* and Barnase (called Barstar) from *Bacillus amyloliquefaciens*, were combined with a tobacco promoter which expresses specifically in the tapetal cells of immature anthers (Mariani *et al.* 1990). This new hybrid system has been applied to spring and winter types of *B. napus* and *B. rapa* (Parker 1993).

1.5.8 Heavy metal tolerance

Misra and Gedamu (1989) have inserted a cloned human metallothionein II gene into *B. napus* using *Agrobacterium*-mediated transformation. Offspring were not affected by toxic levels of cadmium (100 µM $CdCl_2$) while nontransformed plants were severely inhibited in their growth and exhibited leaf chlorosis.

1.5.9 Production of the therapeutic proteins and industrial materials

The use of plants for the large scale production of peptides and proteins has been extensively investigated in recent years. Work in this area has included the production of proteins of potential pharmaceutical interest (Hiatt *et al.* 1989; Vandekerckhove *et al.* 1989; During *et al.* 1990; Guerche *et al.* 1990; Benvenuto *et al.* 1991; Owen et al 1992; DeNeve *et al.* 1993; Firek *et al.* 1993; Conrad and Feidler 1994; Ma *et al.* 1994; Owen 1995; Van Rooijen and Moloney 1995) and enzymes of industrial and agricultural utility (Pen *et al.* 1992, 1993). Vandekerckhove *et al.* (1989) have demonstrated that the small 2S albumin proteins of *B. napus* can be altered to produce the pharmaceutical neuropeptide, Leu-enkephalin. Estimated yield of this recombinant peptide was 15 to

75g per hectare. One of the drawbacks, however, to the use of plants as production vehicles for recombinant proteins is the subsequent cost of processing and purification. In some instances, this limitation has been circumvented by using unpurified proteins in appropriate applications such as the inclusion of phytase in animal feed (Pen *et al.* 1993). For more general use, however improvements in separation and purification schemes, which allow rapid and economical separation of the recombinant proteins from endogenous plant proteins will be required. Van Rooijen and Moloney (1995) have suggested the use of a class of native seed proteins called 'oleosins' or oil-body proteins. Oleosins accumulate only on the surfaces of the oil-bodies and when seeds undergo aqueous extraction, the oil-bodies generally remain intact. On centrifugation, these oil-bodies, with their complement of oleosins may be easily separated from the rest of the cellular extract as an oily 'scum' which floats on the surface (Holbrook *et al.* 1991).

Polyhydroxybutyrate (PHB) and related polyhydroxyalkanoates (PHAs) are biodegradable polyesters with thermoplastic properties that are produced by a wide range of bacteria as storage material (Oeding and Schlegel 1973; Haywood *et al.* 1988,1989; Gibello *et al.* 1995; Rhie and Dennis 1995). The PHB biosynthetic pathway of a bacterium *Alkaligenes eutrophus* consists of three enzymes; β-ketothiolase (*phb*A), acetoacetyl-CoA reductase (*phb*B) and PHB synthase (*phb*C). Of the three enzymes, only the β-ketothiolase is found in the cytoplasm of higher plants. Genes that encode acetoacetyl-CoA reductase and PHB synthase were individually introduced into *A. thaliana* and cross-pollinated F_1 hybrid transgenic plant showed accumulation of PHB

(Poirier *et al.* 1992; Nawrath *et al.* 1994). A similar strategy was also used in oilseed rape to produce PHB (Smith *et al.* 1994).

1.6 Promoter tagging

Three methods are currently available for the isolation of plant genes whose products have not yet been characterized. The first method is based on chromosome walking with the help of cosmid or yeast artificial chromosome cloning systems. The gene is localized on a restriction fragment containing an RFLP marker. This method is laborious and can be impeded by large stretches of repetitive DNA (Brenda *et al.* 1992). The second approach is the genomic subtraction method. This procedure depends on the presence of a characterized large deletion (larger than 5Kb) in the mutant plant genome when compared with the wild type genome (Straus and Ausubel 1990). The third method, known as gene tagging is based on the generation of insertional-mutations by transposons or T-DNA. In this method, a DNA fragment (transposon or T-DNA) is randomly inserted into the target genomic DNA and becomes linked to the DNA sequences of the mutant phenotype. Hybridization of the foreign DNA fragment as a probe to mutant genomic DNA can be used to identify the disrupted gene or its regulatory sequences (Balcells *et al.* 1991). Transposons can move from one DNA site to another, and this approach has been used to generate multiple insertional-mutations from a single transformation event (Gierl and Saedler 1992).

1.6.1 T-DNA as a tool for the isolation of plant promoters/genes

It has been well established that the T-DNA border sequences can be integrated into a plant genome (Koncz *et al.* 1989; Feldman *et al.* 1989). These border sequences can serve as a mutators through insertion and will furthermore act as signals for

identification of the flanking genomic DNA. T-DNA tagging is therefore an efficient method for the creation of mutants and isolation of genes and/or promoters from plants (Koncz *et al.* 1989, Feldman *et al.* 1989). The strategy based on T-DNA insertional mutagenesis may be exploited to identify plant promoters using specially designed T-DNA vectors which contain a reporter gene but lacking transcriptional elements (i.e. promoterless constructs) (Teeri *et al.* 1986; Koncz *et al.* 1989; Kertbundit *et al.* 1991; Lindsey *et al.* 1993; Suntio and Teeri 1994; Topping et al 1994; Fobert *et al.* 1991;1994). If the initiation codon of the promoterless gene is integrated downstream of the gene promoter elements, the reporter gene may be expressed. The resulting hybrid genes produced by T-DNA-mediated promoter tagging, consist of unknown plant promoters residing at their natural location within the chromosome, and the coding sequence of a marker gene located on the inserted T-DNA (Fobert *et al.* 1991).

The most commonly used target plants for promoter tagging have been plants in which an *in vitro* culture system and a good Agrobacterium-mediated transformation system were well established. These include *Arabidopsis thaliana* (Koncz *et al.* 1989; Lindsey *et al.* 1993), *Nicotiana tabacum* (Teeri *et al.* 1986; Fobert 1991;1994) and *Solanum tuberosum* (Lindsey *et al.* 1993).

Most of the promoters identified by T-DNA tagging, control organ or tissue specific expression (Teeri *et al.* 1986; Kertbundit *et al.* 1991; Lindsey *et al.* 1993; Suntio and Teeri 1994; Babic *et al.* 1994). Very limited information on tagged constitutive promoters is currently available (Table 1.6).

Table 1.6 Promoter tagging in plants via *Agrobacterium*-mediated transformation

Target plant	Expression specificity	Reporter/Selection gene	Reference
Arabidopsis thaliana	*	NPTII	Koncz *et al.* 1989
	phloem	GUS/NPTII	Kertbundit *et al.* 1991
	vascular tissue-tapetum, vascular tissue-root, mature pollen	GUS/NPTII	Lindsey *et al.* 1993
	*	GUS/NPTII/BAR	Bouchez *et al.* 1993
	embryo, endosperm, sepal, stem, vascular tissue	GUS/NPTII	Topping *et al.* 1994
Brassica carinata	leaf, flower	GUS::NPTII	Babic *et al.* 1994
Nicotiana tabacum	root	NPTII	André *et al.* 1986
	root, stem	NPTII	Teeri *et al.* 1986
	*	NPTII	Koncz *et al.* 1989
	stem, stem-leaf, stem-root, vascular system	GUS/NPTII	Fobert *et al.* 1991
	root, root-leaf, root-leaf-stamen, root-stamen, leaf-stamen	GUS/NPTII	Lindsey *et al.* 1993
	seed coat	GUS/NPTII	Fobert *et al.* 1994
	shoot apex	LacZ::NPTII	Suntio and Teeri 1994
	constitutive	PAT::NPTII	Bauer-Weston *et al.* 1994
Solanum tuberosum	*	GUS/NPTII	Lindsey *et al.* 1993

* some tagged plants but not specified.
ocs: octopine synthase gene , aadI: spectinomycin and streptomycin resistance gene
BAR: resistance to herbicide phosphinothricin
"/ " stands for separate gene function and "::" for bifunctional that expresses as one gene.

1.6.2 Reporter/Selectable genes for promoterless constructs

The advantage of T-DNA is that it can be manipulated inside of its border sequences. For instance, reporter genes, activated by a promoter in the target DNA, have been engineered into T-DNA (Teeri *et al.* 1986; Koncz *et al.* 1989; Kertbundit *et al.* 1991). The gene *npt*II, which codes for neomycin phosphotransferase II (NPTII) and gives resistance towards aminoglycoside antibiotics, has been used in the tagging of plant genes. Since *npt*II is a selectable maker gene, it is possible to select directly for activation of the reporter. The activity of the *npt*II gene can also be enzymatically assayed. Another reporter gene used for gene tagging is *gus* (Kertbundit *et al.* 1991), which can be histochemically assayed (Jefferson *et al.* 1987), but which can not be used as a selectable marker (Hodal *et al.* 1992).

An ideal construct for tagging should have both easily selectable and histologically assayable properties. Datla *et al.* (1991) fused *gus* and *npt*II genes in-frame and were able to combine the properties of both enzymes in this fused gene system. The construct lacking its own promoter has been successfully used for tagging potential promoters in *Brassica* plants (Babic *et al.* 1994).

The gene which codes for β–galactosidase has been widely used as a reporter gene in microbial, animal and in plant cells (Teeri *et al.* 1989). Suntio and Teeri (1994) have developed an alternative method using the histochemically detectable *lacZ* reporter gene in plants. They constructed a bifunctional reporter gene by fusing *lacZ* and *npt*II genes in-frame. The same constructs code for both a selectable kanamycin resistant

phenotype and a histochemically detectable product. This construct was inserted into a T-DNA vector and used to generate random transcriptional and translational gene fusions to plant genes *in vivo*.

Bauer-Weston *et al.* (1994) developed a bifunctional reporter gene fusing *pat* and *npt*II. Since *pat* is a selectable marker gene which codes for phosphinothricin acetyltransferase that confers resistance to the herbicide phosphinothricin, it is possible to select directly for activation of the reporter. Also the activity of the *pat* gene can be enzymatically assayed (Ridley and McNally 1985; Wohlleben *et al.* 1988; Botterman *et al.* 1991; Oelck *et al.* 1991; Droege *et al.* 1992; Swartz *et al.* 1993). This PAT::NTPII bifunctional reporter system has been successfully used for the identification of putative constitutive promoters from *Nicotiana tabacum* (Bauer-Weston *et al.* 1994).

Bouchez *et al.* (1993) have used the *bar* gene as a selectable marker for the tagging vector, pGKB5. The T-DNA region contained two selectable markers conferring resistance to kanamycin and phosphinothricin under the control of nopaline synthase and the CaMV 35S promoters respectively in the same cassette. A promoterless β–glucuronidase reporter gene was fused to the right border of the T-DNA, in order to identify gene fusions upon insertion in the plant genome. The phosphinothricin resistance trait allowed for selection of transformants in the greenhouse at high density (up to 100-150 seeds/cm^2). This selection strategy represented an improvement over the *in vitro* kanamycin selection, which necessitates sterilization of seeds and use of costly chemicals. This system has been applied to

Arabidopsis thaliana using *Agrobacterium*-mediated *in planta* transformation but no specific expression patterns have been described in the recovered transgenic plants.

CHAPTER 2:

The Development of an Efficient Multiple Shoot Regeneration System for Broccoli (*Brassica oleracea* var. *italica*)

2.1 Introduction

Brassica oleracea is an economically important species. Practically every part of the plant is utilized including the leaves (cabbage, kale), terminal bud (early cauliflower), axillary buds (Brussels sprout), stems (kohlrabi) and flower buds (broccoli). In addition to being generally recognized for their nutritive value, recent reports have indicated that a number of *B. oleracea* varieties have cancer preventive properties (Steinmetz and Potter 1991; Paolini and Legator 1992; Beecher 1994).

The regeneration of plants from leaf cuttings of *B. oleracea* was described years ago (Isabell 1944; Ghosh 1965) and since then several varieties have been examined in a range of tissue culture systems. Kale has been regenerated from stem (Horak *et al.* 1975) and hypocotyl explants (Dietert *et al.* 1982). Shoot regeneration has been demonstrated from a variety of cauliflower explants including the curd (Walkey and Woolfit 1970), root (Grout and Crisp 1980), leaf (Bagga *et al.* 1982) and pedicel (Gorecka 1985). In cabbage, shoot regeneration has been reported from meristem (Walkey *et al.* 1980), pedicel (Dunemann and Grunewaldt 1987), hypocotyl (Bajaj and Neitsch 1975), leaf (Miszke and Skucinska 1976) and root explants (Lillo and Shanin 1986). With Brussels sprout, shoot regeneration from hypocotyl (Dieter *et al.* 1982; Ockendon 1984),

meristem and petiole (Clare and Collin 1974) and root explants (Lazzeri & Dunwell 1984b) has been reported. Dietert *et al.* (1982) and Becker-Zens and Grunewaldt (1984) reported the regeneration of kohlrabi from hypocotyl and pedicel explants.

Broccoli (*B. oleracea* var. *italica*) has been regenerated from a wide range of explants including root segments (Lazzeri and Dunwell 1984a,b; 1986a,b), stem (Johnson and Mitchell, 1978) leaf (Dunwell 1981; Patracek and Sams 1985), hypocotyl (Hui and Zee 1980, Dietert *et al.*, 1982; Robertson and Earle 1986; Lazzeri and Dunwell 1986a,b), flower bud (Pareek and Chandra 1978; Ilzuka *et al.* 1973), pedicel (Anderson and Carstens 1977; Patracek and Sams 1985; Dunemann and Grunewaldt 1987) and peduncle explants (Christey 1989).

Although several methods for plant regeneration have been reported for broccoli, the possibility of developing efficient tissue culture systems suitable for genetic transformation of broccoli has not been systematically investigated. The aim of this study was to develop an efficient shoot regeneration system for cotyledonary petiole and hypocotyl explants from a number of broccoli cultivars which would subsequently be suitable for *Agrobacterium*-mediated transformation.

2.2 Materials and Methods

2.2.1 Plant material

The plant materials used throughout this study are listed in Table 2.1. Most seeds were commercially available from the indicated seed companies. The cultivar 'Shogun' and breeding line 'UL' were kindly provided by Sakata Seeds America, Inc. (California, USA).

2.2.2 Explant preparation

Seeds were surface sterilized for 15 minutes in 50% commercial bleach (Javex[R]) containing a drop of commercial dishwashing detergent, followed by washing with autoclaved water. For germination, seeds were sown onto growth regulator-free, half-strength MS medium with 1% sucrose and 0.6% Phytagar[R] in 60x15 mm petri plates. The seeded plates, with lids removed, were placed onto the upturned lids of a sterile Magenta[R] GA7 boxes and covered with the transparent polystyrene boxes (Figure 2.6A). Containers were incubated in a growth cabinet at $25 \pm 1°C$ with 16h/8h (light/dark) illumination from cold white fluorescent tubes giving an intensity of 120-150 µE.

For preliminary screening, seedlings were harvested seven days after planting. For subsequent studies, five-day-old seedlings were used. Five to seven day old seedlings were found to be optimal for handling and dissection; younger seedlings were too small to handle conveniently. Two types of explants were prepared: (1) 4-5 hypocotyl segments, each segment 7-10mm in length, were taken from each

Table 2.1 Broccoli cultivars/line evaluated in shoot regeneration experiments

Cultivar/line	Supplier
Arcadia	W.H.Perron
Bronzino	Mcfayden Seed Co. Ltd.
Corvet	Stokes Seeds Ltd.
Cruiser	Stokes Seeds Ltd.
Emperor	Stokes Seeds Ltd.
Eureka	Stokes Seeds Ltd.
First Choice	W.H.Perron
Goliath	Stokes Seeds Ltd.
Green Belt	Stokes Seeds Ltd.
Green Comet	Westcan Horticultural Ltd.
Green Duke	Mcfayden Seed Co. Ltd.
Green Hornet	Stokes Seeds Ltd.
Green Sprouting	Westcan Horticultural Ltd.
Green Valiant	Stokes Seeds Ltd.
King Robert Purple	Dominion Seed House
Mariner	Stokes Seeds Ltd.
Packman	Stokes Seeds Ltd.
Paragon	Stokes Seeds Ltd.
Premium Crop	Stokes Seeds Ltd.
Red Broccoli	Dominion Seed House
Romanesco	Dominion Seed House
Shogun	Sakata Seeds America, Inc.
UL	Sakata Seeds America, Inc.
Waltham 29	Early's Farm Garden Center, Inc.

Early's Farm Garden Center, Inc. (P.O.Box 3024, Saskatoon, Saskatchewan, Canada S7K 3S9): Dominion Seed House (Georgetown, Ontario, Canada L7G 4A2); Mcfayden Seed Co. Ltd. (30-9th St. Suite200, Brandon, Manitoba Canada R7A 6N4); Sakata Seeds America, Inc. (105 Boranda Road, Salinas, California 93903, USA); Stokes Seeds Ltd. (P.O.Box 10, St. Catharines, Ontario, Canada L2R 6R6); Westcan Horticultural Ltd. (Bay 5, 6112-30 St.S.E., Calgary, Alberta, Canada T2C 2A6); W.H.Perron (2914 Labelle Blvd., Laval, Quebec, Canada H7P 5R9)

seedling starting about 5mm above the root junction (2) cotyledons were excised leaving 2mm of the petiole attached, as described by Moloney *et al.* (1989).

2.2.3 Media and explant culture conditions

Three basal media were used to culture explants in this study: Murashige and Skoog (1962) inorganic salts and vitamins, (hereafter referred to as MSMS); MS inorganic salts with B5 vitamins as specified by Gamborg *et al.* (1968) (MSB5); and Murashige Minimal Organics medium (Linsmaier and Skoog 1965) (MMO). MSMS and MSB5 media were purchased from Sigma Chemical Co., and MMO was purchased from Gibco/BRL (Appendix 1). Sucrose (3% final concentration), and growth regulators (BA and/or NAA) were added and the pH adjusted to 5.8 before autoclaving at 121°C for 20 min. All media were solidified with 0.6% Phytagar® (Gibco/BRL).

Ten explants (either hypocotyl or cotyledonary petiole) were placed in each 100x15 mm plate containing approximately 30 mL of medium in the case of shoot regeneration experiments. The petioles of the cotyledonary petiole explants were inserted into the regeneration medium so that the cut surface was in contact with the medium. Hypocotyl explants were laid horizontally on the surface of medium. Plates were then sealed with Stretch'n Seal™ wrap. Cultures were maintained in a growth cabinet at 25 ± 1°C with 16h/8h (light/dark) illumination from cold white fluorescent tubes giving an intensity of 120-150 µE. The cultures were scored after four weeks. The mean number of shoots/explant was calculated as the total number of shoots produced in each plate divided by the total number of explants plated. The percentage of explants

responding per treatment was calculated as shooting frequency. Standard errors were also calculated in each case. Root and callus formation during shoot regeneration were observed when shoots were scored.

Preliminary screening

Twenty-two broccoli cultivars (as listed in Table 2.1 with the exception of 'Shogun' and 'UL') were screened on MSB5 medium with 6 growth regulator combinations: 1.0 or 4.0 mg/L BA with either 0, 0.1 or 1.0 mg/L NAA. Thirty explants (both hypocotyl and cotyledonary petiole) were used for each treatment.

Shoot regeneration experiments in detail

Based on the results of the preliminary screening test, four cultivars which responded well ('Cruiser', 'First Choice', 'Green Valiant', and 'Packman') and two additional cultivars ('Shogun' and 'UL') of interest to broccoli breeders were selected for further detailed shoot regeneration experiments. In the case of shoot regeneration experiments from hypocotyl explants, MSB5 medium was used with 16 different growth regulator combinations: four BA concentrations (1, 2, 4, and 8 mg/L) in combination with each of four NAA concentrations (0, 0.01, 0.1, 1.0 mg/L). Fifty hypocotyl explants were used in each treatment.

Cotyledonary petiole explants were tested on three basal media, MSB5, MSMS and MMO. From the results of the hypocotyl regeneration studies, a smaller set of growth regulator combinations was selected for testing regeneration from cotyledonary

petioles to give a total of five different culture conditions: MSB5/BA2/NAA0.05, MSB5/BA4/NAA0.1, MSMS/BA2/NAA0.05, MSMS/BA4/ NAA0.1 and MMO/BA4.5. MSMS basal medium was added based on regeneration results reported in *Brassica carinata* cotyledonary petiole explants (Babic 1994). MMO medium with 4.5 mg/L BA was evaluated as it represents the medium used by Moloney *et al.* (1989) for *Brassica napus* cotyledonary petiole explants. Fifty cotyledonary petiole explants were used in each treatment.

Rooting experiments

Rooting experiments were conducted by placing 30 elongated shoots on MSB5 medium with 0, 0.01, 0.1, or 1 mg/L NAA. Six shoots were cultured in each Magenta[R] GA7 box. Individual rooted shoots were transferred to new rooting medium in baby food jars to encourage growth prior to potting and transfer to greenhouse condition. In order to facilitate hardening for greenhouse growth, freshly potted plantlets were covered with Magenta[R] GA7 boxes for the first week.

2.3 Results

2.3.1 Preliminary screening

The regenerative response of both hypocotyl and cotyledonary petiole explants from all cultivars tested differed widely. Culture variables such as growth regulator combination and type of explant strongly influenced organogenesis. Generally, hypocotyls responded more readily than cotyledonary petioles (Appendix 2). Based on the results with hypocotyl explants, four cultivars which responded well ('Cruiser', 'First Choice', 'Green Valiant', and 'Packman') and two additional cultivars ('Shogun' and 'UL') of interest to broccoli breeders were selected for further detailed shoot regeneration experiments.

2.3.2 Shoot regeneration from hypocotyl explants

Overall, hypocotyl explants from all six lines showed a similar response. The first effect noted was a swelling of the entire explant, followed by further swelling at the both ends of the explants. After seven days, both ends were covered in a small amount of pale green callus. Shoot differentiation was first noted after 10-14 days and after an additional two weeks numerous vegetative buds were present, usually concentrated at both ends of the explants.

Generally, BA alone (without NAA) induced a relatively high frequency of shoot regeneration (80-100%) and shoot number per explant (10-18) in all cultivars. The frequency and the number of shoots per explant increased with increasing BA concentration up to 4 mg/L. However, with the exception of UL, the highest

concentration tested (8 mg/L), slightly reduced the number of shoots produced. Higher concentrations of NAA reduced the mean number of shoots per explant, but this effect could often be partially compensated by higher levels of BA. For example, when 1 mg/L NAA was present, cultivar UL produced 4, 6, 12, and 13 shoots per explant on 1, 2, 4, and 8 mg/L BA respectively. Overall results are summarized in Appendix 3.

Often 100% of the explants regenerated shoots, but the response varied somewhat between cultivars. With 'Cruiser', high shoot regeneration frequencies occurred on all media tested (Figure 2.1). On BA 2/NAA 0 medium, shoot regeneration was obtained on 100% of the explants. The mean number of shoots per explant ranged from 7-18 on all concentrations of BA tested in the absence of NAA. The highest number of shoots per explant (28) occurred with BA 4/NAA 0.01. This medium also gave a 75% shoot regeneration frequency. At higher concentrations of NAA (0.1, 1.0 mg/L), roots and callus were induced at all BA concentrations.

'First Choice' gave a reasonably high regeneration frequency with all media tested, in the range of 50-100%, with a mean of 3-15 shoots per explant (Figure 2.1). The best regeneration frequencies were obtained on BA 2/NAA 0.01, BA 2/NAA 0.1, and BA 4/NAA 0.01 (Figure 2.1). The highest number of shoots per explant (12-15) formed on BA 2/NAA 0, BA 4/NAA 0.01, and BA 8/NAA 0. Root production was highest at all BA concentrations when combined with 0.01 or 0.1 mg/L NAA. Calli formed at lower BA levels when NAA was present.

Most media tested with 'Green Valiant' gave regeneration frequencies of 70-100%. The mean number of shoots per explant ranged from 2-25 (Figure 2.2). High

Cruiser
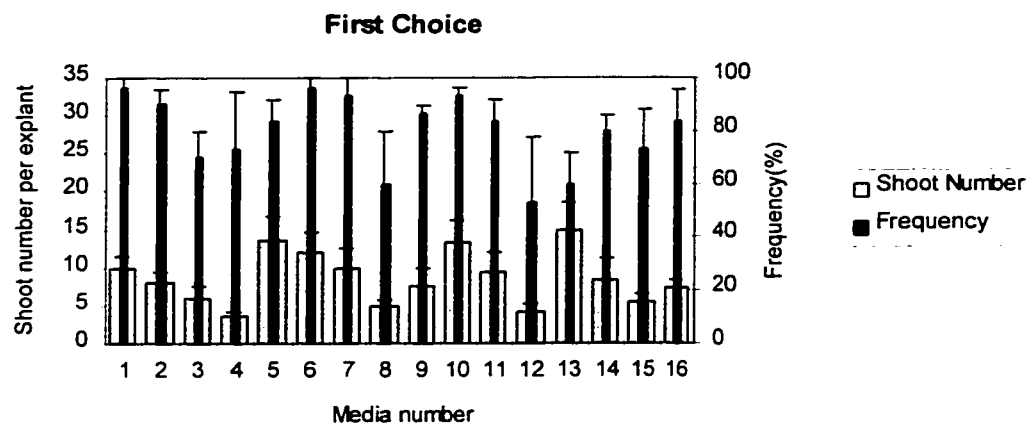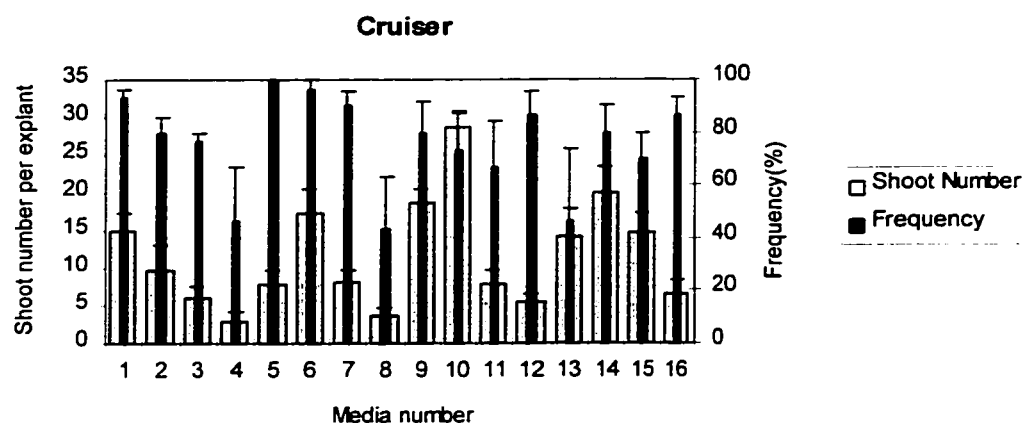
First Choice

Figure 2.1 Shoot regeneration in five-day-old hypocotyl explants of the broccoli cultivars 'Cruiser' and 'First Choice' in various media. Error bars represent standard error of mean (10 explants per plate, 5 plates).

1. BA1/NAA0	2. BA1/NAA0.01	3. BA1/NAA0.1	4. BA1/NAA1
5. BA2/NAA0	6. BA2/NAA0.01	7. BA2/NAA0.1	8. BA2/NAA1
9. BA4/NAA0	10. BA4/NAA0.01	11. BA4/NAA0.1	12. BA4/NAA1
13. BA8/NAA0	14. BA8/NAA0.01	15. BA8/NAA0.1	16. BA8/NAA1

(mg/L), Basal medium: MSB5, pH5.8

concentrations of growth regulators (BA 4/NAA 1, BA 8/NAA 1) resulted in reduced shoot production (less than three shoots per explant). Abundant callus and rooting were observed on 2 mg/L BA with either 0.1 or 1 mg/L NAA.

On all media tested, 'Packman' gave regeneration frequencies of 65-100% and 5-23 shoots per explant (Figure 2.2). The best regeneration response was observed with BA 4/NAA 0, with 23 shoots per explant and 90% regeneration frequency (Figure 2.2). The best callusing and rooting responses were observed with 1 mg/L NAA when combined with either 2 or 4 mg/L BA.

'Shogun' was the least affected by varying the growth regulator combinations, giving good regeneration with all media tested. Regeneration frequencies were 80-100% with 5-16 shoots per explant, except with 1 mg/L NAA combined with 1 or 2 mg/L BA, which gave less than five shoots per explant (Figure 2.3). All BA concentrations, when combined with 1 mg/L NAA, led to high frequency callusing and rooting.

The breeding line 'UL' gave constant response with greater than 80% regeneration frequencies and a mean of 3-20 shoots per explant on all media tested. High NAA concentrations reduced regeneration but this effect could be compensated by elevating BA concentrations (Figure 2.3). All media containing 1 mg/L NAA showed a high frequency of callusing. The highest rooting frequency occurred on BA 1/NAA 0.01.

Overall, 'UL' provided the highest mean number of shoots per explant (15) throughout 16 growth regulator combinations while 'First Choice' provided 8.7. On all

Green Valiant
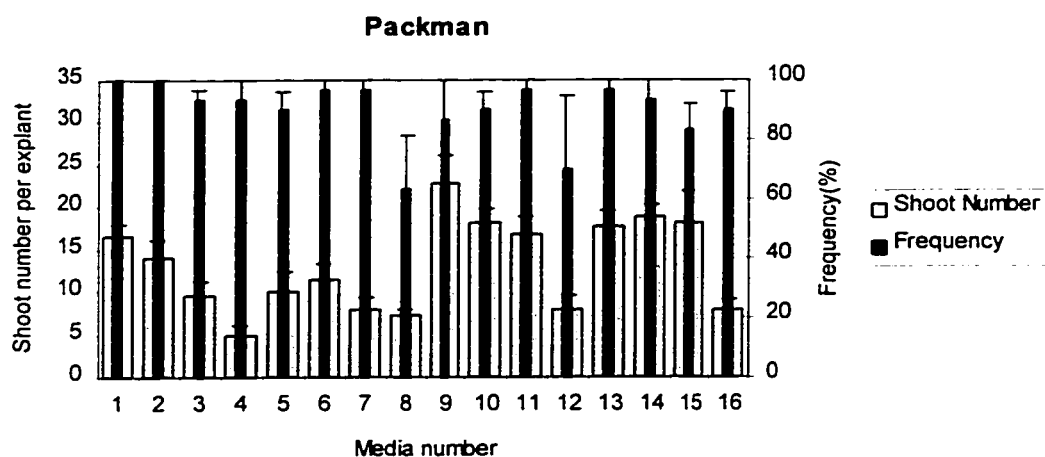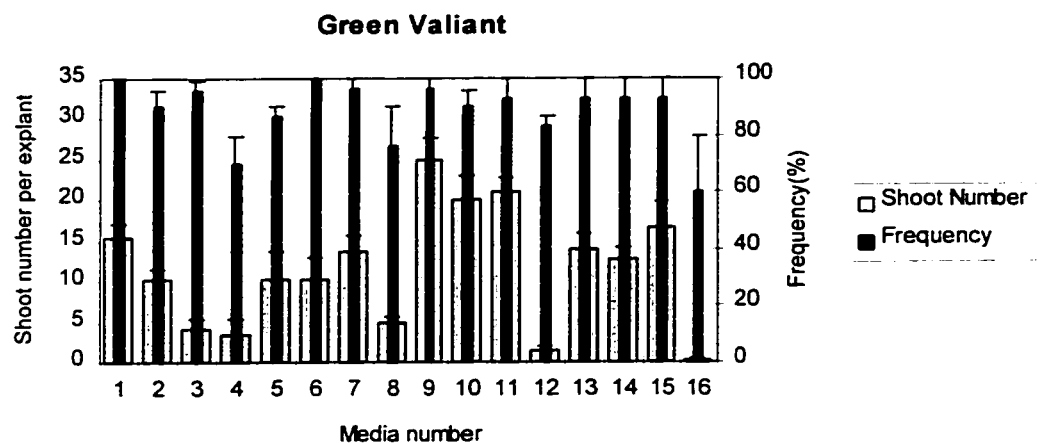
Packman

Figure 2.2   Shoot regeneration in five-day-old hypocotyl explants of the broccoli cultivars 'Green Valiant' and 'Packman' on various media. Error bars represent standard error of mean (10 explants per plate, 5 plates).

1. BA1/NAA0	2. BA1/NAA0.01	3. BA1/NAA0.1	4. BA1/NAA1
5. BA2/NAA0	6. BA2/NAA0.01	7. BA2/NAA0.1	8. BA2/NAA1
9. BA4/NAA0	10. BA4/NAA0.01	11. BA4/NAA0.1	12. BA4/NAA1
13. BA8/NAA0	14. BA8/NAA0.01	15. BA8/NAA0.1	16. BA8/NAA1

(mg/L). Basal medium: MSB5. pH5.8

Shogun

UL
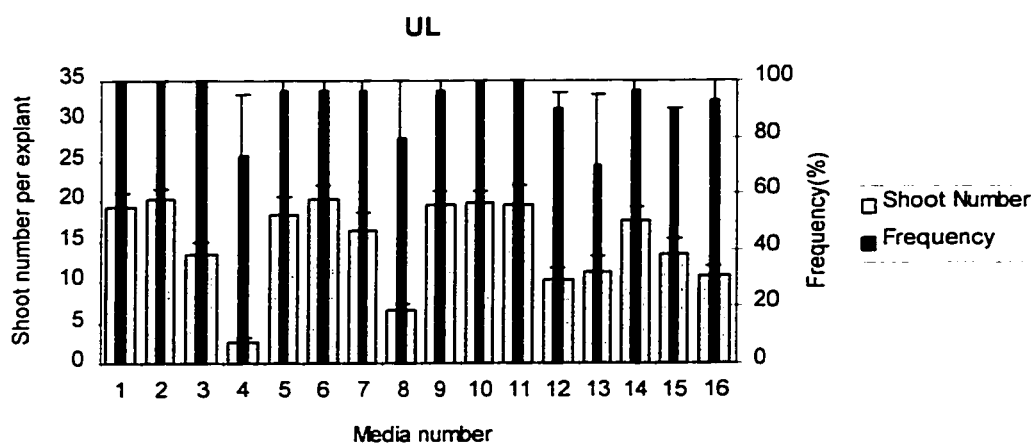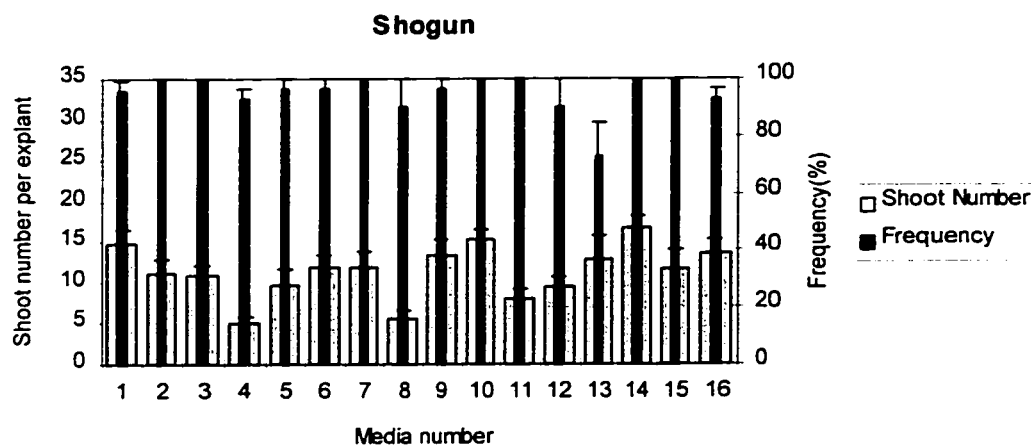
Figure 2.3 Shoot regeneration in five-day-old hypocotyl explants of the broccoli cultivars 'Shogun' and 'UL' on various media. Error bars represent standard error of mean (10 explants per plate, 5 plates).

