Development of a haploid transformation system and overexpression of Phytochrome B gene in *Brassica napus* L.

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Kolitha Bandara Wijesekara
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1. Name of referee: Prof. Dr. Heiko C. Becker
2. Name of referee: Prof. Dr. Petr Karlovsky

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Abbreviations

$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
$Ca(NO_3)_2$	Calcium nitrate
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediamine tetraacetic acid
GA3	Gibberellic acid
GUS	β-Glucuronidase
IBA	Indole-3- butyric acid
kb	Kilobases
LB-Medium	Luria-Bertani medium
MES	Morpholinoethane sulfonic acid
mRNA	Messenger Ribonucleic Acid
NAA	1-Naphthaleneacetic acid
NIRS	Near infrared spectroscopy
OD	Optical density
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
T-DNA	Transfer-DNA
Tris	Trishydroxymethylaminomethane

1. General introduction

Brassica is the most economically important genus in the family *Brassicaceae* (former *Cruciferae*). Cultivated forms of this genus are mainly oil crops and vegetable crops. Among the *Brassica* crops, oilseeds have the highest economic value. The oilseed Brassicas include *Brassica juncea*, *Brassica carinata*, *Brassica rapa* and *Brassica napus* which are collectively and commonly called as oilseed rape. Varieties with low aliphatic glucosinolates and erucic acid in seed oil are referred to as canola which is most often *B. napus*. However, canola-quality *B. rapa* and *B. juncea* varieties are also available (Cardoza and Stewart 2004b). Canola oil is widely used as cooking oil and in making margarine which is very low in saturated fatty acids, making it popular among health conscious consumers. This has made canola the third most important oil crop after soybean and palm oil, in global production (FAO 2006). Canola meal, which is a leftover product after extracting oil, is used as a protein supplement in animal feed. Vegetable Brassicas are a highly diverse group of crops grown worldwide which belong mainly to *Brassica oleracea*, as well as *B. rapa* and *B. napus*. This group includes plants such as broccoli, Brussels sprouts, cabbage, cauliflower, collards, kale, kohlrabi, rutabaga, and turnip (Cardoza and Stewart 2004b).

Because of the high economic importance, more research has been pursued in improving breeding programs with *Brassica* crops. With conventional breeding techniques it takes a minimum of 8-10 generations to develop a new variety with desired traits (Cardoza and Stewart 2004a). An alternative approach of speedy trait development without resorting to conventional breeding techniques would be the use of genetic engineering and biotechnology. Genetic engineering allows transfer of foreign genes with desired traits into plants, regardless of differences in species, genera or even kingdoms. Many genetic improvements to *Brassica* crops with the highest impact have come from genetic transformation. Genetic approaches of *Brassica napus* have mainly focused on improving oil quality (Verwoert et al. 1995, Knutzon et al. 1992) and making it herbicide-tolerant (de Block *et al*. 1989, de Block and de Brouwer 1991) or insect tolerant (Stewart et al. 1996). *Agrobacterium tumefaciens*-mediated transformation is the most widely used method of gene transfer into *Brassica* and it is generally quite efficient and practical for most species in the genus. Frequently, *Agrobacterium*-mediated transformation is carried out using seedling raised hypocotyls (de Block et al. 1989), cotyledons and cotyledonary petioles

(Moloney et al. 1989) owing to the high regeneration ability of these explants. Due to the diploid nature of seedling-raised plant materials, first generation transgenic plants would be hemizygous for the transgene. Consequently, much time and labour has to be invested to produce homozygous plants out of hemizygous plants. This study investigates the potential of using haploid leaf and petiole explants of *Brassica napus* plants propagated *in vitro* for an efficient *Agrobacterium*-mediated gene transfer. Use of haploid materials assures homozygous transgenic plants in one generation after colchicines treatment and also gives greater uniformity among transgenic plants due to the use of cloned plant materials.

Many current *B. napus* cultivars are prone to lodging causing high yield losses (Islam and Evans 1994). Lodging could be decreased by reducing plant height which is often achieved in *B. napus* by applying chemical growth regulators (Gans et al. 2000). This method is expensive and could also interfere with other endogenous plant hormones and metabolic processes (Rademacher 2000). Decrease in plant height has been observed with potatoes (*Solanum tuberosum*) (Thiele et al. 1999) and with tobacco (*Nicotiana tabacum*) (Halliday at al. 1997) after overexpression of *Arabidopsis thaliana* phytochromes B gene (*PHYB*). This study investigates the possibility of reducing plant height by overexpression of the *A. thaliana PHYB* gene in *B. napus* plants.

2. Transformation of haploid explants of *Brassica napus* L. as a rapid method to obtain homozygous transgenic plants

2.1 Introduction

Oilseed rape (*Brassica napus*) is considered to be the most important crop for the production of vegetable oils in Northern Europe, Canada and in China (Downey and Röbbelen 1989). Thus *Brassica napus* is an important target for crop improvements. Several protocols for genetic transformation of *B. napus* have been reported, such as microinjection (Neuhaus et al. 1987, Miki et al. 1989), direct DNA uptake (Guerche et al. 1987, Jardinaud et al. 1993), microprojectile bombardment (Stöger et al. 1995) as well as *Agrobacterium*-mediated DNA transfer (Fry et al. 1987, Pua *et al*. 1987, de Block et al. 1989, Moloney *et al*. 1989, Boulter et al. 1990, Damgaard et al. 1997, Schröder et al. 1994, Stefanov et al. 1994). Out of the above mentioned transformation methods *Agrobacterium tumefaciens*-mediated transformation has proven to be efficient in many *Brassica* species. Using genetic transformation, new traits such as modifications of the oil composition (Verwoert et al. 1995, Knutzon et al. 1992), herbicide tolerance (de Block et al. 1989, de Block and de Brouwer 1991) and altered protein composition (Kohno-Murase et al. 1995, Altenbach *et al*. 1992) have been successfully introduced into this crop.

A wide range of target explants of *B. napus*, including hypocotyl segments (Radke et al. 1988, de Block et al. 1989, Cardoza and Stewart 2003, Khan et al. 2003), cotyledonary petioles (Moloney et al. 1989), thin cell layers (Charest et al. 1988), stem segments (Fry et al. 1987), peduncle segments (Eapen and George 1997) and isolated protoplasts from hypocotyls and mesophyll cells (Thomzik and Hain 1990, Wang et al. 2005) have been transformed using *Agrobacterium tumefaciens* with varying successes in different laboratories. Often these target explants and cells have been obtained from *in vitro* grown seedlings. Owing to the diploid character of these plant materials, first generation transgenic plants (T1) will necessarily be hemizygous for the transformed gene. From the hemizygous loci, the transformed gene would segregate in accordance with Mendelian inheritance in the successive generations. Frequently, transformation with *Agrobacterium tumefaciens* results in insertion of multiple copies of the transgene. When multiple unlinked transgenic copies are present in hemizygous plants, much time and labour has to be invested to produce homozygous plants by repeated selfing or by applying the doubled haploid technology. Conversely, molecular characterization of the transgene copy number

and determination of homo-/ hemizygous state is laborious and not always giving conclusive results.

The use of haploid cells and tissue explants could provide an alternative approach for efficient transformation of oilseed rape. This seems tempting because haploid transgenic plants will become in one step homozygous diploid after colchicine treatment. The transformation of haploid microspores has been reported using the particle gun (Fukuoka et al. 1998) and *Agrobacterium* (Pechan 1989). However, the microspore culture system is very sensitive and the results have been shown to be difficult to reproduce. An alternative to this sensitive system is the use of explants from haploid plants obtained from microspore culture, for *Agrobacterium* transformation. This study investigates the potential of using haploid leaf and petiole explants of *Brassica napus* plants propagated *in vitro* for an efficient *Agrobacterium*-mediated gene transfer.

2.2 Materials and Methods

2.2.1 *in vitro* production and propagation of haploid plants

French spring rapeseed cv. Drakkar plants were grown in a controlled environmental chamber at 17±2 ˚C and a photoperiod of 16/8 hrs day/night. Unopened flower buds of 2-3 mm in length (which corresponds to late uninucleate stage of pollen development), were obtained from the main raceme of these plants. Microspore isolation was carried out according to the method described by Fletcher et al. (1998). Isolated microspores were cultured in liquid NLN medium containing vitamins, macro, and micro elements (Duchefa, Netherlands) and supplemented with 500 mg/l Ca $(NO_3)_2$ and 13% sucrose. The pH was adjusted to 5.8 and the medium was filter-sterilized. Immediately after microspore isolation, microspores were given a heat shock by placing culture plates in an oven at 32 ˚C for 48 hrs in darkness. Thereafter, culture plates were transferred onto a slow rotating shaker (40 rpm) and provided with a photoperiod of 16/8 hrs day/night at 25 ˚C. Well-grown cotyledonary embryos (after 25-28 days from microspore isolation) were transferred onto solid MS medium, where regeneration of embryos into plantlets and root formation was achieved in additional 30 days' time.

Plantlets obtained were analysed for their ploidy level (see below) and afterwards selected haploid plantlets were clonally propagated *in vitro* by transferring the top shoots or stem segments of 1-2 cm in length bearing an auxiliary bud, into agar-solidified hormone-free Murashige and Skoog (1962) basal medium (MS) supplemented with 2% sucrose. Shoots were grown at 25 ˚C with a day length of 16 h. A continuous supply of plantlets was obtained by regular sub-culturing of excised shoot/stem segments on fresh MS medium in every three to four weeks intervals. Leaves and petiole segments obtained from these plants were used as explants in transformation experiments.

2.2.2 Analysis of ploidy level of microspore-derived plants

Fresh young leaf samples (about 1 cm^2) from microspore-derived plantlets were chopped separately using a sharp razorblade in 200 µl of staining solution that contained 1 mg/l of DAPI (4,6-diamino-2-phenyl-indole). Then the re-suspended solution was passed through a 50 µm nylon filter (Nybolt) and diluted with additional 800 µl of staining solution. After that the sample was inserted into the Partec Cell Analyser PAS-II (Partec GmbH, Munster) equipped with a mercury lamp. Data was plotted on a semi-logarithmic scale where the histogram peaks are distributed along the abscissa. Leaf samples taken from seedling-raised *B. napus* plants were used as the diploid standard.

2.2.3 Bacterial strain and plasmid vectors

The binary plasmid system of *Agrobacterium tumefaciens* strains ATHV Rif^R and AGL0 (see Table 2.1) containing binary vectors pPNGUS (provided by Dr. Michael Wallbraun, Centrum Grüne Gentechnik, DLR Rheinpfalz, 67435 Neustadt/Wstr.) and pAK-UGI 9-1 (provided by Dr. Christian Möllers) respectively, were used for transformation.

Table 2.1. *Agrobacterium* strains used in transformation

Agrobacterium strain	Chromosomal		Helper*/Vir*-plasmid	Reference
	background	marker gene*		
ATHV Rif^R	C58	rif	pEHA101	Hellens et al. 2000
AGL0	C58	rif	pTiBo542ΔT-DNA	Lazo et al. 1991

* Antibiotic resistance gene used to select for that strain of *Agrobacterium* or Ti plasmid
Abbreviations: rif, rifampicin resistant

T-DNA region of binary vectors contained chimeric gene construct of *NPTII* gene coding for neomycin phosphotransferase (which allows for the selection of plants resistant to kanamycin) and β-glucuronidase (GUS-Intron) as reporter gene. GUS-Intron reporter gene does not express GUS activity in *A. tumefaciens* cells. Plasmid, transgenes, promoters, terminators, and selection antibiotics in bacteria and in plant were as listed in Table 2.2.

Table 2.2. Binary plasmid, transgenes and selection antibiotics in bacteria and in plants

Binary plasmid	Transgenes (Promoter-Gene-Terminator)	Antibiotic selection	
		in bacteria	in plants
pPNGUS	Pnos-*NPTII*-Tnos PCaMV35S-*GUSi*-T35S	Streptomycin Spectinomycin	Kanamycin
pAK-UGI 9-1	PCaMV35S-*NPTII*-T35S PUbi-*GUSi*-Tnos	Streptomycin Spectinomycin	Kanamycin

Abbreviations: PCaMV35S, cauliflower mosaic virus 35S gene promoter sequence; T35S, terminator sequence of 35S gene; PUbi, sunflower ubiquitin promoter sequence; Pnos, nopaline synthase gene promoter sequence; Tnos, nopaline synthase gene terminator sequence; *GUSi*, β-glucuronidase gene sequence with an intron (Vancanneyt et al. 1990); *NPTII*, neomycin phosphotransferase gene sequence.

2.2.4 Preparation of basic culture media, stock solutions of phytohormones, antibiotics and other reagents

Stock solutions of heat-labile phytohormones, antibiotics and other reagents were prepared beforehand, filter-sterilized and stored in 1 ml aliquots in Eppendorf tubes at -20 ºC (see Table 2.3 and 2.4). Basic culture media such as, MS (Murashige and Skoog 1962) and DKW medium (Driver and Kuniyuki 1984) were prepared in advance and stored in 500 ml blue capped bottles (Scott) until use. Solid media were heated up in a microwave oven to melt. After cooling the medium to about 40-45 ºC, heat labile compounds were added one after the other at desired concentrations with swirling in between, before pouring the medium into sterile petri dishes.

