Transgenic Plant Journal ©2007 Global Science Books

Genetic Transformation of *Brassica oleracea* Vegetables

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ABSTRACT

Brassica oleracea L. with its numerous subvarieties is one of the most important vegetable species in the world. Constant improvement of various agronomical traits is a permanent task of *Brassica* breeders for which methods of genetic engineering have been adopted recently. However transformation in *Brassica* vegetables is not yet a routine. Development of successful transformation protocols based on common transformation procedures has been presented in many reports. Transformation success depends on many factors including genotype, explant type, gene introduction technique and the construct itself. In this review we present recent data on transformation of *Brassica* vegetables using both *Agrobacterium tumefaciens* and *A. rhizogenes* strains, as well as some other alternative approaches.

Keywords: *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, broccoli, Brussels sprouts, cabbage, cauliflower, kale, Savoy
Abbreviations: BA, benzyl adenine; **Bt**, *Bacillus thuringiensis*; **DB**, doubled haploid; **DBM**, diamondback moth; **GFP**, green fluorescent protein; **GM**, genetically modified; **HR**, hairy root; **ICP**, insecticidal crystal protein; **L-PPT**, phosphinotricin; **NAA**, naphthyleneacetic acid; **PEG**, polyethylene glycol; **QTL**, quantitative trait loci; **RC**, rapid cycling; **RT PCR**, real time PCR; **T-DNA**, transfer DNA; (gene abbreviations are listed in **Table 1** legend)

CONTENTS

BRASSICA OLERACEA VEGETABLES

Brassicaceae family includes some 350 genera with 3200 species adapted mostly to mild and colder climate (Janick 1986). Many *Brassica* species have been used by mankind as food since ancient times i.e. 2000 to 2500 BC (Chiang *et al*. 1993). Apart from human consumption and livestock feeding *Brassica* species are also used for industrial non-food processing (Poulsen 1996). The use of oilseed rape as vegetable oil, as well as a fuel source is constantly increasing.

Brassica oleracea is one of the major species of the *Brassicaceae* group (family). It is a highly polymorphic species differentiated into many distinct vegetable and fodder crops/varieties. According to Hodgkin (1995) the most important *B. oleracea* vegetable species are: cabbage (var. *capitata*), Brussels sprouts (var. *gemmifera*), broccoli and Calabrese (var. *italica*), cauliflower (var. *botrytis*), kohlrabi

Received: 30 June, 2007. Accepted: 9 August, 2007.

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(var. *gongyloides*) and kale (var. *medullosa*, *ramosa* and *acephala*). *Brassica* vegetables are considered to be of high nutritional value. They contain high levels of ascorbic acid (vitamin C) and minerals and many important proteins (Munger 1988). American Cancer Society and the USA National Research Council recommended cabbage as a food to reduce the hazard of cancer appearance (Birt 1988).

There are many breeding programs for *B. oleracea* varieties which are on-going. Although aimed at different targets they all have the same goal: to improve the existing genotypes. The sexual incompatibility barrier is sometimes a serious problem which needs to be overcome in order to obtain new hybrid varieties. Much of the current research is mobilized in solving this problem (Dickson and Wallace 1986). Among conventional breeding programs we should mention those aimed to improve the resistance to pests and diseases by hybridization with wild *Brassica* species (Puddephat *et al*. 1996; Obradović *et al*. 2000; Sretenović-Rajičić *et al*. 2000a).

However the most powerful tool which can today be used for breeding and improvement of *Brassica* vegetable species are genetic engineering techniques. They enable fast production of transformed plants by insertion of single genes of interest directly into the cells of perspective genotypes. Many studies dedicated to the production of *B. oleracea* transgenic plants have been published so far and there is a constant need to reviews and classify published results and also to point at leading trends for the future research. We would like to mention papers and reviews by Earle *et al*. (1996), Poulsen (1996), Puddephat *et al*. (1996), Christey (1997), Metz (2001), Christey *et al*. (2001), Paul *et al*. (2002) and Christey and Braun (2004). These reviews contain numerous important data including bacterial strains, plasmids, genes, explant types, transformation efficiency and other. Therefore we shall make a brief general survey of early studies and focus more on reports which appeared in the last decade discussing also trends and prospects for the future research.

THE USE OF *IN VITRO* CULTURE TECHNIQUES

Elaborated protocols which enable shoot regeneration *in vitro* are a basic requirement for the development of procedures aimed at transgenic plant production. *In vitro* culture techniques are here indispensable no matter which transformation method we have in mind except for *in planta* transformation.

Luckily *B. oleracea* varieties can easily be cultured *in vitro* and manipulated. Hypocotyl and cotyledon explants are standard explant sources for the establishment of cultures. Shoot regeneration is possible from various tissues and organs including stems, roots, leaves, flower pedicels, anthers, macro and microspores, callus and cell cultures, thin cell layers and finally protoplasts. Most of this data has been reviewed by Poulsen (1996).

Requirements for *in vitro* culture of *B. oleracea* varieties are simple but diverse. There is not a single protocol common to all varieties indicating a very strong genomic influence. Thus for every new genotype specific protocol needs to be empirically elaborated (optimized).

TRANSFORMATION METHODS (TECHNIQUES)

A characteristic feature of varieties comprising the *B. oleracea* group are differences in parts which are edible and used as food or feed or for industrial processing. In such a diverse group of species it is difficult to formulate a common transformation method or protocol (Earle *et al*. 1996). The most frequently used method for production of transgenic *B. oleracea* plants is *Agrobacterium*-mediated transformation. There have also been studies on the direct insertion of DNA by electroporation and use of polyethylene glycol (PEG) mediated transformation of protoplasts and to a less extent studies on particle bombardment (biolistic transformation).

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation is the main and most successful method for production of genetically modified plants (Tzfira and Citovsky 2006). It was used for transformation of most plant species in the last two decades (Herrera-Estrella *et al*. 2005). *Brassica* vegetables have been successfully transformed both with *A. tumefaciens* and *A. rhizogenes*.

Agrobacterium-mediated transformation is based on the use of extra-chromosomal bacterial DNA present on separate plasmids. Bacteria can replicate a short fragment of plasmid DNA designed T-DNA (T stands for transfer), which can be transferred into plant cells and in their nuclei incorporated into chromosomes. T-DNA thus becomes a constitutional part of plant genome. Placing a gene of interest within the left and right excision border of the T-DNA fragment equipped with a suitable promotor and terminator (regulating elements) enables fast introduction of foreign genes into certain plant genotypes. Although *A. tumefaciens* mediated transformation is a routine technique, the complex mechanism involved in the integration of T-DNA in the host genome is not completely understood (Tzfira *et al*. 2004a, 2004b).

First successful plant transformation were made using wild type strains which in their T-DNA contain a specific class of genes, named oncogenes which after insertion into plant genome enforce cells to produce opine type compounds. Opines are necessary as nutrients for the development of bacteria. We are therefore looking at a highly sophisticated parasitic relationship between plant and bacterial cells. Problem of oncogenes was rather quickly solved by production of "disarmed" plasmids in which oncogenes were removed or inactivated.

First successful transformation of *B. oleracea* varieties using wild type *A. tumefaciens* strain C58 was done by Holbrook and Miki (1985) who obtained normal-looking shoots and whole, rooted plants. Other studies on *A. tumefaciens*-mediated transformation of *B. oleracea* vegetables include reports by Srivastava *et al*. (1988), de Block *et al*. (1989), Toriyama *et al*. (1991), Becklin *et al*. (1993), Berthomieu *et al*. (1994), Ovesna *et al*. (1993), Metz *et al*. (1994,1995a), Passelegue and Kerlan (1996), Bhalla and Smith (1998), Ding *et al*. (1998), Cao *et al*. (1999), Lee *et al.* (2000), Pius and Achar (2000), Radchuk *et al*. (2000), Cogan *et al*. (2001), Kuginuki and Tsukazaki (2001), Chen *et al*. (2001), Gapper *et al*. (2002), Tsukazaki *et al*. (2002), Bhattacharya *et al*. (2002), Sparrow *et al*. (2004a, 2004b), Sretenović-Rajičić *et al*. (2004) and others (**Table 1**) We must point here that most of the transformation research in the family *Brassicaceae* was done on *B. napus* and *B. juncea*, species which are not the subject of our review. However, the results and findings of these studies were always quickly applied in research on *B. oleracea* species.

Explants infected with *A. tumefaciens* by co-cultivation or other methods may regenerate shoots spontaneously or upon treatment with plant growth regulators and various elicitors. Plants which develop from shoots are putative transformants which may contain in their genome functional genes from bacterial T DNA. Not all of this shoots are true transformants. Since shoots are multicellular by origin regenerated shoots are often chimeric, containing both transformed and untransformed cells. Furthermore foreign genes may stop functioning after some time for numerous reasons including rearrangements, silencing and other. Thus although tests may show initial presence and function of foreign genes in regenerated plants (transient expression) stable integration of a gene is considered successful only if it is present and expressed in plants of T_1 generation and further progeny.

Transformation techniques were significantly improved by the introduction of binary vectors which are today routinely used for transformation of *Brassica* vegetables. Here bacterial cell contains two different plasmids, one usually the native plasmid with the functional *vir* region required

Table 1 Summary of *B. oleracea* transformation.