1. BA1/NAA0	2. BA1/NAA0.01	3. BA1/NAA0.1	4. BA1/NAA1
5. BA2/NAA0	6. BA2/NAA0.01	7. BA2/NAA0.1	8. BA2/NAA1
9. BA4/NAA0	10. BA4/NAA0.01	1. BA4/NAA0.1	12. BA4/NAA1
13. BA8/NAA0	14. BA8/NAA0.01	15. BA8/NAA0.1	16. BA8/NAA1

(mg/L), Basal medium: MSB5, pH5.8

growth regulator combinations tested, BA 4/NAA 0.01 gave the best result (19.2 shoots/explant) of all broccoli cultivars tested.

2.3.3 Shoot regeneration from cotyledonary petiole explants

After a week in culture, nodular structures appeared on the cut end of the cotyledonary petiole embedded in the medium. More than 50% of the explants produced 2-5 shoots (more than 1 cm in length) directly from these structures after two weeks. During the same period a mass of callus ($0.5-1.0$ cm^2) formed at the base of the petiole below the surface of the agar from which more shoots developed after four weeks in culture.

In general, the frequency of shoot formation was very similar in all cultivars, but the number of shoots per explant was quite cultivar-dependent. All six cultivars gave a good regeneration response on all media tested, with 60-90% of explants producing shoots. The mean number of shoots per explant ranged from 2-12 and root formation was observed in some cases when shoots were scored (Figure 2.6B).

The best response was found with 'Cruiser', giving more than nine shoots per explant on all media tested while 'First Choice' and 'Packman' gave the lowest response, producing four or fewer shoots per explant (Figure 2.4).

Generally, most cultivars provided good regeneration with MSB5/BA 4/NAA 0.1 while the breeding line 'UL' showed best results with MMO/BA 4.5 while 'First Choice' and 'Packman' were less responsive with MMO/BA 4.5. Shooting frequencies in MMO/BA 4.5 were generally lower than other media.
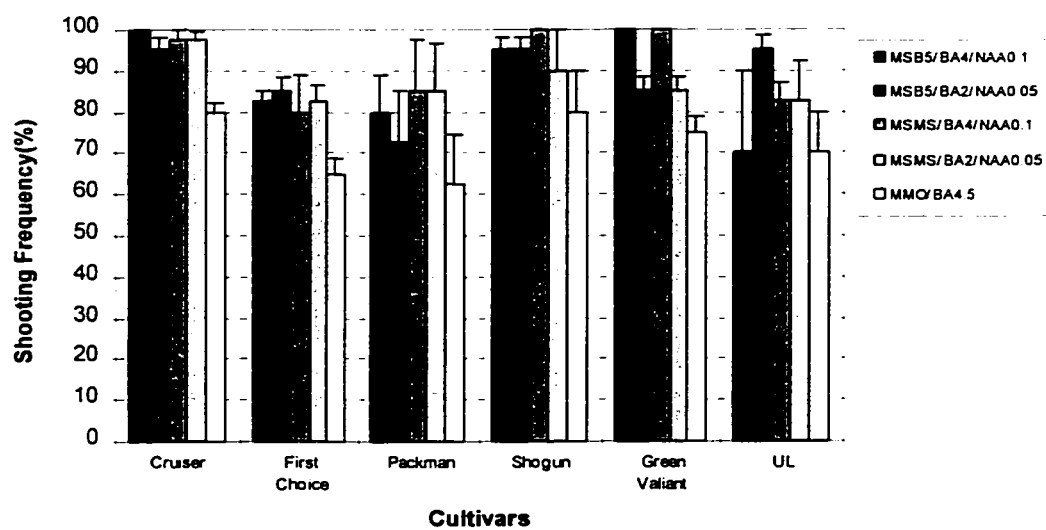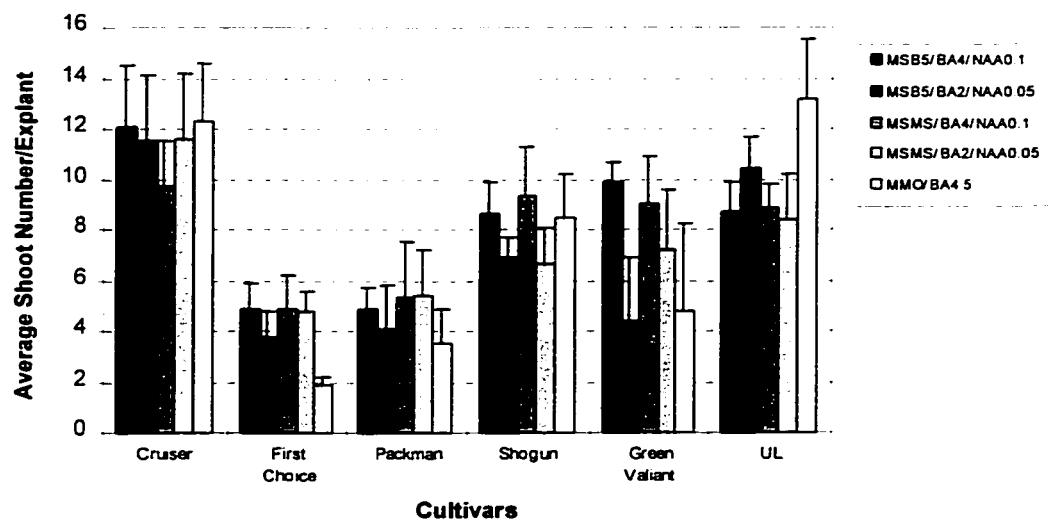
Figure 2.4 Shoot regeneration from cotyledonary petiole explants of the broccoli cultivars (10 explants per plate, 5 plates).

2.3.4 Rooting

After 7 days, small roots were noted and after 3-4 weeks, the root system had developed and plantlets could be transferred to soil (Figure 2.6C,D). Rooting response on the various media was almost identical for all cultivars tested. More than 90% of the shoots rooted on either growth regulator-free medium or 0.01 mg/L NAA. Higher concentrations of NAA tended to reduce rooting efficiency, with 45-64% of explants producing roots on medium containing 1 mg/L NAA, compared to 90-100% with 0.01 mg/L NAA (Figure 2.5).
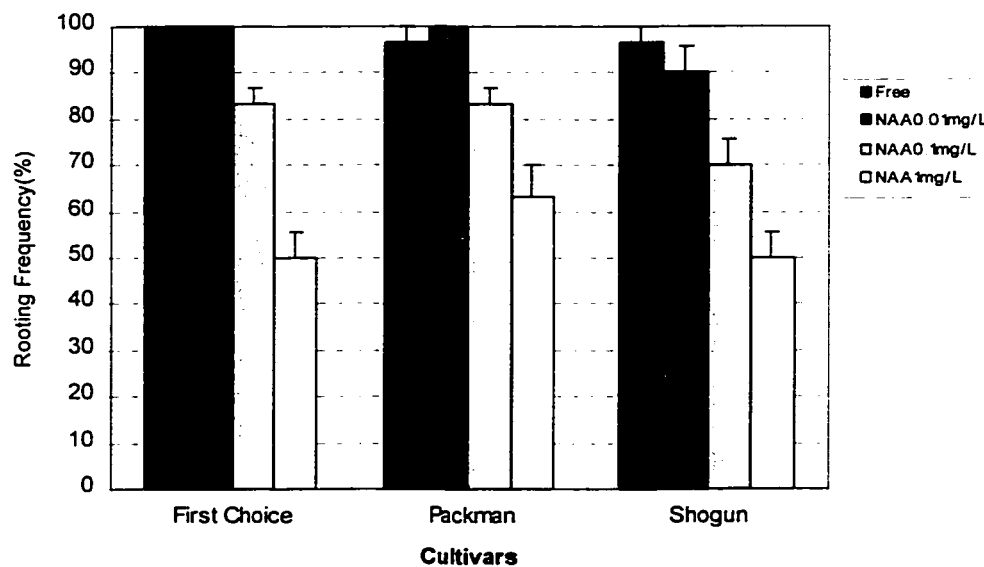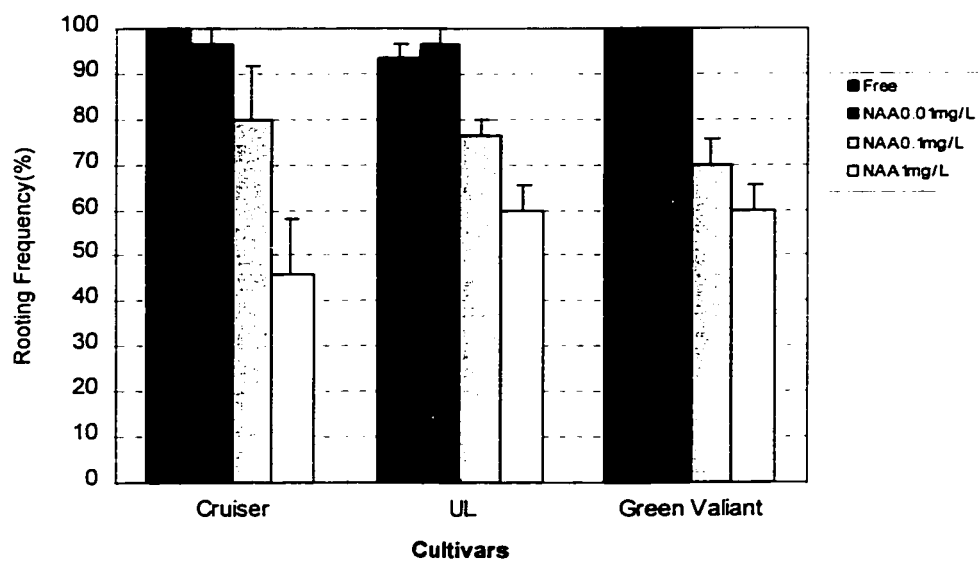
Figure 2.5 Rooting of broccoli shoots in various media. Error bars represent standard error of mean (6 shoots per Magenta® GA7 box, 5 Magenta® GA7 boxes). Basal medium: MSB5, pH5.8
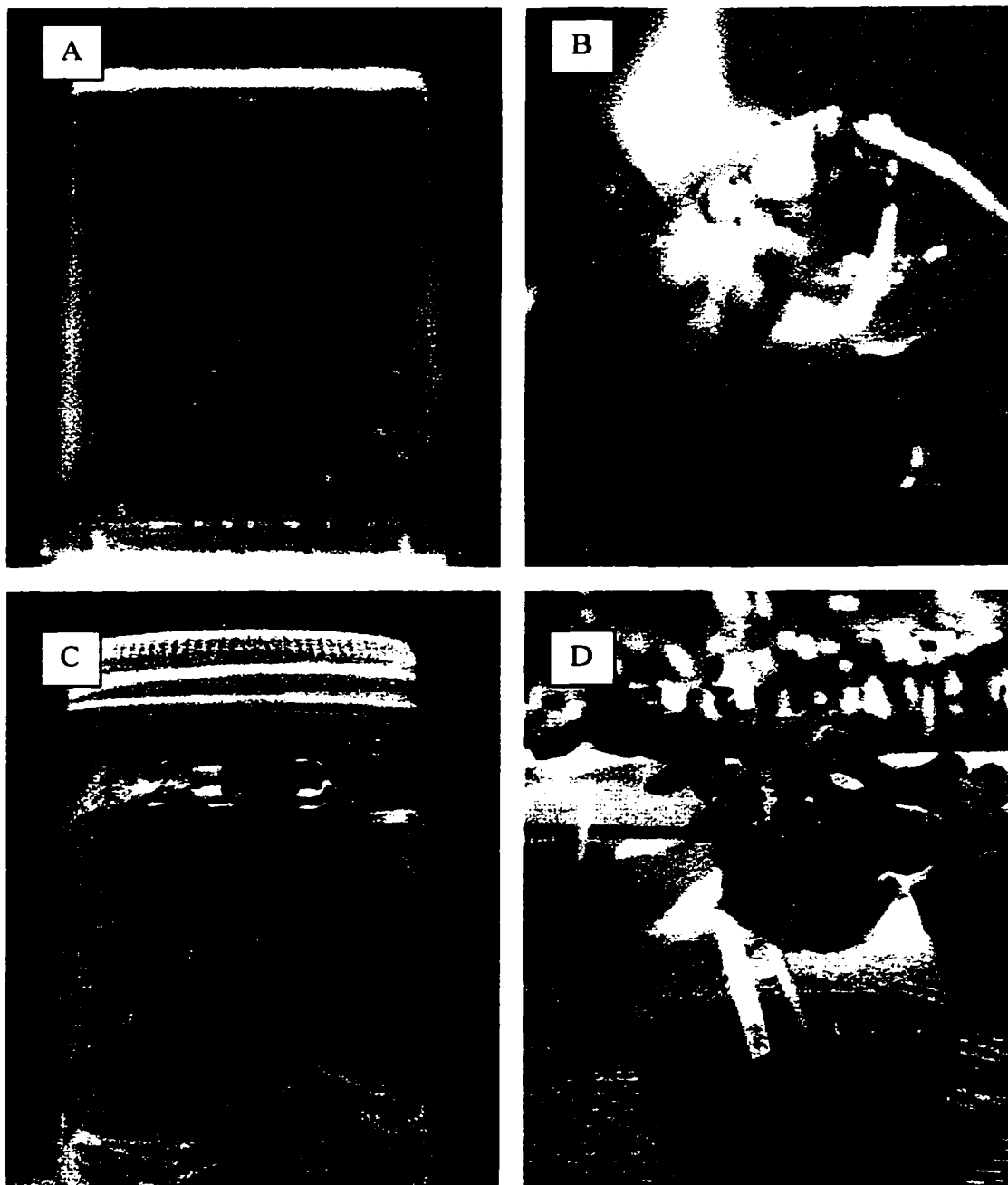
Figure 2.6 Regeneration from cotyledonary petiole explants.

A: Five-day-old seedling of the breeding line UL,   B: Shoot regeneration from cotyledonary petiole on MSB5 medium with BA4/NAA0.1 after 4 weeks in culture,   C: Rooting on MSB5 growth regulator free medium after three weeks in culture, D: R_0 plant in greenhouse, two months after transfer to soil

2.4 Discussion

This study presents the first report of direct shoot regeneration from cotyledonary petiole explants of broccoli (*Brassica oleracea* var. *italica*). Shoot regeneration from hypocotyl explants has previously been reported (Hui and Zee 1980: Dietert *et al.* 1982; Robertson and Earle 1986; Lazzeri and Dunwell 1986a,b).

The present study showed that hypocotyl and cotyledonary petiole explants responded differently to the tested auxin/cytokinin combinations and that each type of explant had a characteristic pattern of response. In contrast to the cotyledonary petiole explants, hypocotyls produced more shoots per explant. This result was similar to that reported by Lazzeri and Dunwell (1986a) who compared hypocotyl segments to root segment explants and observed higher shoot production in hypocotyl explants.

There was no difference in frequency of shoot regeneration from hypocotyl explants taken from 5 or 7 day-old seedlings (based on preliminary screening and detailed subsequent experiments). In contrast, Yang *et al.* (1991) obtained 100% regeneration form hypocotyls from 6-7 day old seedlings but only 18-68% when the seedlings were 4-5 days old. Similarly, Yang *et al.* (1991) obtained better regeneration with older root segment explants (Lazzeri and Dunwell 1984a,b; Kefford and Caso 1972).

Under identical culture conditions, cotyledonary petiole explants taken from five-day-old seedlings (in detailed experiments) gave a higher frequency of shoot formation than those from seven-day-old seedlings (in preliminary screening). Five-day-old seedlings were chosen because of the difficulty in dissecting petioles from younger

seedlings. Furthermore, five-day-old seedlings were found to be optimal for shoot regeneration from cotyledonary petioles of *B. napus* (Dale and Ball 1991; Moloney *et al.* 1989) and *B. carinata* (Babic 1994).

A combination of BA and NAA could be effectively employed to obtain shoot organogenesis in broccoli and it was concluded that other types of growth regulators or media additives were not essential. This conclusion is supported by earlier studies. Lazzeri and Dunwell (1986a) have found that NAA was ten times more active than IBA for inducing shoot regeneration from root explants of broccoli. Similarly, BA was more active than kinetin in shoot induction. Hui and Zee (1978), George and Rao (1980) and Regozinska and Drozdowska (1980) all found combinations of BA and NAA to be more effective than other growth regulators for shoot production from *Brassica* cotyledons. A similar growth regulator combination was optimal for shoot regeneration from root, hypocotyl and cotyledonary petiole explants of *B. carinata* (Jaiswal *et al.* 1987; Yang *et al.* 1991; Babic 1994).

The comparison of organogenesis from hypocotyl and cotyledonary petiole explants of five *Brassica oleracea* var. *italica* cultivars and one breeding line revealed considerable cultivar-dependent variation in regenerative capacity. Direct *in vitro* regeneration of shoots from broccoli cotyledonary petiole explants was observed for the first time. The observed cultivar dependent variation in shoot regeneration was similar to that of other reports (Baroncelli *et al.* 1973; Buiatti *et al.* 1974; Dunwell 1981, Dietert *et al.* 1982).

This study has demonstrated that a relatively simple tissue culture regime could be employed to achieve efficient shoot organogenesis and plant regeneration from hypocotyl and cotyledonary petiole explants of several broccoli cultivars. This system offers a significant advantage for application in *Agrobacterium*-mediated genetic transformation.

CHAPTER 3:

The Development of Efficient Methodology for *Agrobacterium*-mediated Transformation of *Brassica oleracea*

3.1 Introduction

The genus *Brassica* includes many economically important vegetable and oilseed crops. For the improvement of yield and quality of *Brassica* species, genes that may confer potentially useful traits have been introduced by genetic transformation. These traits include: herbicide resistance (DeBlock *et al.* 1989; Mitchell 1993; Oelck *et al.* 1991), insect resistance (Cardineau *et al.* 1993), oil quality (Knutzon *et al.* 1992). controlled genetic male sterility (Leemans 1992; Fabijanski 1992) and protein quality (DeClerq *et al.* 1990; Krebbers *et al.* 1993).

Most of the transformation studies have been carried out with *B. napus* and a number of *Agrobacterium*-mediated transformation methods have been reported for this species (Pua *et al.* 1987; Fry *et al.* 1987; Radke *et al.* 1988; Moloney *et al.* 1989; DeBlock *et al.* 1989; Swanson and Erickson 1989; Mehra-Palta *et al.* 1991; Christey and Sinclair 1992). Transgenic plants have been produced employing *Agrobacterium*-mediated transformation methods with other *Brassica* species, such as *B. rapa* (Radke *et al.* 1992; Christey and Sinclair 1992; Mukopdhyay *et al.* 1992; Swartz *et al.* 1993), *B. juncea* (Barfield and Pua 1991; Deepak *et al.* 1993; Pua and Lee 1995), *B. nigra* (Gupta *et al.* 1993) and *B. carinata* (Narasimhulu *et al.* 1992; Babic 1994).

Among the brassicas, *B. oleracea* (including broccoli, cauliflower, Brussels sprout, kohlrabi, kale and cabbage) constitutes one of the largest group of vegetables on a worldwide basis. There are reports on *Agrobacterium*-mediated transformation of broccoli (Christey and Earle 1989; Hosoki *et al.* 1991), cauliflower (Srivastava *et al.* 1988; David and Tempe 1988; DeBlock *et al.* 1989; Eimert *et al.* 1992), kale (Hosoki *et al.* 1989; Christey and Sinclair 1992) and cabbage (Berthomieu and Jouanin 1992; Berthomieu *et al.* 1994). However, reliable and routine transformation methods are generally not available for this species and it has been considered to be one of the more difficult *Brassica* species to transform (Eimert and Siegemund 1992). Particularly, in the case of broccoli (*B. oleracea* var. *italica*), transformation efficiency has been considered to be relatively low and with the exception of the *Bt* gene (Metz *et al.* 1995) the introduction of agronomically useful traits has not yet been reported.

With a view to developing an efficient *Agrobacterium*-mediated transformation system for broccoli (*B. oleracea* var. *italica*), different parameters were investigated involving tissue culture conditions and the reporter gene constructs such as *gus*, *npt*II and *pat* genes. The reporter gene constructs for this study was chosen based on their selection, biochemical and histochemical properties. The *pat* gene codes for the enzyme, phosphinothricin acetyl-transferase (PAT) which inactivates the non-selective herbicide phosphinothricin by acetylation (Murakami *et al.* 1986). Transgenic plants expressing a *pat* gene can be resistant to doses of phosphinothricin that would be lethal to control plants (DeBlock *et al.* 1987,1989; Oelck *et al.* 1991,1993; Babic 1994; Swartz *et al.* 1993). The expression of the *npt*II gene confers resistance to a broad range

of aminoglycoside antibiotics such as kanamycin (Beck *et al.* 1982), and the *gus* gene codes for β-glucuronidase which provides convenient biochemical and histochemical assays for monitoring expression in transgenic plants (Jefferson 1987). These three genes have been widely used in plant transformation and were therefore chosen in this study for the development of an effective transformation system for *B. oleracea.*

3.2 Materials and Methods

3.2.1 Plant materials

Broccoli (*Brassica oleracea* var. *italica*) cultivars 'Cruiser', 'Green Valiant', 'First Choice', 'Packman'; cauliflower (*B. oleracea* var. *botrytis*) cultivars 'White Summer', 'Cashmere', 'Snow Crown', 'White Top', 'White Fox' and 'Yukon'; cabbage (*B. oleracea* var. *capitata*) cultivar 'Ruby Ball'; kale (*B. oleracea* var. *acephala*) cultivars 'Squire' and 'Green Curled Scotch'; kohlrabi (*B. oleracea* var. *gongylodes*) cultivars 'Early White Vienna' and 'Grand Duke' and Brussels sprout (*B. oleracea* var. *gemmifera*) cultivar 'Pearl' were purchased from Stokes Seed (Ontario, Canada). The broccoli cultivar 'Shogun' and breeding line 'UL' was kindly provided by Sakata Seeds America, Inc. (California, USA). Seeds of the 'Rapid cycling' *B. oleracea* were purchased from the Crucifer Genetics Cooperative (University of Wisconsin-Madison, USA). *B. napus* cultivars including the widely studied cultivar 'Westar' were used as a reference for transformation. Seeds of the *B. napus* cultivars 'Westar', 'Topaz' and 'Cyclone' were kindly provided by Dr. G. Rakow, Agriculture and Agri-Food Canada, Saskatoon, Canada.

3.2.2 Culture media and explant preparation

The various media used in this study are summarized in Table 3.1. Seeds were surface sterilized in 50% commercial bleach (Javex®) for 15 minutes, followed by washing with autoclaved water. For germination, seeds were sown onto growth regulator-free, half-strength MS medium with 1% sucrose and 0.6% Phytagar® in

Table 3.1 Media used in *B. oleracea* var. *italica* transformation

Medium	Modifications as compared to the basal medium
Basal	MSB5 (MS salts with B5 vitamins) Cat. No. M0404, Sigma, USA
Seed germination	Half strength basal medium 1% sucrose, 0.6% agar
Regeneration	Basal medium with 0.5 g/L MES, 4 mg/L BA, 0.1 mg/L NAA 3% sucrose, 0.6% agar, pH 5.8
Co-cultivation	Basal medium with 0.5 g/L MES, 4 mg/L BA, 0.1 mg/L NAA 3% sucrose, 0.6% agar, pH 5.6
Base solution	Basal medium with 0.5 g/L MES, 1 mg/L 2,4-D 2% sucrose, pH5.6
Selection	Basal medium with 0.5 g/L MES, 4 mg/L BA, 0.1 mg/L NAA 3% sucrose, 0.6% agar, pH 5.8 500 mg/L carbenicillin, 20 mg/L kanamycin
Rooting	Basal medium with 0.5 g/L MES, absence of growth regulators 2% sucrose, 0.6% agar, pH 5.8 500 mg/L carbenicillin, 125 mg/L L-ppt

Antibiotics and L-ppt were added after the medium was autoclaved.

60x15 mm petri plates. The seeded plates, with lids removed, were placed onto the uptumed lids of sterile Magenta® GA7 boxes and covered with the transparent polystyrene boxes as shown in Figure 2.6A. Containers were incubated in a growth cabinet at 25 ± 1°C with 16h/8h (light/dark) illumination from cool white fluorescent tubes giving an intensity of 120-150 μE.

Explants were prepared for transformation experiments from five-day-old *in vitro* grown seedlings. Five-day-old seedlings were found to be optimal for handling and dissection; younger seedlings were too small to handle conveniently. Two types of explants were prepared: (1) 4-5 hypocotyl segments (each segment 7-10 mm in length), were taken from each seedling starting about 5 mm above the junction with the root, (2) cotyledonary petiole explants were obtained by removing the cotyledon from each seedling leaving 2 mm of the petiole attached, as described by Moloney *et al.* (1989).

To determine the effect of culturing cotyledonary petiole explants prior to transformation, in some experiments, cotyledonary petiole explants were pre-cultured by embedding the petiole in the agar for two days on the regeneration medium prior to co-cultivation. In the case of hypocotyl explants, only freshly prepared explants were used for transformation.

3.2.3 Bacterial strains and gene construct

Agrobacterium tumefaciens strains GV3101/pMP90 (Koncz and Schell 1986), LBA4404/pAL4404 (Ooms *et al.* 1982) and A281/pEHA105 (Hood *et al.* 1993) harboring the binary vector pRD320 were used for transformation studies. The binary

vector pRD320 was developed by Dr. R. Datla (National Research Council Canada, Saskatoon, Canada). This vector contains a chimeric *gus::npt*II gene (Datla *et al.* 1991) and a *pat* gene under separate control of tandem CaMV 35S promoters each with translational enhancer sequences from alfalfa mosaic virus (Figure 3.1). A stationary phase bacterial culture in LB broth (100 mL) was harvested by centrifugation and resuspended in 10 mL of fresh LB broth with 1% DMSO (final concentration) as a cryo-protectant. Aliquots of 200 μL were dispensed into 1.5 mL micro-tubes. These bacterial cultures were stored at -20 °C until used as a 'seed culture'. For transformation, a tube of seed culture was added to 2 mL Brain Heart Infusion Broth (Difco, USA) supplemented with 2% sucrose, 50 μM acetosyringone (final pH5.6) and incubated overnight at 28°C. Bacterial cell density was in the order of 1×10^9/mL.

3.2.4 Determination of the kanamycin concentration as a selection agent

In preliminary experiments, hypocotyl and cotyledonary petiole explants of the cultivar 'Cruiser' were cultured on the kanamycin containing regeneration medium to determine the amount of kanamycin required as a selection agent. Kanamycin concentrations tested for this experiment were 0, 6.25, 12.5, 25, 50, 75 and 100 mg/L. Hypocotyl segments and cotyledonary petiole explants were taken from the *in vitro* grown five-day-old seedlings. The petioles of the cotyledonary explants were then inserted into the regeneration medium so that the cut surface was in contact with the medium; hypocotyl explants were laid on the surface of medium. Sixteen hypocotyl
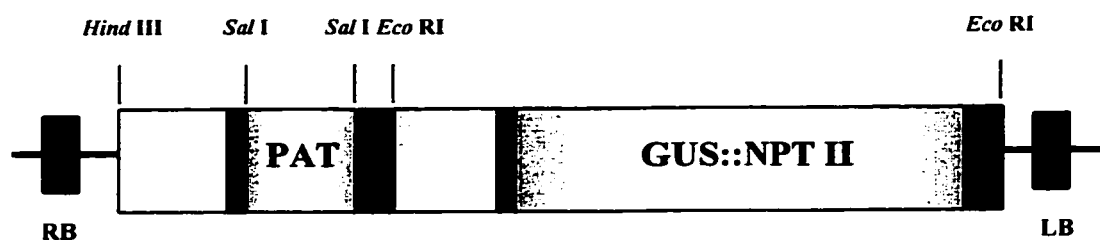
Figure 3.1 Genetic and restriction map of the binary vector pRD320

Tandem 35S promoter: 650bp; AMV translation enhancing leader: 45bp; PAT : 600bp; GUS::NPTII : 2.8Kb; NOS terminator: 250bp; RB: right border of the T-DNA; LB: left border of the T-DNA (from Dr. R. Datla, unpublished data).

explants or ten cotyledonary petiole explants were placed in each 100x15mm sterile petri-plate. Plates were sealed with Stretch'n Seal™ wrap then incubated in a growth cabinet at 25 ± 1°C with 16h/8h (light/dark) illumination from cool white fluorescent tubes giving an intensity of 120-150 μE. The cultures were observed after 3 weeks.

3.2.5 Co-cultivation

Co-cultivation plates were sealed with Stretch'n Seal™ wrap and incubated in a growth cabinet at 25 ± 1°C with 16h/8h (light/dark) illumination from cool white fluorescent tubes, giving an intensity of 120-150 μE unless otherwise stated.

3.2.5.1 Cotyledonary petiole explants

Three kinds of co-cultivation methods were used for cotyledonary petiole explants:

Conventional method

Cotyledonary petiole explants were *Agro*-infected according to the Moloney method (Moloney *et al.* 1989). A thin film of bacterial suspension was used to cover the base of a 5 cm petri-plate. The cut surface of the petiole of fresh or pre-cultured explants was dipped into the bacterial suspension for few seconds. The petioles of the explants were then inserted into the co-cultivation medium so that the cut surface was in contact with the medium. Ten explants were placed in each 100x15mm sterile petri-plate. The plates were incubated for three days in a growth cabinet. After three days, all

explants were transferred to the selection medium by embedding the petiole in the medium.

Filter paper method

Fresh or pre-cultured explants were dipped into the bacterial suspension and then placed on sterilized filter paper (Whatman #4, cat. no. 1004070) overlaying the co-cultivation medium (Figure 3.2A). Twenty explants were placed in each plate. The plates were incubated for various periods (2-4 days) in a growth cabinet. After co-cultivation, all explants were transferred to the selection medium as described above.

Speed transformation method

Overnight bacterial culture (2 mL) was added to 38 mL of base solution in a 100x25mm sterile petri-plate. Up to 500 excised cotyledonary petiole explants were placed in this co-cultivation solution (Figure 3.2B). Five to ten minutes after the last explant was placed in this solution, as much solution as possible was removed by pipette (Figure 3.2C). The bacterial solution (less than 40 mL) was applied to another plate and used for a new batch of experiments. Once prepared, this bacterial solution could be used to treat several thousands of explants by this approach. After removal of the bacterial solution, the lid was replaced and the plates were wrapped loosely with aluminum foil. The plates were incubated in a growth cabinet for various periods (2-4
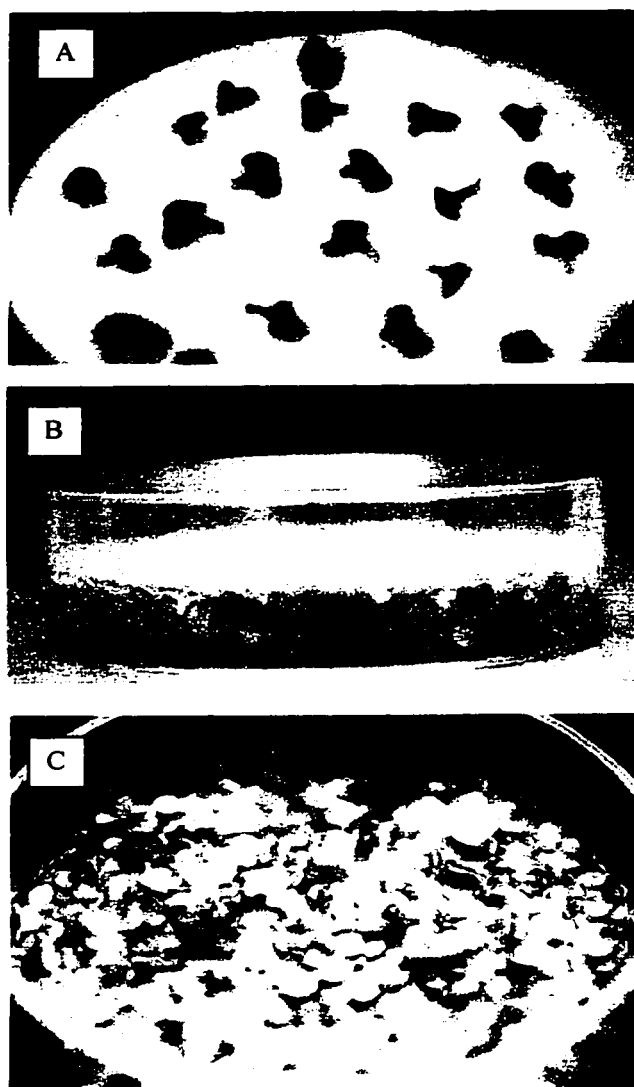
Figure 3.2   Filter paper based co-cultivation (A) and co-cultivation during speed transformation method (B, C)

A: Cotyledonary petiole explants on filter paper in the co-cultivation medium

B: Cotyledonary petiole explants immersed in bacterial solution

C: Cotyledonary petiole explants after removal of the bacterial solution. The explants were co-cultivated in this condition.

days). After co-cultivation, all explants were transferred to the selection medium as described above.

Cold-treatment

Sometimes co-cultivation at a low temperature (cold-treatment) was performed before/after incubation in growth cabinet (25 °C with 16h/8h (light/dark) illumination). A cold chamber (16 hours at 10 °C/ 8 hours at 5 °C with low illumination) and a laboratory refrigerator (4 °C and dark constantly) were used for the cold-treatments. The cold-treatment conditions included; (1) co-cultivation in a growth cabinet for two days, followed by two more days in a cold chamber, (2) co-cultivation in a growth cabinet for two days, followed by four more days in a cold chamber, (3) co-cultivation in a growth cabinet for two days, followed by six more days in a cold chamber, (4) co-cultivation in a cold chamber for four days without incubation in a growth cabinet, (5) co-cultivation in a cold chamber for two days, followed by two more days in a growth cabinet as a reverse sequence.