Table 2.3. Stock solutions of antibiotics used for selection in bacteria and in plants

Antibiotic	Chem. Co.	Stock solution	Final concentration
Streptomycin	Duchefa	100 mg/ml (in H_2O)	300 mg /l
Spectinomycin	Duchefa	100 mg/ml (in H_2O)	100 mg/l
Carbenicillin	Duchefa	50 mg/ml (in H_2O)	500 mg /l
Kanamycin	Duchefa	500 mg/ml (in H_2O)	35 or 50 mg /l
Rifampicin	Duchefa	100 mg/ml (in DMSO)	50 mg/l

Table 2.4. Stock solutions of phytormones and reagents used in callus induction (CIM) and regeneration medium (DKW)

Chemical	Chem. Co.	Stock solution	Final conc.
$AgNO_3$	Riedel	5 mg/ml (in H_2O)	5 mg/l
Acetosyringone	Roth	10mM (in DMSO; 19.6 mg in 10 ml)	100µM/l
BAP	Duchefa	2 mg/ml (solvent, 1M NaOH, diluent H_2O)	2 mg/l
GA_3	Sigma	1 mg/ml (solvent EtOH, diluent H_2O)	0.1 mg/l
IBA	Duchefa	1 mg/ml (1M NaOH, diluent H_2O)	0.1 mg/l
NAA	Duchefa	1 mg/ml (1M KOH, diluent H_2O)	0.1 mg/l
Picloram	Duchefa	1 mg/ml (in H_2O)	0.01 mg/l

Table 2.5. Composition of culture media

Medium	Application	Composition for 1 L of medium
YEB	Growth of *A. tumefaciens*	1 g/l Yeast extract, 5 g/l Meat extract, 5 g/l Peptone (Casein), 0.5 g/l MgSO4 · 7 H2O, 5 g/l Sucrose, pH 7.4
CIM (standard)	Liquid: Infection, co-cultivation, washing Solid: Callus induction	5.3 g/l MS media-mix powder (Duchefa, Netherlands), 300 mg/l Myo-Inositol, 500 mg/l MES, 2 mg/l BAP, 0.001 mg/l Picloram, 5 mg/l AgNO3, 20 g/l sucrose, pH 5.7. Solid medium: 5.4 g/l agarose
CIM-L (for leaf explants)	Liquid: Pre-culture of leaf explants, infection, co-cultivation, washing Solid: Callus induction with leaf explants	5.3 g/l MS media-mix powder (Duchefa, Netherlands), 300 mg/l Myo-Inositol, 500 mg/l MES, 2 mg/l BAP, 0.1 mg/l NAA, 0.1 mg/l GA_3, 5 mg/l AgNO3, 20 g/l sucrose, pH 5.7 Solid medium: 5.4 g/l agarose
CIM-P (for petiole explants)	Liquid: Pre-culture of petiole explants, infection, co-cultivation, washing Solid: Callus induction with petiole explants	5.3 g/l MS media-mix powder (Duchefa, Netherlands), 300 mg/l Myo-Inositol, 500 mg/l MES, 2 mg/l BAP, 0.01 mg/l Picloram , 5 mg/l AgNO3, 20 g/l sucrose, pH 5.7 Solid medium: 5.4 g/l agarose
DKW (standard)	Shoot regeneration (organogenesis)	5.3 g/l DKW medium-mix powder (Duchefa, Netherlands), 20 g/l Sucrose, 1 mg/l BAP, 0.01 mg/l IBA, 0.01 mg/l GA3, 5 mg/l AgNO3, 5.4 g/l agarose, pH 5.7
DKW (modified)	Shoot regeneration (organogenesis)	5.3 g/l DKW medium-mix powder (Duchefa, Netherlands), 20 g/l Sucrose, 1 mg/l BAP, 0.1 mg/l IBA, 0.1 mg/l GA3, 5 mg/l AgNO3, 5.4 g/l agarose, pH 5.7
MS (basal)	Shoot elongation and rooting	5.3 g/l MS media-mix powder (Duchefa, Netherlands), 20 g/l sucrose, 5 g/l agar, pH 5.8

2.2.5 Optimization of callus production and shoot regeneration from leaf and petiole explants

In early experiments, transformation and plant regeneration conditions were carried out according to a standard protocol for hypocotyl segments developed by de Block et al. (1989) and modified by Hüsken et al. (2005). Almost no transformed callus production was obtained with leaf explants using this protocol while poor callus production was achieved with petiole explants. Therefore experiments to optimize callus regeneration were carried out with varying hormone combinations and concentrations.

Picloram concentration of callus induction medium (CIM, see Table 2.5 for composition) in the standard protocol was increased five, ten and twenty times, while keeping the other components of the medium unchanged. Leaves and petioles were obtained from 3-4 weeks old *in vitro* grown plants and were cut into squares of 0.5 cm^2 (leaves) and segments of 0.5-0.7 mm in length (petioles) using a sharp scalpel. Twenty explants of leaf /or petiole were cultured in a single petri dish (which was considered as a replicate) and every treatment was replicated four times within a single experiment. Culture plates were sealed with micropore tapes and incubated in a growth chamber under constant light at 22 ºC. Callus production was determined by assigning a score (from 1 to 5) to each petri dish, after four weeks from culture initiation. Yet, no reasonable increase in callus production was obtained with leaf explants when cultured on CIM medium with increased picloram concentration. Therefore, three different hormone combinations were tried with leaf explants. Experimental design and determination of callus production after four weeks, was as described in the above experiment. Shoot regeneration from green callus was achieved in DKW medium in the standard protocol; however the obtained shoot regeneration frequencies were low. Therefore concentration of phytohormones IBA and GA_3 was increased by five, ten, and twenty times than the concentrations used in the standard protocol. Shoot regeneration efficiency was determined for each treatment combination, after six weeks of culture in the regeneration medium.

2.2.6 Infection and cocultivation of leaf and petiole explants

A single *Agrobacterium* colony formed on freshly streaked selective agar plate (with selective antibiotics) was inoculated into 4 ml of YEB (Yeast Extract Broth) medium (Grimley et al. 1986) with 300 mg/l streptomycin, 100 mg/l spectinomycin and 100 µM

acetosyringone, and grown overnight at 27 ºC on a shaker rotating at 200 rpm in the dark. An aliquot of 100 µl from this solution was used to inoculate 40 ml of YEB medium supplemented with 300 mg/l of streptomycin, 100 mg/l spectinomycin and 100 µM acetosyringone, in a 100 ml Erlenmeyer flask and cultured as above over night. Bacterial solution was then centrifuged in 50 ml sterile plastic centrifuge tubes (Corning) at 6000 rpm for 10 min and the pellet was re-suspended in 3 ml of liquid CIM. Before cocultivation, OD_{600} was measured and adjusted to a final optical density of 0.8-1.0 with approximately 1 x 10^9 bacterial cells/ ml in liquid CIM supplemented with 100 µM acetosyringone. This preparation was used for the inoculation of explants. Excised leaf and petiole segment (squares of 0.5 cm^2 and segments of 5-10 mm) were inoculated separately with *Agrobacterium* suspension in a sterile Petri dish, for 40 min at room temperature with gentle shaking. The segments were subsequently blotted and were co-cultivated on sterile filter papers placed over liquid CIM medium, in a sterile plastic petri dish (9 cm). The number of segments cultured in a single petri dish was 25. Petri dishes were sealed with micropore tape (3M) and incubated for 2 days in a growth chamber under constant light at 22 ºC.

2.2.7 Selection of transformed calli and plants

After two days coculture with *Agrobacterium* and prior to transfer to the selection medium, leaf and petiole segments were washed three times with liquid CIM medium and the final washing was with liquid CIM medium containing 500 mg/l carbenicillin for 30 min. Segments were briefly dried on sterile filter papers and transferred individually to solid CIM-L/ or CIM-P medium supplemented with 500 mg/l carbenicillin and 35 mg/l or 50 mg/l kanamycin. Culture plates were sealed with micropore tape and incubated in a growth chamber at 22º C under continuous light. After 4 weeks on the selection medium, regenerated green calli formed on the explants were separated and transferred individually onto agarose solidified DKW (modified) medium (see Table 2.5 for the composition) supplemented with 500 mg/l carbenicillin, 35 mg/l or 50 mg/l kanamycin and incubated in a growth chamber at 22º C under continuous light. After 6 additional weeks, elongated green shoots (putative transformants) longer than 1 cm were separated from calli and transferred to Magenta boxes containing solid MS medium supplemented with 500 mg/l carbenicillin and 35/or 50 mg/l kanamycin. Three to four shoots were cultured per jar and incubated in a growth chamber at 22º C under continuous light to allow shoot elongation and rooting. Culture plates were scored after 4 weeks from coculture to determine the

percentage of explants with green calli regeneration. Green calli and shoots were further screened for the activity of GUS gene using histochemical GUS assay (see below).

2.2.8 Infiltration of leaf and petiole explants

As one pre-treatment, vacuum infiltration of leaf/petiole explants was performed. Excised leaf and petiole explants were collected separately in a 50 ml centrifuge tubes (Corning) containing liquid CIM-medium with *Agrobacterium*. Tubes were placed in a vacuum chamber and the vacuum was held for 1-2 min with a pressure ranging from 25 to 27 mm Hg. Next, the vacuum was slowly released and the tubes were placed on a slow rotating shaker at 80 rpm for 40 min. The subsequent tissue culture procedure was the same as for those non infiltrated explants.

2.2.9 Preculture of leaf and petiole explants

It was observed that during two days co-cultivation period, a considerable percentage of leaf explants (approximately 25-30 %) displayed tissue browning and sign of wilting and necrosis, due to stress exerted by *Agrobacterium*. Therefore, intact leaves and petioles were subjected to two days preculture period on 8.5 cm sterile filter papers (MN 440, Macherey-Nagel), soaked with liquid CIM-L/or CIM-P medium (see Table 2.5 for media composition) supplemented with 100 µM acetosyringone as a means of hardening the fragile tissue explants, prior to sectioning and subsequent inoculation with *Agrobacterium*. In control experiments, explants were inoculated directly with *Agrobacterium* without a pre-culture period on 8.5 cm sterile filter papers, soaked with liquid CIM-L/or CIM-P medium. In a similar experiment, whole leaves and petioles were subjected to a two days preculture period on agarose solidified CIM-L/or CIM-P medium before sectioning and cocultivation with *Agrobacterium* to determine the effects of liquid and solid medium on preculture of tissue explants.

2.2.10 Histochemical GUS assay

Histochemical GUS assay was carried out as described by Jefferson (1987). Tissue samples taken from putative transformed calli and plantlets were dipped in GUS assay solution containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton-X-100, 0.1% sarcosyl, 10 mM 2-mercapto-ethanol and 1mM X-gluc (5-bromo-4-chloro-3-indolyl-

β-D-glucuronide cyclohexylammonium salt) substrate (X-gluc dissolved in dimethyl sulfoxide) and incubated at 37 ºC overnight. Chlorophyll in tissue samples was de-stained by rinsing them with 70% ethanol.

2.2.11 PCR analysis

Total DNA was extracted from young leaves of kanamycin resistant plantlets using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instruction manual. A sample of 25 ng template DNA was amplified with a thermal cycler-480 (Perkin Elmer, Norwalk, USA) (see below for the composition of PCR mixture and the program used for the PCR reaction) using specific primer combinations for *NPTII* and *GUS* genes (primer combinations used were as listed in Table 2.6). Amplified products were separated in a 1.5% (w/v) agarose gel in 1x TAE buffer at 120 V for two hours, stained with ethidium bromide, visualized under an ultra violet illuminator and recorded with a gel documenter.

Table 2.6. Primers and expected amplification length

Primer	**Sequence**	**Fragment length**
GUS-fw.	5`-CCCTTATGTTACGTCCTGTAGAAACCC -3`	1000 bp
GUS-rev.	5`- CCAATCCAGTCCATTTAATGCGTGGTCG -3`	
NPT-II-fw.	5`-ATCGGGAGCGGCGATACCGTA-3`	700 bp
NPT-II-rev.	5`-GAGGCT ATT CGG CTATGACTG-3`	

fw. Forward primer, rev. Reverse primer

PCR reaction mixture

Component	Volume	Concentration
Template-DNA	1 µl	(25 ng/ µl)
10x PCR-Puffer	2.5 µl	-
MgCl2	1.5 µl	25 mM
Forward-Primer	1.0 µl	10 µM
Reverse-Primer	1.0 µl	10 µM
dNTPs	0.5 µl	1.25 mM
Taq-Polymerase	0.5 µl	1.25 U/µl
H_2O (HPL)	Adjusted	
Total volume	25 µl	

The program for the PCR reaction

1x Denaturing	3 min at 95 °C
35x Denaturing	1 min at 95 °C
Primer annealing temperature	
for *NPTII* primers	1 min at 54 °C
for *GUS* primers	1 min at 60 °C
Extension	2 min at 72 °C

2.2.12 Production of double haploid transgenic plants

To produce double haploid plants, top shoot and stem segments with an auxiliary shoot bud, obtained from haploid transformed plants were treated *in vitro* with colchicine. Top shoots (with primordial leaves only) and stem segments (~1 cm in length) were dipped in MS liquid medium supplemented with 500 mg/l (1252 µM) colchicines in a 100 ml sterile flask and cultured for 24 hrs on a shaker revolving at 200 rpm. After treatment time, segments were washed three times thoroughly with liquid MS medium, briefly dried on a sterile filter paper and cultured on agar solidified MS medium for further growth and rooting. Well grown plantlets with active root growth were transferred into soil (top earth 3: 1compost) in 6 cm x 6 cm black plastic pots and placed in a controlled climate chamber for 4-5 days at 17 ºC with a photoperiod of 16/8 day/night. After that, plants were transferred into the green house and kept under humid conditions for another week. Finally the plants were planted in 11 (length) cm x 9 (width) cm black plastic pots with same soil mixture and grown to maturity.