Agrobac. type [a]	Strain / plasmid	Genes [b]	Explant type	Purpose of research	Authors	Year
	***B.o. italica* – Broccoli**					
A. t.	C58, A208, A277, A6NC, A552 / -	nopaline	Seedling, stem	Tumour induction and regeneration	Holbrook and Miki	1985
A. t.	C58 / pTiC58	nopaline	Stem	Tumour susceptibility	Ohlsson and Eriksson	1988
A. r.	M123 / -	-	Leaf petiole	Technique improv.	Hosoki *et al.*	1991
A. t.	pCIB542 / -	*hpt*, *slg*	Peduncle	Self-incompatibility	Toriyama *et al.*	1991
A. t. and *A. r.*	C58, C58C1, A4 / pGV2260	various	Stem segments, hypocotyl	Technique improv.	Ovesna *et al.*	1993
	82.139, C58, LBA4404	*nptII*, *hpt*, *gus*	Stem	Technique improv.	Berthomieu *et al.*	1994
A. t.	ABI / pMON10517-1	*nptII*, *cry1a(C)*	Peduncle	Resistance - insects	Metz *et al.*	1995b
A. t.	ABI / pMON 10517-1, pMON10837-1	*nptII*, *cry1a(C)*	Peduncle, hypocotyl petioles	Technique improv., resistance - insects	Metz *et al.*	1995a
A. r.	A4T / pART27732, pART278, pLN35	*nptII*, *gus*, EFE, *cry1A*	Cotyledonary petioles, leaves	Technique improv., phenotype stability	Christey *et al.*	1997
A. t.	ABI / pNS6	*hpt*, *cry1C*	Petiole, hypocotyl	Resistance - insects	Cao *et al.*	1999
A. r.	A4T / pLN35	*nptII*, *ACC*	Leaf, cotyledon	Senescence, ethylene production	Henzi *et al.*	1999
A. r.	A4T / -	-	-	Field evaluation -phenotype stability	Christey *et al.*	1999
A. r.	A4T / pLN35	*nptII*, ACC	Leaf, cotyledon petioles	Senescence - ethylene production	Henzi *et al.*	2000b
A. r.	A4T / pART278, pLN35	*nptII*, *gus* ACC	Leaf	Technique improv.	Henzi *et al.*	2000a
A. r.	A4T / pRiA4, pMaspro::GUS	*gus*	Hypocotyl	Phenotype stability	Puddephat *et al.*	2001
A. t.	LBA 4404 / pSG529(+), pSG766A	*nptII*, *ipt*	Cotyledon, hypocotyl	Senescence, cytokinin production	Chen *et al.*	2001
A. t.	LBA 4404 / pBin19 ESR	*nptII*, *endochitinase*	Cotyledon, hypocotyl	Resistance – antimicrobial	Mora and Earle	2001
A. t.	LBA 9402 / pBIN m-gfp5-ER	*gfp*, *nptII*	Hypocotyl	Technique improv.	Cogan *et al.*	2001
A. t.	ABI / pJC12b	*nptII*, *cry1Ab*	Hypocotyl, petiole explants	Resistance – insects	Cao *et al.*	2001
-	-	-	-	Pyramiding insect resistance	Cao *et al.*	2002
A. t.	AGL1, GV3101 KYRT1 / pPN10, pArt27, pPN11, pBJ49	*nptII*, *gus*, *hpt*, *ACC oxidase II*	Hypocotyl, cotyledon petioles	Senescence – ethylene, cytokinin production	Gapper *et al.*	2002
-	*cry* gene pyramiding	-	-	Resistance - insects	Cao and Earle	2003
A. t.	LBA 4404 / pBI-mERS162F, pSM1H-mERS162F	*hpt*, *nptII*, *boers*	Cotyledon, hypocotyl	Senescence – ethylene production	Chen *et al.*	2004
A. t.	LBA4404 / pCAMBIA2200	BoCP5 protein	Hypocotyl	Senescence, cystein protease	Eason *et al.*	2005
A. r.	LBA 9402 / PMBRE/GFP	*gfp*, ACO1&2, ACS1	Leaf	Senescence – ethylene production	Higgins *et al.*	2006
	***B.o. botrytis* – Cauliflower**					
A. t. and *A. r.*	various wild strains / various	opines	Hypocotyl wounding	Initial transformation report	Petit *et al.*	1983
A. r.	various wild strains / various	opines	Hypocotyl	Technique improv.	David and Tempe	1988
-	6042, 6044,C58C1,6046 -	nopaline	Leaf disks, protoplasts	Technique improv.	Eimert and Siegemund	1992
A. t.	C58C1 / pLGVTi23neo	*nptII*, nopaline	Leaf discs	Technique improv.	Srivastava *et al.*	1988
A. t.	C58 B6S3 / pGV3850	*nptII*, nopaline	Hypocotyl, cotyledon	Protoplast, technique improv	Ohlsson and Eriksson	1988
A. t.	C58C1 / pMP90, pGSFR780A	*nptII*, *bar*	Hypocotyl	Technique improv., resistance- herbicides	de Block *et al.*	1989
-	- / pRT55(66,77) gusR	*dhfr*, *hpt*, *gus*, *bar*	Hypocotyl, protoplasts	Technique improv.	Mukopadhyay *et al.*	1991
A. t.	pCIB542 / pKTS8	*dpt*, *SLG*	Peduncle discs	Technique improv., self-incompatibility	Toriyama *et al.*	1991
A. t. and *A. r.*	C58, C58C1, A4 / pGV2260	various	Stem segments, hypocotyl	Technique improv.	Ovesna *et al.*	1993
A. t.	C58C1, 82.139 / pMP90, pKHG	*nptII*, *hpt*, *bar*	Stem wounding	Technique improv.	Passelegue *et al.*	1996
A. r.	A4T / pART27732, pART278 pLN35	*nptII*, *gus*, *EFE*, *cry1A*	Cotyledonary petioles, leaves	Technique improv., phenotype stability	Christey *et al.*	1997
A. t.	LBA4404 / pBI101	*nptII*, *Bcp1*	Hypocotyl cotyledon	Technique improv.	Bhalla and Smith	1998
A. t.	LBA4404 / pBI101/TI	*nptII*, *gus*, *TI*	Hypocotyl	Resistance – insects	Ding *et al.*	1998
A. r.	A4T / -	-	-	Field evaluation, phenotype stability	Christey *et al.*	1999
A. t.	EHA101 / pIG121Hm	*nptII*, *hpt*, *gus*	Hypocotyl	Technique improv.	Kuginuki and Tsukazaki	2001
A. r.	A4T / pRiA4 , pMaspro::GUS	*gus*	Hypocotyl	Phenotype stability	Puddephat *et al.*	2001
A. t.	LBA 9402 / pBIN m-gfp5-ER	*gfp*, *nptII*	Hypocotyl	Technique improv.	Cogan *et al.*	2001
A. t.	GV2260 / pBinAR-Bt	*nptII*, *cry1A(b)*	Hypocotyl	Resistance – insects	Chakrabarty *et al.*	2002

Table 1 (Cont.)

Agrobac. type[a]	Strain / plasmid	Genes [b]	Explant type	Purpose of research	Authors	year
PEG-mediated	- / pRT99GUS	*nptII*, *gus*	Protoplasts	Technique improv.	Radchuk *et al*.	2002
A. r.	LBA9402 / pRD400/GI	*gus*	Hypocotyl	GUS activity variation	Baranski and Puddephat	2003
A. r.	LBA9402 / pSCV1 with 6 different promotors	*gus*	Hypocotyl	Gene expression, promotor activity	Baranski and Puddephat	2004
A. t.	LBA4404 pGA643	*nptII*, *CpTi*	Hypocotyl, cotyledon	Resistance-insects	Lingling *et al*.	2005
PEG mediated	- / pGUS-HYG pZB1	*hpt*, *gus*	Leaf protoplasts	Technique improv.	Nugent *et al*.	2006
	***B.o. alboglabra* – Chinese Kale**					
A. t.	pCIB542 / pKTS8	*hpt*, *SLG*	Peduncle	Technique improv., self-incompatibility	Toriyama *et al*.	1991
	A12DHd x GDDH33 *(B.o. alboglabra × B.o. italica)*					
A. r.	LBA 9402 / pBIN-m-gfp5-ER	*gfp*, *nptII*	Hypocotyl	QTL analysis	Cogan *et al*.	2002
A. r.	LBA9402, C58C1, LBA4404 / pRi 1855, pRD400/GI	*nptII*, *gus*, *gfp*	Hypocotyl	QTL analysis	Cogan *et al*.	2004
A. t.	C58, T37, Ach5 / wild	Nopaline, octopine	Seedling wounding	QTL analysis	Sparrow *et al*.	2004a
A. t.	LBA4404 , EHA101/ p25GI	*nptII*, *gus*	Cotyledonary petioles	QTL analysis	Sparrow *et al*.	2004b
	***B.o. capitata* – Cabbage**					
A. r.	A4, A4H / -	*nptII*, *bpt*	Leaf petiole	Techniques	Berthomieu and Jouanin	1992
A. r.	GV3101, LBA4404, 82.139 / various	*nptII*, *hpg*, *aux2*, *gus*	Stem internode	Techniques	Becklin *et al*.	1993
A. t.	C58, Ach5, B6806 / pKHG	*nptII*, *hph*, *gus*, *aux2*, *cryIC*	Seedling wounding	Technique improv.	Berthomieu *et al*.	1994
A. t.	ABI / pMON 10517-1, pMON10837-1	*nptII*, *cry1a(C)*	Peduncle, hypocotyl petioles	Technique improv., Resistance - insects	Metz *et al*.	1995a
A. r.	A4T / pART27732, pART278 pLN35	*nptII*, *gus*, *EFE cry1A*	Cotyledonary petioles, leaves	Technique improv., phenotype stability	Christey *et al*.	1997
A. r.	A4T / -	-	-	Field evaluation, phenotype stability	Christey *et al*.	1999
A. t.	GV2260, GV3101, DK335 /	*nptII*	Hypocotyl	Technique improv.	Radchuk *et al*.	2000
A. t.	A281, A348, Ach5, A136 / pTiBo542, pGA472	*nptII*, *gus*	Cotyledon	Technique improv.	Pius and Achar	2000
A. t.	LBA4404 / pMOG6-bar, pIH121	*hpt*, *bar*	Hypocotyl	Resistance - herbicides	Lee *et al*.	2000
A. t.	EHA105 / pCry1Ab3, pCry1Ia3	*nptII*, *gus cry1Ab3 cry1Ia3*	Hypocotyl, cotyledon, leaves	Resistance - insects	Jin *et al*.	2000
A. t.	LBA 9402 / pBIN m-gfp5-ER	*gfp*, *nptII*	Hypocotyl	Technique improv.	Cogan *et al*.	2001
A. t.	EHA 101 / pIG121Hm	*nptII*, *hpt*, *gus*	Hypocotyl, cotyledonary petioles	Genotype differences	Kuginuki Tsukazaki	2001
A. t.	LBA4404 / pGAGO	*hpt*, *GO*	Hypocotyl	Resistance-antimicrobial	Lee *et al*.	2002
A. t.	LBA4404, EHA101, EHA105, AGL0 / pIG121Hm	*nptII*, *gus*	Hypocotyl	Transformation techniques	Tsukazaki *et al*.	2002
A. t.	GV2260 / pBT1291, pBin AR	*nptII*	Hypocotyl	Resistance - insects	Bhattacharya *et al*.	2002
A. t.	LBA 4404 / pGR011	*hpt*, *bar*, *BcA9*	Hypocotyl	Polen male sterility	Lee *et al*.	2003
A. t.	GV2260 / pRC-cdh	*nptII*, *betA*	Hypocotyl	Resistance - salt stress	Bhattacharya *et al*.	2004
A. t.	LBA 4404 / pBI121-OCI	*nptII*,*OC-I*		Resistance - insects	Lei *et al*.	2006
A. r.	A4M70GUS / -	*gus*	Hypocotyl, cotyledon	Technique improv.	Sretenović-Rajičić *et al*.	2006
A. t.	AGL1, LBA4404/pDM805, pGKB5	*nptII*, *hpt*, *bar*	Hypocotyl, cotyledon	Technique improv.	Sretenović-Rajičić *et al*.	2007
	***B.o. gemmifera* – Brussel sprouts**					
A. r.	M 123 / -	-	Leaf petiole	Techniques	Hamada *et al*.	1989
A. r.	IFO 13257 / pBI 121	*nptII*, *gus*	Leaf petiole	Techniques	Hosoki and Kigo	1994
A. r.	A4T / pART27732, pART278, pLN35	*nptII*, *gus cry1A*	Cotyledonary petioles, leaves	Technique improv., phenotype stability	Christey *et al*.	1997
A. r.	A4T / -	-	-	Field evaluation -phenotype stability	Christey *et al*.	1999
	***B.o. sabauda* – Savoy**					
A. t.	AGL1, LBA4404 / pDM805, pGKB5	*nptII*, *hpt*, *bar*	Hypocotyl, cotyledon	Resistance- herbicides	Sretenović-Rajičić *et al*.	2004
A. r.	A4M70GUS / -	*gus*	Hypocotyl, cotyledon	Technique improv.	Sretenović-Rajičić *et al*.	2006
	***B.o. acephala*, *ramosa*, *medulosa* – Kale**					
A. r.	M 123 / -	-	Leaf petiole	Technique improv.	Hosoki *et al*.	1989
A. r.	A4T / pKIWI110	*nptII*, *als*, *gus*	Cotyledonary petioles, leaves	Technique improv.	Christey and Sinclair	1992
A. r.	IFO 13257 / pBI 121	*nptII*, *gus*	Leaf petiole	Techniques	Hosoki *et al*.	1994

Table 1 (Cont.)