Vacuum infiltration

In some experiments with speed transformation a vacuum was applied during the *Agro*-infection process (prior to co-cultivation). While the explants were in the bacterial solution, a vacuum was applied for 5 minutes using a vacuum aspirator (Model 7049-50, Cole Parmer, USA). After vacuum infiltration, the vacuum was released very

slowly under aseptic condition. Afterwards, all procedures were followed as described in the speed transformation method.

3.2.5.2 Hypocotyl explants

Hypocotyl explants were transformed according to DeBlock *et al.* (1989) with minor modifications. Fresh hypocotyl explants were immersed in the co-cultivation solution prepared as for the speed transformation method. Explants were co-cultivated in two ways: the filter paper method and the speed transformation method as described above. Twenty explants were laid on the filter paper in the case of the filter paper method. Other procedures were the same as with cotyledonary petiole explants. After co-cultivation, all explants were placed on the surface of the selection medium.

3.2.6 Selection and plant regeneration

The selection medium was the same as the explant regeneration medium except it contained 500mg/L carbenicillin to remove excess bacteria and 20 mg/L of kanamycin for the recovery of putative transgenic shoots.

After 3 to 4 weeks in the selection medium, regenerated green shoots (putative transformants) were excised and transferred to new selection medium to allow shoot elongation. When the shoots were large enough (1.5-2 cm) to be rooted, they were transferred to the rooting medium. Petiole tissues that became necrotic were removed during subculturing.

The cultures were evaluated after 3-4 weeks in the selection medium to determine the percentage of explants that produced green calli or green shoots. Green shoots were screened for the expression of the *gus* gene by the histochemical GUS assay. Transformation frequencies were expressed as the percentage of GUS-positive shoots per total number of explants used. Only one green shoot/explant was considered as a putative transgenic shoot even if there were more than one green shoot/explant.

3.2.7 Cultivation of transgenic plants

As soon as enough root mass was obtained in the rooting medium (containing 125mg/L L-ppt) the plants were transferred to potting mix (Redi-Earth®) supplemented with fertilizer and grown under normal greenhouse conditions. In order to facilitate hardening for greenhouse growth, freshly potted plantlets were covered with Magenta^{k} GA7 boxes for the first week. Under these conditions, plants established rapidly and leaf samples were collected for further assays.

3.2.8 Analysis of putative transgenic plants

Histochemical GUS assay

Histochemical GUS analysis was performed following the method described by Jefferson (1987) with minor modifications. Pieces of green shoot from putative transgenic plants were placed in X-Gluc solution and incubated overnight at 37°C. The samples were bleached with a 10-fold diluted commercial Javex® solution for several hours to remove the pigments. The presence of blue color, an indicator of *gus* gene

expression was regarded as positive evidence of transformation. Details are described in Appendix 4.

NPTII assay

NPTII activity in leaves of the putative transformants was assayed according to Reiss *et al.* (1984) with minor modifications. Leaf tissue (200 mg) was ground in the presence of 50 μL of extraction buffer and 200 μL of ddH$_2$O. The extract was centrifuged for five minutes at maximum speed in a microcentrifuge at 4 °C and the supernatant was transferred to a new tube on ice. Extract (20 μL) was added to 10 μL of reaction buffer and 10 μL ATP solution (3.71 μL cold 100 mM ATP, 5 μL [γ ^{32}P-ATP], 492 μL ddH$_2$O). The reaction mixture was incubated at 37°C for 30 minutes and the reaction was stopped by returning it to ice. This reaction mixture was used for dot blot analysis using Whatman P81 ion exchange paper. The P81 paper was dried after washing then [^{32}P] was visualized by autoradiography (XAR-5-Kodak film overnight) kept at -70 °C for 24 hours. Details are described in Appendix 5.

PAT assay

The PAT assay was performed according to DeBlock *et al.* (1989) with minor modifications. Leaf tissue (200 mg) was ground in the presence of 500 μL of extraction buffer. The extract was centrifuged for five minutes at maximum speed in a microcentrifuge. Extract (20 μL) was added to 5 μL of a 1 mM phosphinothricin stock and 3 μL of ^{14}C-labeled acetyl-CoA. The reaction mixture was incubated at 37 °C for

two hours, and 10 μL was spotted on a silica gel TLC plate. Ascending chromatography was carried out in a 3:2 mixture of 1-propanol and NH_4OH (25% NH_3). [^{14}C] was visualized by autoradiography (XAR-5-Kodak film overnight) kept at -70 °C for 48 hours. Details are described in Appendix 6.

PCR analysis

Two sets of oligonucleotides derived from the *pat* and *npt*II gene sequences were used as primers for PCR. For the *pat* gene, a 22mer "5'-AGACCAGTTGAGATTAGGCCAG" and a 20mer "5'-GCCTCATGCAACCTAA CAGA" (expected product size about 400bp) were used; for the *npt*II gene, two 18mers "5'-GATGGATTGCACGCAGGT" and "5'-TCAGAAGAACTCGTCAAG" (expected product size about 800bp) were used. Plant template DNA was prepared in a micro-tube according to the quick preparation method of Edwards *et al.* (1991). The PCR reaction was carried out in 25 μL volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 400 μM each of dNTPs, 1 μM of each primer, one unit of *Taq* DNA polymerase and 2.5 μL of template DNA. PCR was performed in a thermal cycler (MJ Research, USA) for 35 cycles, with each cycle consisting of 94°C for 30 seconds for denaturing the template, 55°C for 30 seconds for annealing, and 72°C for two minutes for polymerization. At the end of 35 cycles, samples were incubated for 10 minutes at 72°C for final extension and stored at 5°C for further analysis.

DNA preparation for Southern analysis

An efficient and reproducible method for the isolation of high molecular weight DNA, based on CTAB but without using phenol, has been developed in this study (Appendix 7). Two grams of fresh leaf tissue collected from putative transgenic plants were ground with liquid nitrogen, then 5 mL of lysis buffer was added. This was transferred into a 30 mL Oakridge® tube and the final volume was adjusted to 10 mL with lysis buffer, followed by the addition 40 μL of 1% Proteinase K and 2.5 mL of 10% SDS. The contents were gently mixed, and then incubated at 60°C for 30 minutes. After incubation, 2.5 mL of 5M NaCl and 2 mL of 10% CTAB were added, gently mixed, and then incubated for another 30 minutes at 60°C. An equal volume of methylene chloride: iso-amyl alcohol (24:1) was added to this preparation and gently mixed. After centrifugation at 8,000x G for 10 minutes, the supernatant was transferred into a new tube and RNase A was added, and incubated at 37°C for 30 minutes. After this incubation, the methylene chloride: iso-amyl alcohol (24:1) extraction was repeated and the supernatant was transferred into a new tube. The supernatant was mixed with an equal volume of cold isopropanol, the DNA pellet was collected by centrifugation at 8,000x G for 10 minutes, washed once with cold 70% ethanol then dried and dissolved in 1 mL of distilled H_2O.

DNA digestion and Southern analysis

A computer controlled microwave oven-mediated DNA digestion system has been developed in this study (Appendix 8). Ten micro-grams of DNA from each

putative transgenic plant with 10 units of *Hind*III was prepared and used for routine digestion. Reaction tubes were placed in a 900W-0.4 cubic feet microwave oven (Samsung, Korea) and digestion was performed for 35 cycles; each cycle consisted of five seconds with power on and 10 seconds with power off in order to prevent boiling of the reaction mixtures. Genomic DNA digestion was completed within 10 minutes with this system. After digestion, DNA fragments were separated by 0.8% agarose gel electrophoresis, and transferred to a nitrocellulose filter as described by Southern (1975). The PCR amplicon (size about 400bp) of the *pat* gene from pRD320 was used as a hybridization probe. The filter was hybridized with a *pat* gene amplicon labeled with $[\alpha\text{-}^{32}P]dCTP$ using a Random Primer Labeling Kit (Life Technologies, USA). Conditions for hybridization and washing were as described by Maniatis (1982).

Inheritance of GUS activity in R_1 progeny

A solution of 4% NaCl was sprayed onto the inflorescence of transgenic lines (R_0) at flowering to break self-incompatibility. Seeds from self-pollinated transformed plants were germinated on half strength MSB5 medium with 1% sucrose. After 5 days, one cotyledon from each R_1 seedling was stained for GUS expression. Based on these results, segregation ratios for the GUS marker gene were determined. χ^2 values were computed to evaluate segregation ratios by using the MULTICHI program (Appendix 9).

3.3 Results

3.3.1 Determination of the kanamycin concentration appropriate for selection

Plant regeneration from hypocotyl and cotyledonary petiole explants of the untransformed broccoli cultivar 'Cruiser' was inhibited by 6.25 mg/L kanamycin (Table 3.2). Explant tissues swelled and later turned white, but shoot/bud differentiation was not observed. All explants were killed at a kanamycin concentration higher than 25 mg/L. A reduced concentration of 20 mg/L kanamycin was considered to be effective and used in all the subsequent transformation studies for both types of explants.

3.3.2 Transformation of cotyledonary petiole explants

After 10-14 days on the selection medium with 20mg/L kanamycin, green spots appeared at the cut edges of petioles. In some cases, tiny green shoots appeared on white calli after a month on the selection medium (Figure 3.3A). Some of these green spots and/or tiny shoots developed into green shoots suitable for transferring to new selection medium for shoot elongation after a month (Figure 3.3B). Untransformed white calli and/or white/purple colored shoots also developed around the cut surfaces of the petiole.

3.3.2.1 Effect of pre-culture and filter paper

Development of green shoots was influenced by a hypersensitive reaction of the explants (possibly caused by co-cultivation with *Agrobacterium*). The hypersensitive

Table 3.2 Effect of kanamycin on regeneration from *B. oleracea* var. *italica* cv. Green Valiant

Kanamycin (mg/L)	Observations	
	Hypocotyl[a]	Cotyledonary petiole[b]
0	Explants green and swollen, good regeneration of green shoots	Explants green and swollen, good regeneration of green shoots
6.25	6 explants produced white callus 12 explants were a little swollen, but explants turned white 14 explants turned white	Most petioles were swollen; turned white at tip
12.5	12 explants produced white callus 14 explants were a little swollen, but explants turned white 6 explants turned white	Most petioles were swollen; turned white at tip
25	16 explants were a little swollen, turned white 16 explants turned white	All turned black
50	All turned black	All turned black
75	All turned black	All turned black
100	All turned black	All turned black

a: 16 explants per plate with 2 duplications, observations were made after 3 weeks.
b: 10 explants per plate with 2 duplications, observations were made after 3 weeks.
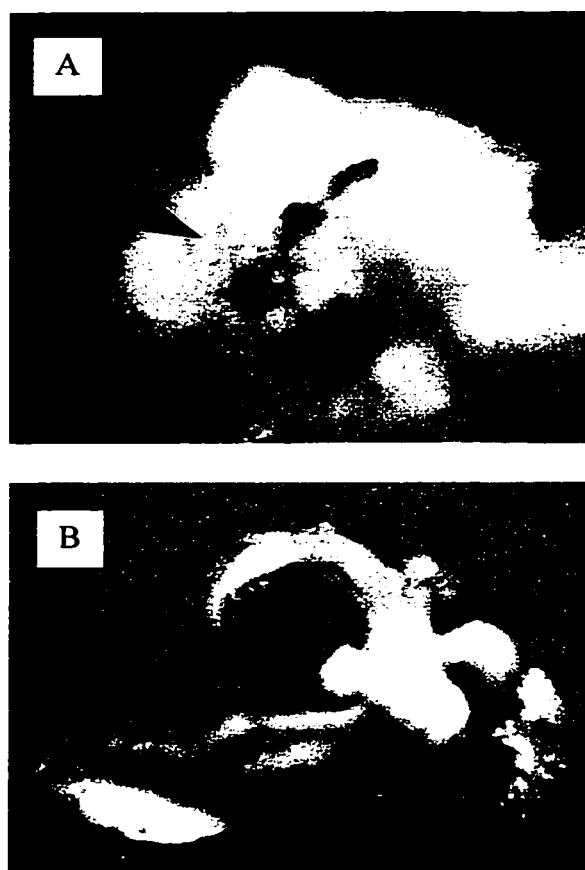
Figure 3.3 Green shoots developing from callus of the broccoli cultivar 'Cruiser' after selection on 20mg/L kanamycin

A: Green shoots appeared on white calli after a month on the selection medium.   B: After a month on the selection medium, rootless shoots developed; These were suitable for transfer to the rooting medium.

reaction was characterized by a severe browning or tissue necrosis at the infection area (i.e. cut ends) of the petioles after co-cultivation with *Agrobacterium* and this inhibited production of green shoots or calli. Co-cultivation of explants on filter paper placed on the regeneration medium helped to reduce the over-growth of *Agrobacterium* and associated tissue necrosis thereby improving the frequency of transformation. The hypersensitive response was considerably reduced by using explants that had been pre-cultured for two days on the pre-culture medium. Pre-cultured explants were more resistant to hypersensitivity.

Fresh and two-day pre-cultured cotyledonary petiole explants of the cultivar 'Cruiser' were transformed using the filter paper method in a preliminary experiment (Table 3.3). Co-cultivation of fresh cotyledonary petiole explants resulted in petiole tip necrosis caused by the hypersensitive response even after co-cultivation on filter paper. When explants were pre-cultured on the regeneration medium for two days, the hypersensitive response was reduced compared to co-cultivation with *Agrobacterium* on filter paper without pre-culturing. With two day pre-cultured explants, more GUS-positive shoots were recovered (1.2%) compared to fresh explants (0.4%) after co-cultivation. As the transformation frequencies with this cultivar were relatively low, the cultivar 'Green Valiant' which had been found to regenerate well in tissue culture (Chapter 2) was used in subsequent detailed studies.

In the case of the cultivar 'Green Valiant', there was no difference in the production of GUS-positive shoots between the conventional and the filter paper method when fresh explants were used (0.4-0.5%) (Table 3.3). However when two-day

Table 3.3 Effect of pre-culture and co-cultivation methods on transformation of cotyledonary petiole explants from five-day-old seedlings of the broccoli cultivars 'Cruiser' and 'Green Valiant'

Pre-culture	Co-cultivation methods	Number of explants	Explants with green shoots(%)	Explants with GUS(+) shoots(%)
cultivar 'Cruiser'				
No	F	1660[a]	0.8	0.4
2 day	F	2200[b]	1.6	1.2
cultivar 'Green Valiant'				
No	C	640[a]	0.7	0.4
	F	670[a]	0.9	0.5
2 day	C	1370[c]	1.1	0.5
	F	2810[d]	2.9	2.2

C: conventional method as Moloney *et al.* described (1989), F: co-cultivation on filter paper
a: total of two experiments, b: total of three experiments, c: total of five experiments, d: total of eleven experiments

pre-cultured explants were used, as shown in the Table 3.3, a higher number of GUS-positive shoots were recovered with the filter paper method (2.2%) compared to the conventional method (0.5%). The transformation frequencies were considerably improved by using the filter paper method with two day pre-cultured explants for the cultivar 'Green Valiant'. Further experiments were conducted based on these conditions.

3.3.2.2 Effect of cold treatment and *Agro*-infection method

Co-cultivation in a growth cabinet (25°C) did not give satisfactory transformation frequencies even by using the filter paper method. In an attempt to overcome this problem, co-cultivation was carried out at a reduced temperature. The rationale for using cold-treatment was based on the following ideas: (1) co-cultivation at low temperatures may inhibit over-growth of *Agrobacterium* thus resulting in less damage to regenerable tissues; (2) low temperatures may inhibit some of the plant's defense mechanisms such as a restriction system against foreign DNA and thus improve the chances of integration for the introduced foreign DNA into plant genome and its expression. In preliminary tests, broccoli seedlings were able to grow normally but slowly even at 4 °C without tissue damage. The temperature for cold-treatment was chosen as 10 °C. At this temperature, plants can still grow but *Agrobacterium* growth is inhibited.

Cotyledonary petiole explants from five-day-old seedlings of the cultivar 'Green Valiant' were co-cultivated either on filter paper or in the conventional way after a two day pre-culture treatment. Plates were placed in the growth cabinet for two days.

Table 3.4 Effect of co-cultivation method with cold-treatment on transformation of two-day pre-cultured cotyledonary petiole explants from five-day-old seedlings of the broccoli cultivar 'Green Valiant' and *B. napus* cv. 'Westar'

Co-cultivation	Number of explants	Explants with green shoots(%)	Explants with GUS(+) shoots(%)
C	580[a]	2.8	1.5
F	2080[b]	6.7	2.5 - 10.0
B. napus cv. 'Westar'	280	6.4	5.0

C: conventional method as described by Moloney *et al.* (1989)
F: co-cultivation on filter papers
a: total of two experiments,   b: total of nine experiments

then subjected to cold-treatment (10 °C) for two more days (Table 3.4). Transformation frequencies with a cold-treatment were consistently higher than previous experiments. In the case of the conventional transformation method, the frequency of GUS-positive shoots was about 1.5% which was higher than without the cold-treatment (0.5%, Table 3.3). When co-cultivations were carried out on filter paper, the frequency of GUS-positive shoots was about 5.2% which was also higher than without the cold-treatment (about 2.2%) (Table 3.3). There was no difference between cold-treatment and untreated groups in producing GUS-negative green shoots ('escapes'). To see if cold-treatment inhibits transformation of other brassicas, transformation of *B. napus* cv. 'Westar' was conducted in the same way as the 'Green Valiant'. This resulted in 5% GUS-positive shoots. Results obtained from this experiment suggest that there was no negative effect of cold-treatment on *Agrobacterium*-mediated transformation of *B. napus*.

To determine the optimum conditions for co-cultivation on filter paper, various cold-treatments were tested using two-day pre-cultured cotyledonary petiole explants from five-day-old seedlings of the cultivar 'Green Valiant'. The four-day cold-treatment (10 °C) produced more GUS-positive shoots (about 2.7%) than that of a two-day treatment (10 °C) (about 1.9%). The six-day treatment (10 °C) reduced the frequency of GUS-positive shoots. It is likely that long-term exposure to low-temperature could reduce genetic material (T-DNA) transfer events from *Agrobacterium* to plant cell. With this reverse sequence of incubation, even more GUS-positive green shoots (5.1%) were produced than normal sequence. It seemed that cold-treatments produced more GUS-positive green shoots when employed immediately after co-cultivation or after a

two-day incubation at 25 °C. Interestingly, a four-day treatment without incubation at 25 °C did not produce any green shoots (Table 3.5).

To determine the effect of further temperature reduction, a 4 °C treatment was conducted using an ordinary refrigerator. This provided a constant 4 °C (in darkness) environment instead of a pulsed temperature (10 °C/5 °C, 16h/8h) with a 16 hours photoperiod. A different method of *Agro*-infection was tested in order to simplify the infection procedure. An overnight-grown bacterial culture (2 mL) and 38 mL of autoclaved distilled water were added to a 100x25mm sterile petri-plate thereby diluting the bacterial concentration by 20 fold. Two-day pre-cultured cotyledonary petiole explants were immersed in this solution for five minutes and then transferred to filter paper as in the other experiments. This type of *Agro*-infection procedure required significantly less time in comparison to dipping of individual explant in the bacterial solution. In this case, co-cultivation was done for two days in a growth cabinet, followed by four more days in refrigerator, based on results from Table 3.5. The 4 °C-treated group showed about 3% GUS-positive green shoots while 10 °C treated group showed a 2.2% response (Table 3.6). It is evident that 4 °C is effective for cold-treatment and light is not strictly required for this treatment. In the case of the simplified procedure of *Agro*-infection, the appearance of the explants after co-cultivation were similar to that of other experiments. Even with a 20 fold dilution of the bacterial concentration, frequencies of GUS-positive green shoots after selection were comparable. These results suggest that the simplified *Agro*-infection procedure can be employed for *B. oleracea* transformation.

Table 3.5 Effect of various cold-treatments during co-cultivation on transformation of pre-cultured cotyledonary petiole explants from five-day-old seedlings of the broccoli cultivar 'Green Valiant' by co-cultivation on filter paper.

Co-cultivation condition[a]	Number of explants[b]	Explants with green shoots (%)	Explants with GUS(+) shoots (%)
25 °C - 2 days/10 °C - **2 days**			
	560	3.3	1.9
25 °C - 2 days/10 °C - **4 days**			
	560	2.9	2.7
25 °C - 2 days/10 °C - **6 days**			
	530	1.3	0.7
10 °C - 4 days			
	560	0	0
10 °C - 2 days/25 °C - 2 days [c]			
	560	6	5.1

a: 25 °C represents growth cabinet conditions while 10 °C represents cold chamber conditions with 10 °C/5 °C (16h/8h), b: total of two experiments, c: incubation in growth cabinet and cold chamber are in reverse sequence.

Table 3.6 Effect of two different low-temperatures during co-cultivation of two-day pre-cultured cotyledonary petiole explants from five-day-old seedlings of the broccoli cultivar 'Green Valiant' on filter paper[a]

Temperature[b]	Number of explants	Explants with green shoots (%)	Explants with GUS(+) shoots (%)
25 °C - 2 days/10 °C - 4 days			
	550	3.6	2.2
25 °C - 2 days/4 °C - 4 days			
	600	4.2	3

a: explants were immersed in bacterial solution instead of single explant dipping for *Agro*-infection. b: 25 °C represents under growth cabinet condition, 10 °C represents under cold chamber condition with 10 °C/5 °C (16h/8h) and 4 °C represents ordinary refrigerator conditions.

3.3.2.3 Effect of explant position during co-cultivation

During co-cultivation, the petiole tips of the cotyledonary explant were placed in contact with the filter paper surface but sometimes their position was accidentally reversed by handling/moving of plates (see Figure 3.4). It was observed that after four days of co-cultivation (two days in a growth cabinet followed by two days in a cold chamber), petiole tips that were in contact with the filter paper surface showed more hypersensitivity (turn blacked and necrotic) while explants in the reverse position had healthy-looking swollen petiole tips (Figure 3.4).

3.3.2.4 Effect of *Agrobacterium* strains

To determine the most effective *Agrobacterium* strain for broccoli transformation, three strains were co-cultivated with two day pre-cultured cotyledonary petiole explants on filter paper (Table 3.7). All strains harbored the same binary vector pRD320. Infections with each strain separately and with combinations of two and three strains were investigated. In the case of mixed infections, equal amounts of each strain were mixed to prepare the bacterial solution.

In the case of strain LBA4404, the conventional transformation method without cold-treatment gave 1.3% GUS-positive green shoots after selection while the filter paper method gave about 0.4%. In this case, the filter paper method was not as good as the conventional method although earlier experiments with GV3101 gave better results with the filter paper method (Table 3.3). However, in both cases, the frequency of production of GUS-positive green shoots with LBA4404 was lower than GV3101.
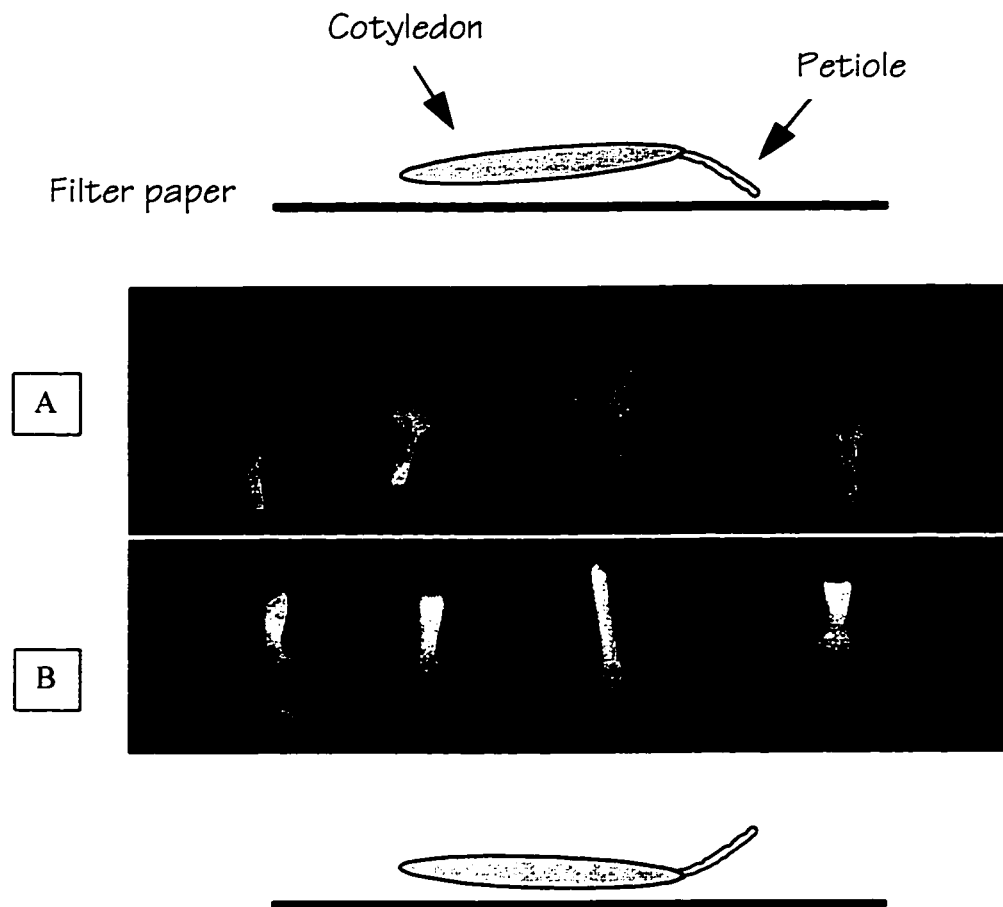
Figure 3.4 Two types of position effects for cotyledonary petiole on filter paper after co-cultivation. Two days treatment in growth cabinet at 25 °C followed by four days in refrigerator at 4 °C.

A: Petioles in contact with the surface of the filter paper   B: Petioles away from the filter paper

Table 3.7 Effect of various *Agrobacterium* strains on transformation of two-day pre-cultured cotyledonary petiole explants from five-day-old seedlings of the broccoli cultivar 'Green Valiant'

Agrobacterium host/ disarmed Ti-plasmid	Number of explants	Explants with green shoots (%)	Explants with GUS(+) shoots (%)
Co-cultivation for two days in a growth cabinet (25 °C)			
LBA4404/pAL4404			
C	540[a]	2.5	1.3
F	940[b]	1	0.4
Co-cultivation for two days in a growth cabinet (25 °C) followed by 2 days in a cold chamber (10 °C)[c]			
GV3101/pMP90	170	7.2	5.3
LBA4404/pAL4404	360	0.8	0.2
A281/pEHA105	200	0	0
Mixed infection			
G+L	240	8.3	5.4
G+A	200	10	8
G+L+A	160	7.5	5.6

C: conventional method as Moloney *et al.* described (1989)
F: co-cultivation on filter paper
a: total of two experiments, b: total of four experiments, c: co-cultivation on filter paper

Infection with each of the strains independently and in combination were carried out using a cold-treatment (Table 3.7). Co-cultivation was done in a growth cabinet for two days, followed by two more days in a cold chamber (10 °C). Amongst single infection experiments, GV3101 was the best strain producing 5.3% GUS-positive green shoots. A281, known as a super virulent strain, did not produce any green shoots. In the case of mixed strain infections, the presence of GV3101 improved the frequency of production of GUS-positive green shoots compared to single infections with either LBA4404 or A281. GV3101 was the most effective for broccoli transformation while the other strains were less effective.

3.3.3 Speed transformation method

The results obtained from regeneration, *Agro*-infection, co-cultivation and selection experiments were integrated to develop an efficient and reliable transformation method. The relevant features of this method are listed as follows: (1) *Agro*-infection was carried out by mass immersion of explants in the bacterial solution instead of dipping individual explants, this saved both time and experimental resources, while still reasonable frequencies (2.2-3%) of GUS-positive green shoots were produced after selection (Table 3.6), (2) an ordinary lab refrigerator (4 °C) was used for cold-treatment giving reasonable frequencies (about 3%) of GUS-positive green shoots after selection (Table 3.6), (3) a source of light was not required during cold-treatment (Table 3.6), (4) it was not necessary for petiole tips to be in contact with the medium during co-cultivation (Figure 3.4A). This transformation procedure also involved the use of fresh

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cotyledonary petiole explants instead of pre-cultured explants. A co-cultivation medium (Base solution) was designed for these experiments (Table 3.1). Plates were protected from light source throughout the whole co-cultivation period by wrapping with aluminum foil. Fifty mg/L L-ppt was used in the rooting medium to remove possible escapes.

3.3.3.1 Effect of *B. oleracea* var. *italica* cultivars

Broccoli cultivars 'Green Valiant', 'Packman', 'First Choice', 'Shogun'. 'Cruiser' and breeding line 'UL' were used in the transformation studies (Table 3.8). The transformation frequencies were cultivar dependent. The number of GUS-positive green shoots produced varied among cultivars (ranged in 0.7-4.5%). In the case of 'UL' and rapid cycling *B. oleracea*, an extreme hypersensitive response was observed. After co-cultivation, most of the explants quickly turned black and finally died but one putative transgenic line was recovered from each cultivar and transferred to soil in the greenhouse.

3.3.3.2 Effect of *B. oleracea* varieties

Cauliflower, cabbage, kale, kohlrabi and Brussels sprout were evaluated with the speed transformation method (Table 3.8). A few cultivars of each variety were also evaluated. The transformation frequencies were variety- and cultivar-dependent. These varieties produced different frequencies of GUS positive green shoots (range of 0.7-10%). In the case of cauliflower, the cultivars 'Cashmere', 'Snow Crown' and 'Yukon' showed high frequencies of GUS-positive shoot production in the range of 6.6-10%. But

Table 3.8 Transformation frequencies of *B. oleracea* varieties and *B. napus* cultivars by the speed transformation method

Variety/cultivar	Number of explants	Explants with green shoots(%)	Explants with GUS(+) shoots(%)
Broccoli (*B. oleracea* var. *italica*)			
Green Valiant	400	6.2	4.5
Packman	400	1.2	0.7
First Choice	400	1.7	1.5
Shogun	200	4.5	2.5
Cruiser	340	1.7	1.5
UL	7758		1 line
Rapid cycling	3120		1 line
Cauliflower (*B. oleracea* var. *botrytis*)			
White Summer	320	2.2	1.2
Cashmere	240	16.6	10
Snow Crown	400	17.5	9.7
White Top	400	14.2	1.2
White Fox	270	4.8	1.5
Yukon	180	8.3	6.6
Cabbage (*B. oleracea* var. *capitata*)			
Ruby Ball	310	3.2	2.6
Kale (*B. oleracea* var. *acephala*)			
Squire	650	9.3	6
Green Curled Scotch	220	3.2	1.3
Kohlrabi (*B. oleracea* var. *gongylodes*)			
Early White Vienna	480	1.8	0.8
Grand Duke	140	2.1	0.7
Brussels sprout (*B. oleracea* var. *gemmifera*)			
Pearl	230	3.0	2.6
B. napus			
Westar	400	4.5	3.2
Topaz	4500		1 line
Cyclone	980		1 line

the cultivars 'White Summer', 'White Top' and 'White Fox' produced low frequencies in the range of 1.2-1.5%. Among *B. oleracea* varieties, kohlrabi (cultivars 'Early White Vienna' and 'Grand Duke') was relatively difficult to transform (range of 0.7-0.8%).

3.3.3.3 Effect of *B. napus* cultivars

B. napus cv. 'Westar' (most commonly used in *B. napus* transformation) and the cultivars 'Topaz' and 'Cyclone' (considered to be cultivars recalcitrant to transformation) were evaluated with the speed transformation method (Table 3.8). The cultivar 'Westar' produced more than 3% GUS-positive green shoots. In the case of the cultivars 'Topaz' and 'Cyclone', they showed an extreme hypersensitive response. After co-cultivation, most of explants turned black and finally died but one putative transgenic line from each cultivar was recovered and transferred to soil in the greenhouse.

3.3.3.4 Effect of vacuum infiltration

Fresh cotyledonary petiole explants from five-day-old seedlings of broccoli (*B. oleracea* var. *italica*) cultivars 'Cruiser' and 'Packman' and *B. napus* cultivar 'Westar' were used for this experiment. After selection, vacuum treated groups showed better results than controls. In the case of the broccoli cultivar 'Cruiser', vacuum treatment gave 3.2% GUS-positive green shoots while the control yielded 1.6% GUS-positive green shoots. In case of *B. napus* 'Westar', vacuum treatment yielded 4.8% GUS-positive green shoots while the frequency in the control was 3.2% (Table 3.9). Although

vacuum infiltration was effective in transformation, fungal contamination was a major problem with this treatment. Many experiments were discarded because of this.

Co-cultivation at 4 °C was attempted with/without vacuum infiltration. Fresh-cut cotyledonary petiole explants (360 explants with vacuum, 290 explants without vacuum) from five-day-old *B. napus* cultivar 'Westar' were used for *Agro*-infection and then co-cultivated at 4 °C for six days without growth chamber incubation. Green shoots were not observed in either group. These results were similar to the results shown in Table 3.5 in which a four day co-cultivation in a cold chamber at 10 °C also blocked the production of green shoots.

3.3.4 Transformation of hypocotyl explants

Hypocotyl explants from five-day-old *in vitro* grown seedlings of the broccoli cultivar 'Green Valiant' were co-cultivated by both the filter paper (1440 explants) and speed transformation (760 explants) method. After co-cultivation, most of the explants turned black and died showing a strong hypersensitive response. GUS-positive green shoots were not obtained in either experiment.

3.3.5 Elongation of putative transformed shoots

Some of the green shoots that were recovered from the selection medium were vitrified. Both vitrified and normal shoots were assayed for GUS activity; normal shoots were transferred to the new selection medium for shoot recovery and shoot elongation. Shoots elongated well in the new selection medium. Growth regulator adjustment was not required for shoot elongation.