2.2.13 Experimental design and statistical analysis

Experiments were repeated four times, unless otherwise stated. Each experiment consisted of at least four petri dishes with each 25 leaf/or petiole explants per explant type, treatment and application. Analysis of variance was based on means of those petri dishes and was performed by the PLABSTAT software (Utz 2001) using the following model:

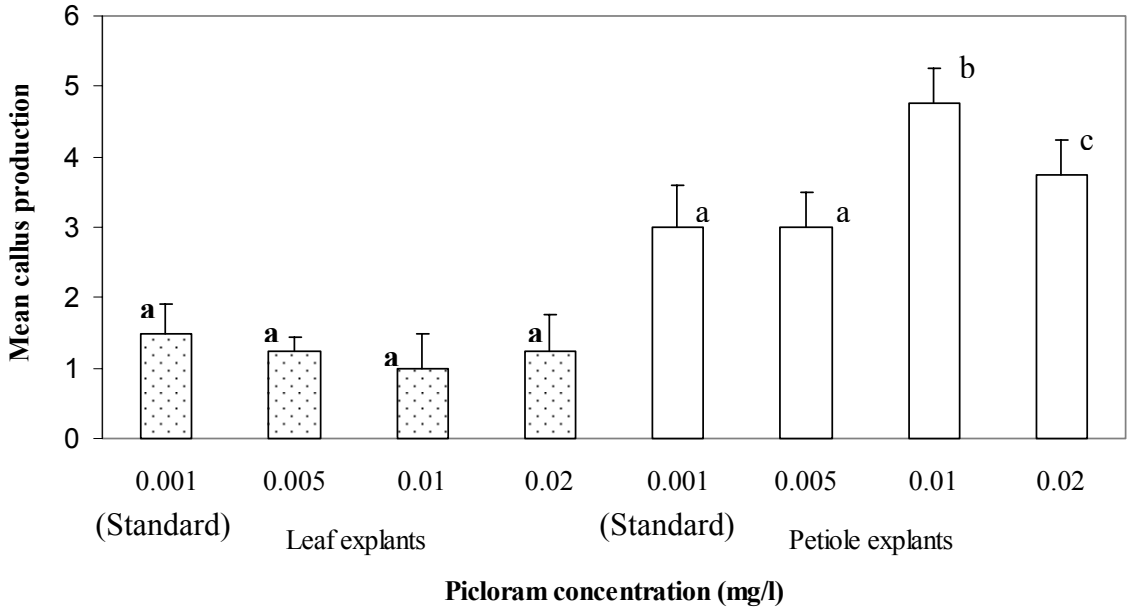
$$Y_{ijkl} = \mu + e_i + t_j + s_k + r_l + et_{ij} + es_{ik} + ts_{jk} + er_{il} + tr_{jl} + sr_{kl} + ets_{ijk} + \varepsilon_{ijkl}$$

where: Y_{ijkl} was observation of explant i at treatment j at application k in experiment l; μ was the general mean; e_i, t_j, s_k and r_l were the effects of explant i, treatment j, application k and experiment l, respectively, along with their respective interactions. ε_{ijkl} was the residual error. The explant type, treatments, and applications were considered as fixed variables. Some experiments had only a treatment and no application. For those experiments the above mentioned model was adapted accordingly. For multiple mean comparisons Duncan's multiple range test at P=0.05 was applied using the Stat-Graphics Plus for Windows 3.0 (Statistical Graphics Corp. 1997).

2.3 Results

2.3.1 Experiments to optimize callus production and shoot regeneration from leaf and petiole explants

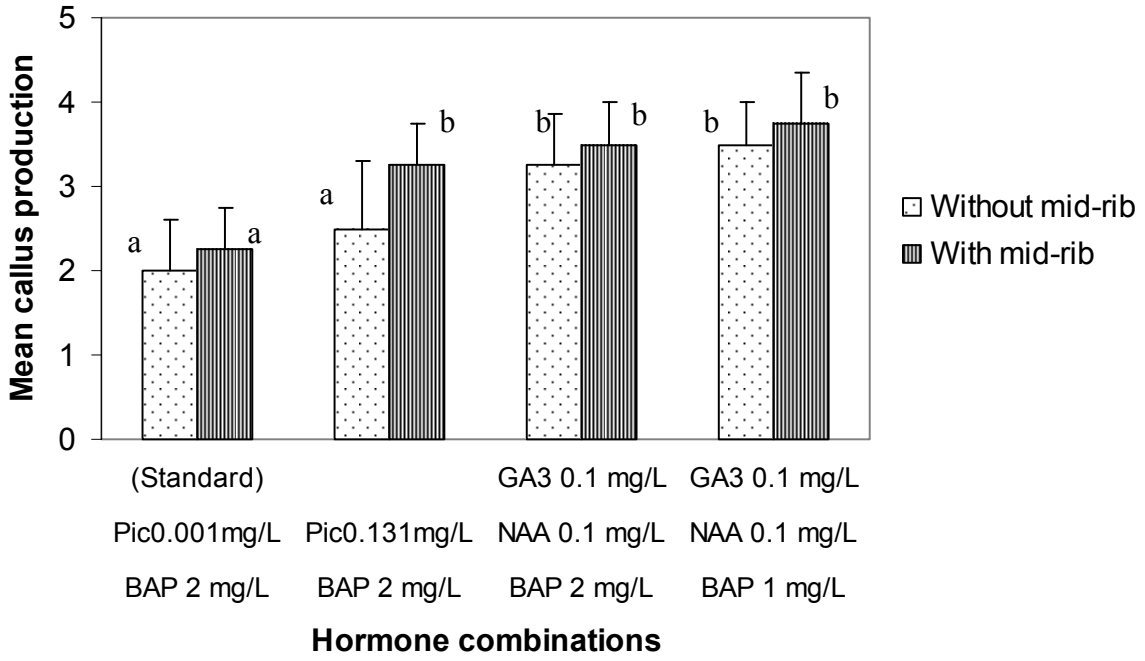
Early experiments conducted with the transformation protocol described by Hüsken et al. (2005) for diploid hypocotyls segments, resulted in poor callus production with petiole explants, but failed to produce any calli with leaf explants (data not shown). Therefore experiments to optimize callus induction and regeneration were carried out with varying hormone combinations and concentrations. Increase in picloram concentration by ten times than the concentration used in the standard protocol (while keeping the other components unchanged), has resulted in optimal callus production with petiole explants (Fig. 2.1). Based on these results, a modified callus induction medium for petiole explants was defined, termed as CIM-P (see Table 2.5 for medium composition) and was used in all subsequent experiments conducted with petiole explants.



1= No callus production, 2= Very poor callus production, 3=Poor callus production, 4=Good callus production, 5= Very good callus production

Fig 2.1. Effect of picloram concentration on callus production with leaf and petiole explants of haploid *B. napus*. Columns denoted by the same letter are not significantly different at P= 0.05 according to Duncan's multiple range test. Results represent means from one experiment with four replications (Petri dishes) with each a sample size of 20 explants. Multiple comparisons of leaf (bold letters) and petiole explants are independent of each other. Vertical bars represent the standard error.

Yet, no or very poor callus production was achieved with leaf explants at all the concentrations of picloram tested (Fig. 2.1). Callus production with leaf explants was significantly increased when BAP concentration was reduced from 2 mg/l to 1 mg/l and picloram was replaced by 0.1 mg/l NAA and GA_3 (Fig 2.2). This hormone combination was chosen for subsequent experiments with leaf explants and termed CIM-L (see Table 2.5 for medium composition). Leaf explants with a part of the mid rib had a positive effect on callus production (Fig 2.2).



1= No callus production, 2= Very poor callus production, 3=Poor callus production, 4=Good callus production, 5= Very good callus production

Fig 2.2. Effect of different combinations of phytohormones on callus production with leaf explants. Columns denoted by the same letter are not significantly different at P= 0.05 according to Duncan's multiple range test. Results represent means from one experiment with four replications (Petri dishes) with each a sample size of 20 explants. Vertical bars represent the standard error

Shoot regeneration medium (DKW) of the standard protocol resulted in poor shoot regeneration from green calli of leaf and petiole explants. To increase shoot regeneration frequency from green calli, concentrations of IBA and GA_3 were increased by five, ten and twenty times than the concentrations used in the standard protocol, while keeping the other components unchanged. Increased phytohormone concentration and origin of callus had a significant effect on shoot regeneration. Calli derived from leaf explants produced a significantly higher number of shoots per callus cultured than those calli with petiole origin (Table 2.7 and Fig. 2.3). Ten times increase in both IBA and GA_3 resulted in significantly higher shoot regeneration from green calli obtained from both leaf and petiole explants (Fig. 2.3). Based on these results, a modified shoot regeneration medium was defined and termed as DKW (modified) and used for all subsequent experiments (see Table 2.5 for medium composition).

Table 2.7. Analysis of variance results for the effect of a phytohormone treatment on shoot regeneration from 8-10 weeks old callus cultures derived from leaf and petiole explants of haploid *B. napus*. (No. of shoots regenerated per cultured callus)

Source	DF	MS	Var. cp	F	LSD5
Explant type	1	1.210	0.0366	30.66**	0.10
Hormone concentration	3	1.574	0.0959	39.89**	0.15
Experiment	7	0.210	0.0214	5.34**	0.30
Explant type x Hormone con.	3	0.023	-0.002	0.6	0.21
Residual error	21	0.039	0.039		

** significant at p=0.01, * significant at p=0.05

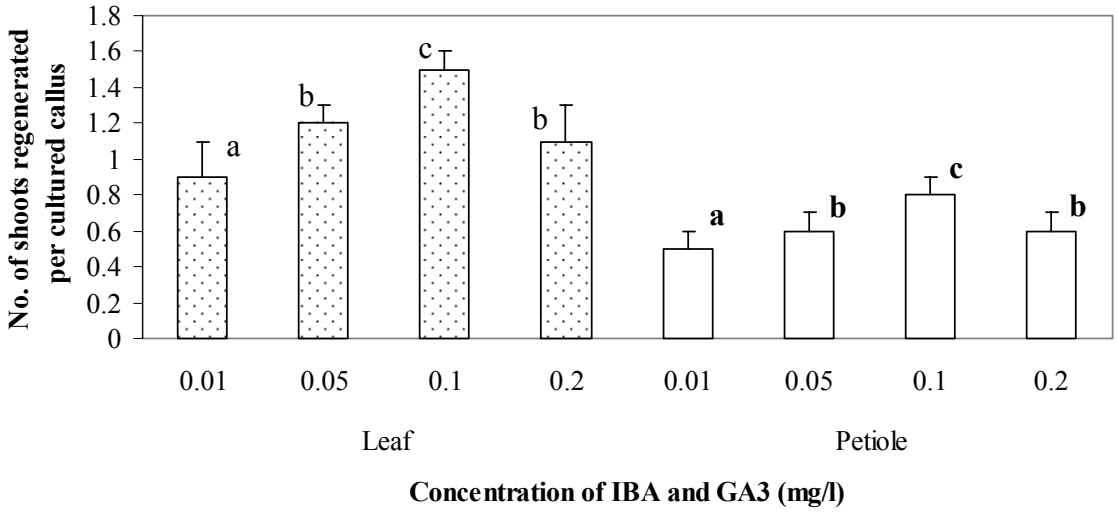


Fig 2.3. Effect of phytohormones on shoot regeneration in 8-10 weeks old callus cultures derived from leaf and petiole explants of haploid *B. napus*. Columns denoted by the same letter are not significantly different at P= 0.05 according to Duncan's multiple range test. Multiple comparisons of leaf and petiole (bold letters) are independent of each other. Vertical bars represent the standard error.

Upon culture in DKW (modified) medium, 53.3% of calli derived from leaf explants produced shoots after six weeks of culture while 40.9% of calli derived from petiole explants produced shoots after the same time period in culture (Table 2.8). When transferred into the solid MS medium, 92.7% of shoots of leaf origin and 90.5% of shoots of petiole origin rooted and became plantlets. 96% of plantlets of leaf origin and 92% of plantlets of petiole origin were found to be haploid after analysis of ploidy of 25 randomly selected plantlets (Table 2.8).

Table 2.8. Shoot regeneration from calli derived from leaf and petiole explants on DKW (modified) medium with 0.1 mg/l IBA and 0.1 mg/l GA3 hormone combination

Callus origin	No. of calli cultured	Shoot initiating calli	Regeneration efficiency (%)	No. of shoots regenerated	Number of plantlets obtained	% of haploid plantlets
Leaf	45	24	53.3	67	55	96.0
Petiole	88	36	40.9	72	53	92.0

2.3.2 Optimization of transformation efficiency

Vacuum infiltration of explants

Histochemical GUS assay was performed after one week from infitration with 50 randomly selected leaf and petiole explants (infiltated and noninfiltated). Vacuum infiltration of leaf and petiole explants (prior to two days cocultivation) with a *Agrobacterium tumefaciens* cell suspension (strain AGL0 pAK-UGI 9-1) has resulted in an increased number of explants with blue transformed areas (GUS^+ explants) as well as the number of blue transformed spots (or GUS^+ spots) per explant (Table 2.9). Vacuum infiltration of leaf explants resulted in 22% increase in GUS^+ explants when compared with the non infiltrated leaf explants while petiole explants showed an increase of 12% in GUS^+ explants after vacuum infiltration (Table 2.9). Infiltration has increased the number of GUS^+ spots in both explant types in general, however leaf explants showed the highest number of GUS^+ spots.

The number of GUS^+ spots exceeded over 20 for some infiltrated leaf explants (see Fig 2.6d at page 27).

However, infiltration with *Agrobacterium* suspension did not increase the percentage of explants with putatively transformed green callus (Table 2.10 and Fig. 2.4).

Table 2.9. Results of histochemical GUS assay of infiltrated and noninfiltrated leaf and petiole explants

Explant type	No. of explants examined	Infiltration	Percentage of GUS^+ explants[§]	Mean number of GUS^+ spots per explant[§]
Leaf	50	with	54	13.8 a
	50	without	32	7.0 b
Petiole	50	with	34	7.2 b
	50	without	22	5.7 c

[§]Histochemical GUS assay was performed one week after infiltration with *Agrobacterium*. Means with the same letters are not significantly different at P= 0.05 according to Duncan's multiple range test.

Table 2.10. Analysis of variance results for the effect of *Agrobacterium* infiltration of explants on the percentage green callus production of leaf and petiole explants with and without kanamycin selection, as determined after four weeks of culture in callus induction medium.