Agrobac. type[a]	Strain / plasmid	Genes [b]	Explant type	Purpose of research	Authors	year
A. r.	A4T / pKIWI110	*nptII*, *gus*, *bar*, *als*	Leaf, cotyledonary petiole	Review, resistance herbicides	Christey and Braun	2001
	LBA 9402 / pBIN m-gfp5-ER	*gfp*, *nptII*	Hypocotyl	Technique improv.	Cogan *et al.*	2001
A. t.	ABI / pNS6, pMON10517	*nptII*, *hpt* *cry1Ac*, *cry1C*	Hypocotyl, cotyledonary petioles	Resistance – insects	Cao *et al.*	2005

[a] Strain type: *A. t.* – *Agrobacterium tumefaciens*, *A. r.* – *Agrobacterium rhizogenes*, PEG mediated – direct transfer

[b] Genes: ACO(n) , ACC, ACS – ACC (1 aminocyclopropane-1-carboxylic acid) oxidase and synthase, *als* – acetolactate synthase, ASpro – asparagine synthethase promoter, *aux2* – auxin synthesis gene, *bar* – phosphinotricin acetyl transferase, bet A – glycinebetain synthetase, BcA9 – *Brassica campestris* tapetum-specific A9 gene, *Boers* – ethylene response sensor gene, CpTi – Cowpea trypsin inhibitor gene, cry (n) – *Baccilus thuringiensis* crystal insecticidal proteins, EFE – ethylene forming enzyme, *gfp* – green fluorescent protein, GO – glucose oxidase, *gus* – β-glucuronidase, *hpt* – hygromycin phosphotransferase, *ipt* – isopentenyltransferase, *nptII* – neomycin phosphotransferase-II, SAG12pro – senescence-associated gene promoter, SLG – self-incompatibility gene, Ti – trypsin inhibitor

for plant cell recognition, association and DNA transfer and the second engineered or binary plasmid with T-DNA region carrying genes of interest with suitable promotors, terminators, selectable markers, reporter genes and a polylinking site. Placement of *vir* genes on separate plasmids is termed "trans" position. The other possibility, co-integrative plasmids where genes of interest are introduced in the T-DNA region of the native, disarmed plasmid has been much less utilized in transformation studies.

Transformation success following *A. rhizogenes*-mediated transformation can be easily confirmed visually by the appearance of hairy roots which do not require addition of exogenous growth regulators for their growth (David and Tempe 1988). Hairy roots have accelerated growth, increased branching and plagiotropic growth (Tepfer 1990). Formation of hairy roots facilitates pre-selection of putative transformants canceling the need for transformation markers.

Spontaneous plant regeneration from transgenic hairy roots of cauliflower was first reported by Petit *et al.* (1983) Further reports of *A. rhizogenes*-mediated transformation of various *B. oleracea* vegetables were contributed by David and Tempe (1988), Hosoki *et al.* (1989), Berthomieu and Jouanin (1992), Hosoki and Kigo (1994), Christey and Sinclair (1992), Christey *et al.* (1997), Henzi *et al.* (1999), Puddephat *et al.* (2001), Baranski and Puddephat (2003), Higgins *et al.* (2006), Sretenović-Rajičić *et al.* (2006) and others (**Table 1**).

Direct gene transfer techniques

Beside *Agrobacterium*-mediated transformation, there are other techniques which enable production of genetically modified plants. These techniques are known as direct gene transfer since they do not require a carrier vector as a mediator. Three of them, PEG-mediated DNA uptake, electroporation and microinjection are founded on the use of protoplast. In absence of cell wall as a protective transport barrier it is much easier for the large DNA molecules to enter the plant cell. Finally there is the biolistic (particle bombardment) transformation method which can also transfer foreign genes directly into plant cells and nuclei.

High-frequency transformation and plant regeneration following PEG-mediated DNA uptake into cauliflower protoplasts was reported by Mukhopadhyay *et al.* (1991). Healthy viable protoplasts were obtained only from hypocotyl explants of six day old seedlings. Transformation efficiency was high (10-33%) and fertile plants transformed with *hpt* (hygromycin phosphotransferase) and *bar* (*pat – phosphinotricin acetyltransferase*) marker genes were produced. Transformation with a construct carrying resistance to methotrexate (*dhfr* - dihydrofolate reductase) was not successful. DNA added directly with PEG decreased cell divisions.

Eimert and Siegemund (1992) investigated and compared PEG-mediated uptake and electroporation of cauliflower protoplasts with plasmids pABDI and pRT103neo. Stable transformation was obtained, but the frequency of calli transformation and shoot regeneration were low in both employed techniques. The frequency of kanamycin resistant calli was 4 x 10^{-5} for PEG-mediated uptake and 3.5 × 10^{-5} for electroporation. Only few shoots were regenerated from transformed calli.

Transformation of cauliflower by direct DNA uptake into mesophyll protoplasts was reported by Radchuk *et al.* (2002). Investigated plasmids (**Table 1**) carried *nptII*, *hpt*, *nisA* and *gus* selectable and reporter genes. Selection pressure was established by addition of 50-100 mgl^{-1} kanamycin or 20-40 mgl^{-1} hygromycin which completely inhibited growth of colonies derived from untreated protoplasts. A total of 18 resistant callus colonies regenerating shoots were obtained. In four investigated plant lines the 3:1 inheritance of transgene was observed in three lines and 1:1 in one plant line. Absolute transformation efficiency was in range 2.9-8.0 × 10^{-7}. Flow cytometry showed that transformed plants had altered ploidy levels. Diploid and tetraploid plants were observed to develop from the same callus.

Biolistic transformation of broccoli was investigated by Puddephat *et al.* (1999). Authors reported only transient reporter gene expression. Pre-culturing of cotyledon leaf discs with hormone-free or callus induction media decreased the transient transformation event.

Nugent *et al.* (2006) reported nuclear and plastid transformation of cauliflower using PEG-mediated uptake of DNA into protoplasts. This is the first report of plastid transformation in a vegetable *Brassica*. Transformation rate for nuclear transformation was (0.3-1.3) × 10^{-5} calculated as resistant colonies per number of treated protoplasts. However, the transformation frequency for plastid transformation was much lower (0.3 × 10^{-6}).

If we summarize and compare results obtained by direct gene transfer and *Agrobacterium*-mediated transformation it is apparent that the former techniques need to be significantly improved if routine applications are expected. Vector-less transfer of DNA may be an advantage enabling faster and less complicated manipulation but accurate DNA insertion provided by *Agrobacterium* transfer mechanism can hardly be achieved by other transformation techniques.

Common improvement strategies for *Agrobacterium*-mediated transformation

In the last decade many studies and small improvements have been made with *A. rhizogenes*-mediated transformation. The first study by Petit *et al.* (1983) already showed the high potential of this approach since the hairy roots which developed at the inoculation site regenerated shoots spontaneously. Drawback of this highly productive method was co-transmission of bacterial *rol* genes responsible for the appearance of plants with altered phenotype. *A. rhizogenes*-mediated transformation is presented in more detail later in a separate chapter.

On the other side techniques employing *A. tumefaciens* have been well elaborated some 10-20 years ago leaving little space for further improvement. However both techniques shared the same goals and common improvement strategies

It seems that two studies dedicated to oilseed rape (*Brassica napus*) which appeared two decades ago signifi-

cantly affected transformation studies of other *Brassica* species. Guerche *et al*. (1987) transformed oilseed rape with *A. rhizogenes* strain A4, and regenerated transformed plants which all belonged to the altered, Rhi phenotype. Transformed phenotype was inherited as dominant giving 3:1 ratio upon self-pollination. The other study by Charest *et al*. (1988) with a number of *A. tumefaciens* strains and pGV3850 provided a large number of transformants which were free of morphological abnormalities and reduced fertility characteristic for plants transformed with Ri plasmids (Guerche *et al*. 1987). Thus the inability of *A. rhizogenes*-mediated transformation to produce transgenic plants of desired genetic fidelity was crucial for the leading position of *A. tumefaciens*-mediated transformation. The study by Metz *et al*. (1995a) on transformation of broccoli and cabbage was the focal point for this line of research.

Since the main problem of *B. oleracea* transformation in general is low transformation efficiency, constant efforts are made to improve this parameter. According to Henzi *et al*. (2000a) increased transformation efficiency can be achieved either by manipulation of explants or bacteria with the aim to enhance virulence. In every study the initial problem is to determine the best type and age of explant and the inoculation procedure. Additional explant manipulations in *B. oleracea* transformation often include: hormonal precultivation treatments, addition of ethylene inhibitors, addition of virulence elicitors and feeder cell layering.

Precultivation treatments

This treatments performed prior to inoculation are done under assumption that explant excision is traumatic *per se* and that it takes some time for the explant to recover before it is exposed to bacteria. Wounded surface of the explant in contact with bacteria can trigger hypersensitive reaction inducing tissue browning and death thereby inhibiting regeneration from transformed tissue (Babić *et al*. 1998). Hypersenitive reaction is considered a natural plant defense mechanism protecting tissues from bacterial infections.

Preculturing is usually done on media supplemented with plant growth regulators that facilitate shoot regeneration or callus development. Thus Srivastava *et al*. (1988) precultured cauliflower leaf explants 24 hours on media with 0.5 mgl^{-1} BA and 1.0 mgl^{-1} NAA. Ovesna *et al*. (1993) observed that cauliflower hypocotyl explants necrotized when co-cultivated with *Agrobacterium* immediately after isolation. Callus formation on explants occurred if they were precultured at least for a day. Precultivation for cauliflower explants was also recommended by Ding *et al*. (1998) and Lingling *et al*. (2005).

Bhattacharya *et al*. (2002, 2004) prior to infection precultured cabbage hypocotyls for a day and Tsukazaki *et al*. (2002) for 3 days. Sretenović-Rajičić *et al*. (2004, 2006, 2007) routinely precultured hypocotyl and cotyledon explants of cabbage and Savoy for 48 h on media supplemented with BA 1.0 mgl^{-1} and IBA 0.5 mgl^{-1}.

In broccoli Metz *et al*. (1995a) showed that seedling explants (hypocotyl and cotyledon) require 2-day precultivation in comparison to peduncle explants which do not require precultivation. Here precultivation may have adverse effect on shoot regeneration increasing at the same time the transformation efficiency of broccoli (Metz *et al*. 1995a). According to Christey and Sinclair (1992) precultivation with or without feeder layers was not advantageous in *A. rhizogenes* mediated transformation of kale (*B.o*. var. *acephala*).