Table 3.9 Effect of vacuum infiltration during speed transformation method on two broccoli cultivars and one *B. napus* cultivar

Vacuum	Number of explants[a]	Explants with green shoots (%)	Explants with GUS(+) shoots (%)
B. oleracea var. italica cv. 'Cruiser'			
NO	520	1.9	1.6
YES	360	5.3	3.2
B. oleracea var. italica cv. 'Packman'			
NO	620	1.4	0.3
YES	360	4.8	2.7
B. napus cv. 'Westar'			
NO	400	4.5	3.2
YES	360	6.1	4.8

a: total of two experiments

3.3.6 Rooting of putative transformed shoots

Elongated shoots (1-2 cm) obtained after transformation were transferred to a growth regulator free rooting medium with 500mg/L carbenicillin and 125mg/L L-ppt. Most of the shoots rooted after a month. Selection was much tighter in the presence of 125mg/L L-ppt than in 20mg/L kanamycin (Figure 3.5A,B). After a week, untransformed 'escapes' turned yellow and died. No escapes were observed in 125mg/L L-ppt. The level of tolerance to L-ppt was dependent on each transgenic line. Some transgenic lines survived for several months in 500mg/L L-ppt while other lines died. Figure 3.5 shows that the transgenic shoot TCR8 survived for several months on 500 mg/L L-ppt while TCR6 died in a week. Rooted shoots were transferred to soil in the greenhouse (Figure 3.6). In some cases, the transgenic plants of broccoli had altered floral structures which resulted in sterility. The most common abnormality was flowers with shortened styles and retarded anther development.

3.3.7 Analysis of putative transformed plants

Analyses were done on ten putative transgenic plants obtained by kanamycin and phosphinothricin selection from the broccoli cultivar 'Cruiser'. These included histochemical GUS staining (Figure 3.7), PCR analysis of *pat* and *npt*II genes (Figure 3.8A,B), Southern analysis (Figure 3.8C), NPTII and PAT enzyme assays (Figure 3.9 A,B) and inheritance of foreign genes in R_1 progeny (Table 3.10).
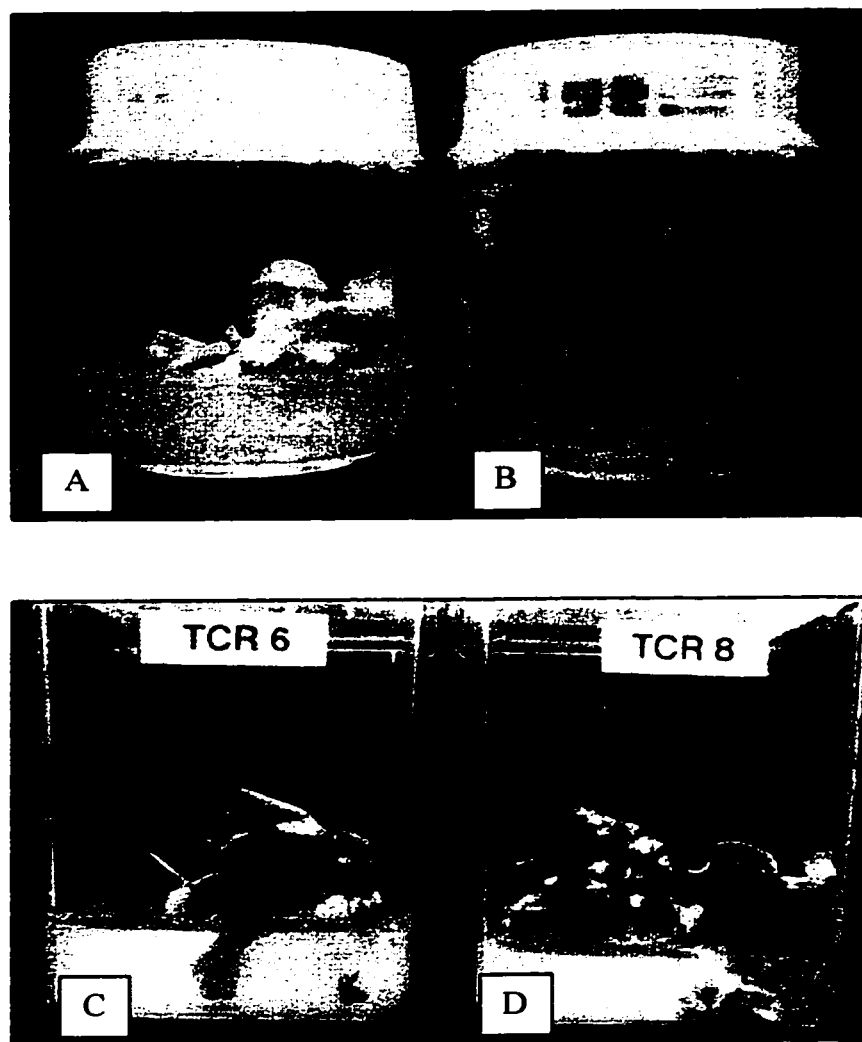
Figure 3.5 Putative transgenic lines of the broccoli cultivar 'Cruiser' in rooting media containing L-ppt.

A: Control plant on 125 mg/L L-ppt rooting medium, B: Putative transgenic line TCR6 on 125 mg/L L-ppt rooting medium, C: Putative transgenic line TCR6 on 500 mg/L L-ppt rooting medium, D: Putative transgenic line TCR8 on 500 mg/L L-ppt rooting medium.
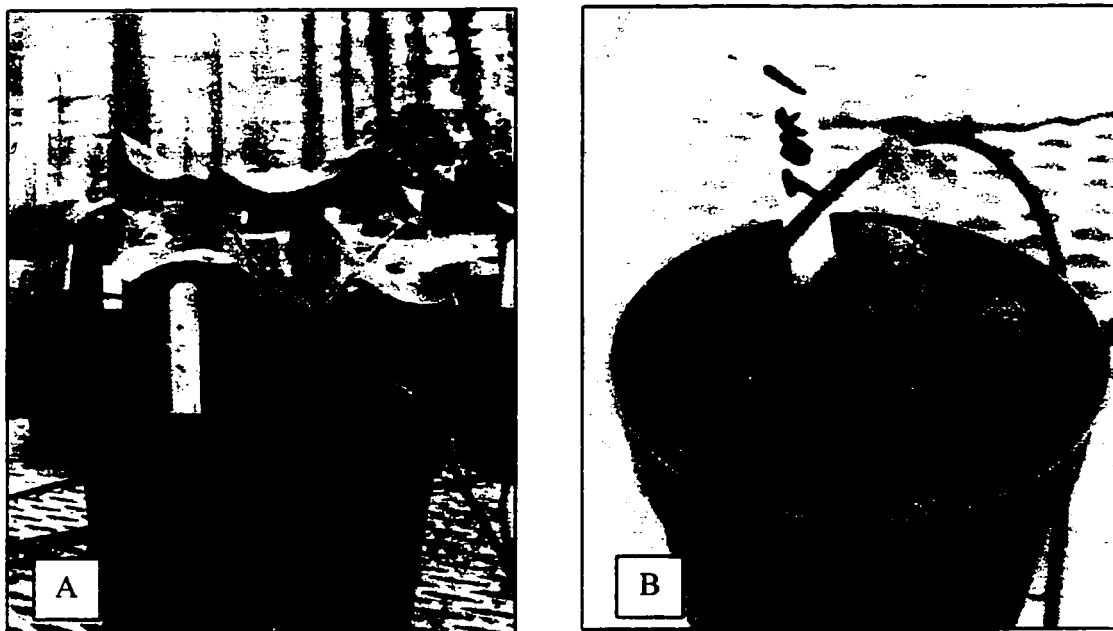
Figure 3.6 Transgenic plants growing in the greenhouse.

Transgenic lines were potted in soil after 2-3 months from co-cultivation. A: Broccoli cultivar 'Cruiser', B: Broccoli breeding line 'UL'

Figure 3.7 GUS histochemical staining of leaf disks of putative transgenic lines of *B. oleracea* var. *italica* cv. 'Cruiser'. Numbers 1-10 stand for transgenic lines TCR1-10; C: control.

Molecular analyses

In the case of PCR analyses, an 800bp fragment and a 400bp fragment were produced with the *npt*II gene and *pat* gene primers respectively (Figure 3.8A,B). All transgenic lines evaluated (TCR1-10) showed these bands consistently.

In the case of Southern hybridization analyses, all transgenic lines (TCR1-10) showed single insertions of various sizes (Figure 3.8C). This suggests that the *pat* gene is integrated into plant genome.

In the case of NPTII enzyme assays, strong signals were obtained in all transgenic lines (TCR1-10), whereas no signal appeared in the control plant (Figure 3.9A). To check the specificity of the dot signal, negative reactions were set up with crude protein extract of transgenic lines without kanamycin in the mixture. No such strength of signals was obtained in the negative reaction.

In the case of PAT enzyme assays, all transgenic lines showed PAT activities (Figure 3.9B). Although the signal was weak, all these plants clearly exhibited strong tolerance to phosphinothricin at the rooting stage.

Inheritance of GUS activity in R_1 progeny

Of 20 transgenic lines tested for segregation analysis, 15 lines showed a 3:1 ratio suggesting a single insertion, one line showed a 15:1 ratio suggesting two insertions and four lines showed an unusual segregation ratio of 2:1 (Table 3.10).
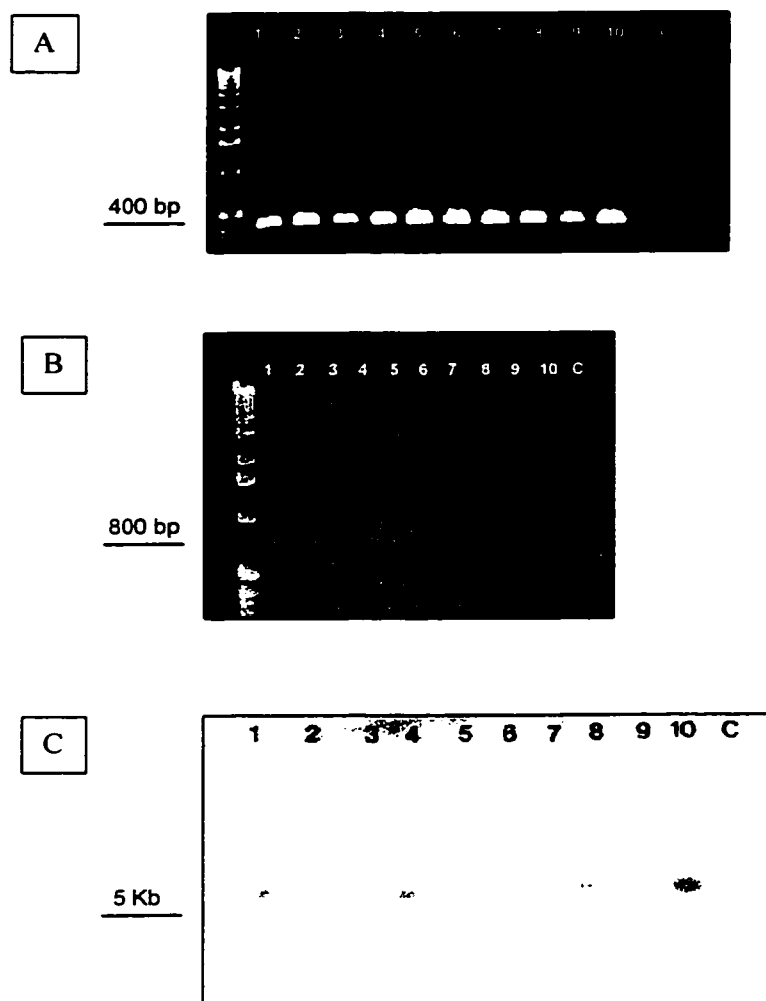
Figure 3.8   Molecular analyses of putative transgenic lines from *B. oleracea* var. *italica* cv. Cruiser.

Numbers 1-10 stand for transgenic lines TCR1-10 while the lane C for control. A: PCR detection of *pat* gene. B: PCR detection of *npt*II gene. C: Southern detection of *pat* gene.
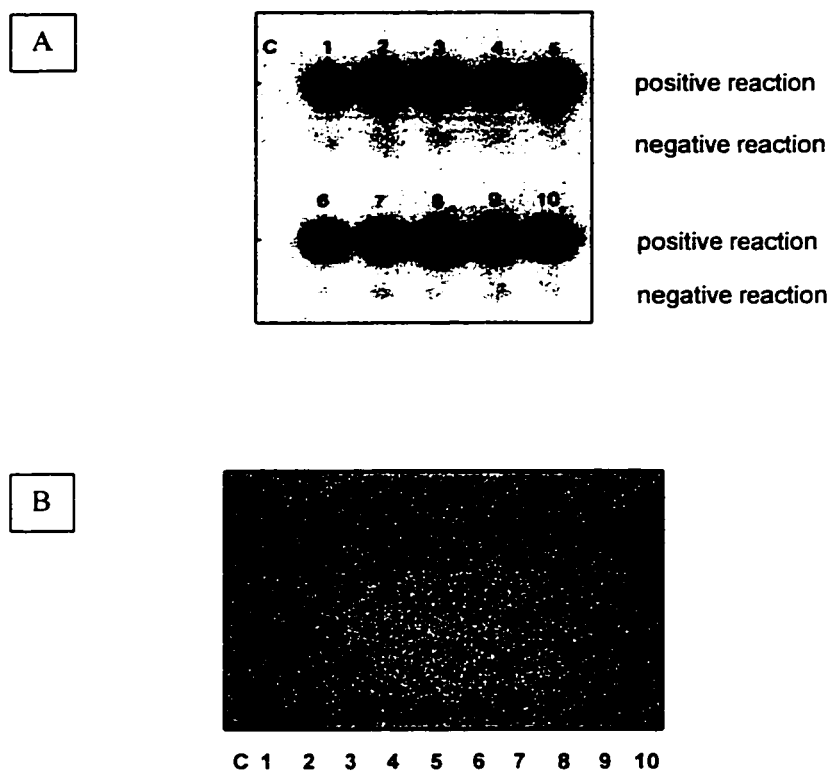
Figure 3.9   NPTII and PAT enzyme assays of putative transgenic lines from *B. oleracea* var. *italica* cv. 'Cruiser'.

Numbers 1-10 stand for transgenic lines TCR1-10 while C for control. A: Dot blot analysis of NPTII enzyme activities of TCR1-10. Rows labeled positive reaction included kanamycin as a substrate while rows labeled negative reaction did not. B: Thin layer chromatography of PAT enzyme activities of TCR1-10. All lines show PAT enzyme activities although weak. The position of acetylated phosphinothricin is marked by the arrow under the strong signals. Strong signals represent unincorporated acetyl CoA.

Table 3.10 Segregations of the GUS activity in progeny (R_1) from transgenic lines of the broccoli cultivar 'Cruiser'

Lines	Tested Seed No.	GUS(+)	GUS(-)	Ratio	χ^2-value[a]
TCR #1	67	53	14	3:1	0.7313
TCR #2	135	88	47	2:1	0.1194
TCR #3	63	53	10	3:1	3.0634
TCR #4	61	48	13	3:1	0.5628
TCR #5	373	247	126	2:1	0.0298
TCR #6	182	136	46	3:1	0.0073
TCR #8	106	95	11	15:1	2.5066
TCR #9	115	87	28	3:1	0.0550
TCR #10	173	128	45	3:1	0.0751
TCR #11	208	153	55	3:1	0.1987
TCR #12	198	147	51	3:1	0.0471
TCR #14	209	134	75	2:1	0.5795
TCR #15	106	86	20	3:1	2.3018
TCR #17	253	184	69	3:1	0.6969
TCR #19	97	77	20	3:1	1.1237
TCR #21	166	124	42	3:1	0.0080
TCR #22	100	63	37	2:1	0.5612
TCR #23	150	112	38	3:1	0.0088
TCR #24	96	74	22	3:1	0.2916
TCR #25	159	118	41	3:1	0.0398

a: significant level was 5%

3.4 Discussion

Transformation in broccoli (*B. oleracea* var. *italica*) has been achieved using a range of explants including peduncle explants (Christey 1989; Christey and Earle 1989; Toriyama *et al.* 1991) and leaf tissues (Hosoki *et al.* 1991). However in most cases, transformation frequencies were lower than other *Brassica* species such *as B. napus* (Radke *et al.* 1988; Moloney *et al.* 1989; DeBlock *et al.* 1989) and *B. carinata* (Babic 1994). Christey and Earle (1989) obtained only one transgenic plant from their study with the cultivar 'Green Comet'; while Toriyama *et al.* (1991) obtained a total of seven transgenic plants from the cultivar 'Green Comet'. Hosoki *et al.* (1991) produced 13 transgenic lines from the cultivar 'Early De Cico' using *A. rhizogenes*-mediated transformation but three out of the 13 lines had an abnormal appearance as is often found in plants transformed with the Ri plasmid (Tepfer 1984). During the preparation of this thesis, a procedure for *A. tumefaciens*-mediated transformation of broccoli with high frequencies was reported using peduncle (5.1% transformation frequency), petiole (1.8%) and hypocotyl (9.8%) explants (Metz *et al.* 1995).

In this study most transformation experiments were conducted with the broccoli cultivars 'Cruiser' and 'Green Valiant' as these cultivars regenerated shoots at a high frequency from hypocotyl and cotyledonary petiole explants (see Chapter 2). Three different transformation methods were used to obtain transgenic broccoli. Tested parameters included explant type (hypocotyl and cotyledonary petiole), use of filter paper during co-cultivation, pre-culture of explants, co-cultivation with various *Agrobacterium* strains, different *Agro*-infection methods, vacuum infiltration during

Agro-infection and co-cultivation at reduced temperatures. Only those parameters producing significant results were described and finally an efficient and simple transformation protocol has been established through those results. Transformation of a number of cultivars and varieties of *B. oleracea* and cultivars of *B. napus* has been successfully demonstrated in this study.

In order to recover transgenic shoots, it is necessary to achieve efficient regeneration from the transformed target cells within the explants. In *B. napus* and *B. carinata*, meristematic activity of vascular parenchyma cells was present at the cut surface of petioles (Moloney *et al.* 1989; Babic 1994). In broccoli, shoots developed directly from the explant and from callus at the cut surface of cotyledonary petioles. To obtain transformed shoots directly from the explants, it is essential that the totipotent vascular parenchyma cells be competent for transformation, and that *Agrobacterium* cells have access to bind to these cells (Hachey *et al.* 1991; Mukhopadhyay *et al.* 1992). In broccoli, in the absence of a selection agent, 2-12 shoots were produced from callus at the cut surface of cotyledonary petioles.

Hypocotyl and cotyledonary petiole explants from *in vitro* grown five-day-old seedlings were used for transformation. Unlike *B. napus* (Radke *et al.* 1988; DeBlock *et al.* 1989), *B. oleracea* var. *botrytis* (cauliflower) (DeBlock *et al.* 1989; David and Tempe 1988) *B. oleracea* var. *italica* (broccoli) (Metz *et al.* 1995) and *B. oleracea* var. *capitata* (cabbage) (Metz *et al.* 1995), transformation with hypocotyl explants was not successful with broccoli in this study. This may be due to the cultivar-dependent phenomenon of transformation (refer to results section in this study). Although Metz *et al.* (1995)

reported a high frequency transformation with hypocotyl explants (9.8%), the target material was limited to only one cultivar ('Green Comet'). In other studies on the cultivar 'Green Comet', peduncle explants were used for transformation and a low frequency of transformation was obtained (Christey 1989; Christey and Earle 1989; Toriyama *et al.* 1991). In comparison to earlier studies, a relatively high frequency of transformation in broccoli was obtained in this study using cotyledonary petiole explants from the cultivar 'Green Valiant' (2.2-10%) as well as the cultivars 'Packman' (0.7%), 'First Choice' (1.5%), 'Shogun' (2.5%), 'Cruiser' (1.5%) and the breeding line 'UL' (1 total), whereas Metz *et al.* (1995) obtained a 1.8% transformation frequency with cotyledonary petiole explants in the cultivar 'Green Comet'.

In many cases $AgNO_3$ has been the preferred agent for improvement of regeneration thus encouraging recovery of transformed shoots in brassicas (DeBlock *et al.* 1989; Mehrapalta *et al.* 1991; Mukopdhyay *et al.* 1992; Babic 1994). Radke *et al.* (1992) found that using $AgNO_3$ in the selection medium did not help transformation of hypocotyl explants of *B. napus*, but did for *B. rapa*, while Metz *et al.* (1995) reported that $AgNO_3$ inhibited shoot regeneration in *B. oleracea* both from control and in transformation experiments. In this study, all transformed shoots were obtained in the absence of $AgNO_3$ and so the presence of this compound does not appear to be a critical condition for broccoli transformation. Metz *et al.* (1995) reported that the use of a tobacco feeder cell-layer was very critical during co-cultivation with *Agrobacterium*. Without the feeder cell-layer, transformation frequencies decreased 10-100 fold, from 5.1% to 0.4% with peduncle explants; from 1.8% to less than 0.1% with petiole explants

and from 9.8% to less than 0.1% with hypocotyl explants. However, good transformation frequencies were obtained without using a feeder cell-layer in this study. Thus a feeder cell-layer is either not as critical as reported by Metz et al. (1995) or its requirement may be cultivar dependent.

One of the other important factors that contributed to a high transformation frequency with broccoli was related to the control of the hypersensitive response of explants to *Agrobacterium* during co-cultivation. In other studies pre-culture and co-cultivation of explants on filter paper were employed to overcome this problem (Dong and McHughen 1991; McHughen 1992; Babic 1994). In this study, co-cultivation on filter paper increased the transformation frequencies from about 0.5% to 2.2% when two day pre-cultured cotyledonary petiole explants were used. Interestingly, filter paper was not very effective when fresh-cut explants were used without pre-culture (transformation frequencies in the range of 0.4 - 0.45%). Furthermore, pre-culture treatment alone did not seem to affect transformation frequencies (0.4% vs. 0.46%). These results are similar to those of Metz *et al.* (1995). They did not obtain transformants when fresh-cut explants were used. In contrast, a pre-culture treatment was not required for peduncle explants (Metz *et al.* 1995). These results suggest that factors that contribute to the hypersensitive response are important in determining the efficiency of transformation.

The chemical 5-azacytidine is a DNA demethylating agent. When incorporated into DNA in place of cytidine, it irreversibly binds DNA methylase, resulting in overall cellular demethylation (Jones 1984). Several studies have suggested a correlation

between DNA demethylation and activation of silent genes after treatment with 5-azacytidine (Shao *et al.* 1995). For example, increased transgene expression was observed in barley (Schulze *et al.* 1991) and tobacco (Palmgren *et al.* 1993). In spite of its potential, as it is a mutagenic toxic compound (Jones 1995), its practical applications are limited. In this study, a cold-treatment was designed to substitute for a chemical treatment such as 5-azacytidine. During the co-cultivation period, the plates were incubated in a cold chamber at 10 °C/5 °C (16h/8h) for 2-4 days after incubation in a growth cabinet at 25 °C for two days. Cold-treatment increased transformation frequencies from 0.46% to 1.5% without filter paper and from 2.2% to 5.2% with filter paper when two day pre-cultured cotyledonary petiole explants were used. With different periods of cold-treatment, a four day cold-treatment was better than a two day treatment while a six day treatment decreased frequencies. A four day incubation in a cold chamber without growth cabinet culture at 25 °C did not stimulate green shoot formation while a two day cold-treatment, followed by a two day incubation in a growth cabinet gave rise to a 5.1% transformation frequency. Failure of shoot formation in this treatment may not be related to transfer of T-DNA but rather to the unfavorable regeneration conditions. Even if transformed shoots were not produced, untransformed escapes should have been produced as these were obtained in all other experiments at a low frequency. Furthermore, in the case of reverse sequence of incubation (i.e. reduced temperature treatment first) transformants appeared at a higher frequency. This may suggest that shoot regeneration from an explant requires stimulation by optimal temperature for a certain period (two days in this study). The reverse sequence of

temperature treatments produced almost three times the frequency of transformation than the usual sequence. It is possible that the cold-treatment stimulated regenerability of explants. Increased pollen embryogenesis in *B. juncea* by low-temperature pre-treatment before a high-temperature stress has been reported (George and Rao 1982). Other studies have also shown increased organogenesis from cold-pretreated explants *in vivo* (Nimi 1978). In some cases *in vitro* cold treatments satisfy dormancy requirements seen *in vivo* (Lazzeri and Dunwell 1986b). It thus appears that cold-treatment may improve the susceptibility of the explant to *Agro*-infection and regenerability of transformed cells as well as repressing the plant's methylation system for better expression of transgenes in the transformed cells. Cold-treatment in a refrigerator (4 °C) appeared to be as effective for transformation as treatment in a cold-chamber (10 °C/5 °C). The 4 °C treatment allows for easy adaptability by using an ordinary refrigerator.

In order to simplify the *Agro*-infection process, it was shown that immersing explants in the bacterial suspension resulted in transformation frequencies similar to those obtained by dipping individual explants into the bacterial solution. This modified *Agro*-infection method significantly reduced the working time during transformation thus this infection method has been employed in developing the speed transformation method.

During co-cultivation of petiole explants on filter paper, hypersensitivity of petiole tip tissues was observed. This resulted in tissue necrosis and reduced transformation efficiency. This phenomenon was rectified by placing the explant in a position in which the tips were turned away from the filter paper and pointing upwards.

Undamaged tissues may have better potential for normal regeneration of green shoots after co-cultivation. This observation suggests that it is not essential for explants to be in contact with the medium during co-cultivation. This information was useful in the development of the speed transformation method which does not involve the use of a solid medium.

Three different *Agrobacterium tumefaciens* strains (LBA4404/pAL4404, GV3101/pMP90, A281/pEHA105) were investigated to optimize the best strain for bacterial-host interaction. Each strain harbored a disarmed Ti plasmid and the same binary vector, pRD320. Previous studies have used host/disarmed Ti plasmid combinations EHA101/pEHA101 for *B. napus* (Radke *et al.* 1988; Moloney *et al.* 1989), GV3101/pMP90 for *B. napus*, *B. oleracea* var. *botrytis* (DeBlock *et al.* 1989) and *B. carinata* (Babic 1994) and LBA4404/pAL4404 for *B. oleracea* var. *italica* (Christey 1989). pEHA105, a disarmed version of pTiBo542 and considered to be "super-virulent" (Hood *et al.* 1986) did not produce any shoots in this study. Infection with LBA4404/pAL4404 resulted in lower frequencies of transformation than those obtained with GV3101/pMP90. Thus GV3101/pMP90 appears to be the most effective for the broccoli transformation.

An efficient, simple and cost-effective transformation protocol has been established by integrating results obtained from this study. Alt-Mörbe *et al.* (1989) have reported on the external factors required for inducing the *vir*G gene which is the major gene for T-DNA processing. The best conditions for *vir*G gene induction were; 2% sucrose, pH5.6 and 25 °C for incubation. Meanwhile, Radke *et al.* (1992) have reported

that pre-treatment of explants on 2,4-D had positive effects on transformation of *B. rapa*. The co-cultivation medium (Base solution) in this study was designed for the speed transformation method (Table 3.1).

For the speed transformation method to be successful it was important to maintain a reduced humidity during co-cultivation. DeBlock *et al.* (1989) and Metz *et al.* (1995) have reported similar phenomenon of this sealing effect during co-cultivation. But unlike this study, they used porous tape throughout whole period of selection. Sealing plates with Stretch'n Seal™ tightly during the selection period seemed to have no significant effect in this study. The other important modification in speed transformation method was use of a vacuum infiltration treatment. Vacuum infiltration was applied during *Agro*-infection with *B. napus* cv. 'Westar' and *B. oleracea* var. *italica* cultivars 'Cruiser' and 'Packman'. In all cases, vacuum infiltration gave a 1.5-8 times higher frequency of transformation. It is possible that the vacuum reduced the surface tension of the bacterial suspension and thus provided better contact between bacterial cells and plant tissues. But a problem associated with vacuum infiltration was a high level of contamination. Even if the vacuum container were surface sterilized with 70% ethanol and negative pressure was released on the clean bench, contamination problems often occurred. It would be beneficial to develop a safe vacuum treatment system that would eliminate the contamination problems, especially those caused by fungi.

The speed transformation method provides an efficient, simple and cost-effective transformation scheme which allows transformation of divergent cultivars from *B.*

oleracea varieties such as broccoli, cauliflower, kale, kohlrabi, cabbage and Brussels sprout. Even though the speed transformation method does not enhance the frequency of transformation, it can still be applied to transform recalcitrant species because it accommodates efficient handling of a large number of explants. This was achieved by integrating crucial factors and removing unnecessary steps thus saving experimental resources and working time (Figure 3.10). A diagrammatic comparison of three different transformation methods and their approximate requirements in terms of experimental resources and working time are presented in Figure 3.11 and Table 3.11. This speed transformation method involved the modification of several steps in transformation such as omitting explant pre-culture and clean-up steps to remove excess *Agrobacterium* after co-cultivation, while employing simplified *Agro*-infection and co-cultivation procedures (Figure 3.11). According to the estimates, the speed transformation method results in a 3-6 fold reduction in experimental resources and work time than other methods (Table 3.11). One of the big advantages of the speed transformation method is that it easily increases sample size, thereby facilitating the production of reasonable numbers of transgenic lines from target plants that respond poorly. This method may be effective with several other members of the *Brassica* evolutionary triangle (U 1935) as well as for rapid screening of several different constructs in a short period. This method may also be useful for promoter tagging experiments, which requires handling of large numbers of explants.

When gene transfer is attempted using explants, it is essential to have a selection scheme whereby transformed cells can be separated from untransformed cells. The *npt*II
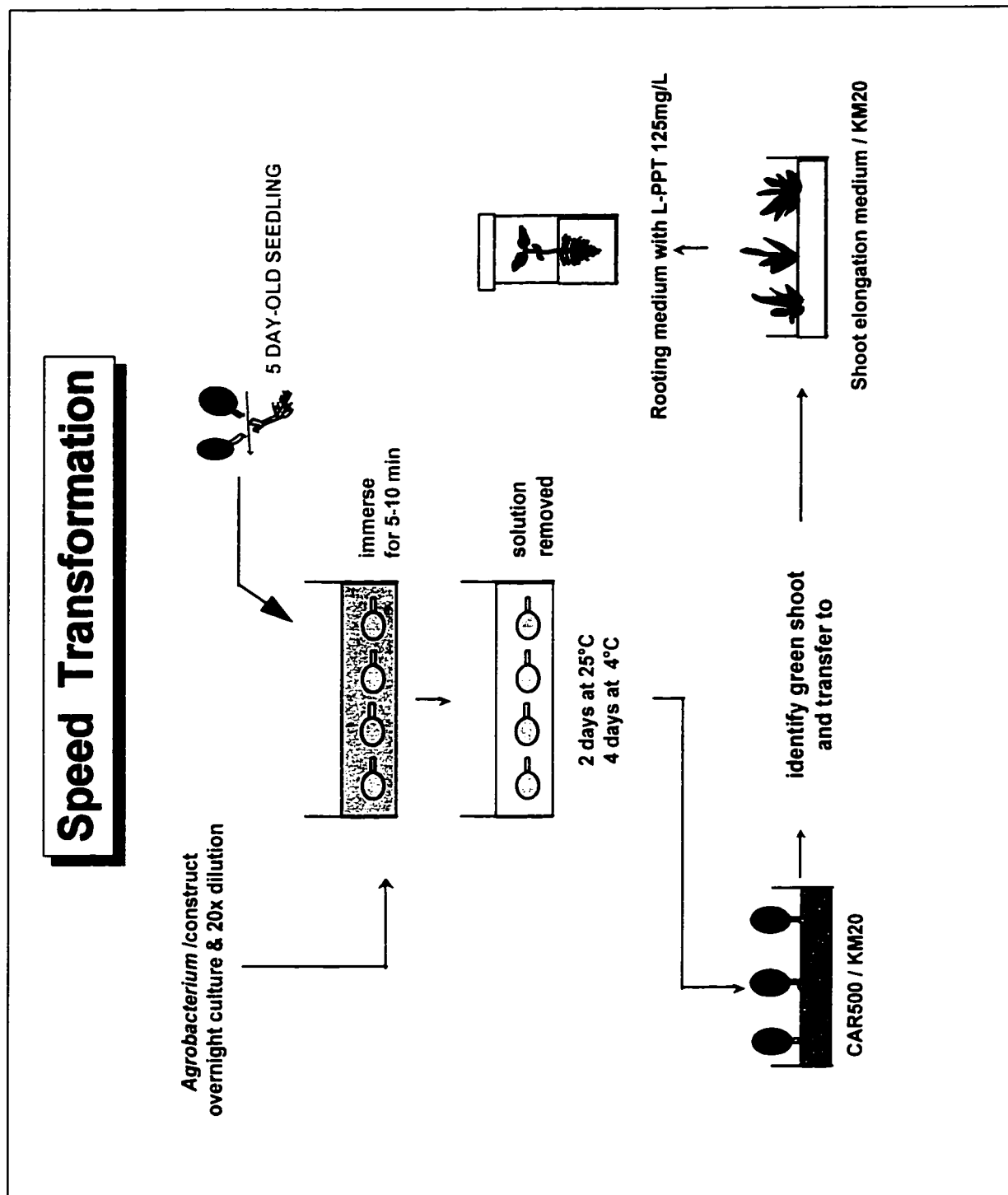
Figure 3.10 Flow diagram of the speed transformation method. See texts for details.
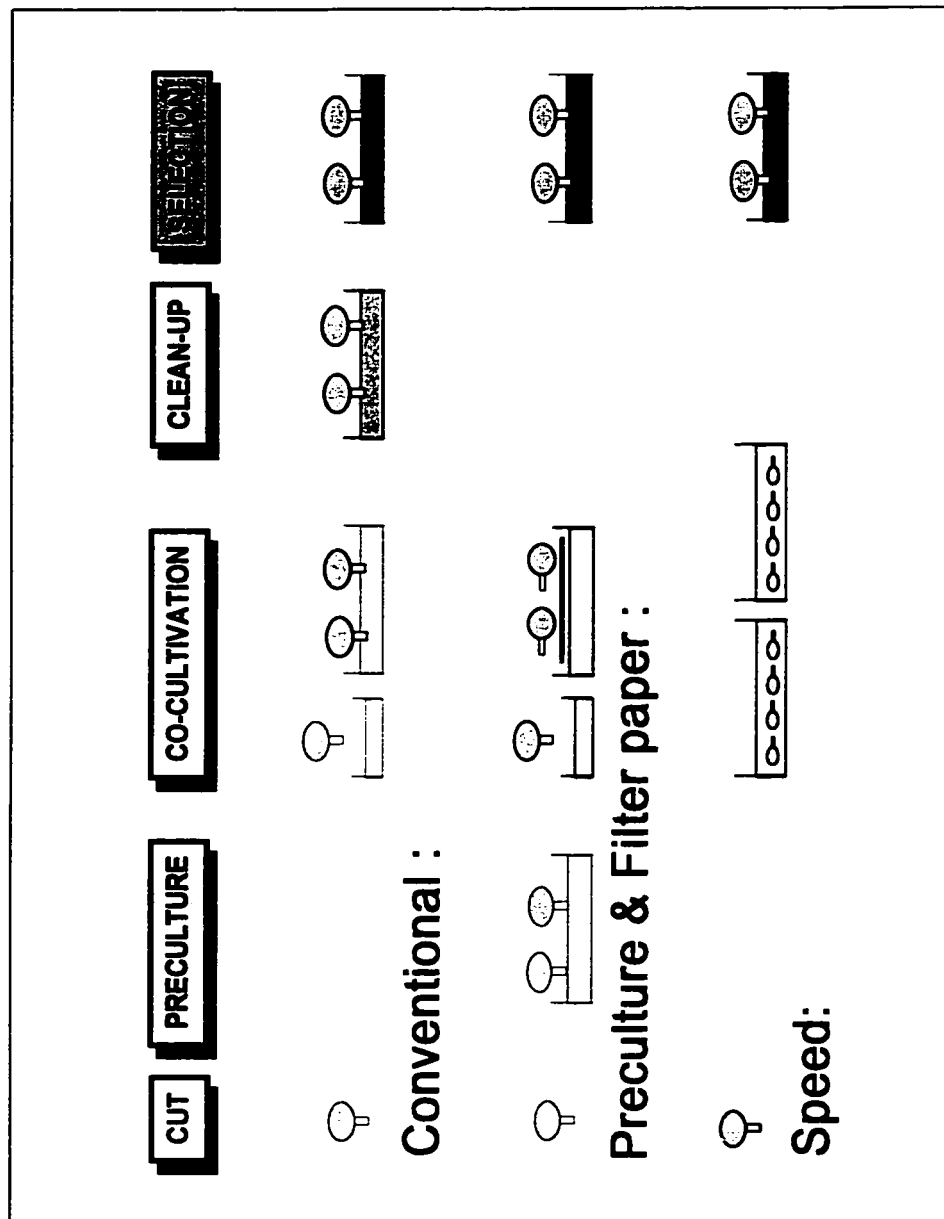
Figure 3.11   Schematic diagram of transformation methods used in this study. The speed transformation method skips many steps such as pre-culture of explants and clean-up compared to other transformation methods with almost same frequencies of transformation.