Source	DF	MS	Var. cp	F	LSD5
Explant type	1	4027.66	124.67	105.54**	3.18
Infiltration	1	0.353	-1.181	0.01	3.18
Kanamycin	1	33659.00	1050.82	882.03**	3.18
Experiment	7	353.74	39.44	9.23*	6.36
Explant x Infiltration	1	220.69	11.40	5.78*	9.00
Explant x Kanamycin	1	4676.65	289.90	122.55**	4.50
Infiltration x Kanamycin	1	48.38	0.638	1.27	4.50
Residual error	25	38.16	38.16		

** significant at p=0.01, * significant at p=0.05

Interaction effects explant type x infiltration were small and not significant. In other words, infiltrated and non-infiltrated explants were found to be equally competent in their ability to produce green calli in the presence of kanamycin. The results also show that the pressure exerted on tissue explants (25 to 27 mm Hg) during vacuum infiltration was not destructive to the tender tissue explants. Significant effects were also found for the explant type and for the interaction effects Explant type x Kanamycin treatment.

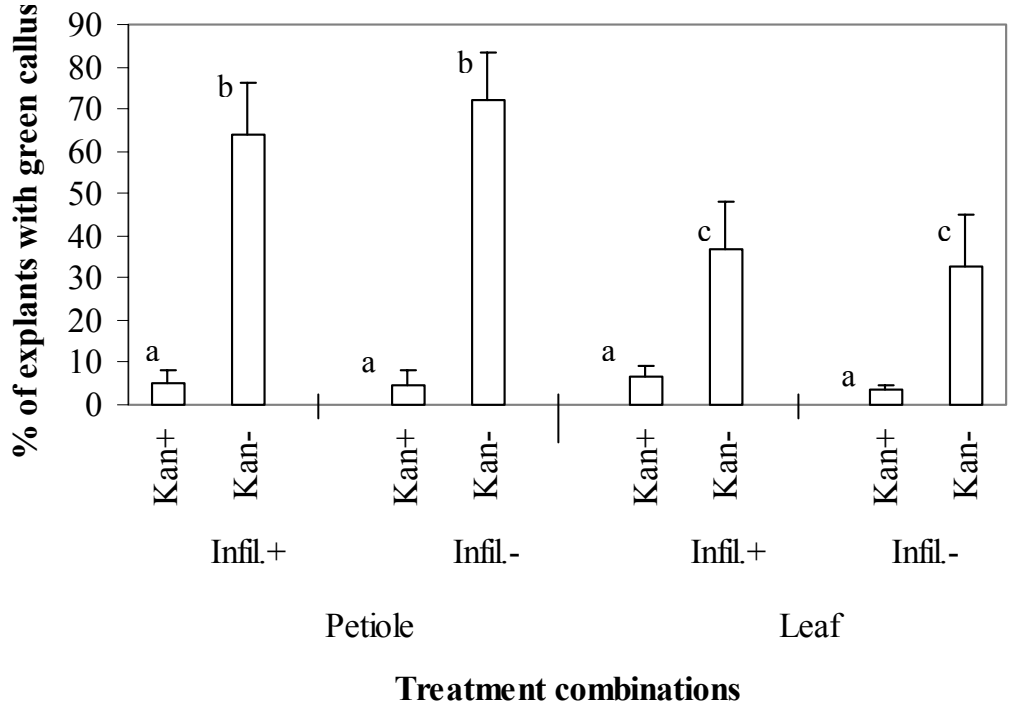


Fig 2.4. Effect of infiltration of explants with a bacterial cell suspension (AGL0 pAK-UGI 9-1) on percentage green callus production of leaf and petiole explants with (Kan+) and without (Kan-) 35 mg/l kanamycin selection, after four weeks of culture in callus induction medium. Columns denoted by the same letter are not significantly different at P= 0.05 according to Duncan's multiple range test. Vertical bars represent the standard error

2.3.3 Effect of filter paper and preculture of explants

It has been obverved in early transformation experiments (conducted with the standard protocol) that, as many as 30% of leaf explants died after the cocultivation period of two days, due to tissue browning and development of necrotic areas on cultured explants. Similarly, about 10% of petiole explants also died during the same culture period due to tissue browning and necrosis (data not shown). Therefore, a two days preculture treatment

was given to intact leaves and petioles before sectioning into segments and subsequent coculture, as a means of hardening the tender tissue explants against stress exerted by *Agrobacterium*. Preculture was performed either on filter papers soaked with liquid CIM-L/or CIM-P, or on solid medium. Explant type, preculture and type of medium (liquid or solid) had significant effect on tissue browning and necrosis as determined after two days from coculture (Table 2.11). Interaction effects Preculture x Explant type were comparatively large.

Table 2.11. Analysis of variance results for the effect of a two days preculture treatment on tissue browning and necrosis of leaf and petiole explants during cocultivation with *Agrobacterium* in liquid or solid medium and on percentage of explants with green callus, after four weeks from coculture (50 mg/l kanamycin selection)

Source	Tissue browning/or necrosis (after two days from coculture)			% of explants with green callus (after four weeks from coculture)		
	DF	Var. cp	F	DF	Var. cp	F
Explant type	1	46.47	254.66**	1	7.92	247.19**
Medium (liquid/solid)	1	6.07	28.03**	1	0.16	5.83*
Preculture	1	35.05	156.95**	1	0.09	3.69+
Experiments	3	0.20	0.40	3	0.10	2.61+
Preculture x Med.	1	2.65	7.21*	1	1.66	26.78**
Preculture x Explant	1	21.17	48.09**	1	1.11	18.39**
Explant x Med.	1	11.80	27.25+	1	1.35	21.92**
Residual error	12	3.59		12	0.54	

** significant at p=0.01, * significant at p=0.05

Preculture of explants in liquid or in solid medium before coculture with *Agrobacterium* significantly reduced the tissue necrosis and explant death in both explant types. Preculture on filter papers soaked with liquid medium had the highest significant effect on both explant types to bring down the tissue necrosis when compared with preculture on solid medium (Table 2.12). Preculture on filter papers in liquid medium reduced necrosis of leaf explants by 65%, while the reduction of necrosis of petiole explants was 35%. Even without two days preculture period, leaf explants cocultivated with *Agrobacterium* on filter

papers soaked with liquid medium, significantly reduced the necrosis of cultured explants by 33%. However, this beneficial effect by liquid culture medium was not significant for petiole explants (Table 2.12). Preculture had an positive effect on green callus (or putative transformed callus) regeneration from leaf and petiole explants, cultured for four weeks in callus induction medium with 50 mg/l kanamycin selection. Based on these results, two days preculture and cocultivation of explants with *Agrobacterium* on filter papers soaked with liquid medium, was adapted for all the subsequent transformation experiments.

Table 2.12. Effect of two days preculture treatment on tissue browning/or tissue death during cocultivation with *Agrobacterium*, and on green callus regeneration with 50 mg/l kanamycin selection. Means denoted by the same letter are not significantly different at P=0.05 according to Duncan's multiple range test.

Explant	Preculture	Medium	Browning or tissue death after two days of cocultivation (%)	Explants with green callus after four weeks of culture under 50 mg/l kanamycin selection (%)
L	+	CIM-L (lq.)	11.0 a	5.4 a
L	+	CIM-L (s)	15.0 b	2.5 b
L	-	CIM-L (lq.)	21.0 c	5.0 a
L	-	CIM-L (s)	31.1 d	4.2 c
P	+	CIM-P (lq.)	7.2 e	9.5 d
P	+	CIM-P (s)	6.7 e	8.6 e
P	-	CIM-P (lq.)	11.0 a	5.4 a
P	-	CIM-P (s)	10.4 a	8.5 e

Abbreviations: lq: liquid medium, s: solid medium, L: leaf explants, P: petiole explants, (+) with two days preculture period, (-) without preculture

Effect of *Agrobacterium* strain/plasmid and acetosyringone

The *Agrobacterium* strain (ATHV Rif^R pPNGUS and AGL0pAK-UGI 9-1), the explant type and the addition of 100 µM of acetosyringone in the culture medium (CIM-L /or CIM-P) during the two days preculture and two days of cocultivation with *Agrobacterium* had a significant effect on the percentage of explants with green callus on both explants types in the presence of 50 mg/l kanamycin selection (Table 2.13 and Fig. 2.5). *Agrobacterium*

strain, acetosyringone, and type of explant had significant individual effects and combine effects on green callus production (Fig. 2.5). Strain AGL0 harbouring plant transformation vector AK-UGI 9-1 resulted in a significantly higher number of kanamycin resistant green calli for both explants types than strain ATHVpPNGUS (Fig. 2.5). Acetosyringone had a significant effect on explant type and *Agrobacterium* strain. Use of acetosyringone increased the green callus production of petiole explants by 35% and leaf explants by 36% (when compared with the corresponding controls without acetosyringone) after coculture with AGL0pAK-UGI 9-1. Explants inoculated with ATHVpPNGUS, the increase in green callus production due to acetosyringone was 12% for petiole explants and 16% for leaf explants (Fig 2.5). Type of explant had a distinct outcome on kanamycin resistant green callus production, where petiole explants produced significantly higher number of calli than leaf explants in all the treatment combinations tested. According to the results obtained with this experiment, 100 µM of acetosyringone was used in the culture medium during two days preculture and two days cocultivation period and transformation was carried out with *Agrobacterium* strain AGL0pAK-UGI 9-1 in all subsequent transformation experiments.

Table 2.13. Analysis of variance results for the percentage of leaf/petiole explants with green callus in dependence on the *Agrobacterium* strain/plasmid and a treatment with acetosyringone

Source	DF	MS	Var.cp.	F	LSD5
Agrobacterium strain	1	87.12	5.41	176.89**	0.54
Explant type	1	158.42	9.87	321.67**	0.54
Acetosyringone	1	26.64	1.63	54.10**	0.54
Experiments	3	4.46	0.49	9.07**	0.76
Agro. Strain x Explant type	1	39.60	4.88	80.42**	0.76
Acetosyringone x Explant type	1	12.5	1.27	25.38**	0.76
Acetosyringone x Agro. strain	1	4.5	0.46	9.14*	0.76
Residual error	12	0.49	0.49		

** significant at p=0.01, * significant at p=0.05

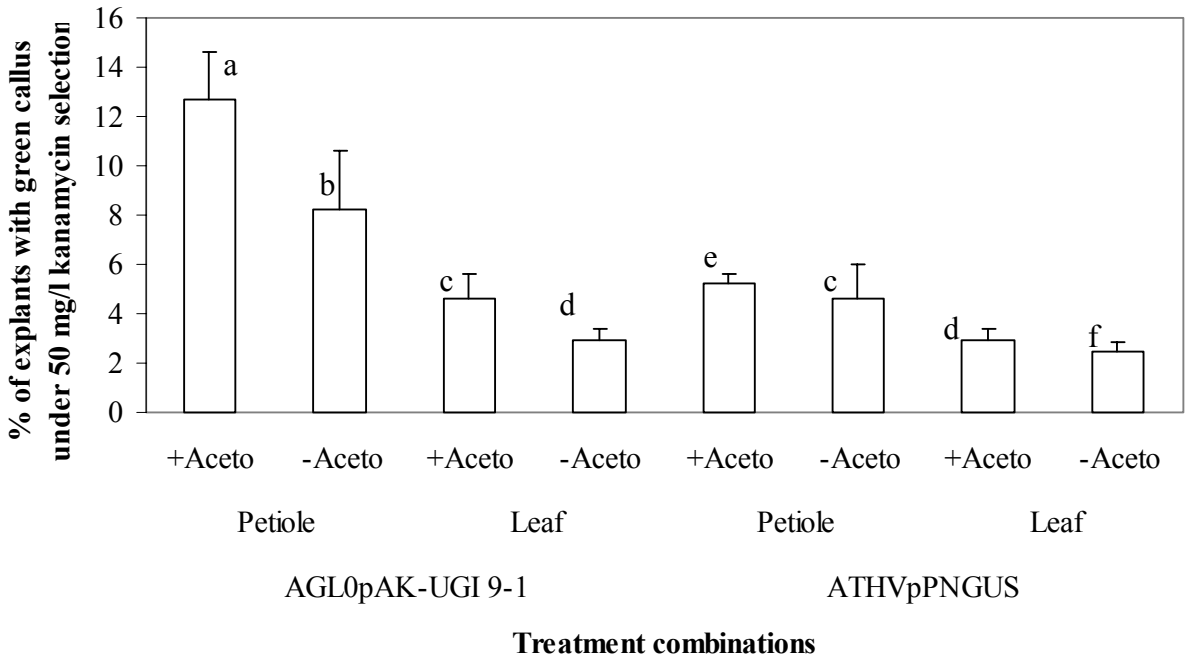


Fig 2.5. Effect of *Agrobacterium* strain/plasmid and use of 100 µM of acetosyringone in the culture medium during two days preculture and cocultivation with *Agrobacterium.* Green callus production of cultured leaf and petiole explants under 50 mg/l kanamycin selection was recorded after four weeks from coculture. Columns denoted by the same letter are not significantly different at P=0.05 according to Duncan's multiple range test. Vertical bars represent the standard error.

Transformation of leaf and petiole explants using modified transformation protocol

Based on the results of above experiments to optimize callus regeneration, organogenesis and transformation efficiency, a modified protocol has been established to obtain transformed plants with haploid leaf and petiole explants using *Agrobacterium* strain AGL0pAK-UGI 9-1. Green callus was induced in the improved callus induction medium (CIM-L and CIM-P) after four weeks from coculture with *Agrobacterium*, under 50 mg/l kanamycin selection. Callus originated mainly from the cut ends or at wounded sites as a result of handling with the forceps. In leaf explants, most of the callus originated from the cut edges of leaf veins. Putative transformed callus sectors were green in colour and healthy, while the non-transformed callus sectors turned whitish or turned necrotic due to the kanamycin toxicity (Fig 2.5a and Fig 2.6a). Out of 625 cocultured leaf explants 31 (5%) produced calli with green sectors while out of 675 cocultured petiole explants 60 (9%) developed green callus sectors (Table 2.14).