Inhibitors of ethylene production

According to van Wordragen and Dons (1992) and Cardoza and Stewart (2004) silver nitrate is an important supplement in media used for *B. oleracea* tissue culture and transformation. It is supposed to stimulate shoot regeneration by inhibition of ethylene production.

de Block *et al*. (1989) recommended silver nitrate as an absolute prerequisite for cauliflower transformation. Early addition of 2-5 mgl^{-1} silver nitrate to the selective medium increased transformation efficiency of hypocotyl explants up to 30% measured as percentage of explants producing rooted transformed shoots. Chakrabarty *et al* (2002) also used silver nitrate at 3-5 mgl^{-1} for transformation of cauliflower.

In broccoli Gapper *et al*. (2002) reported that 29.4 μM silver nitrate had no significant effect on the transformation efficiency.

In cabbage silver nitrate was used for transformation at 2-5 mgl^{-1} by Radchuk *et al*. (2000); 2 mgl^{-1} by Lee *at al*. (2000) and at 3.5 mgl^{-1} by Bhattacharya *et al*. (2002, 2004). In cabbage transformation silver nitrate apparently works well in combination with explant precultivation.

Other ethylene inhibitors which have a positive effect on shoot regeneration in *Brassicaceae* are silver thiosulphate and aminoethoxyvinylglicine (Cardoza and Stewart 2004). Silver thiosulphate at 29.4 μM was used by Ding *et al*. (1998) for transformation of cauliflower.

However there are some limitations and drawbacks for the use of silver nitrate. De Block *et al*. (1989) stated that silver nitrate needs to be added together with carbenicillin which then prevents media from turning brown. Media browning was also reported during cabbage transformation on media supplemented with more than 5 mgl^{-1} silver nitrate (Radchuk *et al*. 2002). It should be noted that silver nitrate needs to be supplemented to media separately by filter sterilization after autoclaving.

There are also reports of the adverse effects of silver nitrate. Metz *et al*. (1995a) reported that addition of 1-10 mgl^{-1} silver nitrate inhibited shoot regeneration both in control and transformation experiments. Sealing plates with porous tape instead of air-tight Parafilm was recommended as a good alternate method for control of ethylene buildup in culture vessels. Finally in cabbage and Savoy cabbage addition of 2 mgl^{-1} silver nitrate significantly decreased callus formation and shoot regeneration capacity of explants during transformation both with *A. tumefaciens* and *A. rhizogenes* (Sretenovic-Rajičić *et al*. 2004, 2006, 2007).

Acetosyringone and virulence elicitors

Wounded plant cells produce compounds which attract *A. tumefaciens* and *A. rhizogenes* and increase their virulence. Acetosyringone has been identified as the major virulence elicitor (Stachel *et al*. 1985) and it can be used as a media supplement to increase transformation efficiency.

In *B. oleracea* transformation studies acetosyringone was at first added to the bacterial suspension several hours prior to infection. Berthomieu and Jouanin (1992) added 20 μM, Christey and Sinclair (1992) 200 μM and Ding *et al*. 50 μM of acetosyringone. Tsukazaki *et al*. (2002) and Chakrabarty *et al*. (2002) obtained high transformation rates with 50 μM acetosyringone supplemented to the co-cultivation medium. Chakrabarty *et al*. (2002) reported that higher acetosyringone concentration (100 μM or more) was detrimental inducing tissue browning. Henzi *et al*. (2000a) optimized several factors which affect the transformation rate of broccoli thoroughly investigating their effects in various transformation stages. According to these results acetosyringone concentration in the co-cultivation media was increased from 50 to final 200 μM. Manopine at 10 μM also significantly increased transformation efficiency alone or even more in combination with 50 or 200 μM of acetosyringone. Arginine supplemented to the selection medium at 0.5 or 1.0 μM concentration further increased the transformation efficiency.

Feeder cell layering

Feeder cell layering a technique originally developed for culture of mammalian cells was later adopted for culture of plant tissues (Street 1973). It promotes cell division and growth of explants (Horsch and Jones 1980). In transforma-

tion experiments it improves the induction of *vir* genes (Fillatti *et al*. 1987) by secretion of virulence inducing phenolics (Veluthambi *et al*. 1987). Feeder cell layering may also reduce growth of *A. tumefaciens* on or around explants during co-cultivation (Niu *et al*. 2000). All these events positively affect transformation efficiency.

Charest *et al*. (1988) employed both preconditioning of explants and feeder cell layering for transformation of *B. napus* explants on its own cell suspensions. Beneficial effect of joint explant precondition and feeder cell layering on the reduction of explant necrosis (hypersensitive reaction) in *Brassica carinata* has been outlined by Babić *et al*. (1998).

Significant increase of hairy root induction after joint co-cultivation of bacteria and plant explants on a layer of tobacco cell suspension was observed by Christey and Sinclair (1992). These results were confirmed by Metz *et al*. (1995a) and became a routine practice in many later studies.

Jin *et al*. (2000) reported that tobacco cell feeder layering was detrimental for cabbage transformation, increasing tissue necrosis and reducing shoot regeneration. Tsukazaki *et al*. (2002) also reported negative effect of tobacco cell feeder layering on transformation efficiency in cabbage. The frequency of GUS positive explants was 2.8% in the presence and 32.7% in the absence of feeder cells.

Improvement of vectors and plasmids

Improvement of vectors and plasmids is always a fast and promising approach for the increase of transformation efficiency. Most studies on *B. oleracea* species were done using LBA4404 (*A. tumefaciens*) or A4 (*A. rhizogenes*) derivatives equipped with binary vectors. When it comes to plasmids the situation is different since many plasmids are available. However, in most studies plasmids are modified in order to accept genes of interest.

Recently more attention has been paid to regulatory sequences. Apart from the widely used CaMV 35S a number of new promotors have been investigated and considered for use. Jin *et al*. (2000) showed that *Bt* cabbage transformed plants with *vspB* promoter from soybean are equally toxic to DBM larvae as those with the same *Bt* gene under the 35S promoter. Cao *et al*. (2001) transformed broccoli with *cry1Ab* controlled by *PR-1a* promoter from tobacco which can be induced by salicylic acid. Baranski and Puddephat (2004) investigated activity of *gus* gene driven by *extAP* from rape, P_SMT_AP from pea, *RBCS3CO* from tomato and *SRS1P* from soybean all introduced into cauliflower seedling explants by *A. rhizogenes*-mediated transformation. This is part of the global trend advocating the use of tissue specific regulatory sequences of plant origin instead of those which are bacterial or viral by origin.

Marker genes

The most frequently used selectable marker genes were the *nptII* (neomycin phosphotransferase II) gene for kanamycin resistance and the *hpt* gene for hygromycin resistance. Genes providing resistance to other antibiotics were less frequently used same as genes providing resistance to herbicides.

Today the use of antibiotic selectable markers is considered not only harmful and detrimental for cultures but also dangerous for the environment being a permanent risk for genomic contamination of various prokaryotes and related wild plants species. The use of PCR in the evaluation of putative transformants can reduce the use of antibiotic resistance markers (Hamill *et al*. 1991; Puddephat *et al*. 1996). Transformation protocols which do no utilize antibiotics as selectable markers are possible as shown by Puddephat *et al*. (2001) and Higgins *et al*. (2006) and this is a clear trend for all further studies.

The most popular reporter gene for transformation of *B. oleracea* species is certainly the *Gus* (β-glucuronidase) gene used as a fast histochemical test for screening putative transformants (Jefferson 1987). At first it was assumed that the β-glucuronidase reaction is restricted to prokaryotes only and that it does not appear in higher plants (Stomp 1992). Hu *et al*. (1990) demonstrated that a transient "false" or "background" GUS reactions occurs in embryos and vegetative organs of many plant species. They classified cabbage and kohlrabi among plants with the most intensive false GUS reaction. Thus the appearance of blue color in the X-gluc test is not necessarily proof for internal β-glucuronidase tissue activity. It can be a staining artifact as showed by Mascarenhas and Hamilton (1992) or even evidence for bacteria persisting in tissue (Stomp 1992). A false-positive GUS reaction can be observed in tissues with increased concentration of peroxidases (Lojda 1970; Guivarc'h *et al*. 1996b) and in cells/tissues with increased lignin content (Guivarc'h *et al*. 1996a). Sretenović-Rajičić (2001) observed a false GUS reaction in a cabbage line known for its increased tolerance to paraquat (Sretenović-Rajičić *et al*. 2000b).

Another useful reporter is the green fluorescent protein (GFP) isolated from marine jellyfish (Chalfie *et al*. 1994; Baulcombe *et al*. 1995; Haseloff and Amos 1995). GFP is rapidly gaining popularity in studies on *B. oleracea* transformation (Cogan *et al*. 2001, 2002, 2004; Oldacres *et al*. 2005; Higgins *et al*. 2006). GFP can be visualized in living cells and even studied by confocal microscopy enabling non-destructive selection of putative transformants. The original gene contained a hidden intron active in eukaryotes cells. Also overexpression of GFP in the cytoplasm adversely affected regeneration of transformed cells. Changes in sequence of the hidden (cryptic) intron and targeting the product into endoplasmic reticulum improved the detection enabling also high shoot regeneration (Haseloff *et al*. 1997).

Quantitative trait loci (QTL) analysis

A totally new approach was developed based on the idea that the genome-dependant effect of transformation efficiency reflects the existence of specific QTL elements. According to this approach it would be possible to obtain genotypes with very high transformation efficiency. In rapid cycling (RC) species it would be possible to create new varieties with improved transformation efficiency with conventional breeding methods. In this line of research studies are done on the progeny of doubled haploid (DH) lines of RC cabbage × alboglabra cross A12DHd × GDDH33 for which detailed chromosome linkage maps are available. Cogan *et al*. (2001, 2002, 2004) and Oldacres *et al*. (2005) for their studies employed *A. rhizogenes* strain 9402 with binary plasmid pBIN-m.gfp-5ER. Sparrow *et al*. (2004a, 2004b) investigated progeny of the same RC DH cabbage × alboglabra crosses using *A. tumefaciens* wild strains C58, T37 and Acph5 (Sparrow *et al*. 2004a) and strain LBA4404 with plasmid p25GI (Sparrow *et al*. 2004b).

Cogan *et al*. (2002) identified three QTLs related to transgenic root production. Linkage groups O1 and O3 contained enhancing alleles from A12DHd while the other parent GDHH33 has an enhancing allele on linkage group O7. QTLs on linkage groups O3 and O7 where also engaged in the enhancement of adventitious root production.

Cogan *et al*. (2004) extended their QTL analysis in cabbage cross A12DHc × GDDH33 using substitution genotypes. It enabled them to analyze all eight possible genotype combination of alleles from QTLs LGO1, LGO3 and LG07 finding that they all participate in stable integrated transgenes 14 days after the transformation event. Same loci were investigated for efficiency of transformation with LBA4404 and C58C1 but due to the high level of variability results were not statistically significant - they were inconclusive. Further analysis showed that *Arabidopsis* chromosome 3 contains a region homologue to the QTLs LGO1 and LGO3.