Table 3.11 Estimated experimental resources and time consumption 'plates (hour-work time)[a] ' for 1,000 cotyledonary petiole explants transformation of *B. napus* cv. Westar in various methods

Steps	Conventional	Pre-culture & filter paper	Speed
Pre-culture	0 (0)	100 (4)	0 (0)
Co-cultivation	100 (4)	50 (3.5)	3 (1)
Clean-up	100 (2.5)	0 (0)	0 (0)
Selection	100 (2.5)	100 (2.5)	50 (2)
Total	*300 (9)*	*250 (9.5)*	*53 (3)*
Transformation frequencies	1 - 10 %[b]	1 - 5 %[c]	1 - 5 %[c]

a: Time consumption only concerns explant handling, preparation of media is not involved. b: personal communications by other researchers; usually 1-3% in my experience (data not shown), c: in this study

gene has proven useful as a selectable marker in *Brassica* species (Radke *et al.* 1988; DeBlock *et al.* 1989; Barfield and Pua 1991; Narasimhulu *et al.* 1992; Mukhopadhyay *et al.* 1992; Gupta *et al.* 1993; Babic 1994) and was also effective for the *B. oleracea* var. *italica* transformation system developed in this study. Transformed calli and shoots remained green while untransformed tissues bleached on a kanamycin-containing medium. Some green shoots regenerated from a kanamycin containing selection medium subsequently tested negative for GUS activity. This has been observed in other studies (Radke *et al.* 1988; Babic 1994) as well. For tight selection, green shoots were selected on a kanamycin-containing medium, followed by rooting on a phosphinothricin-containing medium. As expected PAT-positive shoots remained green and most developed roots whereas PAT-negative shoots turned yellow and died in a week. No escapes were observed in this selection scheme.

Based on Southern analysis and segregation analysis in R_1 progeny by GUS staining, the majority of the transgenic lines represented single locus insertions in the present study contrasting with other *Brassica* transformation studies which have reported a high frequency of multiple insertions (*B. napus*, Fry *et al.* 1987; Moloney *et al.* 1989; DeBlock 1989; *B. juncea*, Barfield and Pua 1991). However Babic (1994) has reported 81% single insertions in a *B. carinata* transformation study. It is possible that insertion number may be related to the selection scheme, the levels of antibiotics used in experiments (discussed by Moloney *et al.* 1989) or recipient genotype. Although transgenic line TCR8 showed a 15:1 segregation ratio, Southern analysis showed a single band. This observation remains unexplained and requires further detailed

molecular analysis. An unusual type of GUS-positive: GUS-negative segregation in the order of 2:1 was found in the progeny of some transgenic lines (Table 3.10). This type of unusual segregation ratios was also found by other researchers with *Arabidopsis* (C. Koncz, Max-Planck-Institute, Germany; N. Chua, The Rockfeller University, USA; M. DeBlock, PGS, Belgium, personal communications), brassica (M. Moloney, University of Calgary, Canada, personal communication) and rice (N. Upadhyaya, CSIRO, Australia, personal communication). Although transgenic lines TCR2 and TCR5 showed the abnormal 2:1 segregation ratio, Southern blotting revealed the presence of single bands (Figure 3.8). As *B. oleracea* is a diploid species, it is possible that the foreign DNA was inserted into an essential plant gene, thereby dominant-homozygous lethality could have been occurred, then the remaining population of dominant-heterozygous and recessive lines exhibited a 2:1 segregation ratio in their R_1 seeds (results from this study and personal communications, C. Koncz, J. Schell, N. Chua, N. Upadhyaya, M. DeBlock and M. Moloney). Gene silencing by suppression or methylation of the foreign genes is also a possible explanation (Assaad *et al.* 1993; Meyer *et al.* 1992; Kooter *et al.* 1993; Yonder and Goldsbrough 1994; Jorgensen 1995).

In some cases, the transgenic plants of broccoli had altered floral structures which resulted in sterility. The most common abnormality was flowers with shortened styles and retarded anthers. Radke *et al.* (1988) have reported similar morphological alterations in *B. napus* at low frequencies. Alterations at a morphological level are expected due to tissue culture effects or the insertion of foreign DNA into the plant genome.

CHAPTER 4:

Promoter Tagging in *Brassica oleracea* var. *italica* and *Brassica napus* Through the Use of a Promoterless *gus::npt*II Fused Gene Construct

4.1. Introduction

One of the goals of plant molecular biology is to obtain a better understanding of the genetic control of the developmental processes that lead to pattern formation, cytodifferentiation and the responses of plants to growth substances and the environment (Topping and Lindsey 1995). Characterization of the genes involved in such processes is expected to play a significant role in the genetic improvement of crop species.

A traditional approach to the isolation of differentially expressed genes involves the use of cDNA libraries. Genes expressed in particular tissues or organs can be identified by hybridization to labeled cDNA probes representing mRNA pools of the different tissues (Maniatis *et al.* 1982). Another important method involves gene tagging by transposable genetic elements (Transposons) (Saedler and Nevers 1985). Transposons are randomly inserted in the genome, resulting in altered phenotypes. The transposon can then be used as a probe to facilitate the molecular cloning of the target sequences whether they are promoters or genes from the phenotypically altered target

material. By using plant transposons as mobile tag elements, it has been possible to isolate regulatory genes (Vollbrecht *et al.* 1991).

A powerful alternative method to transposon tagging is to use T-DNA as an efficient gene tag (Koncz *et al.* 1989). T-DNA tagging is based on the natural process of *Agrobacterium*-mediated transformation in which the single-stranded T-DNA is carried from the bacterium to a wounded dicotyledonous plant cell and inserted into the genomic DNA (Zambryski *et al.* 1988). When transferred to the plant genome, T-DNA has been shown to target frequently into transcriptionally-active sequences (Koncz *et al.* 1989; Herman *et al.* 1990). An advantage of T-DNA is that it can be manipulated inside its border sequences while maintaining its transfer functions. For example promoterless reporter genes which can be activated by a promoter in the target plant genome, have been engineered into T-DNA (Teeri *et al.* 1986; Koncz *et al.* 1989; Kertbundit *et al.* 1991; Topping *et al.* 1991,1994; Lindsey *et al.* 1993; Topping and Lindsey 1995). The gene *npt*II, which codes for neomycin phosphotransferase II (NPTII) and which gives resistance to aminoglycoside antibiotics, has been used in experiments to tag plant promoters. Since *npt*II is a selectable marker gene, it is possible to select directly for activation of the gene. Also the activity of the *npt*II gene can be enzymatically assayed in several ways. Another gene used for promoter tagging is *gus* (Fobert *et al.* 1991, 1994; Kertbundit *et al.* 1991; Lindsey *et al.* 1993; Topping *et al.* 1994), which has the advantage that it can be histochemically assayed to determine the developmental stage and tissue(s) in which a tagged promoter is expressing (Jefferson

1987; Jefferson *et al.* 1987). However the *gus* gene cannot be used as a selectable marker (Hodal *et al.* 1992).

An ideal tagging reporter system would have both of these properties. Datla *et al.* (1991) fused the *gus* and *npt*II genes in-frame and were thereby able to combine the properties of both enzymes. This fused gene codes for a protein which confers both kanamycin resistance and also produces a histochemically detectable GUS product. This gene has been inserted into an *Agrobacterium* T-DNA vector lacking a promoter which was used to generate random transcriptional gene fusions to plant genes *in vivo* (Babic *et al.* 1994). One of the major advantages of this fused gene system is that it can facilitate direct selection and recovery of potential promoter tagged transgenic plants. Additionally, as a component of the same transcriptional product, the GUS histochemical assay will assist in determining temporal and spatial expression properties of the tagged promoter. Most other promoter tagging studies have involved the use of a two-step strategy. In these studies transgenic plants were initially selected on the basis of NPTII activity regulated by the CaMV 35S promoter and then large populations of transgenic lines screened for GUS activity derived through the insertion of a promoterless *gus* gene (Koncz *et al.* 1989; Kertbundit *et al.* 1991; Lindsey *et al.* 1993).

The aim of this study was to evaluate the efficiency of a promoterless *gus::npt*II fused gene system for promoter tagging of *Brassica* species based on a direct selection/screening strategy. By transformation of *Brassica* species with the T-DNA vector, transcriptional *gus* fusions in transgenic plants were obtained by means of the GUS::NPTII system and different types of GUS expression patterns were observed.

4.2 Materials and Methods

4.2.1 Plant materials

'Rapid cycling' *Brassica oleracea*, broccoli (*B. oleracea* var. *italica*) cultivar 'Green Valiant', cauliflower (*B. oleracea* var. *botrytis*) cultivar 'Snow Crown' and *B. napus* cultivar 'Westar' were used in this study. Seeds of 'Snow Crown' were purchased from Stokes Seeds Ltd. and sources of the other materials are described in Chapters 2 and 3.

4.2.2 Culture media and explant preparation

The MSB5 (MS salts with B5 vitamins, Cat. No. M0404, Sigma, USA) medium was used as a basal medium. Modifications were made to this medium and other media used in this study and the details are summarized in Table 4.1. Preparation of cotyledonary petiole explants for *Agrobacterium*-mediated transformation was as described in Chapters 2 and 3.

4.2.3 Bacterial strains and gene construct

For transformation, *Agrobacterium tumefaciens* strain GV3101/pMP90 (Koncz and Schell 1986) harboring the promoterless binary vector pΔGUS::NPTII was used. The promoterless binary vector pΔGUS::NPTII was kindly provided by Dr. R. Datla (National Research Council Canada, Saskatoon, SK, Canada S7N 0W9). This promoterless construct contains a chimeric *gus::npt*II gene (Datla *et al.* 1991) and nopaline synthase terminator in modified pBin19 (Bevan 1984) with synthetic T-DNA

Table 4.1 Media used in brassica transformation

Medium	Modifications as compared to the basal medium
Basal	MSB5 (MS salts with B5 vitamins) Cat. No. M0404, Sigma, USA
Seed germination	Half strength basal medium 1% sucrose, 0.6% agar
Base solution	Basal medium with 0.5 g/L MES, 1 mg/L 2,4-D 2% sucrose, pH5.6
Selection	Basal medium with 0.5 g/L MES, 4 mg/L BA, 0.1 mg/L NAA 3% sucrose, 0.6% agar, pH 5.8 500 mg/L carbenicillin, 20 mg/L kanamycin
Shoot elongation	Basal medium with 0.5 g/L MES, 4 mg/L BA, 0.1 mg/L NAA 3% sucrose, 0.6% agar, pH 5.8
Rooting	Basal medium with 0.5 g/L MES, absence of growth regulators 2% sucrose, 0.6% agar, pH 5.8

Antibiotics were added after the medium was autoclaved.

borders. The 5' end of the *gus* sequence is close to the T-DNA right border sequence. A translation enhancing leader sequence from alfalfa mosaic virus (AMV) was placed between the right border and 5' end of the *gus* sequence to facilitate gene expression through improved translation when this cassette is inserted adjacent to a plant promoter during transformation (Figure 4.1). The bacterial cultures for transformation were prepared as described in Chapter 3.

4.2.4 Co-cultivation

Experiments PT1, 2, 3 and 4 were performed using the filter paper method without cold-treatment and with two-day pre-cultured cotyledonary petiole explants. Subsequent experiments (PT5-14) were performed using the speed transformation method. These tranformation methods are described in Chapter 3.

4.2.5 Selection strategy

After co-cultivation, all explants were directly transferred to the selection medium with 500 mg/L carbenicillin to remove the bacteria and 20 mg/L kanamycin for selection of putative transgenic shoots. After 3 to 4 weeks on the selection medium, regenerated green shoots (putative transformants) were cut and transferred to the shoot elongation medium free of kanamycin. When the shoots had grown to a size (1.5-2 cm in length) sufficient for rooting, they were transferred to rooting medium lacking kanamycin. PCR screening and GUS staining were attempted at the earliest possible stage, while the shoots were in the rooting medium, to confirm that plants were transformed and to detect specific gene expression patterns.
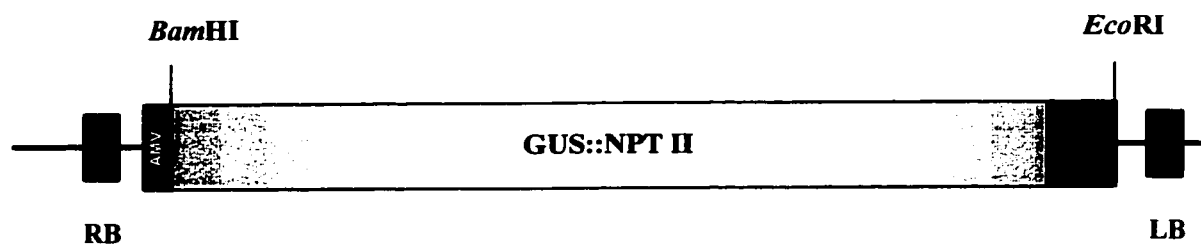
Figure 4.1 Schematic map of transcriptional fusion cassette of vector pΔGUS::NPTII

AMV: Alfalfa mosaic virus translation enhancing leader, 45bp; GUS::NPTII: fused gene of β–glucuronidase and neomycin phosphotransferase II genes, 2.8Kb; Tnos: Nopaline synthase terminator, 250bp; RB: right border of the T-DNA, LB: left border of the T-DNA (Dr. R. Datla, unpublished data).

4.2.6 Cultivation of transgenic plants

As soon as enough root mass was obtained in the rooting medium, the plantlets were transferred to potting mix (Redi-Earth®) supplemented with fertilizer granules under normal greenhouse conditions. In order to facilitate hardening for greenhouse growth, freshly potted plantlets were covered with Magenta® GA7 boxes for the first week. Under these conditions, plants established rapidly and leaf samples were collected for further assays.

4.2.7 Analysis of putative transformants

Histochemical GUS assay

Histochemical GUS analyses were performed as described in Chapter 3. Pieces of tissue from the putative transgenic plants obtained after selection as well as plant organs from plants grown in pots were used to detect specific expression patterns.

PCR analysis

The same protocol as described in Chapter 3 was used to amplify the *npt*II gene from DNA of putative transgenic plants.

DNA preparation, digestion and Southern analysis

DNA preparation, digestion and Southern analysis were performed as described in Chapter 3. The PCR product (size about 800bp) of the *npt*II gene from pRD320 was used as a hybridization probe. To determine insert copy numbers in each transgenic

plant, genomic DNA was digested with the restriction enzyme *Bam*HI. Single digestion with *Bam*HI, *Eco*RI and *Hind*III enzymes and double digestion with *Bam*HI + *Eco*RI, *Bam*HI + *Hind*III and *Eco*RI + *Hind*III combinations were used to generate restriction maps around the GUS::NPTII insert from transgenic lines PT7-1 and PT10-2. Transformation frequencies were expressed as the percentage of Southern-positive plants per total number of explants used in transformation.

Inheritance of GUS activity in R1 progeny

A solution of 4% NaCl was sprayed onto the inflorescence of a transgenic line (R_0) of broccoli to break self-incompatibility. *B. napus* derived transgenic lines were bagged to prevent cross-pollination at flowering without NaCl application. R_1 seeds from self-pollinated transformed plants were germinated on half strength MSB5 medium with 1% sucrose. After five days, whole seedlings were harvested and stained for GUS. Half-seeds were also used for GUS staining. Segregation ratios for GUS activity from these seedlings and seeds were thus obtained. χ^2 values were computed to evaluate segregation ratios by using the MULTICHI program (Appendix 9).

Kanamycin resistance test

To compare the strength of the constitutive promoters tagged in this study, a simple test on kanamycin-containing regeneration medium was performed. Plant materials used in this experiment were PT6-2 and PT7-1 (both obtained from *B. napus* cv. Westar). As controls *B. napus* cv. Westar transgenic line which was transformed

with pRD320 (from Chapter 3) and a *B. napus* cv. Westar transgenic line which was transformed with pYW-ALS3R (kindly provided by J. Hammerlindl, National Research Council Canada, Saskatoon, SK, Canada S7N 0W9). Kanamycin resistance in the promoter tagged lines PT6-2 and PT7-1 would be under the control of tagged plant promoters; in the transgenic line produced with pRD320, the *npt*II gene was regulated by the tandem CaMV 35S promoter and translation enhancing leader from alfalfa mosaic virus (designated as 35S-35S-AMV-NPTII); in the transgenic line produced with pYW-ALS3R the *npt*II gene was regulated by a single CaMV 35S promoter (designated as 35S-NPTII). The medium used in this experiment was Murashige's Minimal Organic salts (Linsmaier and Skoog 1965, referred to as MMO medium, purchased from GibcoBRL, USA, cat.no. 23118-078) (details in Appendix 1) with 4.5mg/L BA and 200mg/L kanamycin. Cotyledonary petiole explants were prepared from five-day-old *in vitro* grown seedlings (R_1 seeds from primary transgenic line) from each transgenic line. Ten explants were inserted in the medium in each petri plate (100x15mm) and sealed with Stretch'n Seal™. Cultures were maintained as previously described for transformation experiments (Chapter 3). Observations were made after three weeks of cultivation.

4.3 Results

4.3.1 Selection strategy and transformation frequency

After 10-14 days on the selection medium with 20mg/L kanamycin, green spots appeared at the cut edges of petioles of some explants. As soon as small shoots appeared, they were excised and transferred to the shoot elongation medium lacking kanamycin. Transferring green shoots as early as possible to a kanamycin-free medium was critical in using this type of promoterless construct. Since the construct, pΔGUS::NPTII used in this study does not have its own promoter, kanamycin resistance was presumably controlled by a promoter from the plant genome. When shoots reached 1-2 cm in length on the shoot elongation medium, pieces of tissue were taken for PCR analysis (data not shown). Subsequently, GUS staining was performed on PCR-positive putative transgenic lines. The expression of the reporter fusion gene will depend on the type of plant promoter present "upstream" of the insertion in each of these transgenic lines, such a promoter might regulate gene expression in a cell, tissue or developmental stage in a specific or constitutive manner. To identify the type of promoter that was tagged, the transgenic lines were analyzed for GUS expression.

Seven putative promoter tagged transgenic plants were identified out of 18,911 *Agro*-infected cotyledonary petiole explants. The average transformation frequency of approximately 0.037% was much lower than in the normal transformation studies conducted previously (Chapter 3). The seven promoter tagged lines recovered included one plant of broccoli and six plants of *B. napus*. The efficiency of recovering promoter tagged lines in broccoli was about 0.012% while in *B. napus* it was 0.056% (Table 4.2).

None of the transgenic lines showed phenotypic abnormalities (transgenic line PT12-2 has not flowered at the time of writing).

4.3.2 Expression pattern analysis by GUS localization

GUS expression patterns were analyzed in various tissues of one broccoli (*B. oleracea* var. *italica*) line (PT10-2) and six *B. napus* lines (PT4-1, 6-1, 6-2, 7-1, 12-1 and 12-2). These tissues included; root, stem, leaf, filament, anther, pollen, style, stigma, sepal, petal and seed tissues. In most cases, primary transformants (R_0) were used for GUS staining. Results are summarized in Table 4.3. Although transgenic lines PT4-1, 6-1 and 6-2 failed to show GUS expression in anther tissue, expression in PT4-1, 6-1, 6-2 and 7-1 was considered to be constitutive while PT10-2 showed expression in all plant parts except the root system. Transgenic line PT12-1 showed GUS expression predominantly in the root tissue, whereas PT12-2 exhibited GUS expression in the phloem tissues of the stem but not in other parts of the plant (Table 4.4).

Table 4.2 Results of transformation of *B. oleracea* and *B. napus* with the promoterless construct pΔGUS::NPTII

Code	Species	Cultivar	No. of Explants	Transgenic line	Frequency(%)
PT1	*B. oleracea* var. *italica*	Green Valiant	2290	0	0
PT2	*B. oleracea* var. *italica*	Green Valiant	2320	0	0
PT3	*B. napus*	Westar	620	0	0
PT4	*B. napus*	Westar	630	1	0.16
PT5	*B. napus*	Westar	1908	0	0
PT6	*B. napus*	Westar	1605	2	0.12
PT7	*B. napus*	Westar	800	1	0.12
PT8	*B. oleracea* var. *italica*	Green Valiant	580	0	0
PT9	*B. oleracea*	Rapid cycling	1680	0	0
PT10	*B. oleracea* var. *italica*	Green Valiant	950	1	0.1
PT11	*B. oleracea* var. *botrytis*	Snow Crown	380	0	0
PT12	*B. napus*	Westar	2288	2	0.1
PT13	*B. napus*	Westar	1340	0	0
PT14	*B. napus*	Westar	1520	0	0
TOTAL			*18911*	*7*	*0.037*

Table 4.3   Histochemical localization of GUS activity in transgenic plants transformed with a promoterless construct pΔGUS::NPTII

Line	Root	Stem	Leaf	Filament	Anther	Pollen	Style	Stigma	Sepal	Petal	Seed
PT4-1	+	+	+	+	-	-	+	+	+	+	+
PT6-1	+	+	+	+	-	-	+	+	+	+	+
PT6-2	+	+	+	+	-	-	+	+	+	+	+
PT7-1	+	+	+	+	+	+	+	+	+	+	+
PT10-2	-	+	+	+	+	+	+	+	+	+	+
PT12-1	+	-	-	N/D	N/D	N/D	N/D	N/D	N/D	N/D	-
PT12-2	-	+	-	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D

+: Activity detected, -: No activity detected, N/D: not determined

Table 4.4 Expression patterns of promoter tagged transgenic plants

Transgenic line	Species	Expression pattern
PT4-1	*B. napus*	constitutive
PT6-1	*B. napus*	constitutive
PT6-2	*B. napus*	constitutive
PT7-1	*B. napus*	constitutive
PT10-2	*B. oleracea* var. *italica*	shoot specific[a]
PT12-1	*B. napus*	root predominant
PT12-2	*B. napus*	stem-phloem predominant

a: expressed in most parts of the plant except the root system

PT4-1, PT6-1, PT6-2

Interestingly, these three lines of *B. napus* cv. Westar showed similar GUS expression patterns. GUS staining was observed in the root, stem and leaf and also in most parts of the flower with the exception of pollen grains. Staining at specific stages of microsporogenesis was not attempted. R_1 seeds showed GUS activity and R_1 seedlings also showed GUS activity in root, hypocotyl and cotyledons similar to the expression patterns observed in the primary transgenic lines (Figure 4.2). The expression patterns observed in these lines suggest that the tagged promoters have a pattern of expression similar to CaMV 35S which is often considered to be a constitutive promoter but in fact is not usually active in pollen grains.

PT7-1

This line was derived from *B. napus* cv. Westar. GUS activity was expressed in all tested tissues or organs including pollen. R1 seeds and seedlings also showed strong intensities of GUS staining as in the primary transgenic plant (Figures 4.3, 4.4, 4.5, 4.6). These observations suggest that the GUS::NPTII insert was fused to a strong constitutive promoter in the transgenic plant.

PT10-2

This line was derived from the broccoli cultivar 'Green Valiant'. Strong GUS staining was observed throughout the primary transgenic plant except the root system

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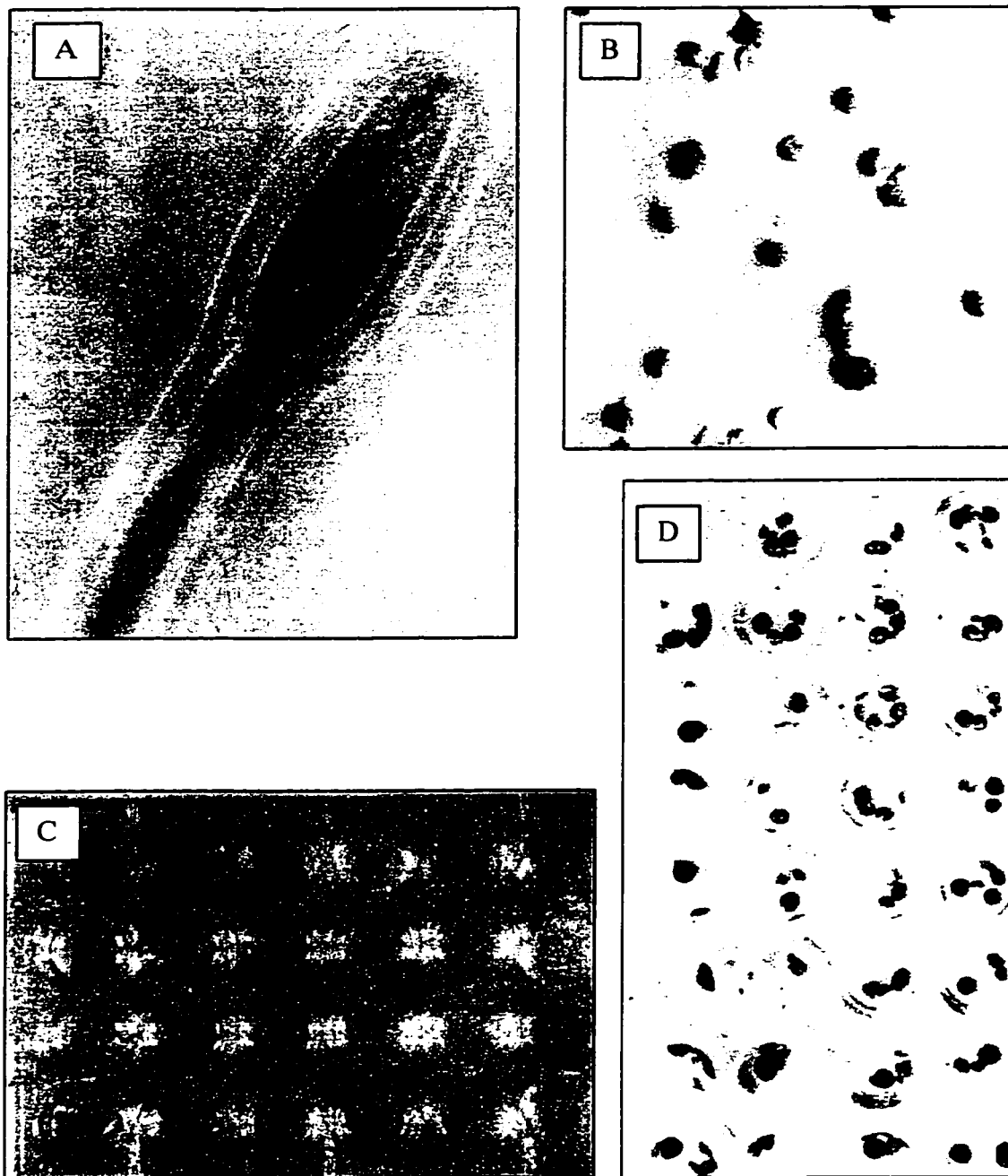
Figure 4.2 GUS activity in various tissues of the promoter-tagged transgenic line PT6-2 (*B. napus* cv. Westar)

A: The filament of the primary transgenic line shows GUS activity but the anther does not; B: Pollen grains from A, GUS activity was not detected; C: Cotyledon, hypocotyl and root of R_1 seedlings show GUS activity and segregate in a 3:1 ratio; D: Seeds from the R_1 plant show GUS activity and also segregate in a 3:1 ratio.
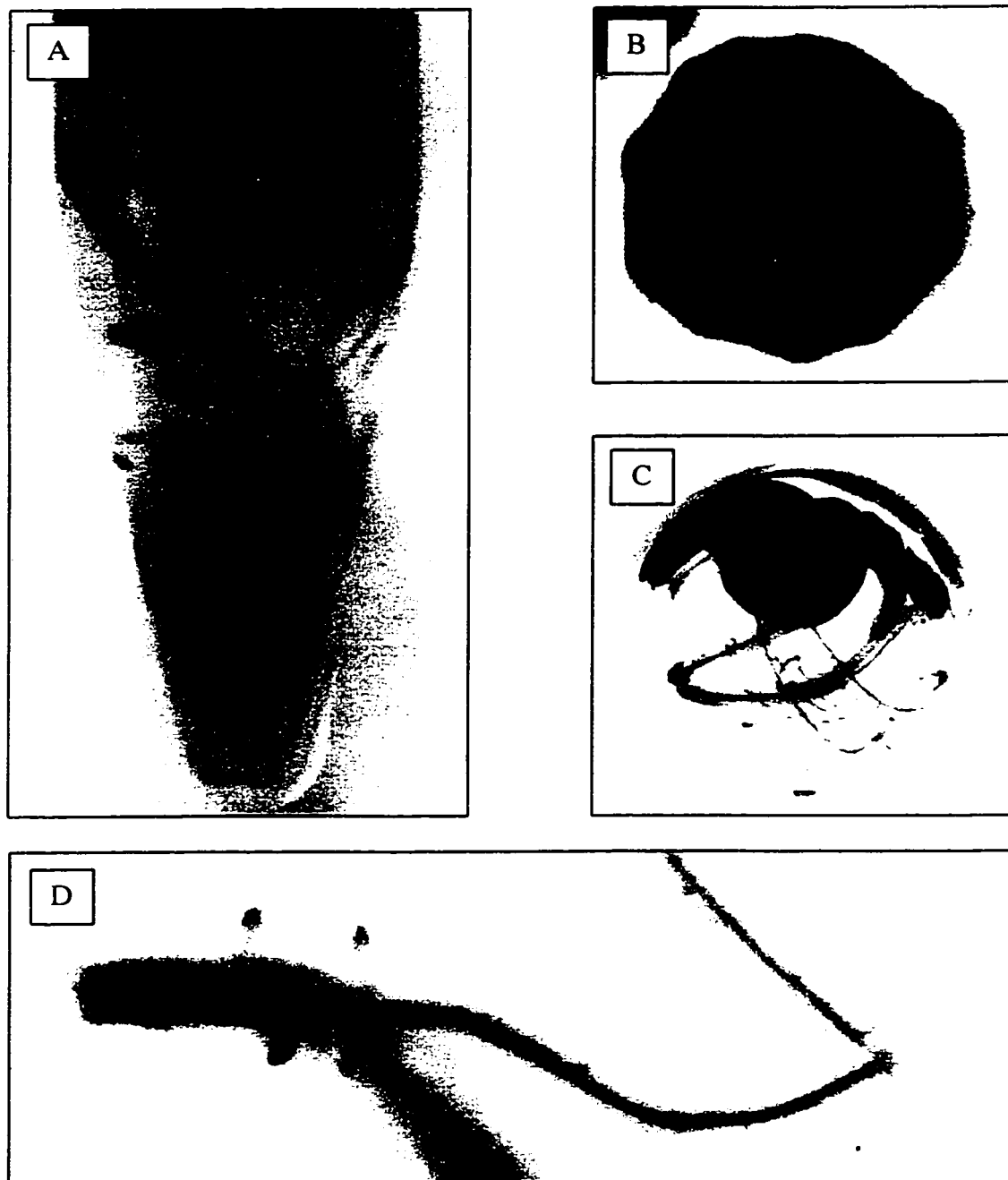
Figure 4.3 GUS activity in various tissues of the promoter-tagged transgenic line PT7-1 (*B. napus* cv. Westar)