Table 2.14. Transformation efficiency of haploid leaf and petiole explants of *B. napus* cv. *Drakkar* transformed with *Agrobacterium* strain AGL0pAK-UGI 9-1 with two days preculture.

Explant	No. explants cocultured	No. of Kan^R green callus	No. of Kan^R shoots regenerated	No. of rooted shoots in MS+Kan. 50	Transformation efficiency (TE) (%)
Leaf	625	31	18	2	1.8
Petiole	675	60	28	6	2.9

Abbreviations, Kan^R: Kanamycin resistant, MS+Kan. 50: MS medium supplimented with 50 mg/l kanamycin.

TE= number of kanamycin resistant shoots x 100/number of explants cocultured

GUS assay conducted with representative explants with green callus showed that, all leaf explants assessed and 90% of petiole explants assessed were having transformed sectors. This suggest that selection with 50 g/l kanamycin was adequate to eliminate possible escapes or non-transformed callus, in early stages of callus proliferation. Green shoot buds (GSBs) became visible as miniature protuberances on green callus sectors after second week of culture in DKW (modified) medium and by the fourth week well developed GSBs could be seen. In additional two weeks time, shoots with 2-3 primodial leaves ranging from 5 to 10 mm in length were observed (Fig 2.5c and Fig 2.6c).

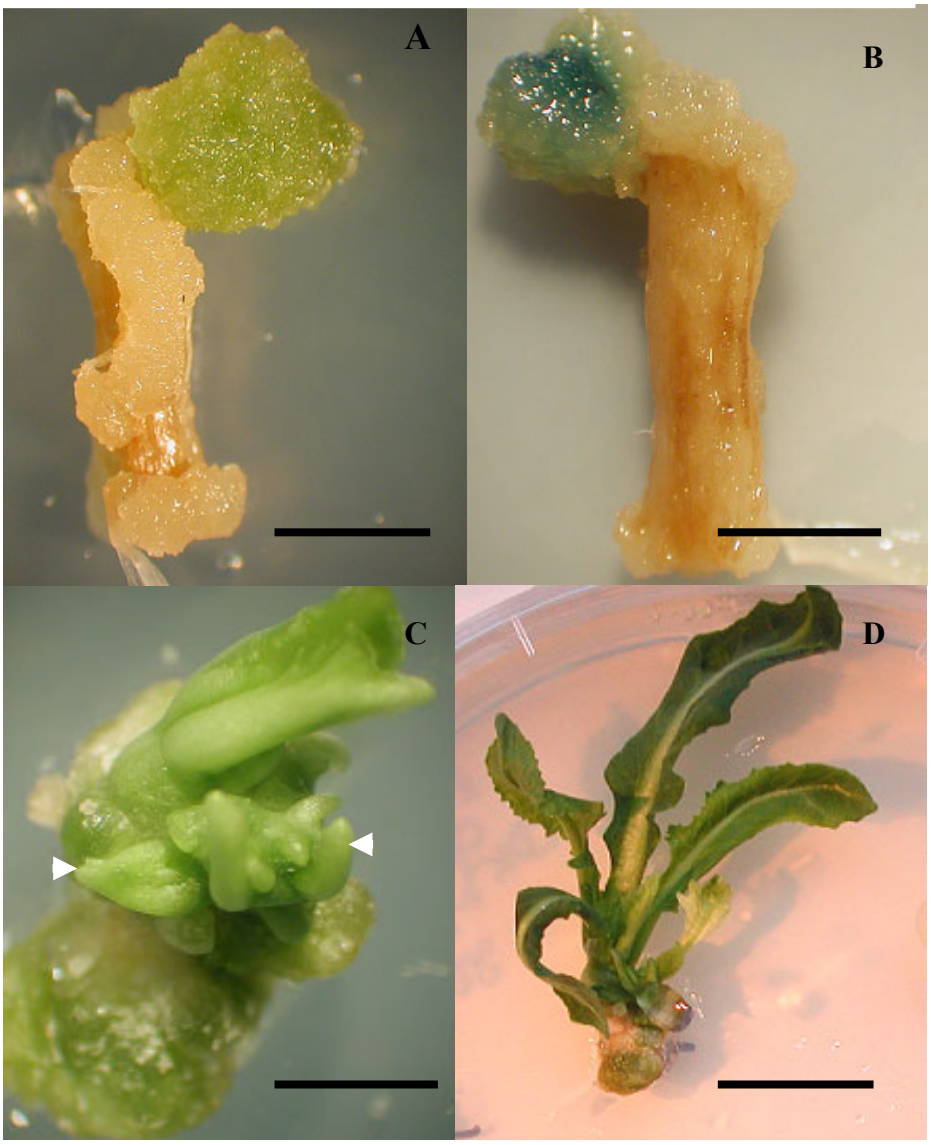


Fig 2.5. Shoot regeneration from kanamycin resistant callus derived from petiole explants inoculated with *A. tumefaciens* strain AGL0 pAK-UGI 9-1.

(A) Green callus regeneration from the cut surface of the petiole, after four weeks of culture in CIM-P medium under 50 mg/l kanamycin selection. (B) Petiole explant with GUS^{+} callus (blue colour). The light areas on the explant are non-transformed callus tissues. (C) Formation of green shoot buds (arrow heads) and shoots regeneration from green callus cultured in DKW (modified) medium (with 50 mg/l kanamycin selection) after 6-8 weeks from coculture. (D) A well developed kanamycin resistant shoot, after 10 weeks from inoculation. (*Scale bar= 5 mm*)

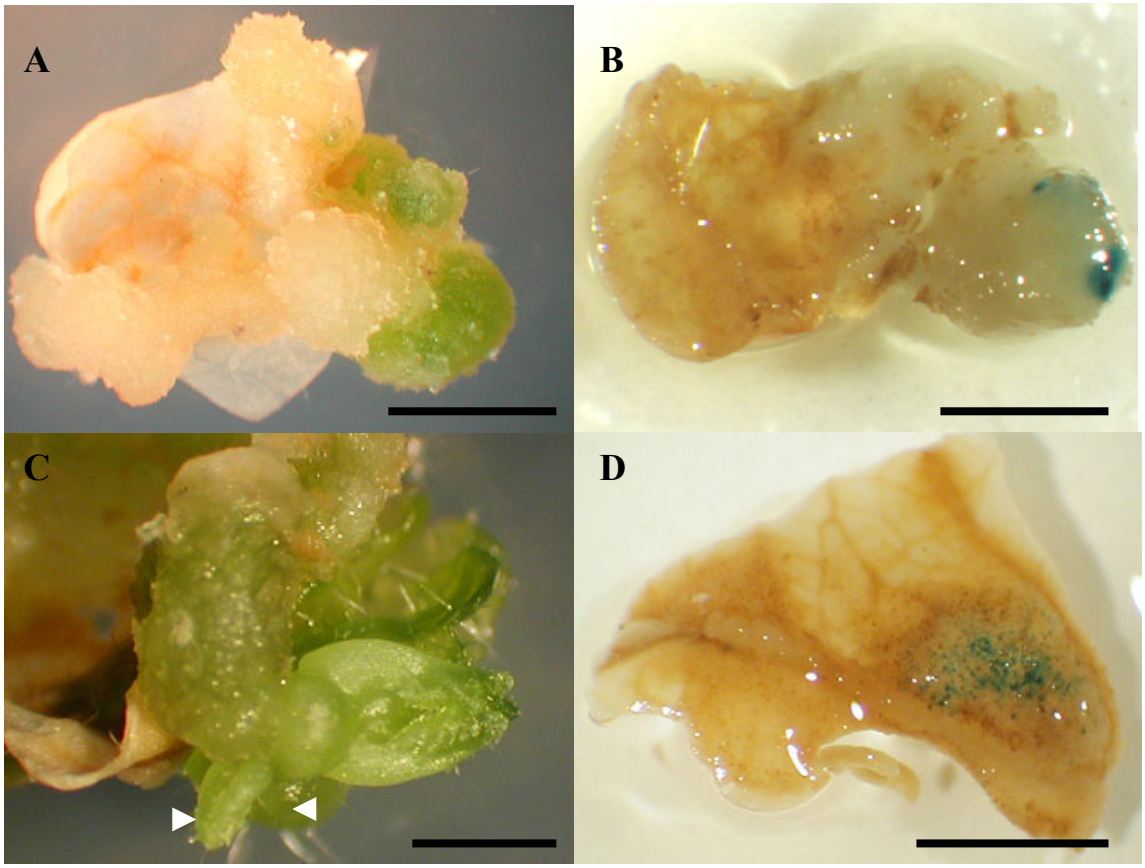


Fig 2.6. Histochemical GUS assay and shoot regeneration from kanamycin resistant callus derived from leaf explants inoculated with *A. tumefaciens* strain AGL0 pAK-UGI 9-1.
(A) Green callus regeneration from leaf explant, after four weeks of culture in CIM-L medium with 50 mg/l kanamycin selection. (B) Leaf explant with GUS+ callus (blue colour). The light areas on the explant are non-transformed callus tissues. (C) Formation of green shoot buds (arrow heads) and shoot regeneration from green callus cultured in DKW (modified) medium (with 50 mg/l kanamycin), after 6-8 weeks from coculture. (D) A leaf explant subjected to GUS assay after one week from vacuum infiltration with *A. tumefaciens* cell suspension. Note the numerous blue transformed spots in the leaf blade. (*Scale bar= 5 mm*)

35% of green calli of leaf origin (11 out of 31 transferred) produced shoots in DKW (modified) medium while 30% of calli of petiole origin (18 out of 60 transferred) responded by producing shoots. Conversion of GSBs into well developed shoots was 72 % for the calli of leaf origin and 64% for the calli of petiole origin. Both types of calli underwent the successive developmental stages of shoot organogenesis either simultaneously or without a noteworthy difference in time. No stuctural or morphological dissimilarity was observed between regenerated shoots of leaf and petiole origin.

Upon transfer to MS (basal) with 50 mg/l kanamycin, 11 shoots of leaf origin and 19 shoots of petiole origin showed further growth and remained green. Two shoots of leaf origin and six shoots of petiole origin rooted in MS (basal) with 50 mg/l kanamycin and developed into plantlets (Table 2.14).

Ploidy, GUS assay, and PCR analysis of putative transformed plants

Analysis of ploidy of rooted plants showed that all of them were haploids. Histochemical GUS assay performed with rooted putative transformed plants were all found to be negative. Amplification of template DNA extracted from four rooted plantlets in 50 mg/l kanamycin containing MS medium and five kanamycin resistant plantlets (they did not produce roots in MS with kanamycin, but in MS without kanamycin) with *GUS* primers resulted in an expected 1000 bp fragment for all the plantlets tested and for the plasmid pAK-UGI 9-1, which was the positive control. No amplification was obtained with DNA from non transgenic control plant (Fig. 2.7).

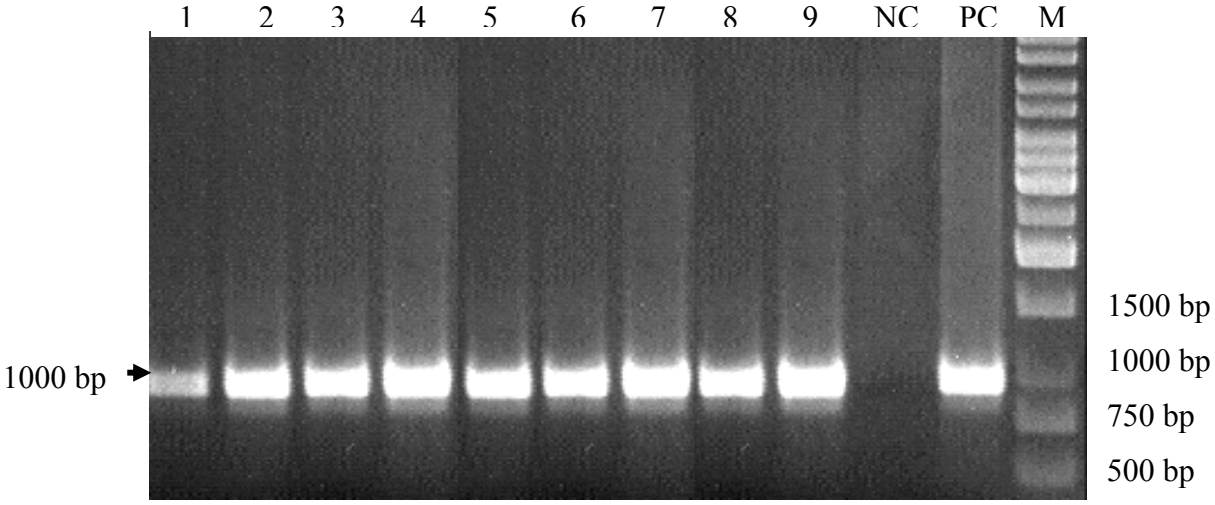


Fig. 2.7. PCR detection of *GUS* gene in *Brassica napus* cv. Drakkar transformed with binary vector system of *A. tumefaciens* strain AGL0 pAK-UGI 9-1.

Lanes 1-9: Kanamycin resistance T1 plants (1-4: T1 plants rooted in MS medium with 50 mg/l Kanamycin, 5-9: Kanamycin resistant T1 plants rooted in MS medium without 50 mg/l kanamycin), NC: non-transformed plant as the negative control, PC: plasmid pAK-UGI 9-1 as the positive control, M: DNA size marker.

Similarly, amplification of total DNA with *NPTII* primers resulted in an expected band of 700 bp with all the putative transgenic plants and with positive control (plasmid pAK-UGI 9-1) while no amplification product was obtained with non transgenic control plant (data not shown). Culture of leaf samples taken from PCR positive plants in YEB medium confirmed that plants were not contaminated with *Agrobacterium*.