Sparrow *et al*. (2004a) performed an 8 × 8 dialel analysis of genotypes showing different transformation efficiency with wild type *A. tumefaciens* strains. Parents with low ef-

ficiencies produced progeny which also had low transformation efficiency. Also parents with high transformation efficiency produced progeny with high transformation efficiency. GDDH33 was highly susceptible to *A. tumefaciens* with 92% seedlings producing crown galls while A12DHd was less susceptible failing to produce crown galls with octopine strain Ach5 and nopaline strain T37. Crown galls at low frequency (8%) appeared only upon inoculation with strain C58. A QTL was identified in the central part of linkage group O9.

Sparrow *et al*. (2004b) studied phenotypic markers which can indicate genotypes with a high transformation rate. They found a highly significant correlation between the crown gall formation and GUS expression which would suggest that the genetic control of crown gall formation is related to infection susceptibility and not to gall formation. In genotypes in which explants induced blackening of media (dominant trait) transformation and regeneration of transgenic shoot was not possible. Another interesting trait was the mode of shoot regeneration, callus vs. tissue swelling, the former being more favorable for successful transformation. Pooling of such "desirable" characteristics may greatly assist in breeding genotypes with increased transformation efficiency.

Oldacres *et al*. (2005) studied the formation of adventitious and transformed roots in the DH cauliflower line Niche, DH Brussels sprout line Gower and 48 lines of their progeny transformed with *A. rhizogenes* strain LBA 9402 with GFP as a marker gene. QTL analysis showed that both production of adventitious and transformed roots are controlled by loci at the same position within the genome. They observed that genotypes exhibiting high adventitious root production showed also high transgenic root production, allowing selection of lines with higher transformation efficiency than in the parent lines.

Transformation of local DH lines was investigated by Tsukazaki *et al*. (2002). Hypocotyl explants were inoculated by co-cultivation with LBA4404, EHA101, EHA105 and AGL0, all containing plasmid pIG121Hm. Overall transformation success under optimal conditions including treatments with 50 μM acetosyringone was 3.1%. PCR analysis of T1 plants of transformant MP22-0 containing a single copy of the *gus* gene suggested a 3:1 segregation ratio. Transformation efficiency decreased when tobacco suspension feeder cell layering was used.

A. RHIZOGENES STUDIES

In the last decade *A. rhizogenes*-mediated transformation was a popular technique used in many studies providing good results with different *B. oleracea* vegetable species (Christey *et al*. 1997; Henzi *et al*. 2000a; Puddephat *et al*. 2001; Higgins *et al*. 2006). Techniques elaborated in these studies can be considered as routine i.e. suitable for fast introduction of foreign genes in genotypes of commercial interests.

Among many available *A. rhizogenes* strains the agropine type A4 found the widest application appearing in most transformation studies (Puddephat *et al*. 1996). Plasmids of strain A4 can express their virulence after *trans*-conjugation into *A. tumefaciens* cells (Petit *et al*. 1983). Thus *A. tumefaciens* cells containing *A. rhizogenes* plasmids induce hairy roots in susceptible plants. A4 and related strains were improved by addition of specialized plasmids (binary vector) or rarely by co-integration of genes in plasmids of the original strains. Contrary to *A. tumefaciens*, plasmids in *A. rhizogenes* are not disarmed. Presence and activity of their oncogenes (*Rol* genes) are responsible for the appearance of plants with altered phenotype of roots and whole plants named HR (hairy root) or Ri phenotype. Phenotype alterations although undesirable may have potential application in plant improvement since in the horticultural industry morphological alteration like dwarfing, increased rooting, altered flowering, wrinkled leaves and/or increased branching are desirable (Christey 2001). HR roots have accelerated growth, increased branching and plagiotropic growth (Tepfer 1990). Plants expressing *rol* genes usually have stunted shoot growth, reduced apical dominance, changes in the morphology of leaves and flowers and decreased fertility (Tepfer 1989).

The virulent plasmid of strain A4 named pRiA4b has been thoroughly studied and its physical map was constructed by Huffman *et al*. 1984. Further research by White *et al*. 1985 showed that T DNA of pRiA4b consists of two separate fragments designed as TL and TR DNA. Using transposon insertion they identified four morphogenic loci on the TL DNA designed as *RolA-D* and two *Rol* loci on TR DNA designed as *tms1* and *tms2*. Further analysis of pRiA4b and related pRiHRI TL DNA performed by Slightom *et al*. (1986) provided complete nucleotide sequence for pRi HRI TL DNA. They also identified 18 open reading frames on the Tl DNA among which *Orf 10*, *11*, *12* and *15* correspond to *rolA-D* respectively (Christey 2001). Function of individual *Rol* genes has been thoroughly investigated (Spano *et al*. 1988; Leach and Aoyagi 1991).

The TL region seems to be the more efficient root-inducing T-DNA segment (Petersen *et al*. 1989). However a plant transformed with agropine type plasmids like A4 may contain TL, TR or both TL and TR DNA fragments integrated in its genome. The presence of different T-DNA fragments and the differential expression of their *Rol* genes are supposed to be the main reason for the large phenotype variations observed among plants transformed with *A. rhizogenes* (Christey 2001).

According to Christey (1997) the main advantage of *A. rhizogenes*-mediated transformation is that transgene plants can be obtained without selection agents. Primary selection can be done on basis of morphological characteristics (growth habit) and the absence of selective agents can increase co-transfer rate of genes present on the second, binary vector. Production of chimeric plants characteristic for *A. tumefaciens* transformation is unlikely. Clones consisting only of transformed cells can be obtained after several subculture cycles and shoot regeneration which occurs from them is not associated with cytological problem inferred in long term callus cultures. In plants where Ri phenotype appearance is undesirable it is still possible to obtain phenotypically normal transgenic plants since two T-DNAs can segregate at meiosis in subsequent generation.

However there are several problems in this interesting approach. Inoculation with *A.rhizogenes* produces also a large percentage of adventitious, non-transformed roots (Christey and Sinclair 1992; Higgins *et al*. 2006). Also the two T-DNAs present in the same bacterial cell during inoculation have a tendency to get incorporated in the same loci in the host genome (Hosoki and Kigo 1994). Since they are co-integrated, frequency of their segregation is very low. Still segregation of two T-DNAs in T_1 generation and formation of plants with normal phenotype has been reported by Puddephat *et al.* (2001) and Higgins *et al*. (2006). Christey (2001) points that the appearance of cauliflower plants with normal phenotype observed in field evaluation studies (Christey *et al*. 1999) may not be result of independent segregation but of gene silencing.

Christey *et al*. (1997) investigated *Ri*-mediated transformation using strain A4T with binary vectors in a number of *Brassica* species including: broccoli, Brussels sprout, cauliflower, cabbage, rapid-cycling cabbage and Chinese cabbage. In addition to the *npt*II gene providing kanamycin resistance and the *gus* gene for identification of putative transformants, binary vectors contained either the *cry1A(c)* gene from *B. thuringiensis* providing insect resistance (pART 27732) or the tomato ethylene-forming enzyme gene in antisense orientation (pLN35). Inoculation based on previous studies (Christey and Sinclair 1992) was done by brief dipping of cotyledon petioles and leaf explants in bacterial suspension. Explants blotted on filter paper were co-cultivated for 48 h and then transferred to cefotaxime-containing media. Successful transformation was obtained with all investigated cultivars and lines. Selective kanamycin

supplemented media were used to separate the poorly growing A4T transformed hairy roots from the fast growing binary vector transformed hairy roots. Shoot regeneration LS-5 medium containing 5 mgl^{-1} BA and 5 mgl^{-1} NAA enabled shoot regeneration of all investigated genotypes. Some of them, like rapid cycling cabbage and cauliflower, regenerated shoots spontaneously. Plants were fertile enabling transmission of foreign genes into the T_1 progeny. All plants showed *Ri*-induced morphological changes which varied from barely noticeable in broccoli to heavily wrinkled leaves in cauliflower. Transformation efficiency was calculated as the number of hairy roots obtained per total number of co-cultivated explants. Depending on genotype measured efficiencies were: Broccoli (1%, 4% and 8%), Brussels sprouts (6%), cabbage (18%, 28% and 33%), cauliflower (12% and 15%), Chinese cabbage (27% and 43%). Southern analysis performed for some genotypes indicated insertion of a high and variable number of NPTII copies in transgene plants (1-9+). Progeny analysis in most cases demonstrated transmission of a single transgene copy but Southern analysis in most samples indicated presence of multiple copies. Authors considered several different explanations for this discrepancy. First, multiple copies of T-DNA may be inserted at the same site in which case they would behave as a single insertion event in the progeny analysis. It is also possible that some copies of the inserted T-DNA are not expressed in the progeny.

Christey *et al*. (1999) investigated field performance of previously produced transgenic *B*. *oleracea* species. In each of the 21 studied transgene lines there was little phenotypic variation suggesting the complete dominance of HR (hairy root) genes. In six transgenic broccoli lines three had a severe HR phenotype, two were with slight HR and one with normal phenotype. In cabbage all six transgenic lines showed slight to moderate HR phenotype. In a single line of Chinese cabbage HR phenotype was absent. In contrast the single transgenic line of Brussels sprouts was with severe HR phenotype. Finally in seven transgenic cauliflower lines four lines showed severe HR phenotype, two lines had normal phenotypes and one contained both normal plants and severe hairy phenotypes. PCR analysis of *rol*B and *rol*C presence performed on six normal and five lines with HR phenotype confirmed that in cauliflower line P4/Bt#7 both *rol* genes were absent. Since lines corresponded to the T_2 generation plants this case is a clear proof for independent gene segregation.

Puddephat *et al*. (2001) studied transformation of cauliflower and broccoli. In the T_1 progeny, beside plants with HR phenotype, in some transgene lines they observed plants with normal phenotype appearing at Mendelian ratios. Appearance of plants with normal phenotype was attributed to the independent segregation of genes transferred by the binary vector and helper plasmid. Such outcome is possible only if binary vector and helper plasmid genes get inserted independently. Study was done using *A*. *rhizogenes* strain A4T with pRiA4 helper plasmid and pMaspro:GUS binary vector. Inoculation explants were young seedlings co-cultured with bacterial suspension. Hairy roots were screened for GUS activity and transgenic plants were produced without the use of selective antibiotics. Overall transformation efficiency was 8.3% for cauliflower and 3.03% for broccoli hypocotyl explants. PCR was used to confirm the presence of *gus* and *rolB* genes from the vector and Ri T-DNA and the absence of *virD* gene to confirm absence of contaminating bacteria. Southern analysis of root clones indicated integration of 1 to 5 *gus* copies.

Christey and Sinclair (1992) observed that inoculated explants produce a mixture of transformed and normal roots which could be visually distinguished after 3 weeks of growth in culture on basis of their growth habits. Transgenic roots grew much faster with abundant branching and characteristic plagiotropic growth. Furthermore, the use of selective markers present on binary vectors enable separation of hairy roots produced by native plasmids (non resistant) and binary vector (resistant).