A: Primary transgenic plantlet grown on rooting medium shows GUS activity in roots. root tips, stem and leaf; B: Primary transgenic plant in greenhouse shows GUS activity in all stem tissues; C: GUS-positive R_1 seedling; D: Root and root tips of a GUS-positive R_1 seedling
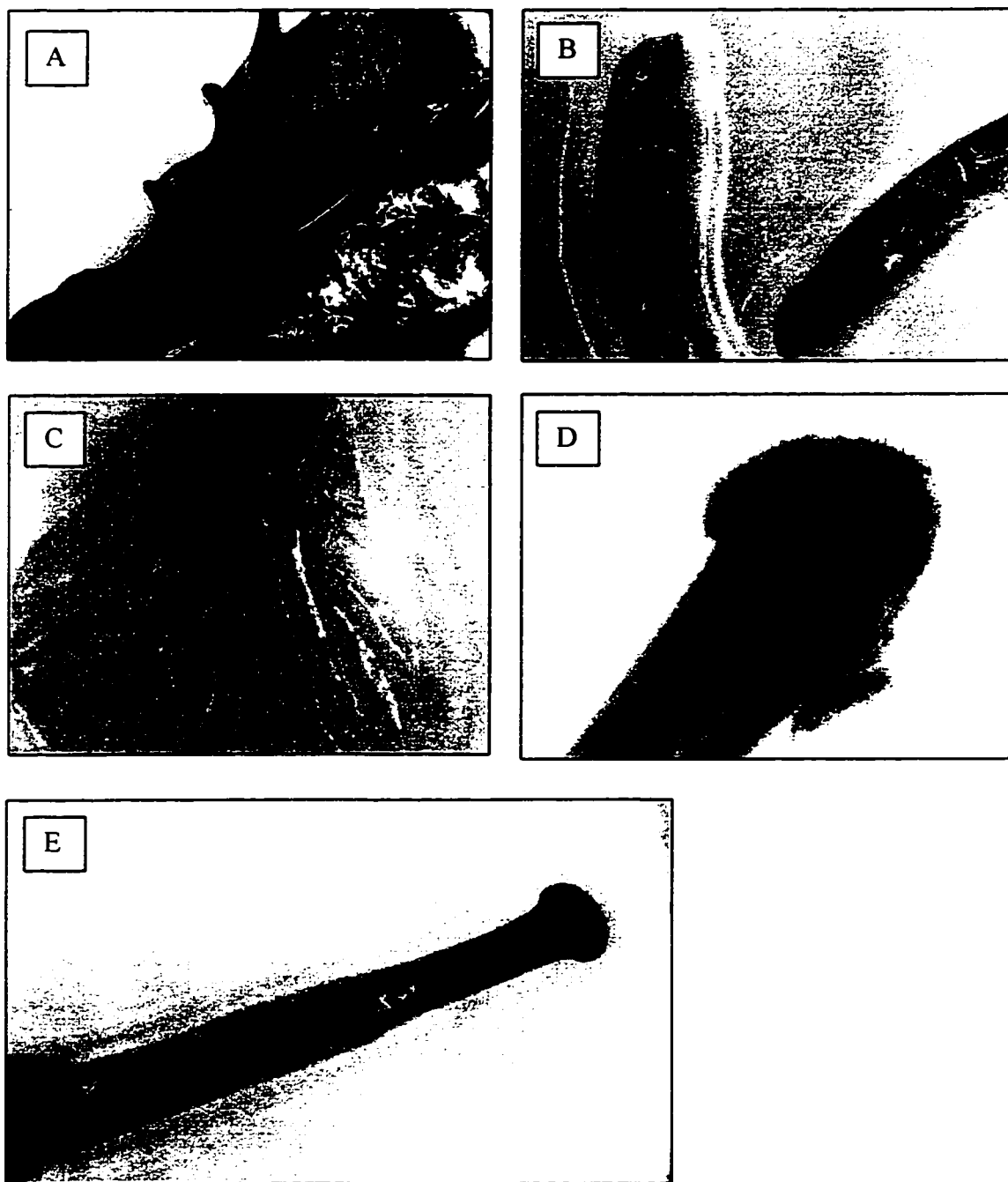
Figure 4.4 GUS activity in various tissues of the promoter-tagged transgenic line PT7-1 (*B. napus* cv. Westar), grown in the greenhouse

A: Leaf; B: Sepal; C: Petal; D: Stigma; E: Style
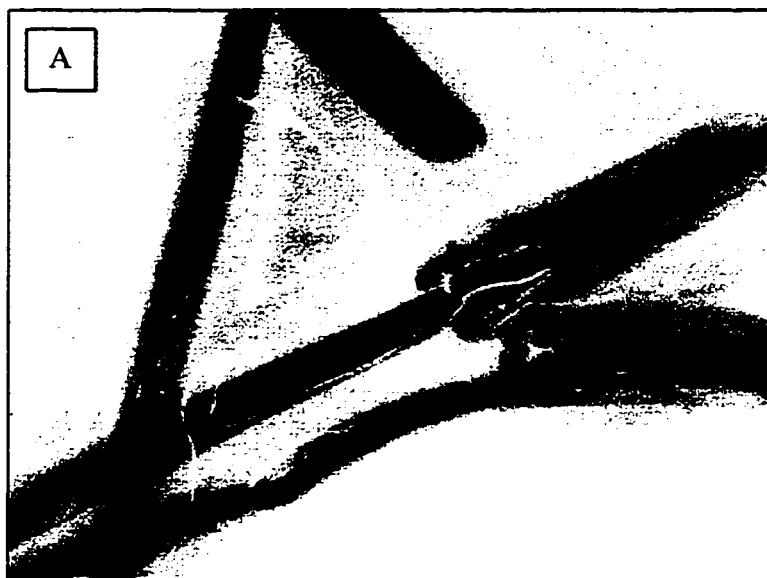
Figure 4.5 GUS activity in various tissues of R_1 plant of the promoter-tagged transgenic line PT7-1 (*B. napus* cv. Westar)

A: Filament and anther;   B: Pollen grains from A; GUS activity in all pollen grains indicates that this is a homozygous line.
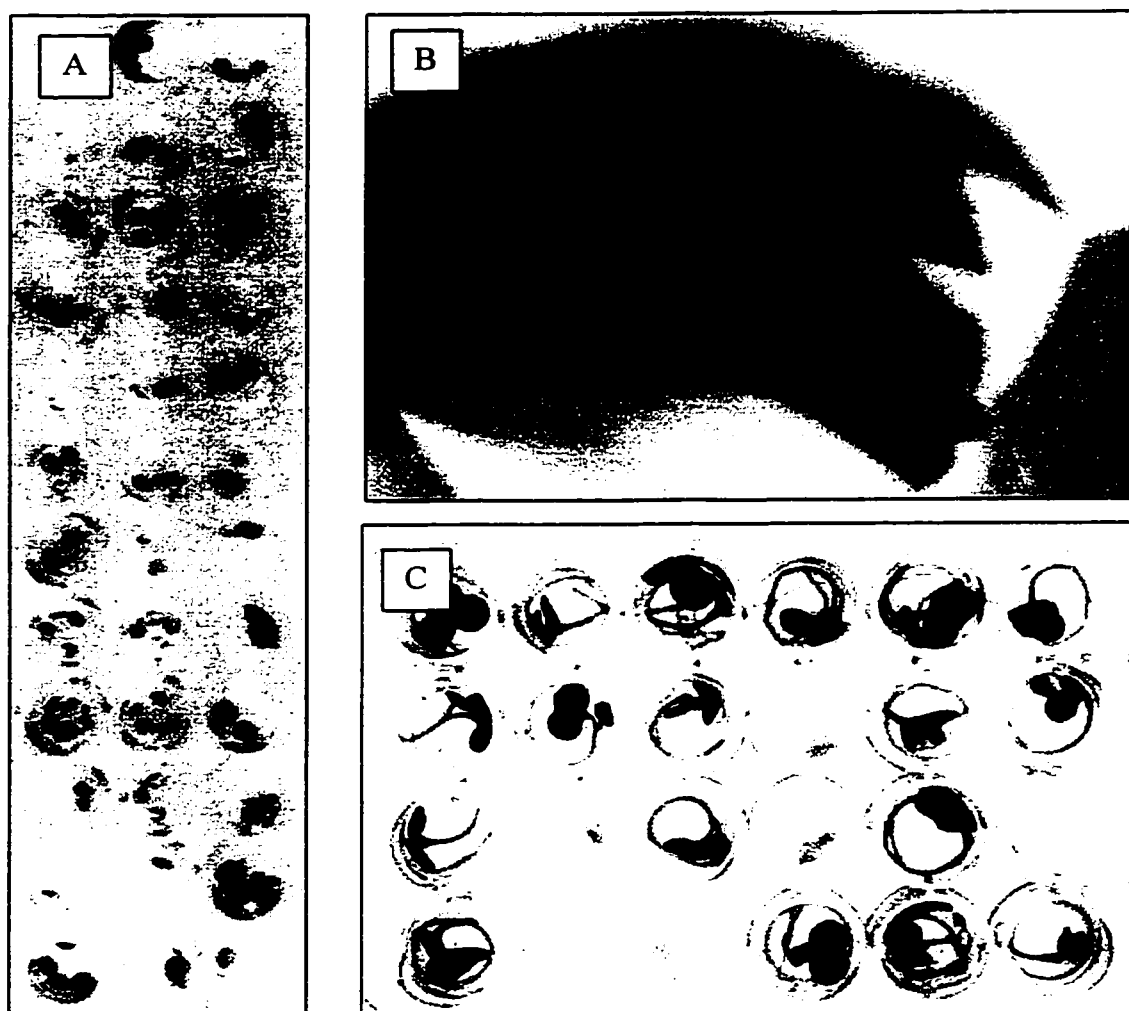
Figure 4.6 GUS activity in various tissues and segregation ratio in R_1 plant of the promoter-tagged transgenic line PT7-1 (*B. napus* cv. Westar)

A: Seeds showing GUS activity, represent a 3:1 segregation ratio; B: Seed coat and cotyledons show GUS activity; C: Seedlings segregate in a 3:1 ratio and exhibit GUS activity in the root, hypocotyl and cotyledons

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when grown *in vitro*. A mature, greenhouse grown plant also showed strong expression patterns in all parts of the plant including pollen grains with the exception of the root system. Seed staining revealed that all tissues including the seed coat, cotyledons and hypocotyls were GUS-positive with the exception of the region of the hypocotyl from which the root would develop. The R_1 seedlings showed GUS activities in hypocotyl and cotyledons but not in the root system (Figure 4.7, 4.8). This tagged promoter was therefore considered to exhibit shoot specific expression.

PT12-1

This line was derived from *B. napus* and exhibited GUS activity predominantly in the root system including root hairs. Expression was also observed in auxiliary bud regions on stem. No GUS activity was observed in R_1 seeds. In R_1 seedlings, GUS activity was observed only in the root system. This expression pattern was similar to the primary transgenic plant. GUS staining of the roots indicated that the staining intensity and pattern were irregular (Figure 4.10).
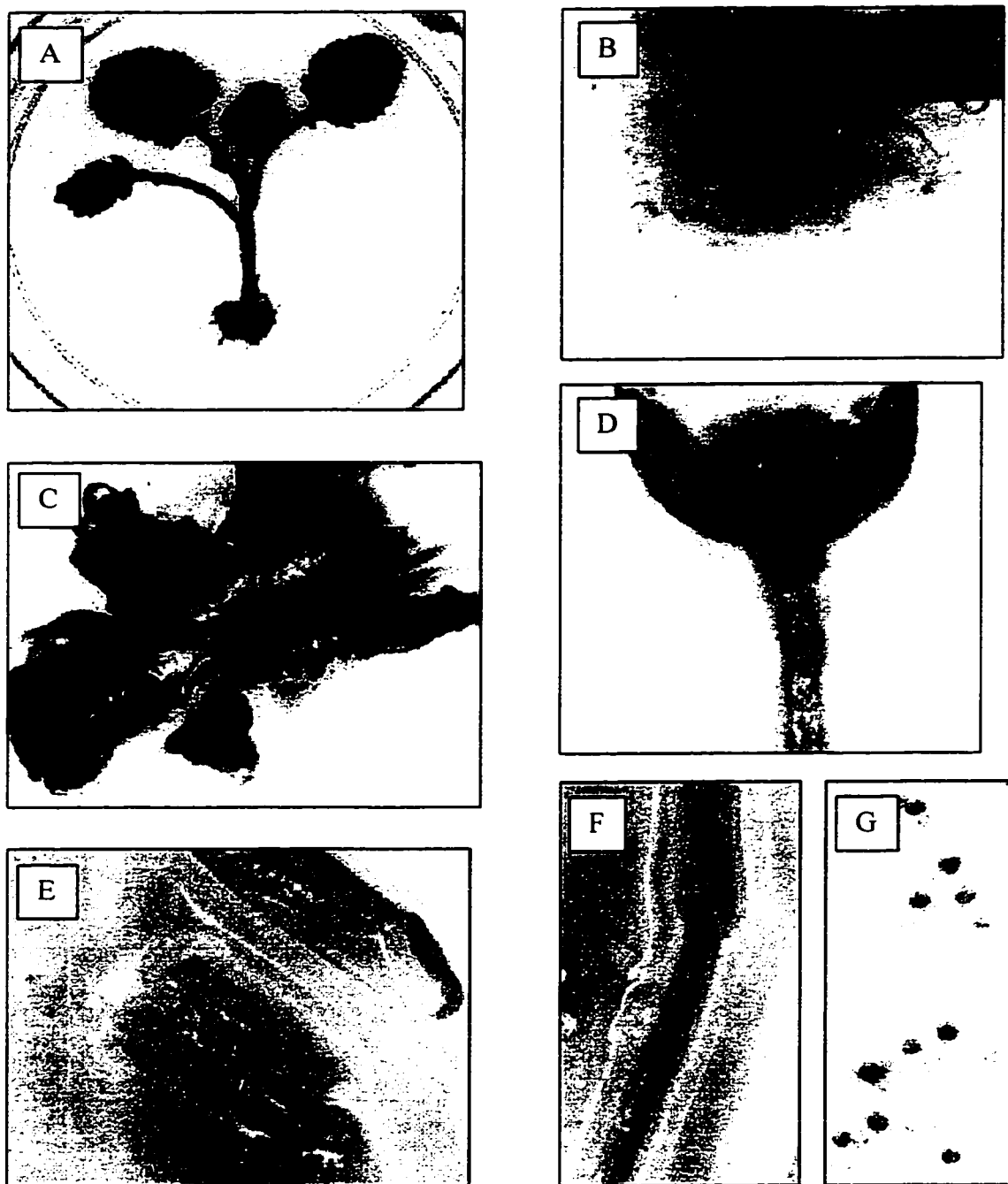
Figure 4.7 GUS activity in various tissues of the primary promoter-tagged transgenic line PT10-2 (*B. oleracea* var. *italica* cv. Green Valiant)

A,B: Plantlet grown on rooting medium; GUS was not detected in the roots; C: Leaf: D: Pedicel and receptacle; E: Filament and anther
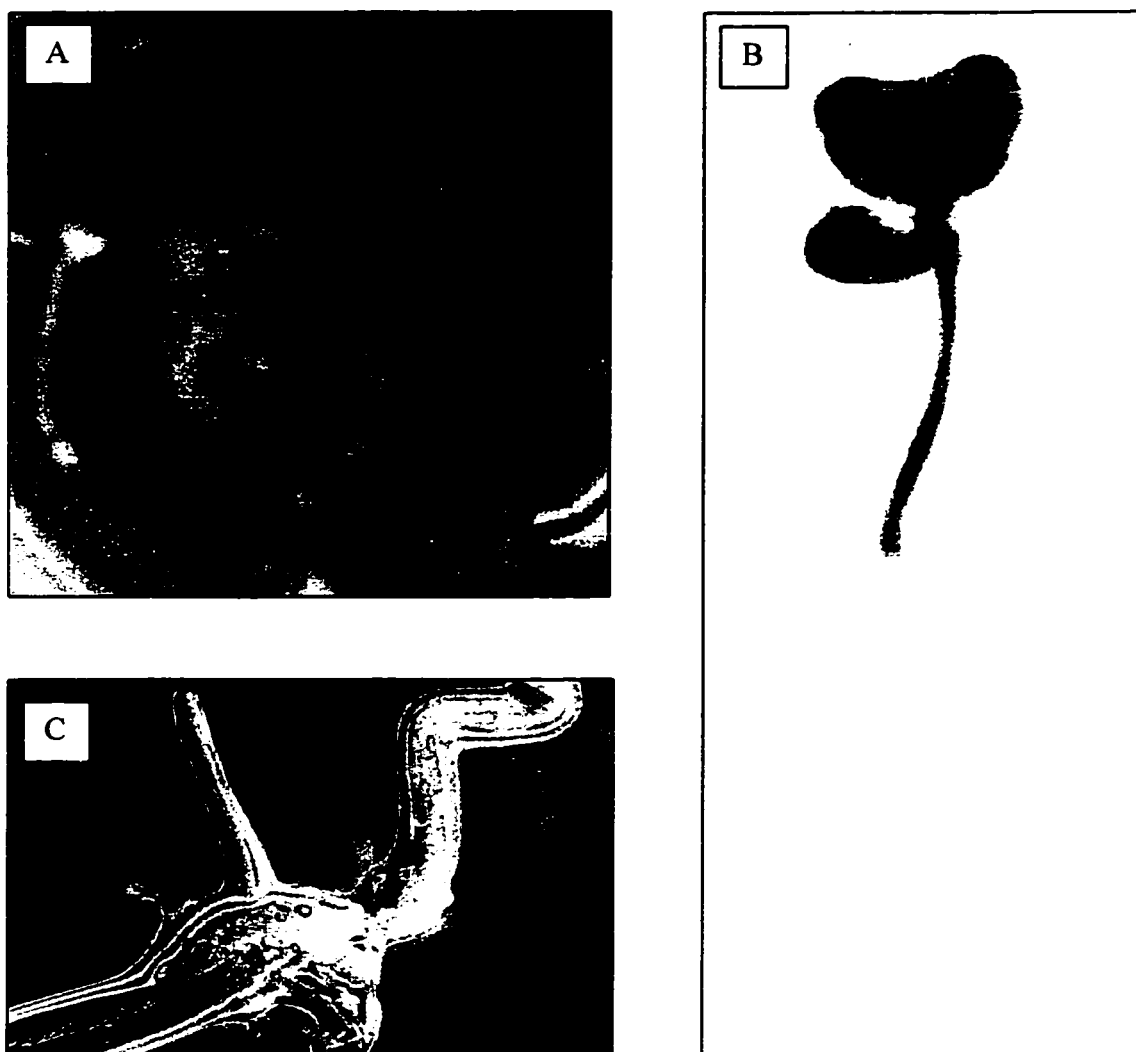
Figure 4.8 GUS activity in various tissues of R_1 plant of the promoter-tagged transgenic line PT10-2 (*B. oleracea* var. *italica* cv. Green Valiant)

A: Seed and cotyledon show GUS activity; B: Seedling of R_1 indicating the absence of GUS activity in the root; C: Absence of GUS activity in root tips
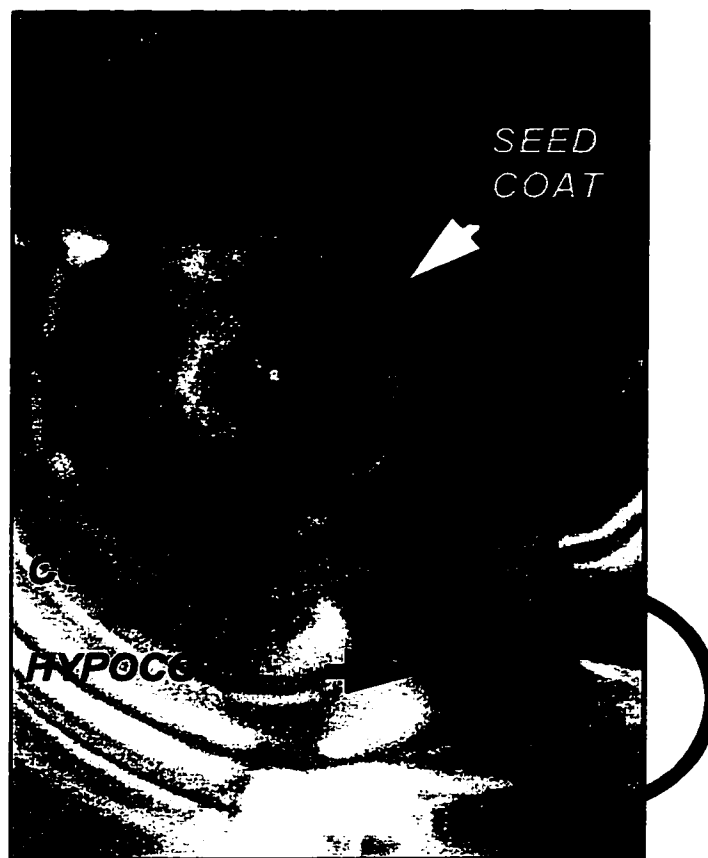
Figure 4.9 GUS activity in R₁ seed of of the promoter-tagged transgenic line PT10-2 (*B. oleracea* var. *italica* cv. Green Valiant)

Transgenic line PT10-2 expressed GUS in most plant tissues and organs except in the root. Staining of seeds revealed that GUS activity was present in seed coat, cotyledon, and hypocotyl but not in the end of hypocotyl which will develop into the root (circled).
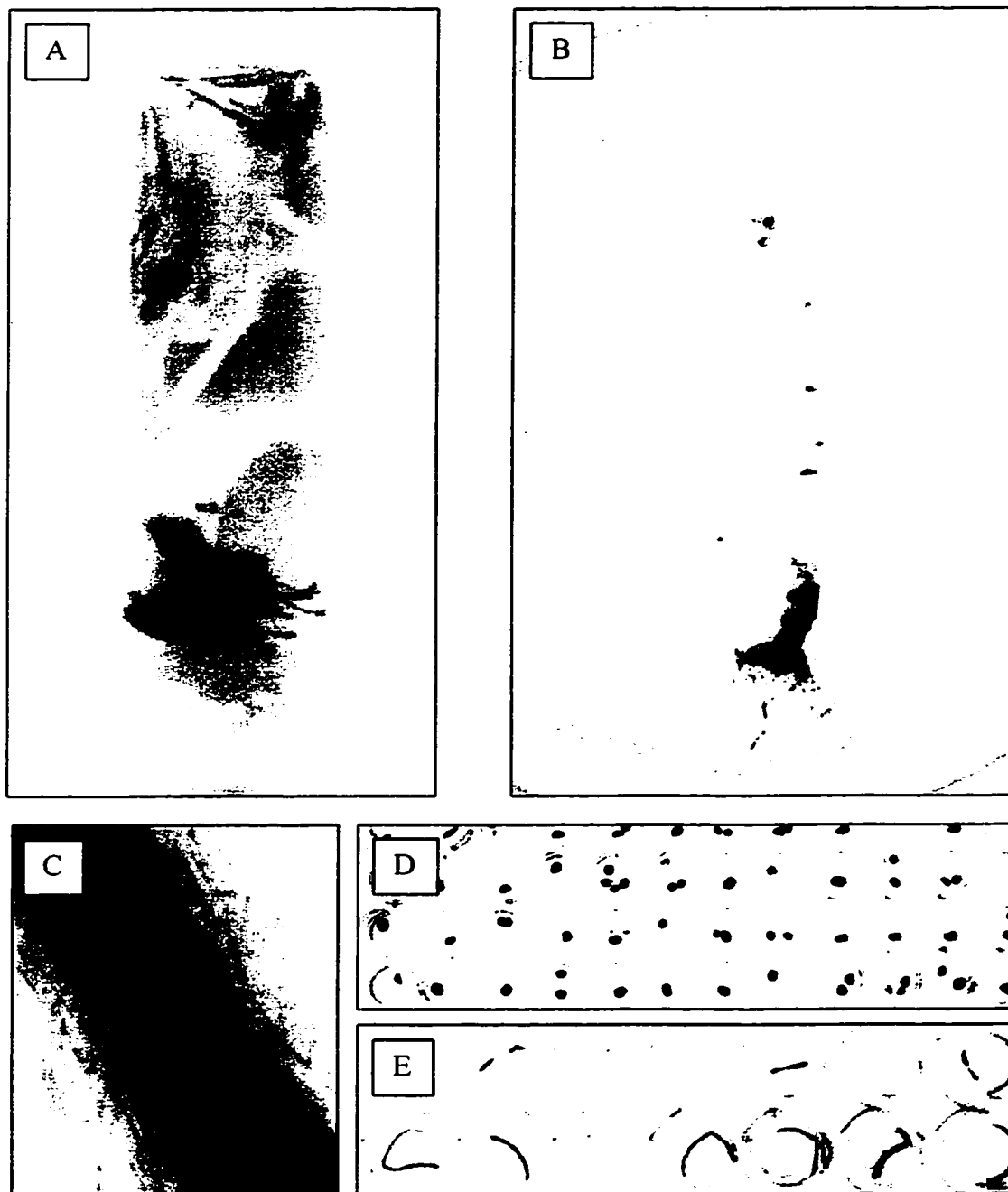
Figure 4.10   GUS activity in various tissues of the promoter-tagged transgenic line PT12-1 (*B. napus* cv. Westar)

A: Primary transgenic plantlet grown in the rooting medium shows GUS activity predominantly in the root system. B: Primary transgenic plantlet grown in the rooting medium shows GUS activity predominantly in the root area and at the nodal junctions. C: Magnified picture of B, strong GUS activity in the root meristem and root hairs; D: GUS activity is absent in R_1 seeds. E: GUS activity is revealed in seedling roots.

PT12-2

This line was derived from *B. napus* and exhibited GUS activity predominantly in the stem. Staining of cross and longitudinal stem section revealed that GUS activity was localized in the phloem. GUS activity was not detected in the petiole, leaf and root (Figure 4.11). This line was transferred to soil and further detailed analyses are in progress.

4.3.3 Inheritance of GUS activity and Southern analysis

The T-DNA copy number of transgenic lines showing GUS activities was determined by segregation of the GUS activity encoded by the *gus::npt*II gene, and by Southern hybridization analysis. The interpretation of GUS activity patterns in promoter-tagged transgenic plants, and subsequent cloning of the tagged promoter is greatly facilitated in lines that contain only a single copy of T-DNA.

R_1 progeny from five selfed primary transformants (PT4-1, 6-1, 6-2, 7-1, 10-2) showed segregation ratios of 3:1 (GUS-positive : GUS-negative) (Table 4.5). These lines were therefore expected to contain a single T-DNA insert and these were further characterized by Southern analysis to confirm T-DNA copy number.

Genomic DNA from each of the seven transgenic lines was digested with the restriction enzyme *Bam*HI to generate T-DNA border fragments and hybridized with a *npt*II probe. These results suggested that most of the transgenic lines (6 out of 7) contained a single copy of the full-length T-DNA insert, however PT12-1 appears to have five copies (Figure 4.12). All transgenic plants analyzed to date have had
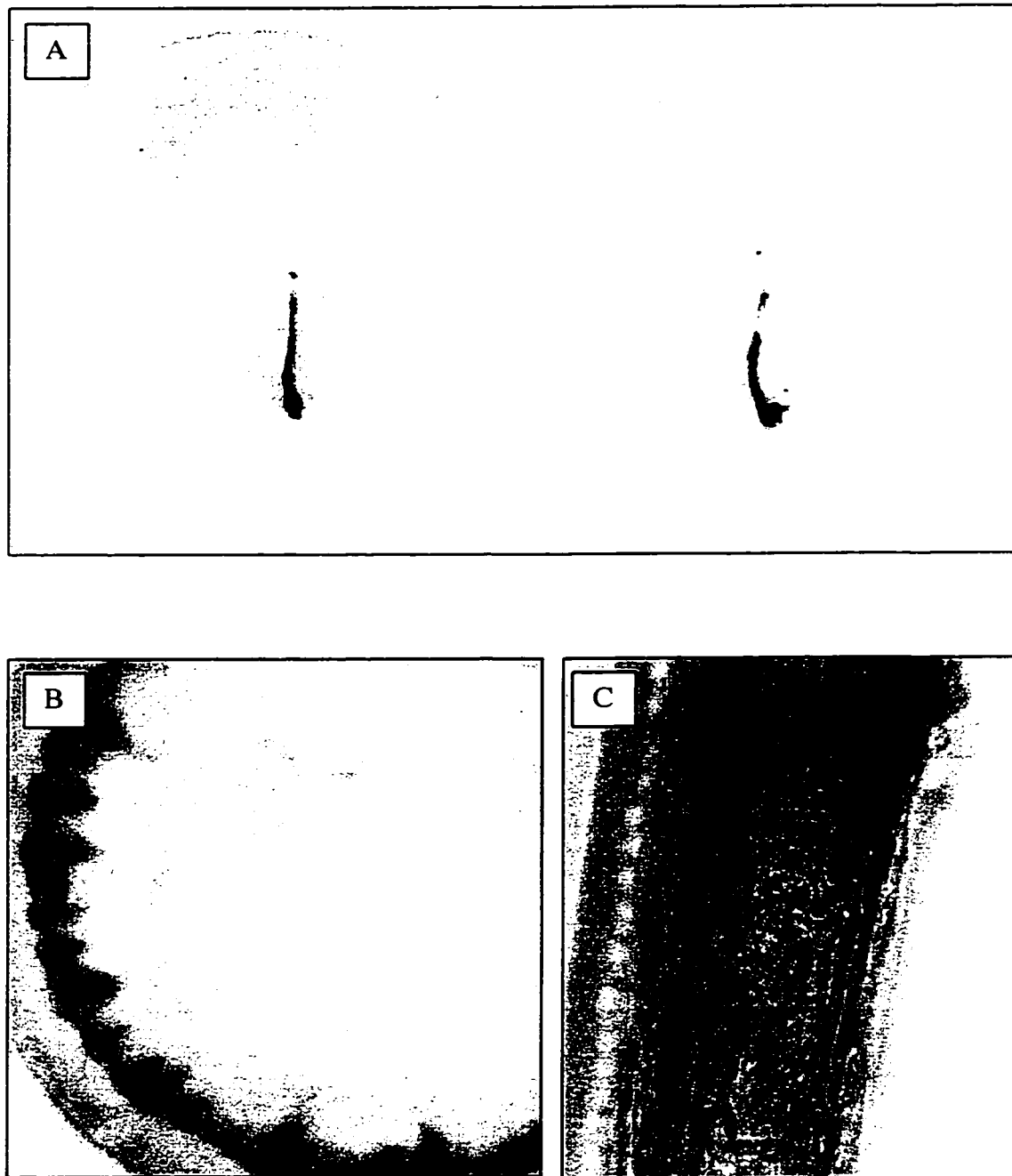
Figure 4.11 GUS activity in various tissues of the primary promoter-tagged transgenic line PT12-2 (*B. napus* cv. Westar)

A: GUS activity is exhibited predominantly in stems but not in roots, petioles and leaves (grown in the rooting medium). B: Cross-section of stem; only the phloem tissues show GUS activity in the stem (grown in the greenhouse) C: Longitudinal-section of stem (grown in the rooting medium)

Table 4.5   Segregation for GUS staining in R_1 progeny of promoter tagged transgenic lines

Transgenic line	Number of seed tested	GUS(+)	GUS(-)	Ratio	χ^2-value[a]	Number of insertion[b]
PT4-1	63	47	16	3:1	0.0015	1
PT6-1	43	32	11	3:1	0.0023	1
PT6-2	96	76	20	3:1	1.0138	1
PT7-1	51	38	13	3:1	0.0019	1
PT10-2	53	42	11	3:1	0.6477	1
PT12-1	N/D	-	-	-	-	5
PT12-2	N/D	-	-	-	-	1

a: significant level was 5%,   b: results from Southern analysis (Figures 4.12 and 4.16),   N/D: not determined
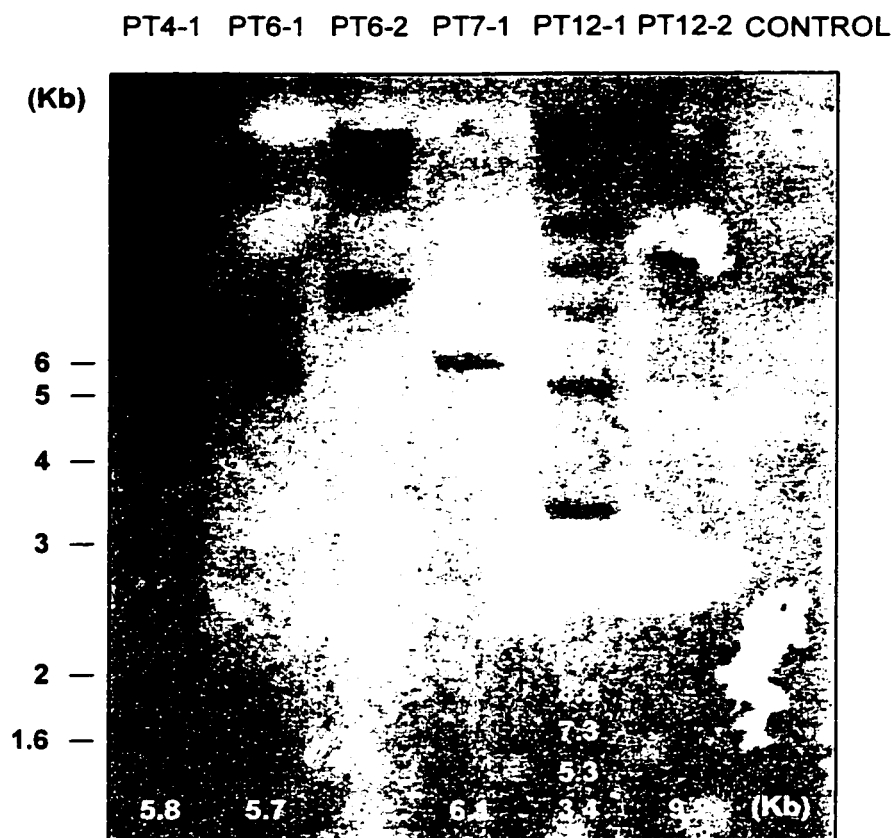
Figure 4.12   Southern analysis of promoter tagged lines (except PT10-2, see Figure 4.16) transformed with pΔGUS::NPTII.

The 10μg genomic DNA sample obtained from each line was digested with BamHI to generate a GUS::NPTII-containing fragment.   Transgenic line PT12-1 contains five copies of GUS::NPTII insert, while other lines contain one insertion.

different sizes of flanking junction fragments, suggesting that the T-DNA integrated into different sites in the plant genome. These results also suggest that although transgenic lines PT4-1, 6-1 and 6-2 exhibit similar constitutive GUS expression patterns, their tagged promoters are likely different.