2.4 Discussion

2.4.1 Optimization of callus production and shoot regeneration

One of the main prerequisites of a successful plant transformation protocol is the development of an efficient regeneration system from those specific tissues that can be infected by *Agrobacterium*. Tissue culture requirements to achieve better plant regeneration from a given tissue explant could vary among cultivars or even genotypes (Kazan et al. 1997). Therefore, it would be difficult to adapt a previously described transformation protocol to another system as it is, without modifications and improvements. Efficient plant regeneration from leaves of *B. napus* has been reported (Dunwell 1981, Akasaka-Kennedy et al. 2005), however, a successful plant transformation using these protocols hitherto is not known. Development of a plant regeneration protocol alone may not necessarily guarantee a successful transformation with that protocol. Stress exerted by *Agrobacterium* during coculture and detrimental effects by selective agents such as kanamycin and hygromycin may greatly influence the plant regeneration frequency. Therefore, it is important to test the developed plant regeneration protocol under the conditions required in a standard transformation procedure and keep on introducing improvements to the protocol to achieve better transformation efficiency.

This study is a simultaneous approach to develop an efficient plant regeneration protocol for haploid leaf and petiole explants and to regenerate transformed plants from infected tissue explants using *Agrobacterium*-mediated gene transfer method. With this protocol plant regeneration from leaf and petiole explants had been achieved in two phases: a callus regeneration phase and a shoot regeneration phase. Callus regeneration was achieved in defined callus induction media with specific hormone combinations for leaf and petiole explants. The optimal callus production was achieved with leaf explants in CIM-L medium while CIM-P medium gave the optimal callus production with petiole explants. Shoot organogenesis from green transformed calli was achieved in DKW (modified) medium

where 53% of leaf derived calli and 41% of petiole derived calli produced shoots. 96% of plantlets derived from leaf explants and 92% of plantlets derived from petiole explants remained haploid (Table 2.8). It is important that regenerated plantlets remain haploid in order to produce homozygous double haploid transgenic plants. Silver nitrate ($AgNO_3$) which is a potent ethylene inhibitor was added in 5 mg/l concentration to both callus induction (CIM) medium as well as to organogenesis medium (DKW) taking into consideration the previous reports of improved organogenesis with *Brassica napus* in the presence of $AgNO_3$. Addition of $AgNO_3$ has significantly increased the green bud formation and shoots regeneration from rapeseed leaves (Akasaka-Kennedy et al. 2005). Medium supplemented with $AgNO_3$ has also resulted in high shoot regeneration in *B. napus* with hypocotyls segments (Schröder et al. 1994, Eapen and George 1997, Cardoza and Stewart 2003) and mesophyll protoplasts (Hu et al. 1999).

1.4.2 Optimization of transformation efficiency

Effect of filter paper, preculture of explants and the use of acetosyringone

In this study, leaf explants were found to be very sensitive to cocultivation with *Agrobacterium* and turned necrotic very easily. For petiole explants, the tissue necrosis was not as high as for leaf explants (which was ~30%) but remained at a considerable percentage of ~10%. Tissue necrosis was surmounted in this study by preculture of whole leaves with petioles in CIM for two days, before sectioning into segments and coculture with *Agrobacterium*. Preculture was found to be more effective on filter papers soaked in liquid CIM than in solid medium. Filter paper acts in this case as a paper wick connecting the medium and cultured explants and thus allowing an efficient supply of essential nutrients to the tissue explants and keep cultured explants moist all the time to avoid a possible dehydration or wilting. Tissue necrosis of leaf explants could be reduced by 65% after two days preculture on filter papers (Table 2.12). Considering the positive effects of using filter papers on liquid medium, cocultivation was also carried out on filter papers soaked in liquid medium. Preconditioning of hypocotyl explants for 72 h before cocultivation with *Agrobacterium* has resulted in a higher number of transgenic plants in *B. napus* (Cardosa and Stewart 2003). Cocultivation of explants on filter paper has increased the transformation efficiency from 10% to more than 20% for cotyledonary petioles in *B. carinata* (Babic et al. 1998). Wounding of tissue explants before inoculation with *Agrobacterium* has been considered to be necessary for the release of *vir*-genes inducing phenolic compounds (Binns 1990). Therefore, preculture in this study was performed with

intact leaves and petiole explants, unlike preculture of cut hypocotyl segments as reported by Cardosa and Stewart (2003). Precultured explants were dissected into segments just before coculture with *Agrobacterium.* This simple modification allows the release of *vir*-inducing compounds at the time of *Agrobacteriu*m infection and also prevents a possible tissue browning due to early release of phenolic compounds. Other than wound released plant phenolic compounds, chemicals such as acetosyringone is also found to be enhancing the ability of *Agrobacterium tumefaciens* to transform host plants, thus incorporated either in culture medium used for cocultivation of *Agrobacterium* with plant tissue explants (Godwin et al. 1991, Henzi et al. 2000) or alternatively has been used to precondition tissue explants prior to inoculation with *Agrobacterium* (Guivarc'h et al.1993). In this study, the use of 100 µM of acetosyringone during preculture and coculture with *Agrobacterium* resulted in a significant increase in putative transformed callus production in both explant types; petioles and leaves. However, the beneficial effects of acetosyringone apparently fluctuate along with *Agrobacterium* strain and plasmid that has been used to inoculate tissue explants. Differences in induced virulence amongst different *Agrobacterium*-strains after cocultivation with 200 µM with acetosyringone have been reported by Godwin et al. (1991). Improved transformation efficiency after inclusion of acetosyringone in coculture medium has been reported in *B. oleracea* (Henzi et al. 2000, Tsukayaki et al. 2002), *B. campestris* (Zhang et al. 2000), *B. napus* and in *B. juncea* (Charest et al. 1989).

Infiltration of tissue explants

Infiltration of leaf and petiole segments with *Agrobacterium* cell suspension has increased the number of transformed sectors per explant (as confirmed by GUS assay after one week from coculture), but majority of these transformed sectors did not proliferate into green callus. Consequently tissue infiltration in this study did not help to increase the number of explants with transformed callus. It could be possible that not all of those transformed cells at the beginning, have the equal capacity to proliferate and develop into callus. Differential callus proliferating ability of leaf tissue cells of *B. napus* has been reported (Akasaka-Kennedy et al. 2005) where cells of vascular parenchyma cells exhibited the highest proliferation and callus production ability. This observation is in agreement with the findings of this study where most of the transformed callus originated from the cut edges of leaf veins. GUS staining of infiltrated leaf explants showed that most of the transformed cells were in the middle of the leaf blade and not in the vascular regions with higher regeneration ability (Fig. 2.6d). It could be important to have a higher number of

transformed cells with ability to undergo cell proliferation in order to achieve a higher number of transgenic calli.

Using the protocol developed in this study, transformed calli expressing GUS gene were obtained from haploid leaf and petiole explants after coculture with *Agrobacterium tumefaciens* strain AGL0 pAK-UGI 9-1. Kanamycin resistant transformed plants with *NPTII* and *GU*S genes (confirmed by PCR) have been obtained after organogenesis of transformed calli. A transformation efficiency (TE) of 1.8 was obtained with leaf explants and TE of 2.9 was obtained with petiole explants (TE= number of kanamycin resistant shoots x 100/ total number of explants cultured). Nevertheless, rooting was achieved only with 8 kanamycin resistant shoots when cultured in MS with 50 mg/l kanamycin (2 leaf-origin plantlets and 6 petiole-origin plantlets). PCR amplification with DNA from four rooted shoots and five non-rooted but kanamycin resistant shoots (they rooted in MS without kanamycin) confirmed the presence of both transgenes (*NPTII* and *GUS*) in all plantlets tested. Healthy shoots that displayed an active growth under 50 mg/l kanamycin selection could therefore be considered as transformed, even though they failed to produce roots in the presence of kanamycin. Transformation efficiency in this study was determined taking the number of kanamycin resistant shoots into consideration and not the number of rooted shoots (Table 2.14). These kanamycin resistant but non-rooted shoots in the presence of kanamycin readily produced roots upon transfer to hormone-free solid MS without kanamycin. Surprisingly none of the kanamycin resistant shoots gave positive results after GUS assay, suggesting a possible GUS gene inactivation. It has been revealed in several reports that, transgenes can undergo silencing after integration into host genome (Ingelbecht et al. 1994, Matzke et al. 1994, Balandin and Castresana 1997, Vaucheret et al. 1998). Suppression of the expression of transgene could happen transcriptionally or post-transcriptionally (Vaucheret et al. 1998). Methylation of promoters and gene sequence has been related to transgene silencing in several cases (Kilby et al. 1992, Matzke et al. 1994, Balandin and Castresana 1997). Transgene silencing could either be transmitted stably through several generations or could be meiotically reversible (Vaucheret et al. 1998). In many cases post-transcriptional gene silencing has shown to be developmentally regulated and not heritable. In transgenic tobacco, silencing of a β-1, 3-glucanase has been overcome during seed formation (Balandin and Castresana 1997). In a similar situation, transgenic double haploid plants of *B. napus* produced after inoculation of microspore-derived embryos with *Agrobacterium*, failed to express the transgene (*NPTII*) in primary

transformants, but restored *NPTII* activity has been observed with seed raised plants of T2 generation (Swanson and Erickson 1989). In this study, GUS expression was observed with callus of both leaf and petiole origin and GUS gene silencing was observed only with regenerated shoots and plantlets. This could therefore be a case of developmentally regulated or post-transcriptional gene silencing. Rooted plants in kanamycin have already been treated with colchicine to produce double haploid transgenic plants and are currently in the process of acclimatization. T2 plant generation would be analyzed to determine whether GUS activity is restored after meiosis and seed set. Expression of an integrated transgene could be established by studying either mRNA (to determine whether gene is transcribed) or protein accumulation (to determine whether mRNA is translated to produce a protein). RNA analysis could provide useful information on transcript accumulation and stability and is often used when protein analysis (western blot or ELISA) is not possible. Molecular techniques such as RT-PCR (Reverse Transcriptase PCR) could be used as a rapid and reliable method to establish the presence or absence of a specific transcript. RT-PCR technique utilizes extracted mRNA (or total RNA) from transformed plants to produce copies of cDNA (complementary DNA) of the transgene in the presence of enzyme reverse transcriptase. Analysis of putative transformed plants with RT-PCR could therefore be an important future perspective of this project.

2.4.3 Advantages of using haploid plant materials for transformation

One of the main advantages of haploid transformation is that, the transformants will have the transgene permanently fixed (homozygous after cholchicine doubling). Successful transformations via *Agrobacterium* using microspore-derived embryos have been achieved with *Brassica napus* (Swanson and Erickson 1989), *Nicotiana tabaccum* and *Datura innoxia* (Sangwan at al. 1993). Although reported to be successful, one major disadvantage commonly shared in these works is that, no assessment has been made to establish the ploidy of haploid embryos before cocultivation with *Agrobacterium*. If those embryos used for cocultivation turned out to be autodiplodized, which is a common phenomenon amongst microspore-derived embryos (Möllers et al. 1994), they would inevitably produce hemizygous transgenic plants. From the hemizygous loci, transgene would segregate following Mendelian genetics.

Use of haploid plant material as in this study could shorten the time taken to obtain fertile transgenic plants with *B. napus* cv. Drakkar when compared with diploid plant materials (Fig. 2.8).

Haploid system	**Time (weeks)**	**Diploid system**	**Time (weeks)**
Transformation experiments and plantlet regeneration	16	Transformation experiments and plantlet regeneration	16
↓		↓	
Colchicine treatment, transfer into green house	2	Acclimatization and transfer into green house	2
↓		↓	
Delay due to colchicine treatment	4	Harvest seeds of T1 hemizygous transgenic plants	16
↓		↓	
Harvest seeds from homozygous transgenic DH plant	16	Segregation analysis with T2 plant population	16
↓		↓	
Replicated trials with homozygous T2 plants		Replicated trials with T3 plants (homozygous?)	
Estimated time	9.5 months		12.5 months

Fig. 2.8. Comparison of the time required for the production of homozygous transgenic plants using haploid and diploid explants as starting material

Therefore, use of haploid materials should accelerate transgenic breeding programs with oilseed rape. On the other hand, use of clonally propagated plant materials assures high genetic uniformity among transformed plants. Transformed plants will differ from their non transgenic counterparts only at the transformed loci. This would be a definite advantage in morphological and physiological studies that requires a high degree of genetic uniformity amongst transgenic plants. However, transformation protocol described in this study needs establishment of haploid plants via microspore culture beforehand, which could take up to 3 to 4 months. Once produced, haploid transgenic plants should be made diploid to obtain fertile transgenic plants (by colchicine treatment), which takes 3 to 4 weeks before the establishment of plants in the green house. Colchicine treatment to produce double haploids at best results in 20-50% doubling rate (Möllers et al. 1994) and could lead to chimera formation or polyploidization. Haploid plant regeneration protocol described in this study resulted in relatively higher shoot regeneration efficiency 53% for leaf derived calli and 41% for petiole derived calli without *Agrobacterium* infection. Nevertheless, the transformation efficiency achieved with haploid leaf (1.8%) and petiole (2.9%) explants

was still low when compared with transformation efficiency of 3.6% in the standard protocol by Hüsken et al. (2005). However, the difference in starting plant materials could not be ruled out in this comparison, where standard protocol uses diploid hypocotyl explants while current study uses vegetative plant materials such as leaf and petioles. Seedling-raised hypocotyl and cotyledonary explants are shown to yield high regeneration efficiencies in *Agrobacterium* mediated transformation of *B. napus* (Moloney et al. 1989, Khan et al. 2003). Transformation frequencies obtained with leaf and petiole explants in this study could not be directly compared since there is no report available to our knowledge for a successful transformation using these plant materials in *B. napus*.