Henzi *et al*. (1999, 2000a) improved the protocol for *A*. *rhizogenes*-mediated transformation of broccoli. Leaf explants cut in half were dipped in bacterial suspension for 5 min, blotted dry and placed on sterile filter paper floated on top of *B*. *campestris* or tobacco feeder cell layer. Various virulence elicitor compounds were also investigated. Addition of 200 μM acetosyringone in the medium for bacterial growth, 10 μM manopine and 50 μM acetosyringone in B *campestris* feeder cell layers used for co-cultivation and addition of 1 μM arginine in the media for elimination of bacteria significantly improved transformation success of broccoli which reached 33% of inoculated explants (Henzi *et al*. 2000a).

Higgins *et al*. 2006 used *A*. *rhizogenes* mediated transformation to study transformation of broccoli with ACC synthase 1 and ACC oxidases 1 and 2. Their results are presented in the next chapter dedicated to post harvest senescence. They showed that using *A*. *rhizogenes* co-transformation system it is possible to produce marker-free plants which contain only the construct of interest.

Sretenović-Rajičić *et al*. (2006) presented research on *A*. *rhizogenes*-mediated transformation of elite domestic cabbage and Savoy cabbage. Bacterial strain A4M70GUS contains pRiA4 plasmid with a co-integrated *gus* gene. This is a rare report on the transformation with cointegrated plasmids. The protocol enabled a very high hairy root production from hypocotyl and cotyledon explants followed by spontaneous shoot regeneration in all three investigated genotypes. Overall transformation efficiency expressed as percentage of explants producing hairy roots was 92.3% for Savoy and 64.4% and 87.2% for the two cabbage lines. Spontaneous regeneration of shoots on the hormone-free medium was 1.3% for Savoy and 9.3% and 2.6 for cabbage. All cabbage plants regenerated from HR cultures were GUS positive and 55% plants of Savoy cabbage. PCR analysis confirmed the presence of the *gus* gene in shoots regenerated from HR cultures same as in T_1 plants.

POST HARVEST SENESCENCE

One of the main aims in the current transgenic research of *B*. *oleracea* vegetable species is to gain access to the regulation of post-harvest senescence. This can be done by transferring genes which affect biosynthesis and metabolism of cytokinins and ethylene. Apart from the direct gene overexpression in target tissues it is possible to modulate gene expression by insertion of genes in an antisense orientation and use of specific promotors which can activate genes at the appropriate moment. Genes commonly transferred include *ACC* (1-aminocyclopropane-1-carboxylix acid) oxidase, *ACC* synthase, *ipt* gene for cytokinin synthesis and others. Most of the studies in post harvest senescence have been performed on broccoli.

Henzi *et al*. (1999, 2000b) employed *A*. *rhizogenes*-mediated transformation to study post harvest senescence in broccoli. *A*. *rhizogenes* strain A4T contained binary vector pLN35 with *ACC* oxidase gene in antisense orientation which is supposed to down regulate post-harvest ethylene synthesis. High transformation efficiency was obtained, 35% for cv. 'Shogun' and 17% for cv. 'Green Beauty' (Henzi *et al*. 1999). Southern analysis revealed full insertion of ACC oxidase gene in 15 investigated plants among which only four displayed more than one integrated band (Henzi *et al*. 1999). Shogun transformant *Sh/2* had a statistically significant decrease of ethylene production showing 91% reduction 96 h after harvest, in comparison to the non-transformed control. Also transgene lines *D/1* and *D/2* of cv. 'Dominator' showed significant improvement in head color relative to the control. Authors suggest that broccoli senescence has two ethylene bursts and that the *ACC* antisense oxidase gene inhibits only the second one. Morphological characterization of transformants (Henzi *et al*. 2000b) showed high variability among transformants similar to the those described previously by Christey *et al*. (1999). However 27% of ACC transgene broccoli clones had a normal

phenotype.

Gapper *et al*. (2002) further investigated means to alter post-harvest senescence in broccoli. In addition to antisense *ACC* gene, plants were also transformed with the *SAG12-IPT* gene construct which alters cytokinin biosynthesis. Transformation was done using *A. tumefaciens* strains AGL1, GV3101 and KYRT1 with several different binary vectors among which plasmid pN10 with antisense *ACC* oxidase gene and pN11 with *SAG12-IPT* cytokinin construct. *A. tumefaciens* was chosen with the aim to avoid aberrant plant formation connected with the use of *A. rhizogenes*-mediated transformation. A total of 53 transgenic lines of broccoli were obtained among which 26 antisense ACO lines, 8 pARt27 controls, 13 SAG12-IPT lines and 6 pBJ49 controls. Overall transformation efficiency for both cotyledonary petiole and hypocotyl explants (1.0%) was obtained from 5501 inoculated explants. Transformation rate equaled the number of independent transgenic lines obtained per 100 explants inoculated. The addition of silver nitrate to the medium had no significant effect on transformation efficiency. Southern analysis confirmed the transgenic nature of PCR-positive plants and estimated insertion of plasmid DNA equal to one, three and five copies of a single gene. Some plants with abnormal phenotypes were registered in all groups of transformants showing reduced shoot, leaf or root growth and alterations in shape. However the majority of transgenic lines appeared phenotypically normal in culture.

Chen *et al*. (2001) studied the effect of cytokinin-synthesizing *ipt* gene on the post-harvest yellowing in broccoli. LBA 4404 with plasmids pSG529(+) and pSG766A both containing the *ipt* gene were used for inoculation by co-cultivation and vacuum infiltration. In transformed plants postponed yellowing of detached leaves and florets was evident but it varied among different transformant lines. Delayed yellowing of leaves and florets appeared to be independent traits. From 140 kanamycin resistant plant brought to maturity 43 postponed yellowing in detached leaves and 22 in floret heads with only 10 transformants manifesting delayed yellowing in both leaves and florets. Transformation efficiency using cotyledon, hypocotyl and peduncle explants varied from 0.6 to 14.7%. Calculation was done on basis of kanamycin resistant regenerants per total. After selection at 75 mgl^{-1} kanamycin 60% of surviving plantlets were positive both for *nptII* and *ipt.* Over 200 kanamycin resistant plants were produced. Authors also obtained high transformation 23% (39/168) with vacuum infiltration of peduncle explants with pSG766A. Vacuum infiltration of seedling explants was not efficient due to their poor survival rate. Southern analysis demonstrated presence of one to four restriction fragments among transformants. Results indicated possible T-DNA rearrangement and perhaps even gene silencing since the high proportion of transformants was possibly not active.

Chen *et al*. (2004) investigated post harvest senescence of broccoli as affected by a mutant *boers* gene which induces ethylene insensitivity. This gene was derived from an *ERS*-type ethylene receptor gene cloned from broccoli (Chen *et al.* 1998). It was constructed by replacing isoleucine (Ile 62) with (Phe) phenylalanine. Transformation was performed with LBA 4404 harboring plasmids pSMY1H-mERS162F (with *hpt*) and pB1-mERS162F (with *nptII*). Transgenic plants manifested retarded yellowing but their response varied with transgenic lines. Yellowing in most transgenic lines was delayed for a day or two. Some transformants with the transgene inserted failed to show the retarding effect indicating gene silencing. In most transformants multi-site integration and DNA rearrangements had occurred. Some morphological and anatomical alterations in transformants were also observed. Transformed plants were fragile and perished from manipulation injuries. Authors concluded that the retarding effect of the mutant *boers* gene on the yellowing of detached leaves and florets was not as good as the one previously obtained with *ipt* transformation (Chen *et al*. 2001). Putative transformation efficiency was up to 6.9% for pSMY1H-mERS162F and up to 2.7% in pB1-mERS162F.

Higgins *et al*. (2006) transformed the GDDH33 broccoli line with *ACC synthase 1* and *ACC oxidase 1* and *2* genes which can extended the shelf life of heads. Transformation was *A. rhizogenes*-mediated with LBA9402 and several different constructs providing an overall transformation efficiency of 3.26% (150/4599) calculated from the number of excisable GFP-fluorescent roots and total number of explants. Agro-pine type pRi1855 was co-integrated to contain GFP which proved to be a reliable non-destructive marker for identification of transgene plants. Constructs containing clones of cDNA *ACC oxidase 1* and *2* (ACO1 and ACO2) and *ACC synthase* (ACC1) were made and used in experiments. Transgenic ACO1A line produced 564 seeds of which 69 were sown and 67 germinated. There was a high percentage (57%) of double-positive plants in which GFP and ACO1A T-DNA co-segregated. Southern analysis of these plants showed four copies of GFP and 2 copies of ACO1A. The important finding was two marker-free plants containing only ACO1A T-DNA. Thus although ACO1 and GFP co-segregated, 3% of the progeny was marker-free. Post harvest ethylene production in plants transformed with ACO1A and ACO2A was reduced in relation to non-transformed harvested buds and equaling the non-harvested control. A relation between reduction in post harvest ethylene production and chlorophyll loss was established. Authors believe that the use of transgenic plants may increase the shelf-life of broccoli for at least 2 days. Shoot regeneration from hairy roots was the limiting factor in this study. Only 12% of transgenic hairy roots developed into plantlets giving the overall transformation/regeneration rate of one transgenic plant per 200 inoculated explants. The average time laps between explant inoculation and production of mature broccoli head was 385 ± 21 day.

Eason *et al*. (2005) used *Agrobacterium*-mediated transformation to produce broccoli plants with down-regulated BoCp5, protein which during senescence normally gets up-regulated. BoCp5 is an early cystein protease involved in programmed cell death of broccoli florets. Reduction of the harvest-induced expression of BoCp5 was achieved in all four analyzed transgenic lines but the relative accumulation and expression pattern of BoCp5 messenger RNA was different in each transgenic line. Post harvest floret senescence was delayed in three antisense lines. Twenty-six independently transformed lines were identified by PCR after selection on kanamycin. Overall transformation efficiency was 0.9%, 26 plants from 2831 explants. Transformation was higher with hypocotyl (2.35%) than with cotyledon explants (0.11%).

INSECT RESISTANCE

The use of transformation techniques for creation of species resistant to insect attack is one of the most promising approaches of modern agriculture. Outstanding results can be obtained by mobilization of genes encoding insecticidal crystal proteins (ICP) from *Bacillus thuringiensis* (Bt) into genomes of cultivated plants. In *B. oleracea* species first studies started by Bai *et al*. (1993), Metz *et al*. (1995b) were soon accompanied by many more including Cao *et al*. (1999), Earle *et al*. (2000), Chakrabarty *et al*. (2002), Cao *et al*. (2005), Christey *et al*. (2006), and others. Resistance to insect attack was also attempted by transfer of trypsin inhibitor, cystein proteases and other substances detrimental to insects.

Bt insecticidal crystal proteins (ICP) offer excellent protection against certain insect species. However there is a constant danger that populations of resistant insect may appear as in cases of fields previously sprayed with spores of *B. thuringiensis* subsp. *kurtstaki*. To prevent or to delay appearance of resistant insects, special strategies have been devised including refuges planted with insect non-resistant species and others. In the meantime genes encoding ICP have been significantly improved providing high protein ex-

pression in plant tissues.