4.3.4 Kanamycin resistance test

Among the transgenic lines exhibiting constitutive expression, PT7-1 consistently showed stronger GUS staining compared to PT6-2 (Figure 4.13). This would suggest that the tagged promoter in PT7-1 was stronger than the promoter tagged in PT6-2. To compare the strength of constitutive expression of the tagged promoter in PT6-2 and PT7-1 a regeneration test on 200mg/L kanamycin-containing medium was carried out along with other transgenic lines as described in Material and Methods. In this experiment, all the petiole tips of the control 'Westar' turned black (Figure 4.14A). A transgenic line obtained with pYW-ALS3R that contains a single CaMV 35S promoter (35S-NPTII-Tnos), showed swollen petiole tips with a little callus but no shoots (Figure 4.14B). Plants transformed with pRD320 that contained the tandem CaMV 35S promoter with translation enhancing leader from alfalfa mosaic virus (35S-35S-AMV-GUS::NPTII-Tnos) and explants from PT7-1 showed good regeneration and produced green shoots (Figure 4.14C,D). Explants from PT6-2 showed similar results to explants with a single CaMV 35S promoter (Figure 4.14E). Explants of transgenic line PT7-1 exhibited very strong regeneration when placed on
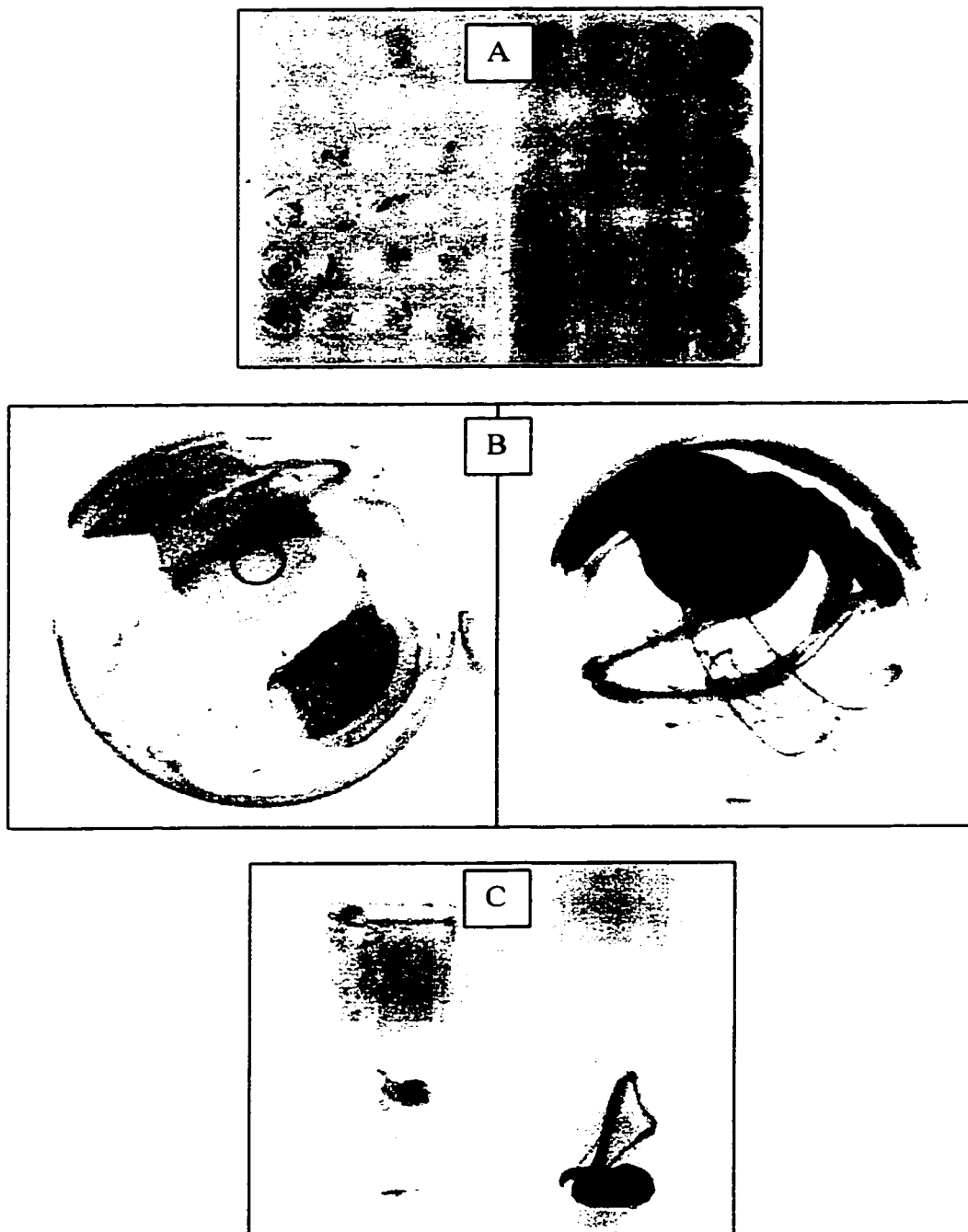
Figure 4.13 Comparative strength of GUS activity shown in R_1 seedlings of PT6-2 (on the left) and PT7-1 (on the right). Materials were treated at the same developmental stage in all three (A,B,C) tests. PT7-1 consistently exhibited stronger GUS staining intensity than PT6-2.
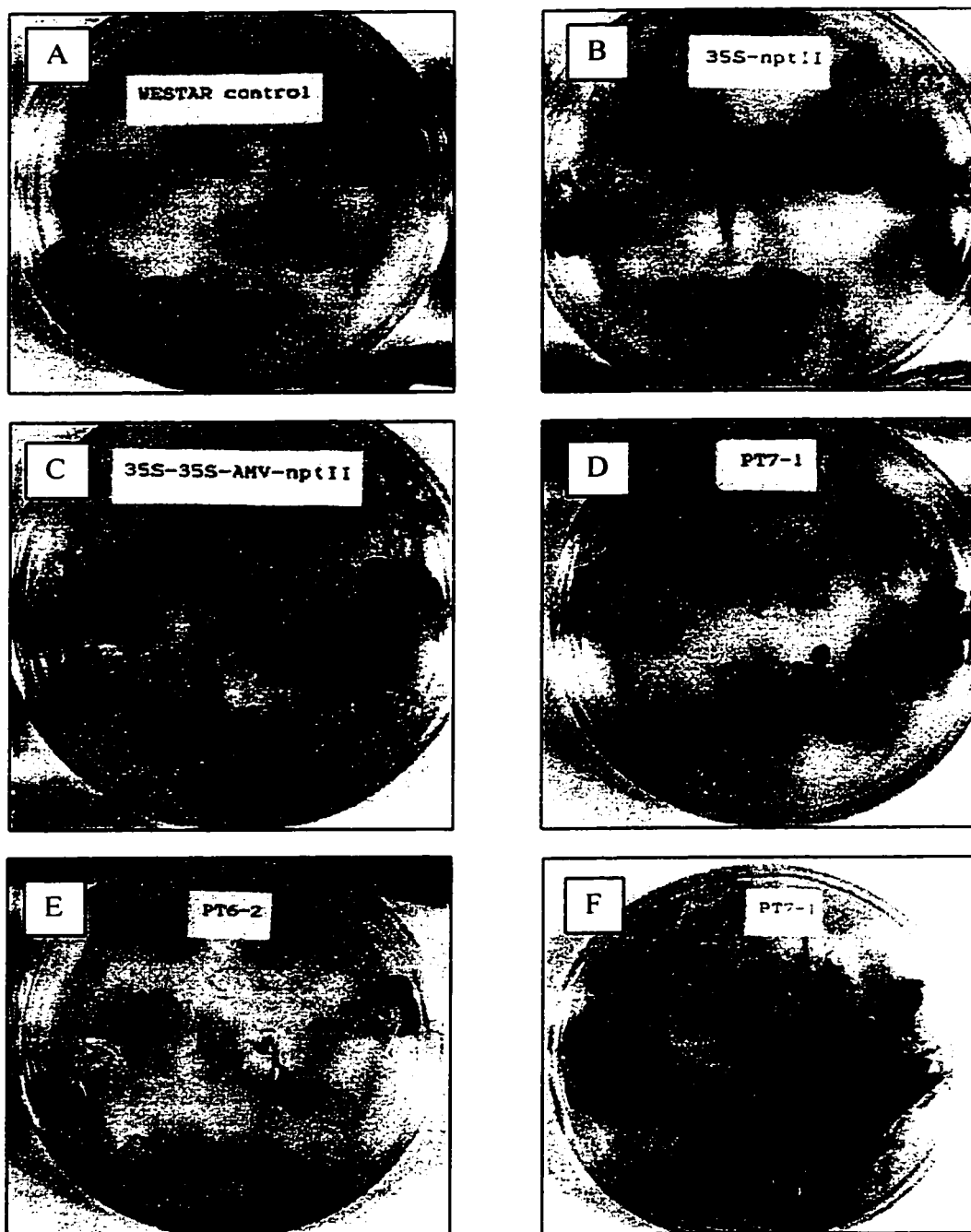
Figure 4.14 Regeneration tests using cotyledonary petiole explants from R_1 seedlings of PT6-2 and PT7-1 on 200 mg/L kanamycin-containing medium.

A: 'Westar' control, B: Transgenic plant obtained with pYW-ALS3R (35S-NPTII-Tnos), C: Transgenic plant obtained with pRD320 (35S-35S-AMV-GUS::NPTII-Tnos), D: Transgenic line PT7-1, E: Transgenic line PT6-2, F: Transgenic line PT7-1 on 100 mg/L kanamycin.

regeneration medium containing 100 mg/L kanamycin (Figure 4.14F). These results confirm that the promoter from PT7-1 is a stronger constitutive promoter than that of PT6-2 and that this promoter is as strong or possibly stronger than the CaMV 35S promoter.

4.3.5 Restriction map construction

In order to characterize plant promoters from the T-DNA tagged transgenic lines, a restriction map must be constructed prior to isolation of the putative promoter region from the genomic DNA of the transgenic line. Single and double digestions with the restriction enzymes *Bam*HI, *Eco*RI and *Hind*III were used to generate restriction maps around the GUS::NPTII for the transgenic lines PT7-1 (constitutive expression) and PT10-2 (shoot specific expression).

In the case of PT7-1, an *Eco*RI site was found 1.5 (Kb) away from the 5' end of the GUS::NPTII insert in the upstream area that may include the tagged promoter while a *Bam*HI site was located 2.2 (Kb) away from the 3' end of the GUS::NPTII insert that may include part of the target gene disrupted by the insertion (Figure 4.15). In the case of PT10-2, another *Eco*RI site was found 4 (Kb) away from the 5' end of the GUS::NPTII insert in the upstream area that may include the tagged promoter while a *Bam*HI site was located 6.3 (Kb) away from the 3' end of the GUS::NPTII insert that may include the target gene disrupted by the insertion (Figure 4.16).
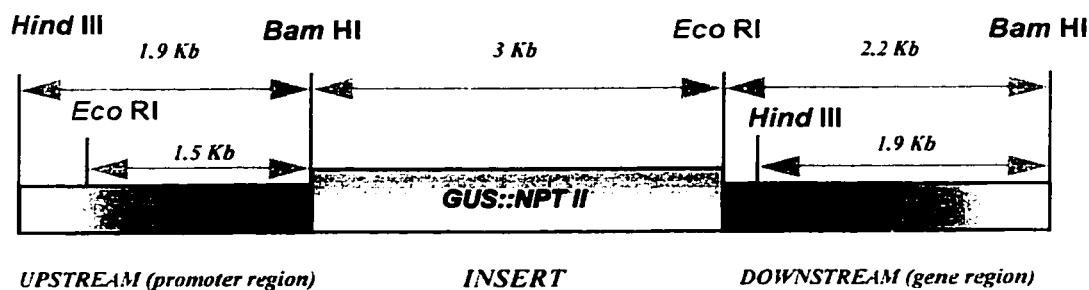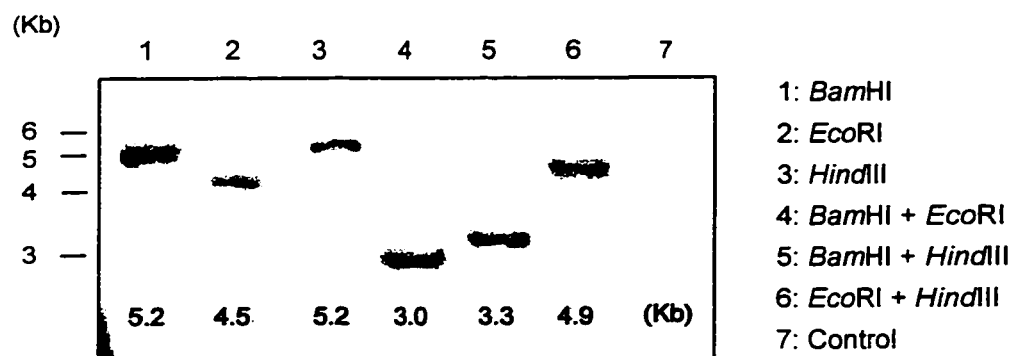
Figure 4.15 Restriction map of PT7-1 (*B. napus* cv. Westar) based on Southern analysis with three restriction enzymes *Bam*HI, *Eco*RI and *Hind*III

Single digestion with each enzyme and double digestions with combinations of each enzyme were performed to generate a restriction map of adjacent upstream and downstream areas of the GUS::NPTII insert. About 800bp of PCR amplified DNA fragment from *npt*II gene of pRD320 was used as a probe for hybridization.
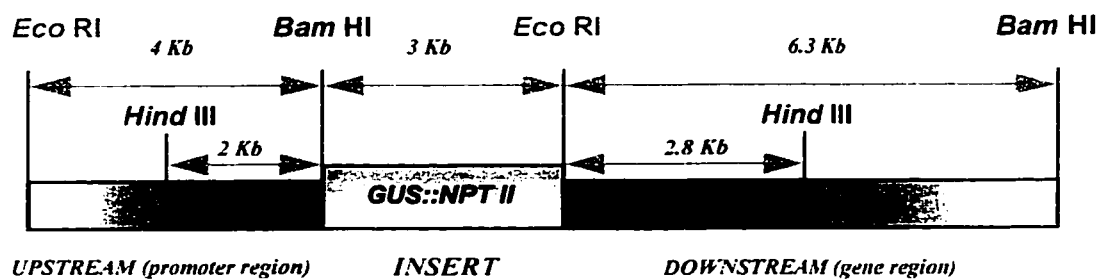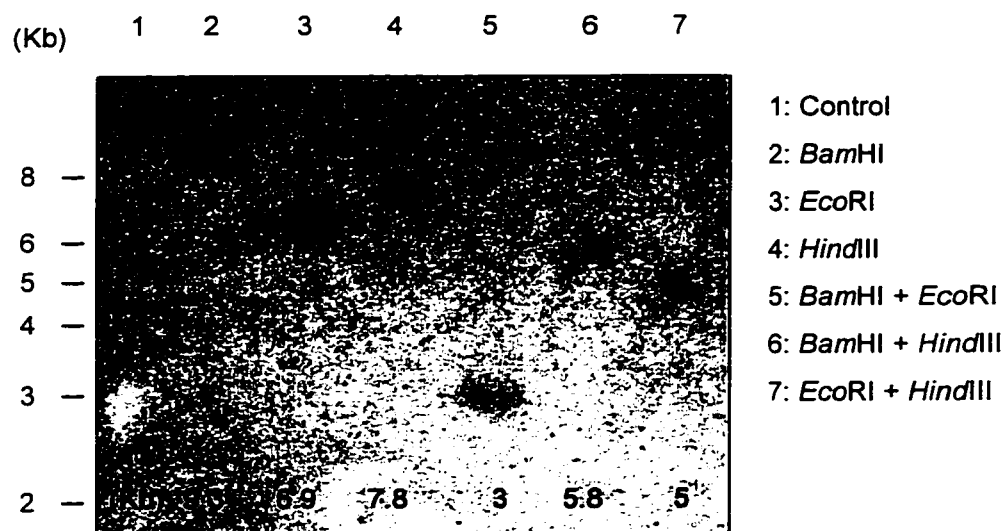
Figure 4.16 Restriction map of PT10-2 (*B. oleracea* var. *italica* cv. Green Valiant) based on Southern analysis with three restriction enzymes *Bam*HI, *Eco*RI and *Hind*III

Single digestion with each enzyme and double digestions with combinations of each enzyme were performed to generate a restriction map of adjacent upstream and downstream areas of the GUS::NPTII insert. About 800bp of PCR amplified DNA fragment from *npt*II gene of pRD320 was used as a probe for hybridization.

4.4 Discussion

Promoter tagging represents an effective method of identifying and cloning plant genes and may be particularly useful for identifying genes that are expressed in restricted cell types or that are expressed during short periods of development (Koncz *et al.* 1989, 1990; Kertbundit *et al.* 1991; Topping *et al.* 1991; Walden *et al.* 1991, 1994; Lindsey *et al.* 1993, 1995). A strategy based on T-DNA insertional mutagenesis may be exploited to identify plant promoters using specially designed T-DNA vectors which contain a reporter gene without transcriptional elements (i.e. promoterless constructs) located close to the border sequences (Teeri *et al.* 1986; Koncz *et al.* 1989; Kertbundit *et al.* 1991; Lindsey *et al.* 1993; Suntio and Teeri 1994; Topping et al 1994; Fobert *et al.* 1991;1994). If the coding sequence of the promoterless reporter gene is integrated downstream of a native gene's promoter elements, the reporter gene will be transcribed and expressed. The resulting hybrid genes produced by T-DNA-mediated promoter tagging, consist of unknown plant promoters residing at their natural location within the chromosome, and the coding sequence of a marker gene located on the inserted T-DNA (Fobert *et al.* 1991). The plant promoter sequences controlling the expression of these gene fusions can subsequently be isolated and further analyzed by standard molecular genetic techniques. This T-DNA based promoter tagging system requires *Agrobacterium*-mediated transformation as a prerequisite and therefore, application of this system is limited to few species in spite of its powerful potential for promoter studies (Table 1.6). The most commonly used target plants represent species such as *Arabidopsis thaliana* (Koncz *et al.* 1989; Lindsey *et al.* 1993), *Nicotiana tabacum* (Teeri

et al. 1986; Fobert 1991;1994) and *Solanum tuberosum* (Lindsey *et al.* 1993) in which an *in vitro* culture system was well established with a high transformation frequency by *Agrobacterium.* The objective of this study was to evaluate the feasibility of the application of a T-DNA based promoter tagging strategy with the genus *Brassica.*

Routinely, one of the simplest methods of plant transformation is explant inoculation. This involves the incubation of excised explants with *Agrobacterium* containing the appropriate transformation vector, followed by culturing the explants on media that contain a selection agent to recover transformed cells or callus from which plants can be regenerated by appropriate methods. Transformant selection can be applied at the initiation of callus formation as well as at the stage of transfer of shoots to the rooting medium. While this approach has been used by several workers for promoter/gene tagging, generation of transgenic lines is labor intensive and it requires the production of a large number of independent transgenic plants (Walden *et al.* 1991; 1994). As the genus *Brassica* includes many important crop plants, the isolation of promoters from these species could have several applications in genetic engineering of useful traits. The promising results obtained from the speed transformation method (see Chapter 3) made it feasible to attempt tagging experiments in *Brassica* species. This protocol was successfully employed to produce seven promoter tagged transgenic plants lines from *B. oleracea* and *B. napus.*

More than 18,000 cotyledonary petiole explants were infected with *Agrobacterium* strain GV3101/pMP90 harboring the promoterless construct pΔGUS::NPTII. A chimeric kanamycin-resistance gene present in this vector allowed

for the selection of transgenic plants. As soon as green shoot buds appeared on explants, they were transferred to a kanamycin-free shoot elongation medium. Because kanamycin resistance is expressed at the early stage of cell division and callus formation (even though the tagged promoters possess tissue specific regulatory properties), it is critical to make selections during the early stage of shoot differentiation in order to optimize recovery of tagged lines. Although this promoterless bifunctional gene system requires more attention during the selection stage, it requires less labor than the two step selection system used by others in previous studies (Koncz *et al.* 1989; Kertbundit *et al.* 1991; Lindsey *et al.* 1993).

One transgenic line from broccoli and six transgenic lines from oilseed rape (*B. napus*) were recovered as promoter tagged transgenic plants. Although the overall frequency was very low (0.037%), it was possible to produce promoter tagged plants in *Brassica* species using the speed transformation method developed in this study. According to researchers who have used similar strategies, a 100-fold reduction (compared to normal transformation) in transformation frequency has been experienced in promoter tagging experiments with *A. thaliana*, *N. tabacum* and *B. carinata* (personal communications by R. Datla, B. Weston-Bauer and V. Babic, National Research Council, Saskatoon, Canada). As a successful promoter tagging event requires insertion of the T-DNA based reporter gene adjacent to the promoter in the plant genome and in the correct reading frame, such an event happens less frequently than an ordinary transformation event which can occur randomly anywhere in the plant genome, having its own promoter in the insertion cassette.

Four out of the seven promoter-tagged transgenic plants exhibited constitutive expression while others were root predominant, phloem predominant and shoot specific. The promoter in transgenic line PT7-1 showed GUS activity in pollen unlike the widely used CaMV 35S promoter that does not express well in pollen (Hoekstra and Bruinsma 1979; Mascarenhas and Hamilton 1992). This promoter may therefore be considered to be truly constitutive.

Based on Southern analysis and segregation data, six out of seven transgenic lines had single copy insertions while one had five inserts. These results suggest that this tagging system using a GUS::NPTII bifunctional promoterless construct provides a high frequency of single copy insertions (85%). This is preferred for cloning the tagged promoters. The observation in this study contrast that of other studies that have reported a high frequency of multiple insertions. More than 67% of multiple insertions in *Arabidopsis* and *Nicotiana* (Koncz *et al.* 1989), 40-50% in *Arabidopsis* (Topping *et al.* 1994) and 67% in *Nicotiana* and 48% in *Arabidopsis* (Lindsey *et al.* 1993) have been reported with various types of promoterless constructs. Multiple insertion events in some of these promoter tagging experiments may be due to the specific construct, the transformation method, the target plant or combinations of these factors (Walden *et al.* 1991; Topping and Lindsey 1995).

Constitutive expression in the tagged lines (PT6-1 and PT7-1) was further investigated to find their relative strength in comparison with the most widely used CaMV 35S constructs. Based on a shoot regeneration test on 200 mg/L kanamycin, the transgenic line PT6-2 showed almost the same regenerability as transgenic plants

containing a single CaMV 35S promoter. Another transgenic line, PT7-1 exhibited levels of regenerability comparable to transgenic lines with a tandem CaMV 35S promoter enhanced by AMV. In tests with transgenic line PT7-1 on 100 mg/L kanamycin, shoot regeneration was virtually unaffected. This suggests that the insert in the transgenic line PT7-1 may be downstream of a strong constitutive promoter that can be used as an alternative to the CaMV 35S promoter.

Most of the promoters so far identified by T-DNA tagging methods exhibit organ or tissue specific expression (Teeri *et al.* 1986; Kertbundit *et al.* 1991; Lindsey *et al.* 1993; Suntio and Teeri 1994; Babic *et al.* 1994) with limited information on the identification of constitutive promoters available. Promoter tagged lines investigated in this study could potentially provide new constitutive promoters for crop genetic engineering. As a part of characterization of promoters from the lines PT7-1 (constitutive) and PT10-2 (shoot specific), restriction maps adjacent to GUS::NPTII inserts have been generated. Further detailed analyses will be undertaken.

CONCLUSIONS

The Development of an Efficient Multiple Shoot Regeneration System in Broccoli (*Brassica oleracea* var. *italica*)

1. An efficient and reproducible multiple shoot regeneration system based on *in vitro* organogenesis has been established with five broccoli cultivars and one breeding line using hypocotyl and cotyledonary petiole explants from *in vitro* grown seedlings.

2. Culture variables such as the ratio of BA to NAA and type of explant strongly influenced organogenesis from hypocotyl and cotyledonary petiole explants.

3. Hypocotyl explants were superior to cotyledonary petiole explants in terms of frequency of responding explants as well as shoot number per explant.

4. The highest percentage of shoot formation (100%) and greatest average number of shoots per explant (28) were observed with the cultivar 'Cruiser' from hypocotyl explants. The cultivar 'Cruiser' gave the highest regeneration frequency (100%) and greatest mean number of shoots per explant (12) from cotyledonary petiole explants among tested cultivars.

5. Shoots rooted with up to 100% frequency after 4 weeks of culture on growth regulator free MS basal medium, giving rise to plants that appeared normal in development.

6. The shoot organogenesis and plant regeneration methodology developed should have wide application in propagation and genetic transformation studies.

The Development of Efficient Methodology for *Agrobacterium*-mediated Transformation of *Brassica oleracea*

1. An *Agrobacterium*-mediated transformation system based on the use of cotyledonary petiole explants from *in vitro* grown seedlings was developed to transform broccoli.

2. Transgenic plants were obtained at frequencies of 1-10% by co-cultivation with *A. tumefaciens* carrying a plasmid coding for β-glucuronidase (GUS), neomycin phosphotransferase (NPTII) and phosphinothricin acetyl transferase (PAT) which confers herbicide (phosphinothricin) tolerance under the independent regulation of the cauliflower mosaic virus (CaMV) 35S promoter.

3. The hypersensitive response of explants was reduced after co-cultivation with *Agrobacterium* on filter paper, thereby increasing the transformation frequency.

4. Pre-culturing of cotyledonary petiole explants on regeneration medium for two days prior to co-cultivation generally enhanced transformation frequency.

5. Cold-treatment of explants during the first few days of co-cultivation and vacuum infiltration improved transformation frequency.

6. An efficient *Agrobacterium*-mediated transformation method termed the Speed Transformation Method was developed with significant modifications of conventional methods to facilitate handling very large numbers of explants in a short period of time.

7. Transformation of other *B. oleracea* varieties such as cauliflower, kale, kohlrabi, cabbage and Brussels sprout was also successful with this method.

8. Segregation analysis for the presence of GUS gene in R_1 progeny of transgenic broccoli showed that 75% of transgenic lines considered a single insertion event.

9. The genetic transformation methodology developed in this study should have widespread application in crop biotechnology as well as in fundamental studies of plant molecular biology.

Promoter Tagging in *Brassica oleracea* var. *italica* and *Brassica napus* Through the Use of a *gus::npt*II Fused Gene Construct

1. An *Agrobacterium*-mediated transformation system in broccoli and *B. napus* has been used in a promoter tagging study employing a *gus::npt* II promoterless fused gene construct.

2. Seven promoter tagged transgenic lines were produced through the use of the promoterless construct and GUS expression analyses revealed that the tagged putative promoters included constitutive, root predominant, phloem predominant and shoot specific types.

3. Promoter tagging frequency was 0.012% with broccoli and 0.056% with *B. napus*.

4. Based on *in vitro* tolerance to kanamycin, the strength of a putative novel constitutive promoter (from PT7-1) identified in this study was at least as strong as the CaMV 35S promoter.

5. Future studies on isolation and characterization of these promoters could contribute to the development of additional strategies for the regulation of transgenes in transgenic plants.

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APPENDICES

APPENDIX 1

Basal plant tissue culture media (mg/L)

COMPONENT	MSB5[a]	MSMS[b]	MMO[c]
Ammonium Nitrate	1650.0	1650.0	1650.0
Boric Acid	6.2	6.2	6.2
Calcium Chloride Anhydrous	332.2	332.2	332.2
Cobalt Chloride.6H_2O	0.025	0.025	0.014
Cupric Sulfate.5H_2O	0.025	0.025	
Cupric Sulfate Anhydrous			0.016
Na_2.EDTA	37.26	37.26	37.25
Ferrous Sulfate.7H_2O	27.8	27.8	27.8
Magnesium Sulfate Anhydrous	180.7	180.7	180.7
Manganese Sulfate. H_2O	16.9	16.9	16.9
Molybdic Acid(Sodium Salt).2H_2O	0.25	0.25	0.25
Potassium Iodide	0.83	0.83	0.83
Potassium Nitrate	1900.0	1900.0	1900.0
Potassium Phosphate Monobasic	170.0	170.0	170.0
Zinc Sulfate.7H_2O	8.6	8.6	5.37
ORGANIC SUPPLEMENTS			
Glycine (Free Base)	2.0		
myo-inositol	100.0	100.0	
i-inositol			100.0
Nicotinic Acid (Free Acid)	1.0	0.5	
Pyridoxin.HCl	1.0	0.5	
Thiamin.HCl	10.0	0.1	0.4

a: MSB5 medium was acquired from Sigma, USA, Cat.No. M0404.
b: MSMS medium was acquired from Sigma, USA, Cat.No. M5519.
c: MMO medium was acquired from GibcoBRL, USA, Cat.No. 23118-078.

APPENDIX 2

Regeneration response of hypocotyl (H) and cotyledonary petiole (P) explants from 22 broccoli cultivars plated on a medium* with 6 different growth regulator combinations. The first number is the mean value of shoots per explant and the number in brackets is the percentage of responding explants.

Cultivar	BA 1/NAA 0**		BA 1/NAA 0.1		BA 1/NAA 1	
	H	P	H	P	H	P
Arcadia	9.7(100)	1.1(50)	0.4(20)	3.3(50)	4.3(80)	0.0(0)
Bronzino	2.6(20)	0.0(0)	0.0(0)	1.7(20)	0.0(0)	0.6(20)
Corvet	0.0(0)	3.2(40)	0.0(0)	0.0(0)	0.0(0)	0.0(0)
Cruiser	16.9(100)	3.1(20)	4.7(40)	6.4(70)	2.0(40)	0.4(10)
Emperor	0.8(10)	0.0(0)	0.0(0)	1.4(50)	0.4(20)	1.4(40)
Eureka	4.4(50)	13.9(90)	5.5(60)	5.7(90)	2.7(40)	0.0(0)
First Choice	21.3(100)	0.5(10)	13.9(100)	4.3(50)	5.5(60)	0.3(10)
Goliath	1.1(40)	0.7(20)	0.5(20)	0.0(0)	2.4(70)	1.2(40)
Green Belt	3.1(62)	8.5(75)	1.8(40)	0.7(10)	2.0(20)	0.0(0)
Green Comet	7.9(80)	7.3(60)	7.3(70)	2.0(50)	3.0(20)	1.9(40)
Green Duke	0.3(20)	0.2(10)	3.3(40)	0.0(0)	1.4(10)	1.5(60)
Green Hornet	2.6(30)	0.7(10)	1.7(40)	1.8(30)	1.1(20)	2.6(30)
Green Sprouting	5.0(60)	0.0(0)	11.0(70)	1.2(20)	1.4(20)	0.0(0)
Green Valiant	21.8(100)	0.0(0)	12.1(90)	0.8(20)	1.8(50)	0.4(30)
King Robert	3.8(50)	1.9(20)	3.7(40)	0.8(30)	3.6(50)	4.4(50)
Mariner	0.3(10)	0.0(0)	1.0(10)	3.6(60)	0.0(0)	0.6(20)
Packman	14.4(100)	0.9(30)	21.3(100)	3.1(50)	3.8(90)	3.1(50)
Paragon	2.3(60)	0.0(0)	0.7(20)	0.2(10)	0.7(20)	0.2(10)
Premium Crop	4.2(60)	4.3(70)	1.2(30)	1.7(40)	0.0(0)	0.0(0)
Red Broccoli	2.7(20)	5.4(100)	5.4(40)	5.0(100)	1.3(10)	2.8(70)
Romanesco	1.3(20)	4.3(70)	2.3(20)	2.6(50)	0.0(0)	2.2(70)
Waltham 29	9.4(80)	3.8(20)	1.1(40)	5.5(70)	0.5(20)	0.6(30)
MEAN	*6.2(53.2)*	*2.7(31.5)*	*4.5(40.4)*	*2.4(39.5)*	*1.7(29.0)*	*1.1(28.1)*

* Basal medium: MSB5/pH5.8, 30 explants per cultivar for preliminutesary screening;
** Growth regulator (mg/L)

continued from previous page

Cultivar	BA 4/NAA 0		BA 4/NAA 0.1		BA 4/NAA 1	
	H	P	H	P	H	P
Arcadia	9.6(60)	10.9(90)	7.1(40)	5.1(70)	0.0(0)	2.6(70)
Bronzino	0.0(0)	0.8(20)	0.0(0)	1.2(10)	0.0(0)	2.3(40)
Corvet	0.0(0)	3.3(60)	1.1(20)	0.0(0)	0.0(0)	0.0(0)
Cruiser	12.8(70)	12.9(40)	9.8(80)	0.0(0)	7.8(80)	1.3(20)
Emperor	2.1(30)	1.3(30)	0.0(0)	1.2(30)	0.0(0)	1.7(30)
Eureka	9.3(60)	9.2(70)	4.9(40)	7.6(100)	0.4(10)	2.8(60)
First Choice	13.6(80)	0.0(0)	20.7(100)	0.0(0)	15.6(70)	6.7(10)
Goliath	8.8(90)	0.5(10)	0.0(0)	1.4(30)	1.4(40)	4.2(80)
Green Belt	8.5(90)	5.6(50)	4.5(40)	0.0(0)	1.7(20)	0.0(0)
Green Comet	24.6(80)	24.1(60)	0.9(30)	6.6(70)	10.5(80)	2.3(70)
Green Duke	1.6(40)	0.4(10)	0.0(0)	0.0(0)	6.2(40)	2.3(60)
Green Hornet	2.3(30)	0.9(10)	2.0(30)	2.0(30)	2.4(60)	2.4(30)
Green Sprouting	9.3(40)	6.1(60)	6.3(50)	1.0(10)	2.6(50)	0.0(0)
Green Valiant	12.8(80)	5.2(60)	23.0(100)	0.0(0)	4.0(60)	0.6(20)
King Robert	5.8(60)	0.3(10)	7.4(50)	4.0(50)	3.6(50)	4.6(70)
Mariner	4.2(60)	0.0(0)	8.4(80)	3.5(70)	5.0(60)	2.6(80)
Packman	23.7(100)	1.8(40)	16.9(100)	3.1(60)	9.0(100)	3.0(80)
Paragon	4.7(40)	0.0(0)	1.0(30)	0.7(10)	3.7(40)	5.9(90)
Premium Crop	4.7(40)	2.2(20)	2.4(30)	0.7(20)	0.0(0)	1.1(20)
Red Broccoli	2.0(10)	4.6(80)	3.2(10)	3.3(80)	4.8(20)	5.1(90)
Romanesco	8.9(50)	4.6(60)	3.8(10)	2.0(40)	0.9(30)	1.5(50)
Waltham 29	12.5(100)	15.1(80)	8.2(80)	6.0(70)	0.0(0)	1.4(30)
MEAN	*8.3(50.5)*	*5.0(39.0)*	*6.0(41.8)*	*2.2(34.0)*	*3.6(36.8)*	*2.5(45.4)*

Cultivar	OVERALL MEAN	
	H	*P*
Arcadia	*5.2(50)*	*3.8(55)*
Bronzino	*0.4(3.3)*	*1.1(18.3)*
Corvet	*0.2(3.3)*	*1.1(16.7)*
Cruiser	*9(68.3)*	*4(26.7)*
Emperor	*0.6(10)*	*1.2(30)*
Eureka	*4.5(43.3)*	*6.5(68.3)*
First Choice	*15.1(85)*	*2(13.3)*
Goliath	*2.4(43.3)*	*1.3(30)*
Green Belt	*3.6(45.3)*	*2.5(22.5)*
Green Comet	*9(60)*	*7.4(58.3)*
Green Duke	*2.1(25)*	*0.7(23.3)*
Green Hornet	*2(35)*	*1.7(23.3)*
Green Sprouting	*5.9(48.3)*	*1.4(15)*
Green Valiant	*12.6(63)*	*1.2(21.7)*
King Robert	*4.7(50)*	*2.7(38.3)*
Mariner	*3.2(36.7)*	*1.7(38.3)*
Packman	*14.9(98)*	*2.5(51.7)*
Paragon	*2.2(35)*	*1.2(20)*
Premium Crop	*2.1(26.7)*	*1.7(28.3)*
Red Broccoli	*3.2(18.3)*	*4.4(86.7)*
Romanesco	*2.9(21.7)*	*2.9(56.7)*
Waltham 29	*5.3(53.3)*	*5.4(50)*

APPENDIX 3

Regeneration response of hypocotyl explants from five broccoli cultivars and one breeding line plated on a medium[*] with 16 different growth regulator combinations[**].