2.5 Conclusions

This study has established a working protocol to obtain callus and plant regeneration with haploid plant material of *B. napus* cv. Drakkar. Transgenic calli expressing GUS activity were obtained with leaf and petiole explants after coculture with *Agrobacterium tumefaciens.* Kanamycin resistant haploid transgenic plants were regenerated from callus produced on leaf and petiole explants with kanamycin selection. The presence of selectable marker gene (*NPTII*) and reporter gene (*GUS*) in putative transformed plants was confirmed with PCR. Expression of *GUS* gene was not detected with plants which were found to be kanamycin resistant and PCR positive for the *GUS* gene. Whether meiosis could restore the *GUS* gene activity could be established by studying double haploid transformed plants in the T2 generation. Haploid transformation should produce genetically uniform plants differing only at the transgenic loci and enable easy evaluation of physiological and agronomical effects caused by the introduced gene. Haploid transformation is relatively fast and avoids formation of hemizygous transformants. The transformation method described in this study could be extended to other important crops in *Brassicaceae*, *Solanaceae* and *Poaceae* in which pollen embryogenesis has already been established.

3. Overexpression of *Arabidopsis* phytochrome B in *Brassica napus* L.

3.1 Introduction

Plant development is strongly influenced by environmental factors. Sunlight is perhaps the most decisive abiotic factor that influences the growth and development of plants. Plants possess at least two types of photoreceptors: red (R) and far-red (FR) light absorbing phytochromes and the UV-A/blue light-absorbing cryptochromes, whose primary physiological role is the acquisition of signals from the light environment (Ballaré 1999). The phytochrome family of photoreceptors plays a vital role in modifying the developmental and physiological responses of plants to the changing light environment (Smith and Whitelam 1997). The phytochrome molecule is a dimer of two identical apoproteins of approximately 120 kD, each covalently attached to a linear tetrapyrrole pigment called chromophore (Furuya 1993, Rockwell et al. 2006). Chromophore is synthesized in chloroplasts and assembled with apoproteins in the cytosol, forming the inactive red light absorbing Pr conformation of phytochromes. Pr form is considered as biologically inactive and is converted to an active Pfr form upon absorption of red light. Pr and Pfr forms are inter-convertible by sequential absorption of R and FR light (Quail 1991). Under daylight conditions (which is rich in red light), phytochrome is mostly converted to the Pfr form. Pfr form is unstable compared to Pr form and is converted slowly back to Pr form under high FR conditions or at darkness (Smith 1995). Plants absorb red light for photosynthesis and reflect and transmit FR. Therefore, conversion of Pfr into Pr could also occur under the shade of neighbouring plants. This conversion triggers a series of plant responses called shade avoidance responses such as promotion of stem elongation, reduction in leaf thickness, reduced chlorophyll content, increased apical dominance, reduced branching and accelerated flowering (Casal et al. 1987, Halliday et al. 1994, Smith and Whitelam 1997). Shade avoidance responses help plants to beat the competition exerted by other pants and is solely stimulated by reduction in R: FR ratio, which occurs under dense plant populations (Smith and Whitelam 1997). Hence the primary, if not solely, physiological function of plant phytochrome seems perception of changes in R to FR ratio and let the plant detect shade and presence of neighbouring plants.

Evidence suggests the existence of multiple, distinct, but structurally conserved species of phytochromes of which, the apoproteins are encoded by a small family of divergent genes (Quail 1991, Furuya 1993 and Smith 1995). In the model plant *Arabidopsis thaliana*, five genes encoding five distinct phytochromes (phyA to phyE) have been identified and cDNA

of all five have been sequenced and characterized (Sharrock and Quail 1989, Clack et al. 1994). The polypeptides encoded by *PHYB* and *PHYD* genes share approximately 80% amino acid sequence identity and are more related to *PHYE* with approximately 55% identity than they are to either the *PHYA* or *PHYC* proteins (with approximately 47% identity) (Mathews and Sharrock 1997). The different phytochrome family members were shown to have distinct, sometimes overlapping and even antagonistic roles in mediating R and FR effects on plant development (Smith et al. 1997, Whitelam and Devlin 1997). Depending on the stability of Pfr state, two pools of phytochromes could be identified; light labile and light stable phytochromes (Furuya 1993, Smith 1995). Phytotochrome A (phyA) is light labile and accumulates predominantly in etiolated plant tissues while phytochromes B (phyB) and phytochrome C (phyC) are relatively light stable, with phyB accumulating as the major phytochrome in light grown or in green tissues (Somers et al. 1991).

Much of the existing knowledge on distinct roles by individual phytochromes in plant development has come from studying mutants that are null for a particular phytochrome or from analysis of transgenic plants overexpressing a particular member of phytochromes (Whitelam and Harberd 1994, Smith 1995, Greef 1996, Smith and Whitelam 1997). Mutants that are deficient in a particular phytochrome exhibit a light-insensitivity under irradiation conditions in which that phytochrome is functional under normal circumstances. Likewise, transgenic plants that overexpress a particular phytochrome display light-hypersensitivity under the same irradiation condition (Halliday et al. 1997). Mutants lacking phyB or functional form of it, such as *hy3* (now *phyB*) mutant in *Arabidopsis* (Reed 1993), *ein* mutant in *Brassica rapa* (Devlin et al. 1997) and *tri* mutant in tomato (van Tuinen et al. 1995) are insensitive to red light while transgenic *Arabidopsis* plants overexpressing an oat *PHYB* cDNA are hypersensitive to red light for the same morphological responses (Wagner et al. 1991, Nagatani et al. 1993). Light grown *ein* mutant seedlings of *B. rapa* demonstrated an elongated hypocotyl phenotype similar to *phyB* mutant of *Arabidopsis* which also showed elongated and early flowering phenotype, typical of shade avoidance responses of wild type seedlings grown under a low R/FR ratio or end of the day FR treatment (Devlin et al. 1997, Reed et al. 1993). These observations suggest that phyB plays a predominant role in mediating plant responses to vegetational shade. However, evidences are emerging to suggest the participation of other phytochromes (such as phyC) in shade avoidance responses (Smith and Whitelam 1997). In response to shade, plants allocate more assimilates for stem elongation at the expense of leaf and root development and resort to accelerated flowering that results in reduced seed set, truncated

fruit development and low rate of seed germination (Halliday et al. 1994). An alternative transgenic approach to achieve improved agricultural productivity with crop plants has been suggested by overexpression of phytochrome genes, *PHYA* and *PHYB* (Smith 1992, Robson and Smith 1997). Overexpression of phytochrome genes effectively disables the shade avoidance responses of plants under dense populations and this may lead to higher proportion of assimilates been allocated to economically important plant parts (Smith 1995). Overexpression of oat (*Avena sativa*) *PHYB* cDNA in tobacco (*Nicotiana tabacum*) has resulted in a reduced stem growth and a greater stem to fresh leaf biomass ratio at high planting densities resulting up to 20% improvement in harvest index in the field (Robson et al. 1996). Overexpression of *Arabidopsis PHYB* cDNA in potato (*Solanum tuberosum*) has resulted in semidwarf plants with higher photosynthetic performance, longer lifespan of transgenic plants and greater biomass production and tuber yields (Thiele et al. 1999).

Oilseed rape (*Brassica napus* L.), a natural amphidiploid derived by hybridization of *B. rapa* and *B. oleracea*, is a renowned crop worldwide as edible oil as well as a source of protein animal feed. Thus oilseed rape is an important target for crop improvements. Lodging or the collapse of plants and pods falling below the cutter level at harvest is a serious problem in oilseed rape. Many current *B. napus* cultivars are prone to lodging and should lodging occur early in the crop season during flowering or siliques development, high yield losses could be anticipated (Islam and Evans 1994). Lodging could be decreased by reducing plant height which is often achieved in *B. napus* applying chemical growth regulators (Gans et al. 2000). However, this could be counterproductive since growth regulators are also found to be interfering with other endogenous plant hormones and metabolic processes (Rademacher 2000). Furthermore, success with growth regulators on plant height depends on the time of application, and late applications may leave high amounts of chemical residues in the grain yields (Gans et al. 2000). Two dwarf mutants; rapid cycling *dwarf* (Zanewich et al. 1991) and *bzh* (Foisset et al. 1995) have been identified in *B. napus*. However, these mutants are known to possess several undesirable pleiotropic effects and their field performance are not yet been well documented (Muangprom et al. 2006).

Reduced plant height observed with potato (Thiele et al. 1999) and with tobacco (Halliday at al. 1997), as a consequence of overexpression of *Arabidopsis PHYB* cDNA, may provide useful alternative for *B. napus* to achieve shorter plants which under field conditions would reduce lodging. This study reports the morphological and physiological implications of phyB overexpression in transgenic *B. napus* plants. This study was carried out with diploid

plant material because the haploid transformation protocol described in chapter one was not fully established and optimized at the beginning of this study.

3.2 Materials and methods

3.2.1 *Agrobacterium* strain and plasmid vectors

The binary plasmid system of *Agrobacterium tumefaciens* strain C58C1 ATHV Rif^R containing the helper plasmid pEHA101 and the binary vector p35SA.thPhyB was used for transformation. The plant expression vector p35SA.thPhyB (derived from pBIN19), harbours the *Arabidopsis thaliana PHYB* gene under the control of CaMV35S promoter and the *NPTII* gene under the control of the *nos* promoter on the T-DNA (Fig.3.1).

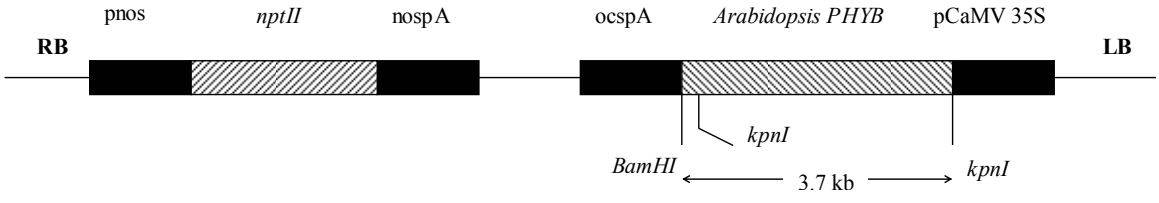


Fig. 3.1 Schematic diagram of the T-DNA region of the binary vector p35SA.thPhyB. Vector contains the coding region of the neomycin phosphotransferase gene (*NPTII*) under the control of nos promoter and coding region of the *Arabidopsis PHYB* regulated by CaMV35S promoter and pAOCS terminator of octopine synthase gene. LB, RB, Left and right border sequences from the vector T-DNA.

3.2.2 Multiplication and isolation of vector

The vector was introduced first into *Escherichia coli* strain XL1-Blue (Bullock et al. 1987) by electroporation and multiplied. *E. coli* was grown in 10 ml of LB medium (see Table 3.1 for the composition) supplemented with 50 mg/l kanamycin and 100 mg/l rifampicillin (Sigma) at 37 °C for 12-16 hours with shaking at 200 rpm on a rotary shaker. Bacteria cells were pelletted by centrifugation for 1 min at 10,000 x g in several 1.5 ml microcentrifuge

tubes and the supernatant was discarded. Plasmid was re-isolated from these bacterial cells using E.Z.N.A Plasmid Miniprep Kit 1 (Classic Line) (PQLab Biotechnologie GmbH) according to the manufacturer's instruction manual. Isolated plasmid was collected in TE buffer (pH 8.0) and stored at 4 °C until use.

Table 3.1. Composition of culture media

Medium	Application	Composition for 1 L of medium
YEB	Growth of *A. tumefaciens*	1 g/l Yeast extract, 5 g/l Meat extract, 5 g/l Peptone (Casein), 0.5 g/l MgSO4 · 7 H2O, 5 g/l sucrose, pH 7.4
LB	Growth of *E. coli*	1 g/l Yeast extract, 5 g/l Peptone (Casein), 10 g/l NaCl, pH 7.0 - 7.5
CIM	Liquid: Infection, co-cultivation, washing	5.3 g/l MS media-mix powder (Duchefa, Netherlands), 300 mg/l Myo-Inositol, 500 mg/l MES, 2 mg/l BAP, 0.001 mg/l Picloram,
	Solid: Callus induction	5 mg/l AgNO3, 20 g/l sucrose, pH 5.7. Solid medium: 5.4 g/l agarose
DKW	Shoot regeneration (organogenesis)	5.3 g/l DKW medium-mix powder (Duchefa, Netherlands), 20 g/l sucrose, 1 mg/l BAP, 0.01 mg/l IBA, 0.01 mg/l GA3, 5 mg/l AgNO3, 5.4 g/l agarose, pH 5.7

3.2.3 Preparation of competent cells of *Agrobacterium tumefaciens*

4 ml of YEB medium was inoculated with 40 µl of bacteria suspension (from stock culture) in a 40 ml conical flask and grown at 27 °C for 8 hours with shaking (200 rpm). Next, this liquid was transferred into 400 ml of YEB medium (see Table 3.1 for the composition) in a 1000 ml conical flask and grown overnight under the same growth conditions as before. Optical density of the bacterial suspension was measured at OD 600 nm and adjusted to 0.5-0.8. After that, the suspension was placed on ice for 15 min and centrifuged at 4000

rpm at 4 °C for 15 min. Supernatant was discarded and the bacterial pellet was kept on ice to cool down to 4 °C. Pellet was re-suspended in 400 ml of cold water (pre-cooled to 4 °C) and centrifuged at 4000 rpm for 15 min. Supernatant was discarded, pellet was re-suspended in 200 ml of pre-cooled water at 4 °C and centrifuged at 4000 rpm for 15 min. The pellet was re-suspended in 8 ml of Glycerine (filter sterilized and pre-cooled to 4 °C) and centrifuged at 4000 rpm for 15 min. Finally, the pellet was re-suspended in 0.8-1.2 ml of Glycerine (filter sterilized and pre-cooled to 4 °C) distributed into 1.5 ml microcentrifuge tubes (40 µl of bacterial suspension in each), immediately cooled down with liquid nitrogen and stored at -70 °C.