Cao *et al*. (1999) transformed broccoli with an improved synthetic *cry1C* gene previously used to establish insect resistance in alfalfa and tobacco (Strizhov *et al*. 1996). Transformation was done with *A.tumefaciens* strain AVI harboring pNS6 according to Metz *et al.* (1995b). A strong correlation was found between the levels of mRNA, production of Cry1C protein and insect control. Plants producing high mRNA levels were toxic to larvae. Total Cry1C production in tissues reached 0.4% of total soluble proteins. Study demonstrated that high production of Cry1C protein can protect transgenic broccoli not only from susceptible but also from diamondback moth (DBM) larvae resistant to CRY1A and CRY1C. Transformation efficiency was 5.25% corresponding to 21 Cry1C transgenic plants derived from 400 cotyledon and hypocotyl explants.

Jin *et al*. (2000) transformed cabbage with two different *cry* genes, synthetic *cry1Ab3* and wild type *cry1Ia3*. Transformation initially performed according to Metz *et al*. (1995a) was done with EHA105 and pTiBo542 super-virulent plasmid and Bt genes introduced into pBI121 to produce pCry1Ab3 and pCry1Ia3. Explant types included hypocotyls, cotyledons and leaves and their petioles. Plants transformed with the wild-type *cry1Ia3* were susceptible to DBM (diamondback moth) larvae while those with the synthetic *cry1Ab3* gene were highly resistant. Examination of susceptible transformants containing the wild-type gene showed presence of numerous truncated *cry1Ia3* transcripts. mRNA truncation was probably results of AT-rich sequences producing a premature polyadenylation signal for the end of transcription at the 950 downstream position. Transformation procedure was thoroughly investigated and modified but a total of 15 independent transformed lines and transformation efficiency which was less than 1% reflects genomic problems involved in cabbage transformation. Problems encountered were high explant necrosis which required reduction of the concentration of bacterial co-cultivation suspension, exclusion of tobacco feeder cell layering and agar concentrations increased to 1%. Cabbage explants were also sensitive to kanamycin and resistant shoots were obtained only after kanamycin concentration was reduced to 10 mgl^{-1}.

Cao *et al*. (2001) transformed broccoli with cry1Ab controlled by the PR-1a promoter from tobacco which can be induced by salicylic acid and related chemicals. Hypocotyl and cotyledon explants were inoculated with *A. tumefaciens* strain ABI harboring pJC12B vector. Transformed plants induced with BTH (1, 2, 3-benzothiadiazole-7-carbothioic acid S-methyl ester) showed good protection against DBM larvae. However control plants which were not chemically induced also exhibited some protection against insects. Results indicate that the PR-1a promoter is "leaky" producing Cry1Ab protein without chemical induction. Overall transformation frequency was 6.1% calculated as 49 transgenic kanamycin resistant plants obtained from 800 explants. Nine out of the ten investigated transgenic lines exhibited 3:1 segregation ratio suggesting *nptII* integration into a single locus. One line had a 15:1 ratio indicating two unlinked genes.

To overcome the insect resistance problem Cao *et al*. (2002) incorporated two slightly different *Bt* genes in the same plant species (pyramiding). This was done by classic hybridization of broccoli cv. 'Green' comet with transgene plants carrying *Cry1Ac* (Metz *et al*. 1995b) and *Cry1C* gene (Cao *et al*. 1999). Plants equipped with both *Bt* genes caused rapid and complete mortality of DBM larvae feeding on their leaves. This study gives a good example how once established and recognized GM plants may be efficiently used by conventional breeding methods to fortify resistance in their hybrids.

Bhattacharya *et al*. (2002) transformed "Golden Acre" a popular Indian cultivar of cabbage with the synthetic *cryIA*(*b*) *Bt* gene and investigate resistance of transformants towards DBM (*Plutella xylostella*). They used *A. tumefaciens* strain GV2260 with plasmids pBT1291, pBinAR and their derivatives. Explants comprising hypocotyl segments were precultured for a day and co-cultivated in bacterial suspension for three days in darkness. Cefotaxime was used to remove bacteria. Selective shoot regeneration was done in presence of 25 mgl^{-1} kanamycin. Larvae feeding on transgenic plant leaf discs were severely stunted in growth demonstrating active *Bt* mediated resistance in plants. Larval mortality ranged from 51.84 to 74.06%. Fifteen kanamycin resistant plants were recovered among which six were normal-looking. There is no data on transformation efficiency. Three independent transformant lines showed single-copy transgene insertion.

Chakrabarty *et al*. (2002) incorporated *cry1A*(*b*) into cauliflower using GV2260, LBA 4404, A208 and EHA105 with plasmids p35GUSINT and pBinAR-Bt. Explant age affected transformation efficiency. Thus all young, 4 day-old hypocotyls explants perished from necrosis and the 7 days-old explants gave the best results. Hypocotyls which were not precultured for a day or two perished from the hypersensitive reaction. Strain GV2260 was superior in comparison to others. Transformation efficiency calculated on basis of GUS staining ranged from 2.5 to 22.6%. Southern analysis revealed integration of transgenes at one to three loci. RT PCR showed transcriptionally active *cry1A*(*b*) in the plant genome. The highest mortality of DBM larvae feeding with leaf disks was 85.7% after 48 h.

Cao *et al*. (2005) extended their research to collards (*B. oleracea* var. *acephala*). Using the well established protocols (Metz *et al*. 1995a; Cao *et al*. 1999) they created a number of transgenic collard cultivars containing *Bt* genes *cry1C* or *cry1Ac*. Bt gene integration was confirmed by PCR and Southern analysis. Although the production of *Bt* proteins was variable plants which expressed *Bt* genes caused mortality in susceptible DBM larvae. Thus all Champion and MGG plants with moderate or high levels of Cry1C protein caused 100% mortality of susceptible and Cry1A resistant larvae. The overall transformation rate based on hygromycin or kanamycin resistance ranged from 0.8% to 3.6%.

Zhao *et al*. (2005) studied the appearance of resistance against *Bt* insecticidal toxins in DBM larvae feeded with leaves from plants transformed with a single or two dissimilar *cry* genes. Data which they collected indicate that the excellent resistance offered by two dissimilar *cry* genes pyramided in a plant species is jeopardized if plants transformed with single *cry* genes are grown at the same location.

Ding *et al*. (1998) used *A. tumefaciens* LBA4404 with pBI121 to introduce a trypsin inhibitor gene (*ti*) from local sweet potato into cauliflower cultivars. Trypsin inhibitor (TI) belongs to protease inhibitors which can be used to combat insect pests. In storage roots of Taiwan sweet potato TI accumulates presenting more than 80% of all soluble proteins. Hypocotyl explants precultured for 3 days on callus inducing medium supplemented with 29.4 μM silver ions provided high transformation rates which in some cultivars exceeded 95%. Regenerated shoots in early stages of development were sensitive to the selective agent. Thus the lack of selective agent in the first week after co-cultivation followed by progressive increase of selective pressure provided optimum conditions for selection of transgenic shoots. More than 100 transgene plants were obtained. Functional integration of TI in transformed plants was demonstrated by TI activity assay. PCR and southern analysis showed a 0.66-kb DNA segment corresponding to the TI gene sequences present in putative transformants and not in control plants. *In planta* feeding bioassays showed that TI transformed cauliflower plants have a high degree of insect protection.

Lingling *et al*. (2005) transformed cauliflower with a trypsin inhibitor originating from cowpea, Transformation was done with *A. tumefaciens* strain LBA4404 with pGA643 which was modified to contain *nptII* and the *CpTi* gene of interest (not stated clearly). A total of 14 transgenic plants was produced and analyzed by PCR and Southern showed presence of *CpTi* gene integrated in the cauliflower genome. Data concerning the *CpTi* gene were not presented.

These preliminary results showed that transgenic plants exhibited some resistance to *P. rapae*.

Lei *et al*. (2006) in a short study presented transformation of self-incompatible cabbage line 131-A with *A. tumefaciens* LBA 4404 and plasmid pBI121-*OCI* containing *OC-I* oryzacystatin, cystein proteinase (protease) inhibitor gene from rice. Transformation procedure has been studied in detail. Hypocotyl explants, 2 day preculture, 4 day co-cultivation with bacteria, Petri dish sealing tape which is not airtight and delayed application of antibiotic selection of regenerated shoots were all crucial for successful transformation. After 4 days of precultivation 79% hypocotyl explants regenerated shoots. Also hypocotyl explants produced 4.7% kanamycin resistant calli after 4 days of co-cultivation. Transgenic nature of putative transformant was confirmed by PCR analysis, Southern hybridization and field performed resistance analysis. Transformed plants expressed increased resistance to insect larvae in comparison to control plants.

HERBICIDE RESISTANCE

Among the first genes of interest which were introduced into *B. oleracea* vegetable species was the *bar* gene providing resistance to phosphinothricin (glufosinate) which is the active principle of the herbicide Basta® (de Block *et al*. 1989; Mukhopadhyay *et al*. 1991). Providing commercial cultivars with herbicide resistance can offer a substantial advantage reducing the crop production costs.

Lee *et al*. (2000) introduced *bar* gene into cabbage using LBA4404 with pMOG6-Bar. Plant transformation in this and two latter studies of same authors (Lee *et al*. 2002, 2003) was done according to Metz *et al*. (1995a). Transformation efficiency was 5% (75/1500) calculated on basis of hygromycin resistant calli produced from inoculated explants. Forty transgene plants were brought to maturity. Transformed plants were resistant to Basta® spraying in contrast to controls which perished 10-20 days after spraying. Southern blot analysis hybridization of the *bar* probe with genomic DNA of transformants showed 2.0 kb band absent in control. Genomic DNA digested with *Hind*III hybridized with *npt* probe showed 1 to 5 integrated gene copies. Five out of 40 transformants were not able to grow showing severe leaf malformations. The same abnormal phenotype was earlier observed in non-transformed plants regenerated from callus indicating a callus related variation. T_1 progeny showed very strong herbicide resistance and a Mendelian type segregation (3:1) indicating stable single gene insertion.

In a study on forage and vegetable *Brassica* species Christey and Braun (2001) transformed CN95 kale (*B. oleracea* var. a*cephala)* with *A. rhizogenes* strain A4T containing binary vector pMOA4 with chimeric *bar* gene. All six transgenic hairy root lines regenerated shoots. Selection for Basta resistance was not done until shoot regeneration was obtained. Resistance of putative transformants was evaluated according to the leaf-piece assay prior to transfer of plants to greenhouse. Leaf pieces were exposed to media containing 10 mgl^{-1} Basta® for 7 days. In the field test transformed kale plants manifested *Ri* phenotype morphology comprising stunted growth and wrinkled leaves.