Shoot number per explants

Media No.	Combination	Cruiser	First Choice	Green Valiant	Packman	Shogun	UL	*MEAN*
1	BA 1/NAA 0	15.0	10.0	15.3	16.5	14.9	19.3	*15.2*
2	BA 1/NAA 0.01	9.7	8.0	10.4	14.0	11.2	20.2	*12.3*
3	BA 1/ NAA 0.1	6.0	6.0	4.1	9.6	11.0	13.4	*8.4*
4	BA 1/NAA 1	3.0	3.6	3.5	5.0	5.0	2.6	*3.8*
5	BA 2/NAA 0	7.9	13.6	10.2	10.0	9.7	18.4	*11.6*
6	BA 2/NAA 0.01	17.3	12.0	10.4	11.5	12.0	20.2	*13.9*
7	BA 2/NAA 0.1	8.1	10.0	13.7	8.0	11.8	16.5	*11.4*
8	BA 2/NAA 1	3.8	5.0	4.8	7.2	5.6	6.5	*5.5*
9	BA 4/NAA 0	18.7	7.7	25.0	22.8	13.3	19.5	*17.8*
10	BA 4/NAA 0.01	28.6	13.3	20.0	18.3	15.3	19.9	*19.2*
11	BA 4/NAA 0.1	7.9	9.5	21.0	16.9	8.0	19.7	*13.8*
12	BA 4/NAA 1	5.6	4.3	1.5	8.0	9.4	10.2	*6.5*
13	BA 8/NAA 0	14.2	15.0	14.0	17.8	13.0	11.3	*14.2*
14	BA 8/NAA 0.01	19.9	8.4	12.8	18.8	16.7	17.7	*15.7*
15	BA 8/NAA 0.1	14.7	5.6	16.6	18.3	11.7	13.4	*13.4*
16	BA 8/NAA 1	6.7	7.3	0.3	8.0	13.5	10.7	*7.8*
MEAN		*11.7*	*8.7*	*11.5*	*13.2*	*11.4*	*15.0*	

* Basal medium: MSB5/pH5.8, 50 explants per cultivar for detailed experiments
** Growth regulator (mg/L)

Shooting frequencies (%)

Media No.	Combination	Cruiser	First Choice	Green Valiant	Packman	Shogun	UL	*MEAN*
1	BA 1/NAA 0	93.3	96.6	100.0	100.0	96.0	100.0	*97.7*
2	BA 1/NAA 0.01	80.0	90.0	90.0	100.0	100.0	100.0	*93.3*
3	BA 1/ NAA 0.1	76.6	70.0	96.0	93.3	100.0	100.0	*89.3*
4	BA 1/NAA 1	46.7	73.0	70.0	93.3	93.3	73.3	*74.9*
5	BA 2/NAA 0	100.0	83.3	87.0	90.0	96.6	96.6	*92.3*
6	BA 2/NAA 0.01	96.6	96.6	100.0	96.6	96.6	96.6	*97.2*
7	BA 2/NAA 0.1	90.0	93.3	96.6	96.6	100.0	96.6	*95.5*
8	BA 2/NAA 1	43.3	60.0	76.0	63.3	90.0	80.0	*68.8*
9	BA 4/NAA 0	80.0	86.6	96.6	86.7	96.6	96.6	*90.5*
10	BA 4/NAA 0.01	73.3	93.3	90.0	90.0	100.0	100.0	*91.1*
11	BA 4/NAA 0.1	66.7	83.3	93.3	96.7	100.0	100.0	*90.0*
12	BA 4/NAA 1	86.6	53.3	83.3	70.0	90.0	90.0	*78.9*
13	BA 8/NAA 0	46.7	60.0	93.3	96.6	73.0	70.0	*73.3*
14	BA 8/NAA 0.01	80.0	80.0	93.3	93.3	100.0	96.6	*90.5*
15	BA 8/NAA 0.1	70.0	73.3	93.3	83.3	100.0	90.0	*85.0*
16	BA 8/NAA 1	86.6	83.3	60.0	90.0	93.0	93.3	*84.4*
MEAN		*76.0*	*79.7*	*88.7*	*90.0*	*95.3*	*92.5*	

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APPENDIX 4

Histochemical β-glucuronidase (GUS) assay
(modified from Jefferson 1987)

REAGENTS

X-Gluc assay buffer:

8.8 g	Na_2HPO_4
5.24 g	$NaH_2PO_4.H_2O$
10 mL	0.1 M $K_3[Fe(CN)_6]$
10 mL	0.1 M $K_4[Fe(CN)_6].3H_2O$
40 mL	0.5 M EDTA..Na_2

bring up to 1000 mL with ddH_2O and adjust to pH 7.0

To prepare a working solution: weigh out 50 mg X-Gluc into a microtube, dissolve in 1 mL NN-dimethylformide, then add to 100 mL X-Gluc buffer and mix well

PROTOCOL

Dispense 100 μL X-Gluc solution into each well of a 96 well plastic plate. Place a piece of leaf tissue from putative transgenic plants into each well then add a drop of Tween-20 into each well. Apply a vacuum treatment for five minutes. Seal the plate with Saran Wrap® and incubate overnight at 37 °C. Remove X-Gluc staining solution from the well and add 10-fold diluted commercial bleach (Javex®) solution and leave at room temperature for several hours then observe.

APPENDIX 5

Neomycin phosphotransferase II (NPTII) activity assay
(modified from Reiss *et al.* 1984)

REAGENTS

Extraction Buffer:

3.32 g $NaH_2PO_4.H_2O$
0.23 g $Na_2HPO_4.H_2O$
0.5 mL   Triton X-100
0.5 g   Sarcosyl (n-lauroyl sarcosine)
0.35 mL β-mercaptoethanol
1.46 g   EDTA (free acid)

pH to 7.0 with NaOH and bring up volume to 100 mL with ddH$_2$O, stored at 4 °C

Reaction Buffer:

0.67 mL 1M Tris-maleate buffer, pH7.1
           12.1 g   Tris base
           11.6 g   maleic acid
           19.8 mL 5N NaOH, make to 100 mL with ddH$_2$O

0.42 mL 1M MgCl2
1.0 mL   4M NH4Cl
17 μL   1M dithiothreitol (DTT)
7.49 mL ddH$_2$O

Split into 2x 5 mL aliquots. To one, add 40 μL 100 mg/mL kanamycin sulfate (positive reaction). To the other, add 40 μL ddH$_2$O (negative reaction). Make these buffers up fresh and store on ice or at 4 °C

ATP solution:

3.71 μL   cold ATP (100 mM)
5 μL   [γ ^{32}P-ATP] (3000Ci/mmol; 10 μCi/μL)
492 μL   ddH$_2$O

make up immediately before use

PROTOCOL

Extract preparation:

Place approximately 0.2 gram of leaf tissue into a microtube and keep on ice. Add 50 μL of extraction buffer and grind with a pellet pestle. Add 200 μL ddH$_2$O and grind until tissue is throughly homogenized. Centrifuge for 5 minutes with maximum speed using a microcentrifuge at 4 °C and transfer supernatant to a fresh tube on ice.

Protein quantification:

The total amount of protein in each sample is determined relative to bovine serum albumin (BSA) using a Bradford's assay (Bradford 1976).

Reaction:

For each plant sample, prepare (on ice) two reactions in microtube: one with kanamycin positive reaction buffer and one with kanamycin negative reaction buffer.

> For each reaction:
> > 20 μL plant extract (40 μg of protein/20 μL sample)
> > 10 μL reaction buffer (kanamycin positive and negative reaction buffer)
> > 10 μL ATP solution

Flick tubes gently to mix and incubate at 30 °C for 30 minutes. Stop reactions by returning them to ice. During the incubation, assemble the Dot Blot Apparatus with 3 layers of Whatman P81 ion exchange paper on top of one layer of Whatman 3MM paper (cut to fit apparatus).

Pipette reaction mixture to the wells of the Dot Blotter and allow the liquid to be completely absorbed by the paper layers. Discard the 3MM paper to the radioactive waste and wash the three P81 papers as follows:

> 2x 5 minutes in ddH$_2$O at room temperature
> 1x 45 minutes in 0.5 mg/mL Proteinase K solution (25 mg Proteinase K,
> > > 2.5 mL of 20% SDS, 47.5 mL ddH$_2$O)

Dry the papers, cover with Saran Wrap® and autoradiograph with Kodak X-OMAT AR film. Expose papers to the film at -70°C for 24 hours, and then develop.

APPENDIX 6

Phosphinothricin acetyltransferase (PAT) activity assay
(modified from DeBlock *et al.* 1989)

REAGENTS

Extraction buffer: for 10 mL

    5 mL   100 mM Tris-Cl, pH 7.5
    40 μL   500 mM EDTA, pH 8.0
    1.5 mL   1 mg/mL leupeptin (frozen stock in ddH$_2$O)
    600 μL 5 mg/mL BSA (frozen stock in ddH$_2$O)
    3 mL   1 mg/mL DTT (frozen stock in ddH$_2$O)

immediately before use, add 50 μL freshly prepared 30 mg/mL PMSF (stock made up in isopropanol: 0.003 g PMSF in 10 μL isopropanol); Poisonous!
PMSF (Phenylmethylsulfonyl Fluoride) : inhibitor of serine proteases such as
                                  trypsin and chymotrypsin

12% TCA

Developing solvent for TLC:

    60 mL   1-propanol
    10 mL   NH$_4$OH
    30mL   ddH$_2$O

dispense into a TLC tank, which has a good seal (stop cock grease) on the lid

PROTOCOLS

Extract preparation:

Place approximately 0.2 gram of leaf tissue into a microtube and keep on ice. Add 500 μL of extraction buffer and grind with a small pestle. Centrifuge for 5 minutes at maximum speed in a microcentrifuge at 4 °C and transfer supernatant to a fresh tube on ice.

Protein quantification:

The total amount of protein in each sample is determined relative to bovine serum albumin (BSA) using Bradford's assay (Bradford 1976).

Reaction:

All samples were standardized to 50 μg of total protein in 20 μL reaction buffer. All standardized samples were kept on ice at all times.

For each reaction:

20 μL	plant extract (50 μg protein/20 μL)
5 μL	1 mM L-ppt (frozen stock made up in 50 mM Tris-Cl, pH 7.5)
3 μL	^{14}C-acetyl CoA (58.1 mCi/mmol: NEN)

Mix well and incubate samples at 37 °C for 2 hours

Following incubation, add 22 μL of 12% (w/v) TCA (trichloroacetic acid) to each tube and mix well. Let sit for two minutes at room temperature (to precipitate proteins) and then centrifuge at maximum speed in a microcentrifuge for three minutes at room temperature.

Spot 10 μL of the supernatant (3x 3.3 μL aliquots) onto a flexible Whatman Silica Gel (PE SIL G, Cat. No. 4410221) plate (1 cm from the bottom of the plate). Allow each aliquot to dry before applying the next one. Also spot a set of standards (10 μL per standard) onto each plate as a reference. Ensure the spots are dry before placing the TLC plate upright in a TLC chamber containing the developing solvent. Let the solvent front migrate for approximately 4 hours.

Dry the TLC plate, cover with Saran Wrap® and autoradiograph with Kodak X-OMAT AR film. Expose papers to the film at -70°C for 2 days, and then develop.

APPENDIX 7

Method for plant DNA extraction using CTAB without phenol

SUMMARY

Current DNA technologies in crop improvement and phylogenetic studies require an efficient and reliable procedure for DNA extraction from plants. Isolation of high quality plant DNA is known to be difficult because of the presence of contaminating polysaccharides (Delaporta *et al.* 1983; Hattori *et al.* 1987; Rowland and Nguyen 1993). To overcome this problem, an efficient and reproducible method for the isolation of high molecular weight DNA based on the CTAB procedure (Murray and Thompson 1980; Doyle and Doyle 1987) but without using phenol has been developed. This method minimizes polysaccharide contamination and replaces chloroform with the less toxic methylene chloride (dichloromethane) (Maloisel and Perbal 1990). The phenol extraction step was removed and Oil-Red (Elder and Turner 1993) was used to improve the visualization of the organic:aqueous interface. DNA yield from this method was approximately 1-2 mg per 2 grams of leaf tissue and the 260nm/280nm ratio was higher than 1.8 on average and the preparations are therefore highly suitable for Southern blotting, RFLP analysis and DNA fingerprinting. This method worked well with *Brassica napus*, *B. oleracea*, *B. rapa*, *Arabidopsis thaliana* and *Nicotiana tabacum* and may be applied to other plant species.

REAGENTS

1%   Proteinase K
10% CTAB
5M   NaCl
70% Ethanol
Iso-propanol

Lysis buffer:

0.88 g	NaCl
1.68 g	EDTA.Na$_2$
0.12 g	Tris-base
1000 mL	ddH$_2$O
(pH 7.2-7.5)	

RNase A: 10 mg/mL in 10 mM Tris.Cl (pH7.5), 15 mM NaCl, boiled for 5 minutes

Methylene Chloride:iso-amylalcohol (24:1)

add trace amount of Oil Red-O to make this solution a red wine color;
this color will then distinguish two phases. Methylene chloride is a less toxic
than chloroform.

PROTOCOL

grind 2 grams of leaf tissue in liquid nitrogen

add 5 mL lysis buffer and prepare a slurry; decant into a 30 mL Oakridge® tube or equivalent, and bring to 10 mL with lysis buffer

add 40 μL of 1% Proteinase K

add 2.5 mL of 10% SDS

incubate at 60°C for 1 hour, mix gently from time to time

add 2.5 mL of 5M NaCl and 2 mL of 10% CTAB, gentle mix

incubate at 60°C for 30 minutes, mix gently from time to time

spin down at 8,000x G at 5°C for 5 minutes

transfer supernatant into a new 30 mL Corex® tube

add same volume of Methylene Chloride:iso-amylalcohol (24:1)

spin down at 8,000x G at 5°C for 10 minutes

transfer upper phase into a new tube

add 20 μL RNase A, incubate for 30 minutes at 37°C

add same volume of Methylene Chloride:iso-amylalcohol (24:1)

spin down at 8,000x G at 5°C for 10 minutes

transfer upper phase into a new tube

add same volume of cold isopropanol, gentle mix

spin down at 8,000x G at 5°C for 15 minutes, then wash once with cold 70% ethanol

dry and dissolve in ddH₂O

APPENDIX 8

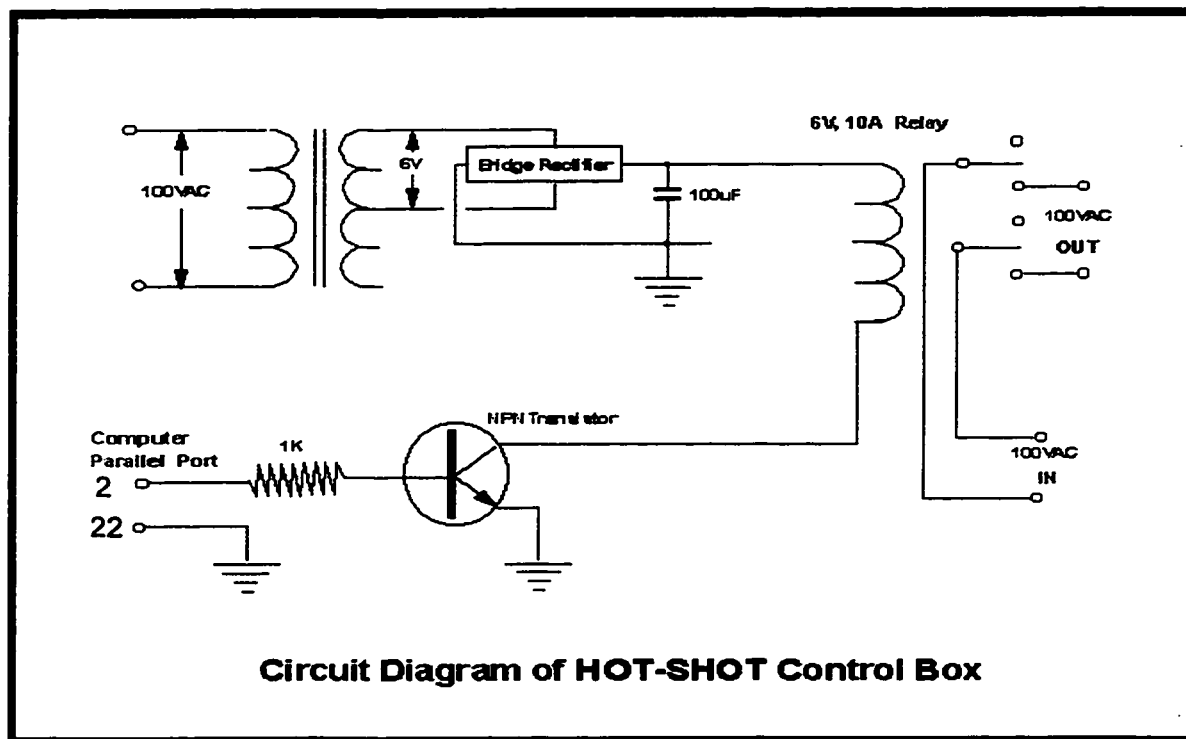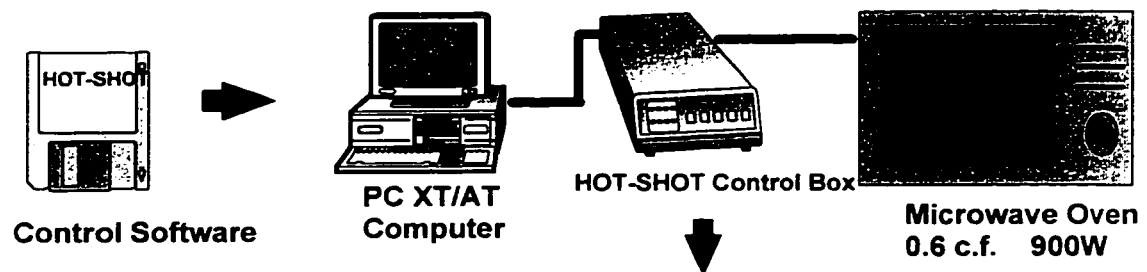## HOT-SHOT: Automation of microwave oven mediated
DNA digestion

SUMMARY

Site specific cleavage of DNA by restriction endonucleases followed by electrophoretic

fractionation of the   resulting fragments is used routinely in laboratories to study

restriction fragment length polymorphism (RFLP) and DNA fingerprinting, and in other

molecular biology investigations.   Traditionally, digestions of DNA with restriction

endonucleases are carried out in a buffer containing salts at 37°C. The incubation time

varies with the type and quality of DNA and the kind of restriction endonuclease used.

Although the digestion of plasmid DNA with certain enzymes can be achieved in 1 hour

using a large amount of enzyme, genomic DNA digestions generally require overnight

incubation. Since digestion of DNA with restriction endonucleases is a vital but time-

consuming technique, it would be useful to develop an efficient and more rapid

procedure.   Jhingan (1992) investigated various conditions for microwave oven-

mediated DNA digestion. Even if this technique can reduce reaction time, this requires

laborious key-pad punching steps to achieve 20-40 cycles of Power On/Power Off. In

order to avoid this, an automatic control system based on an IBM PC system has been

developed. The control unit is simple and inexpensive to make. The control Program is

written in Quick-BASIC and compiled into an executable file. A microwave oven (0.4 cubic feet, 900 W output power) with a 30 minutes timer switch was used in this study. Conditions for various restriction enzymes were established by Jhingan (1992) and this study focused on the automation of this process.

Circuit Diagram of HOT-SHOT

Control Software

PC XT/AT Computer

HOT-SHOT Control Box

**Microwave Oven
0.6 c.f.   900W**

Circuit Diagram of HOT-SHOT Control Box

HOT-SHOT Control Program Written in Quick-BASIC

```
10 OUT &H378, 0
20 CLS : LOCATE 15, 35: COLOR 20, 3: PRINT "<< HOT SHOT >>"
30 LOCATE 17, 18: COLOR 1, 3: PRINT "(C) Copyright 1993, All Right Reserved by Sun Lee"
40 FOR QQ = 1 TO 5000: NEXT
50 COLOR 3, 0: CLS : LOCATE 2, 1: PRINT "PLEASE ENTER EACH PARAMETERS . . . ."
60 LOCATE 7, 1: COLOR 4: INPUT "> SHOOTING TIME (SEC) "; ST
70 LOCATE 9, 1: COLOR 2: INPUT "> INTERVAL TIME (SEC) "; IT
80 LOCATE 11, 1: COLOR 5: INPUT "> CYCLE NUMBER "; CN
90 CLS : LOCATE 1, 1: COLOR 2, 0: PRINT "<< PROGRAMMED ENVIRONMENT >>"
100 LOCATE 2, 1:
110 PRINT : PRINT "+ SHOOTING TIME : "; ST; " (SEC)"
120 PRINT "+ INTERVAL TIME : "; IT; " (SEC)"
130 PRINT "+ CYCLE NUMBER  : "; CN; " (CYCLES)"
140 LOCATE 12, 32: COLOR 7: PRINT "<< WORKING ENVIRONMENT >>"
150 F = F + 1
160 IF F > CN THEN 410
170 LOCATE 15, 32: COLOR 5: PRINT "+ CYCLE NUMBER : "; F: SOUND 780, 1
180 REM
190 REM          SHOOTING TIME CONTROL
200 REM
210 REM
220 TIME$ = "00:00:00"
230 S$ = RIGHT$(TIME$, 2)
240 S = VAL(S$)
250 IF S > ST THEN 340
260 OUT &H378, 1
270 LOCATE 17, 30: COLOR 20: PRINT "+ NOW, SHOOTING!!! : "; S; " (SEC)          "
280 GOTO 230
290 REM
300 REM
310 REM          INTERVAL TIME CONTROL
320 REM
330 REM
340 TIME$ = "00:00:00"
350 S$ = RIGHT$(TIME$, 2)
360 S = VAL(S$)
370 IF S > IT THEN 150
380 OUT &H378, 0
390 LOCATE 17, 30: COLOR 3: PRINT "+ having a break... : "; S; " (SEC)          "
400 GOTO 350
410 OUT &H378, 0
420 LOCATE 20, 30: COLOR 17, 2: PRINT "***THE OPERATION IS OVER***"
430 LOCATE 22, 18: COLOR 13, 7: PRINT " If you want another operation, hit anykey now !"
440 COLOR 7, 0
450 WHILE INKEY$ = ""
460 FOR SO = 650 TO -650 STEP -7
470 SOUND 780 - ABS(SO), .3
480 SO = SO - 2 / 650
490 NEXT SO
500 WEND
510 CLEAR : GOTO 50
```

DNA Digestion Pattern by HOT-SHOT System

B: *Bam* HI

E: *Eco* RI

H: *Hind* III
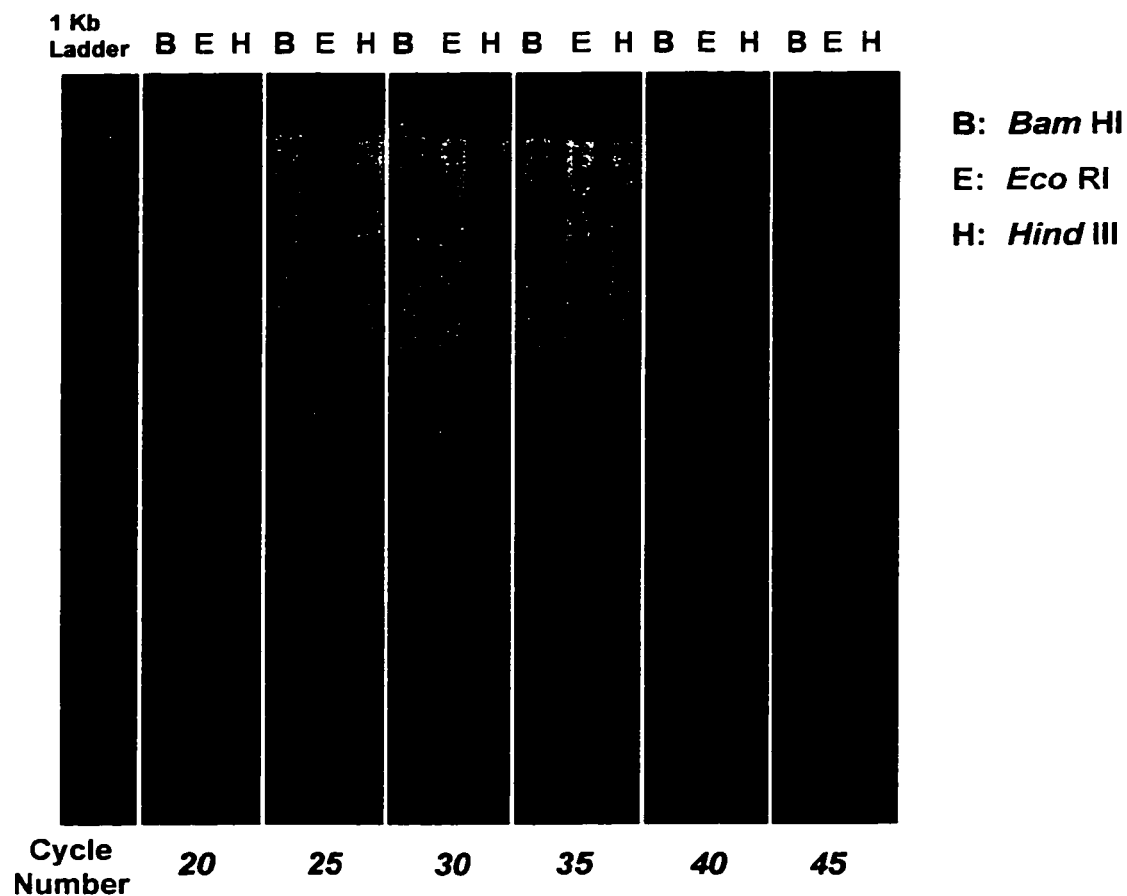
Each reaction mixture was prepared in the same way as conventional digestion using 10μg of *Brassica oleracea* total DNA.

Power On **:** *5 seconds*
Power Off **:** *10 seconds*
Cycle number **:** *varied (20-45)*

A 0.4 cubic feet, 900 W simple microwave oven with a 30min timer was used for this demonstration. Operating conditions may be different according to different type of microwave oven.

The HOT-SHOT control device and program were able to automate microwave oven-mediated DNA digestion successfully.

APPENDIX 9

MULTICHI: Multiple chi-square test program for progeny segregation analysis of transgenic plants

SUMMARY

Segregation in progeny of selfed transgenic plant which has a single foreign gene insertion is expected to follow a 3:1 ratio. In some cases unusual segregation ratios like 1:1, 2:1, 15:1 and 63:1 have been observed. A chi-square (χ^2) test is required to analyze these results. Sometimes it is necessary to calculate several different segregation ratios separately such as 1:1, 2:1, 15:1, 63:1 when segregation does not fit a 3:1 ratio. This may be time consuming work even if there are available package softwares on statistics or spreadsheet programs. A convenient PC based program has been developed to make transgenic seed segregation analysis easy and simple. Input data are total number of progeny tested and number regarded as dominant. This program computes chi-square values for 1:1, 2:1, 3:1, 15:1 and 63:1 segregation ratios with a 5% level of significance.

MULTICHI Program Written in Quick-BASIC

```
10 CLEAR : CLS
20 LOCATE 8
30 PRINT "   ==========================================================="
40 PRINT "         Multiple, Chi-square test for Transgenic Seed Segregation"
50 PRINT "   ==========================================================="
60 PRINT : PRINT
62 PRINT "                                    Sun Lee"
64 PRINT
112 PRINT
114 PRINT "                               (April, 1994)"
116 PRINT "                                 ver. 2.1"
117 PRINT : PRINT
118 PRINT "                         press any key to start ......"
120 WHILE INKEY$ = ""
122 WEND
130 CLS : LOCATE 2
140 INPUT "> JOB TITLE "; TT$
150 PRINT
160 INPUT "> TESTED SEEDS NUMBER "; N
170 PRINT
180 INPUT "> OBSERVED VALUE OF DOMINUTESANCE "; OD
190 PRINT
200 ORR = N - OD
222 IF N < 200 THEN YT = .5
230 REM
232 REM RATIO 1:1
234 REM
236 ES = N / 2: ER = ES
238 CH1 = ((OD - ER - YT) ^ 2 / ER) + ((ORR - ES - YT) ^ 2 / ES)
240 REM
242 REM RATIO 2:1
244 REM
246 ES = N / 3: ER = ES * 2
248 CH2 = ((OD - ER - YT) ^ 2 / ER) + ((ORR - ES - YT) ^ 2 / ES)
250 REM
252 REM RATIO 3:1
254 REM
256 ES = N / 4: ER = ES * 3
258 CH3 = ((OD - ER - YT) ^ 2 / ER) + ((ORR - ES - YT) ^ 2 / ES)
260 REM
262 REM RATIO 15:1
264 REM
266 ES = N / 16: ER = ES * 15
268 CH5 = ((OD - ER - YT) ^ 2 / ER) + ((ORR - ES - YT) ^ 2 / ES)
270 REM
272 REM RATIO 63:1
274 REM
276 ES = N / 64: ER = ES * 63
278 CH6 = ((OD - ER - YT) ^ 2 / ER) + ((ORR - ES - YT) ^ 2 / ES)
```

```
300 CLS
320 PRINT TAB(10); "  ** "; TT$; " **"; "  ("; TIME$; "  "; DATE$; ")"
330 PRINT
340 PRINT TAB(10); "+ SIGNIFICANT LEVEL = 5%"
342 PRINT TAB(10); "+ DEGREE OF FREEDOM = 1"
343 PRINT TAB(10); "+ DOMINUTESANT = "; OD; ",  RECESSIVE = "; ORR; ", TOTAL = "; N
344 PRINT
346 R$ = "> SEGREGATION RATIO  "
350 C$ = "> CHI-SQUARE = "
360 PRINT TAB(10); R$; "1:1"
370 PRINT TAB(10); C$; CH1;
380 IF CH1 <= 3.84146 THEN PRINT ",  AGREE!": GOTO 400
390 PRINT ",  DISAGREE///"
400 PRINT
410 PRINT TAB(10); R$; "2:1"
420 PRINT TAB(10); C$; CH2;
430 IF CH2 <= 3.84146 THEN PRINT ",  AGREE!": GOTO 450
440 PRINT ",  DISAGREE///"
450 PRINT
460 PRINT TAB(10); R$; "3:1"
470 PRINT TAB(10); C$; CH3;
480 IF CH3 <= 3.84146 THEN PRINT ",  AGREE!": GOTO 500
490 PRINT ",  DISAGREE///"
500 PRINT
510 PRINT TAB(10); R$; "15:1"
520 PRINT TAB(10); C$; CH5;
530 IF CH5 <= 3.84146 THEN PRINT ",  AGREE!": GOTO 550
540 PRINT ",  DISAGREE///"
550 PRINT
560 PRINT TAB(10); R$; "63:1"
570 PRINT TAB(10); C$; CH6;
580 IF CH6 <= 3.84146 THEN PRINT ",  AGREE!": GOTO 600
590 PRINT ",  DISAGREE///"
600 LOCATE 23
602 PRINT "                                "
610 INPUT "                    PRINT (Y/N) : "; PR$
620 IF PR$ = "Y" OR PR$ = "Y" THEN 800
624 IF PR$ = "N" OR PR$ = "N" THEN 627
626 GOTO 600
627 LOCATE 23
628 PRINT "                           "
629 LOCATE 23
642 PRINT "                            "
644 INPUT ; "              ANOTHER ANALYSIS (Y/N) : "; AA$
646 IF AA$ = "Y" OR AA$ = "Y" THEN 130
648 IF AA$ = "N" OR AA$ = "N" THEN 900
650 GOTO 629
800 LPRINT TAB(10); "  ** "; TT$; " **"; "  ("; TIME$; "  "; DATE$; ")"
802 LPRINT : LPRINT
804 LPRINT TAB(10); "+ SIGNIFICANT LEVEL = 5%"
806 LPRINT TAB(10); "+ DEGREE OF FREEDOM = 1"
808 LPRINT TAB(10); "+ DOMINUTESANT = "; OD; ",  RECESSIVE = "; ORR; ", TOTAL = "; N
810 LPRINT : LPRINT : LPRINT
812 R$ = "> SEGREGATION RATIO  "
```

```
814 C$ = "> CHI-SQUARE = "
816 LPRINT TAB(10); R$; "1:1"
818 LPRINT TAB(10); C$; CH1;
820 IF CH1 <= 3.84146 THEN LPRINT ",  AGREE!": GOTO 824
822 LPRINT ",  DISAGREE///"
824 LPRINT : LPRINT
826 LPRINT TAB(10); R$; "2:1"
828 LPRINT TAB(10); C$; CH2;
830 IF CH2 <= 3.84146 THEN LPRINT ",  AGREE!": GOTO 834
832 LPRINT ",  DISAGREE///"
834 LPRINT : LPRINT
836 LPRINT TAB(10); R$; "3:1"
838 LPRINT TAB(10); C$; CH3;
840 IF CH3 <= 3.84146 THEN LPRINT ",  AGREE!": GOTO 844
842 LPRINT ",  DISAGREE///"
844 LPRINT : LPRINT
846 LPRINT TAB(10); R$; "15:1"
848 LPRINT TAB(10); C$; CH5;
850 IF CH5 <= 3.84146 THEN LPRINT ",  AGREE!": GOTO 854
852 LPRINT ",  DISAGREE///"
854 LPRINT : LPRINT
856 LPRINT TAB(10); R$; "63:1"
858 LPRINT TAB(10); C$; CH6;
860 IF CH6 <= 3.84146 THEN LPRINT ",  AGREE!": GOTO 864
862 LPRINT ",  DISAGREE///"
864 LPRINT : LPRINT : LPRINT : LPRINT : LPRINT : LPRINT
866 GOTO 627
900 END
```

Example of Output Sheet Produced by MULTICHI Program

** TCR1 **   (02:48:38 01-29-1996)

+ Significant level = 5%

+ Degree of Freedom = 1

+ Dominant = 53 ,   Recessive = 14 ,   Total = 67

> Segregation Ratio   1:1

> Chi-square = 22.71642 ,   disagree///

> Segregation Ratio   2:1

> Chi-square = 4.867537 ,   disagree///

> Segregation Ratio   3:1

> Chi-square = 0.7313433 ,   AGREE!

> Segregation Ratio   15:1

> Chi-square = 22.40298 ,   disagree///

> Segregation Ratio   63:1

> Chi-square = 150.8806 ,   disagree///