Lee *et al*. (2003) made a construct in which a cytotoxic diphtheria toxin A-chain gene (*DTx-A*) was driven by promoter of tapetum specific *BcA9* gene isolated from Chinese cabbage. Activity of this construct following *Agrobacterium* mediated transformation into cabbage according to Lee *et al*. (2000) was studied through the activity of a *bar* gene inserted in the same construct to function as a reporter gene. Construct when active induced male sterility. Transformed plants were additionally resistant to Basta®. From 1600 hypocotyl explants 50 hygromycin calli were obtained. A total of 15 transgenic shoot were obtained providing 13 transgenic plants which were cultivated to maturity. Male sterile flowers were observed in all transgenic plants which otherwise appeared to be of normal phenotype. Male sterile Basta® resistant transgene plants were crossed with pollen from wild-type plants producing more than 100 seeds. Southern analysis of this T_2 progeny showed that two copies of *DTx-A* transgene were integrated into genome and stably inherited through the next generation.

Sretenović-Rajičić *et al*. (2004) elaborated a transformation protocol for Savoy cabbage (*B. oleracea* var. *sabauda)* using *A. tumefaciens* strains AGL1/pDM805 and LBA4404/pGKB5. Both plasmids contained *bar* gene. Delayed selection pressure was achieved by addition of 10 mgl^{-1} phosphinotricin (L-PPT). Protocol provided efficient transformation of Savoy inbred line Gg-1 and transgene expression in T_0 and T_1 plants which were resistant to Basta® spraying. Transformation efficiency expressed as percentage of explants regenerating shoots after L-PPT selection was 58% for AGL1/pDM805 and 25% for LBA4404/pGKB5.

Christey and Sinclair (1992) introduced into kale (*B. oleracea* var. *acephala*) a mutant acetolactate synthase gene (*als*) providing resistance to chlorsulfuron. Plants survived selection on media containing 10 µg chlorsulfuron but the PCR could not discern *als*-transformed from non-transformed plants.

ANTIMICROBIAL RESISTANCE

Mora and Earle (2001) transformed broccoli with an endochitinase gene from *Trichoderma harzianum*. Construct containing endochitinase cDNA *hEn42* was transferred into pBin19 to obtain pBIn19ESR incorporated into LBA4404 and used in experiments. Transformation was done according to modified techniques of Moloney *et al.* (1989), and Metz *et al*. (1995a). Overall transformation efficiency was 6.8% (19/280) calculated on basis of *nptII* assay and kanamycin resistance. Transgene plants were analyzed by PCR and blot analysis. The expected 42 kDa endochitinase band was detected in T_0 plants but not in the controls. Transformed plants selected according to their kanamycin resistance manifested 14 to 37 times higher endochitinase activity in mature leaves than in the control. Endochitinase activity of T_0 and T_1 plants decreased when they reached flowering stage. Transgene plants had less severe disease symptoms when infected with *Alternaria brassicicola* but not when they were infected with *Sclerotinia sclerotinorum*.

Polyploid plants were highly susceptible regardless of their endochitinase levels. Also plants with two inserts of *nptII* transgene did not have higher endochitinase activity than plants with a single insert resulting perhaps from position effects or partial gene silencing.

Lee *et al*. (2002) transformed cabbage with a glucose oxidase gene (GO) from *Aspergilus niger*. Gene generated by PCR with *Aspergillus* genomic DNA as template was inserted in pGA1182 binary vector to obtain pGAGO incorporated in LBA4404 and used in experiments. Plant transformation was done as previously reported (Lee *et al*. 2000). Ten independent T_0 transformants selected on hygromycin were used to obtain T_1 and T_2 seeds. High variation in the GO activity was detected in different T_1 lines and T_2 progeny. The number of gene copies from 5 present in T_0 changed to 3 in T_1 generation. Such findings indirectly indicate unstable inheritance due to copy segregation or partial gene silencing. Transgene cabbage plants showed significantly increased tolerance to black rot disease (*Xanthomonas campestris*) but there was also significant growth retardation and reduced fertility.

SELF INCOMPATIBILITY

Important trait which can be introduced in *Brassica* species by transgene techniques is altered self-incompatibility. Most *Brassica* species have sporophytic self-incompatibility which is a great barrier in commercial hybrid production.

Genetic transformation techniques have been used to overcome incompatibility and study the incompatibility mechanism achieving new breeding strategies in many plant species. Toriyama *et al*. (1991) transformed cauliflower

with *S*-locus gene from *B. campestris* in order to change self-incompatibility phenotype.

Bhalla and Smith (1998) optimized a protocol for (simple and efficient) transformation of cauliflower using *A. tumefaciens* strain LBA4404 with a pollen-specific *Bcp1* gene in antisense orientation linked to a pollen specific promoter (*Lat52*) which induced sterility in 50% pollen. This enables particular nuclear male sterility useful for hybrid seed production. Procedure is based on the use of cotyledon and hypocotyl explants with good timing and transfer to separate media for co-cultivation, callus induction, shoot induction, shoot outgrowth, selection of transformed shoots and root induction. Use of silver nitrate, delayed kanamycin selection and a BA + NAA + GA_3 shoot regeneration combination is also recommended. Overall transformation efficiency was 6.49% (61/939) according to kanamycin resistance. Molecular analysis done by PCR detected fragment corresponding to *nptII* in transformants and not in the control. Southern blot analysis of four putative transgene plants showed that three of them had a single T-DNA insertion while the fourth had two T-DNA copies inserted.

Lee *et al*. (2003) produced plants with male sterile flowers using a construct in which a cytotoxic diphtheria toxin A-chain gene (*DTx-A*) was driven by promoter of tapetum specific *BcA9* gene. Transformed plants were morphologically normal. *BcA9* gene was expressed only in anther tissue and it did not affect the vegetative growth.

SALT STRESS TOLERANCE

Bhattacharya *et al*. (2004) investigated production of cabbage plants with increased tolerance to salt stress by transfer of the *bet*A gene from *E. coli*. Gene is involved in oxidation of choline into glycinebetaine, substance which can act as an osmoprotectant increasing the salt stress tolerance of plants. Transformation was done using same protocols as in Bhattacharya *et al*. (2002) and chimeric pRC-cdh binary vector mobilized in *A. tumefaciens* strain GV2260. Twelve plantlets regenerated on selective medium rooted under kanamycin selection produced true transformants. The number of *bet*A gene insertion in transgenic lines varied from 1 to 4. Southern analysis revealed that most of the transformants contained more than one copy of the insert. Single gene insert was confirmed in two plants. Transformed plants exhibited increased tolerance to NaCl stress.

PROSPECTS FOR FUTURE RESEARCH

For most *B. oleracea* vegetable species transformation protocols can still be improved and their optimization remains a constant goal for future research. Even today transformation of broccoli and cauliflower seems to be a much easier task than the transformation of cabbage, which is still highly genotype specific (Jin *et al*. 2000; Pius and Achar 2000; Kuginuki and Tsukazaki 2001; Sretenović-Rajičić *et al*. 2004). Kuginuki and Tsukazaki (2001) observed significant varietal differences in cabbage. They obtained transgene plants from only four out of 13 studied cabbage lines and cultivars with the highest transformation frequency of 4.7% in cv. Matsunami P22 which is a DH line established from microspores of cv. Matsunami. It is also interesting to note the absence of transformation studies performed on kohlrabi (*B. oleracea* var. *gongyloides*).

The importance of genotype in the transformation event has been amply demonstrated by Cogan *et al*. (2002) and Sparrow *et al*. (2004b). Identification of QTLs (quantitative trait loci) connected with increased efficiency of transgene plant production will result in the creation of specialized genotypes. Some of them could be designed with the aim to introduce QTLs by conventional breeding techniques into genotypes recognized as recalcitrant for transgene manipulations.

Our knowledge of mechanisms underlying the transfer of T-DNA into plant genome on molecular level is still far from being satisfactory and needs to be improved. Extending our knowledge in this direction will help us to increase control over transformation techniques (Tzfira and Cytovski 2006). Useful explanation of the T-DNA transfer at the current stage of knowledge has been presented by Zupan *et al*. (2000).

It is to be expected that many more genes of non-plant origin like *gfp* will be used in future transgene research. Higher biomass producing *B. oleracea* species are also candidates to harbor genes for production of special metabolites. However they will always be less interesting than the oilseed *Brassica* species which are already in wide commercial use as transgene crops. Fast growing species like kohlrabi could be used for phytoremediation upon transfer of metal hyperaccumulating systems functional in related *Alyssum* species.

Avoiding genes for antibiotic resistance is a clear trend for all plants which are intended for commercial production. We can expect new less invasive and more friendly marker genes. However marker free selection is the ultimate goal. This means that transgene cultivars prepared for commercial exploitation will contain no marker genes, although they can be used through the construction procedure and then excised from plant genome at appropriate time. There is no point in creation of commercial transgene cultivars which would contain unnecessary portions of bacterial genome like antibiotic resistance genes and their promotors (Veluthambi *et al*. 2003). Apart from vectors which independently segregate after meiosis, marker-free transformation can be obtained by other means like transposons and recombination systems which remove marker and other genes after stable integration (Ebinuma *et al*. 1997; Lyznik *et al*. 2003).

In general plant transformation techniques can be also used to: increase biomass production oriented towards production of biofuels (Sticklen 2006) and bio-polymers, increase of drought tolerance and tolerance to abiotic stress (Vinocur and Altman 2005; Umezawa *et al*. 2006), introduce multiple genes creating dual resistance modes Castle *et al*. (2004, 2006), Cao *et al*. (2002), enable resistance to viruses Prins (2003) and other purposes.

Plastid transformation is a bright and promising approach in plant transformation (van Bel *et al*. 2001; Daniell *et al*. 2005) which was already used for transformation of some *Brassica* species (Zubko *et al*. 2001; Hou *et al*. 2003). Although there is currently only one report of plastid transformation in *B. oleracea* vegetable species (Nugent *et al*. 2006) we can soon expect more contributions employing this technique.

Finally it is possible to transform plants without elaborated techniques. *In planta* transformation performed by dipping intact flowers in the bacterial suspension has been known for some time. According to Cardoza and Stewart (2004) it was achieved in several *Brassica* species including *B. napus* and *B. rapa* ssp. *chinensis*. The success of this novel and unusual transformation method indicates that there are still possibilities for radical improvement of transformation methods intended for *Brassica* species.

CONCLUSION

A. tumefaciens and *A. rhizogenes* mediated transformation of *B. oleracea* vegetable species in the last decade entered a mature phase in which transferring genes of interest is done by routine techniques. In most of these techniques transformation efficiency is still low leaving ample space for further improvement. It is apparent that the available transformation techniques are most successful with broccoli and Brussels sprouts and least satisfactory with cabbage.

The use of antibiotic selectable markers is constantly decreasing in favor of other, less harmful markers. Among reporter genes GUS histological assay is loosing popularity and the non-invasive GFP is gaining. However marker-free selection is a general trend.

Most studies are dedicated to transfer of genes providing resistance to insects and herbicides. There is also an

increased number of studies aimed at modification of post-harvest senescence. It is reasonable to expect that some of these traits will appear in commercial cultivars when they fulfill tests and requirements proscribed for transgenic crops release.

Expansion of new techniques can be expected specially plastid transformation and to a less extent transformation *in planta*.

ACKNOWLEDGEMENTS

This work was sponsored by the Serbian Ministry of Science and Environmental Protection, Grant No. 143026B.

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