3.2.4 Introduction of plant expression vector into *Agrobacterium tumefaciens* strain

40 µl of bacterial cell suspension was mixed with 1 µl of isolated plasmid in TE buffer (concentration 10-100 ng/µl), mixed well, and placed on ice for 1 min. This liquid was then transferred into a sterilized plastic cuvette and subjected to an electric pulse of 2.5 kV (at 200 Ω) using a pulsar (BioRad, USA). After that, liquid was transferred into a sterilized 15 ml centrifuge tube, added 450 µl of YEB medium, mixed well and incubate at 27 °C for 3 hours with shaking at 200 rpm. 200 µl aliquots from this suspension was transferred onto selection plates (containing 25-30 ml of agar solidified YEB medium with 50 mg/l kanamycin and 50 mg/l rifampicillin in 90 mm Petri dishes), spread well, and incubated at 27 °C for 3 days. Transformed bacterial cells were obtained from isolated single colonies and grown further in new selection plates for several times before establishing stock cultures with them.

3.2.5 Preparation of glycerine stocks of transformed *Agrobacterium tumefaciens*

4 ml of YEB medium with selective agents (50 mg/l kanamycin and 50 mg/l rifampicillin) was inoculated with transformed *Agrobacterium* cells obtained from an well isolated colony on the selection medium, and grown overnight at 27 °C with shaking. 150 µl aliquots of overnight grown bacterial suspension was distributed among 1.5 ml microcentrifuge tubes containing 85 µl of filter sterilized glycerine, mixed well, immediately cooled with liquid nitrogen and stored at -70 °C.

3.2.6 Preparation of bacterial cell suspension for transformation of plant tissues

A single bacterial colony formed on solid YEB selection medium was inoculated into 4 ml of YEB medium with selective antibiotics and 10 µM acetosyringone and grown overnight at 27 ºC on a shaker rotating at 200 rpm. 100 µl aliquot of this solution was added into 40 ml of fresh YEB medium with selective antibiotics and 100 µM acetosyringone and grown overnight under the same conditions as above. The bacteria suspension was centrifuged at 6000 rpm for 10 min and the pellet was re-suspended in liquid callus induction medium (CIM), consisting of MS salts and vitamins (see Table 3.1 for the composition), and diluted to a bacterial cell density of 1 X 10^9 cells/ ml. This preparation was left for 3 hours at room temperature and subsequently used for inoculation of plant tissues.

3.2.7 Plant material

Hypocotyl explants obtained from *in vitro* grown seedlings of spring oilseed rape *Brassica napus* cultivar Drakkar, (Serasem Company, France) was used for transformation experiments. Seeds were surface sterilized with 5% (w/v) solution of Ca(OCl)2 for 30 min with continuous shaking in a 50 ml sterile bottle (Duran). After that seeds were rinsed three times thoroughly with sterile distilled water and dried on sterile filter papers. Sterilized seeds were plated onto agar solidified MS (basal) medium in 9 cm Petri dishes and grown *in vitro* at 25 ºC and 16/8 hours day/night photoperiod provided by cool fluorescent tubes (Osram, Germany) for 5-6 days.

3.2.8 Inoculation of hypocotyls explants

Hypocotyls of *in vitro* grown seedlings were cut into 5-7 mm segments under aseptic conditions and inoculated with *Agrobacterium* cell suspension in a sterile Petri dish placed on a slow rotating shaker at 80 rpm for 40 min at room temperature. Segments were briefly dried on sterile filter papers and transferred onto CIM medium solidified with 5 g/l agarose in 9 cm Petri dishes. Plates were sealed with Micropore tape (3M) and incubated for two days at 22º C. After two days of co-cultivation the segments were washed tree times with liquid CIM medium and the final washing was with 500 mg/l carbenicillin. Segments were briefly dried on sterile filter paper and transferred onto fresh solid CIM selection medium supplemented with 500 mg/l carbenicillin and 35 mg/l kanamycin. The number of segments cultured in a single Petri dish was 25. Plates were sealed with Micropore tape and incubated at 22º C under continuous light for four weeks.

3.2.9 Callus and plantlet regeneration

The number of green calli formed on hypocotyls explants in selection medium after four weeks were recorded separately for each Petri dish and expressed as a percentage value. Green calli were then separated from the explants and transferred onto DKW medium (see Table 3.1 for the composition) supplemented with 500 mg/l carbenicillin und 50 mg/l kanamycin. Plates were incubated at 22 ºC under continuous light condition and calli were transferred onto fresh medium in every 2-3 weeks intervals. Regenerated green shoots were separated from callus masses and transferred onto hormone free agar solidified MS medium supplemented with 500 mg/l carbenicillin und 50 mg/l kanamycin, in which rooting and plantlet regeneration were achieved. Well grown plantlets were transferred into soil (top earth 3: 1 compost) in 6 cm x 6 cm black plastic pots and placed in a controlled climate chamber for 4-5 days at 17 ºC with a photoperiod of 16/8 hours day/night. After that, plants were transferred to the greenhouse and kept under humid conditions for another week. Finally the plants were planted in 11cm (length) x 9cm (width) black plastic pots with same soil mixture and grown to maturity.

3.2.10 Isolation of total DNA from transgenic plants

100 mg fresh weight of young leaves obtained from putative transformed plants was used as starting material for DNA isolation. Harvested plant tissue samples were immediately frozen in liquid nitrogen and stored at -70 ºC in microcentrifuge tubes until use. Tissue samples were crushed in liquid nitrogen to obtain a fine powder using a mechanical mixture mill with a plastic pestle. Isolation of total DNA was done using DNeasy Plant Mini Kit (Qiagen GmbH) following the manufacturer's instruction manual. 400 µl of buffer AP1 and 4 µl of RNase (a stock solution of 100 mg/l) were added into ground tissue sample and vortexed vigorously. Next, the mixture was incubated at 65 ºC for 10 min to achieve lyses of cells while mixing 2-3 times during incubation by inverting the tube. 130 µl of AP2 solution was added to the tube, mixed well, and incubated on ice for 5 min. Mixture was then transferred into QIAshredder spin column and centrifuged at full speed of the microcentrifuge. This step binds DNA to the spin column and in the following step bound DNA was eluted with 50 µl of AE buffer (preheated to 65 ºC) into a fresh 1.5 ml microcentrifuge tube and stored at 4 ºC until further use.

AP1-Buffer: 50 mM Tris-HCl (pH 8.0)
10 mM EDTA
100 µg/ml RNase A
AP2-Buffer: 200 mM NaOH
1% (w/v) SDS
AP3-Buffer: 3 M Calciumacetate (pH 5.5)
TE-Buffer: 10 mM Tris-HCl (pH 8.0)
1 mM EDTA

3.2.11 Determination of DNA concentration and PCR analysis

Fluorometer was turned on 20 min before to allow the instrument to warm up. Gain and MED (medium) was adjusted to read 00000. 0.1µg/ml Hoechst 33258 (Bio-Rad) dye was prepared by mixing 1.4 µl of dye (stock solution of 1 mg/ml), 1.4 µl of 10x TEN buffer and 12.6 ml of sterile distilled water. Standard solutions of 20, 50 100, 200, 500 and 1000 ng DNA were prepared by mixing calf thymus DNA from stock solutions of 100 and 10 µg/ml with 2 ml of 0.1µg/ml Hoechst dye. Machine was calibrated using these samples at ambient temperature. 2 µl extracted total DNA from transformed plants was mixed with 2 ml of 0.1 µg/ml Hoechst dye in a clean cuvette, inserted into the machine and relative fluorescence unit (RFU) was recorded. The final DNA concentration for each sample was determined by dividing the RFU value by 2 (since 2 µl of extracted DNA was initially mixed with the dye).

PCR was performed in a reaction mixture containing about 25 ng plant genomic DNA, 125 µM of each dNTP, 10 µM of each primer and 1.25 U/ µl of Taq-polymerase. PCR analysis was carried out under standard conditions with 1 min denaturation, 1 min annealing, and 2-min extension at 94°C, 60°C and 72°C, respectively, for 35 cycles. The sequences of the PCR primers were as listed in Table 3.2. Primer design for *A. thaliana PHYB* gene was done by the web based Primer3 program (http://frodo.wi.mit.edu). Search was started with CaMV35S and *PHYB* gene coding sequence as the query. Designed primer pair amplifies the CaMV35S promoter and part of the *PHYB* gene. Amplified products were separated in a 1.5% (w/v) agarose gel in 1x TAE buffer at 120 V for two hours, stained with ethidium bromide, visualized under an ultra violet illuminator and recorded with a gel documenter.

Table 3.2. Primers and expected amplification length

Primer	Sequence	Fragment length
35S-x PHY B-fw.	5`- ATGGGTGCAGGTGGAAGAATG -3`	800 bp
35S PHY B-rev.	5`- TTCTTTCACCATCATCATATCC-3`	
NPT-II-fw.	5`-ATCGGGAGCGGCGATACCGTA-3`	700 bp
NPT-II-rev.	5`-GAGGCTATTCGGCTATGACTG-3`	

fw. Forward primer, rev. Reverse primer

3.2.12 Morphological characterization of primary transformants (T1 plants)

Out of 21 putative transformed plants regenerated (with 50 mg/l kanamycin selection) two transgenic lines, 5 and 9 were selected for further experiments on the basis of showing bands with both *npt*II and *PHYB* primers. Ten *in vitro* propagated clones of each line (5 and 9) and ten non transformed plants (hypocotyl raised) were established in the greenhouse in 11 cm (length) x 9 cm (width) plastic pots and grown to maturity under long day conditions (from Sep 06 to Dec 06). Transformed and non transformed plants used in this experiment were simultaneously raised and have gone through similar *in vitro* and *in vivo* culture phases. Transgenic plants were compared with control plants in randomized experiment for morphological, phenological, and agronomical traits.

Plant height was measured from the soil to the apex with a ruler at bolting, at first flowering and at maturity. Date of flowering was recorded after daily observations for each plant and expressed as the number of days taken from sowing to appearance of the first open flower. Leaf thickness was measured with a micrometer at four random points on leaf blades of 3rd and 4th leaf (from plant apex). Fresh leaf weight per cm^2 was determined by measuring four randomly cut leaf discs of 1 cm^2 from 3rd and 4th leaves of each plant.

Chlorophyll content was measured at bolting and at flowering for each plant with a chlorophyll meter (SPAD-502, Konica Minolta sensing, Inc.). Chlorophyll content of three randomly selected plants from two primary transgenic lines, T5(1) and T9(1) and from three corresponding control plants was recorded in weekly intervals from the time of

transfer into the greenhouse until plants attained maturity (marked by siliques turning from green to yellowish brown).

3.2.13 Greenhouse experiments with T2 plants

The main shoot of T1 plants was self pollinated to produce T2 seeds. Around 200 T2 seeds from each transgenic line T5 and T9 were sown alongside with 40 non-transgenic seeds in the greenhouse. Hypocotyl length of the growing seedlings was recorded at the first and second week after sowing. All seedlings (206) from line 5 were grown into maturity. 50 shortest and 50 tallest seedlings were selected from line T9 after two weeks and grown into maturity. All plants were grown in 11cm (length) x 9cm (width) plastic pots (containing a mixture of top earth 3: compost 1) and arranged in randomized experiment so that two non-transgenic control plants were present in each row.

3.2.14 Measurement of hypocotyl length and kanamycin sensitivity assay of T2 seedlings

A sample of 100 seeds obtained from each of the self pollinated transgenic T5 and T9 plants and from a corresponding control plant were grown separately *in vitro* for 7 days in complete darkness or in white light (at 16/8 hrs day/night light photoperiod), after which hypocotyl length was determined for each seedling. Next, the apical shoot (with a small part of the hypocotyl) was cut from all seedlings and cultured separately in solid MS medium (basal) supplemented with 100 mg/l kanamycin and grown for four weeks under 16/8 hours day/night light condition. Those shoot apexes which showed continuous growth (with adventitious root formation) were recorded as kanamycin resistant (Kan^R) and those shoots died without further growth as kanamycin sensitive (Kan^S). Total DNA was extracted from ten selected shortest seedlings, ten intermediately tall seedlings and ten tallest seedlings, of a seven days old *in vitro* grown seedling population of 100, from transgenic line T5 and amplified with *PHYB* primers to determine the inheritance of *PHYB* gene. Similarly, total DNA from 20 selected shortest and 20 tallest greenhouse grown plants of T5(2) and T9(2) populations (selection criterion was the seedling height after one week) was isolated and amplified with *PHYB* primers to determine the inheritance of phyB gene.

3.2.15 Statistical analysis

Statistical analysis of data was performed using the Stat-Graphics Plus for Windows 3.0 software (Statistical Graphics Corp. 1997). Data were presented as means and standard deviation separately for transgenic and non-transgenic plants for a given trait. Means were separated using Duncan's multiple range test at P=0.05.

3.3 Results

3.3.1 Transformation and plant regeneration

Sixteen putative transformed clones, named T1(1) to T16(1), were obtained (with 50 mg/l kanamycin selection) from 1335 infected hypocotyl segments which corresponds to a mean transformation efficiency of 1.2 %.

3.3.2 PCR analysis

PCR analysis of the total DNA extracted from the 16 putative transformed plants confirmed the presence of both *NPTII* and *PHYB* genes in two transgenic clones, T5(1) and T9(1). These two transgenic clones were selected to be used in further studies. Amplification of total DNA with the *NPTII* primers gave a 700 bp band for the clone T5 and T9 and for plasmid p35SA.thPhyB which was the positive control (PC), (Fig. 3.2b). When the *PHYB* primers were used for amplification, an 800 bp band was observed for the same clones and the plasmid p35SA.thPhyB (Fig. 3.2a). No bands were obtained with an untransformed plant used as the negative control (NC).

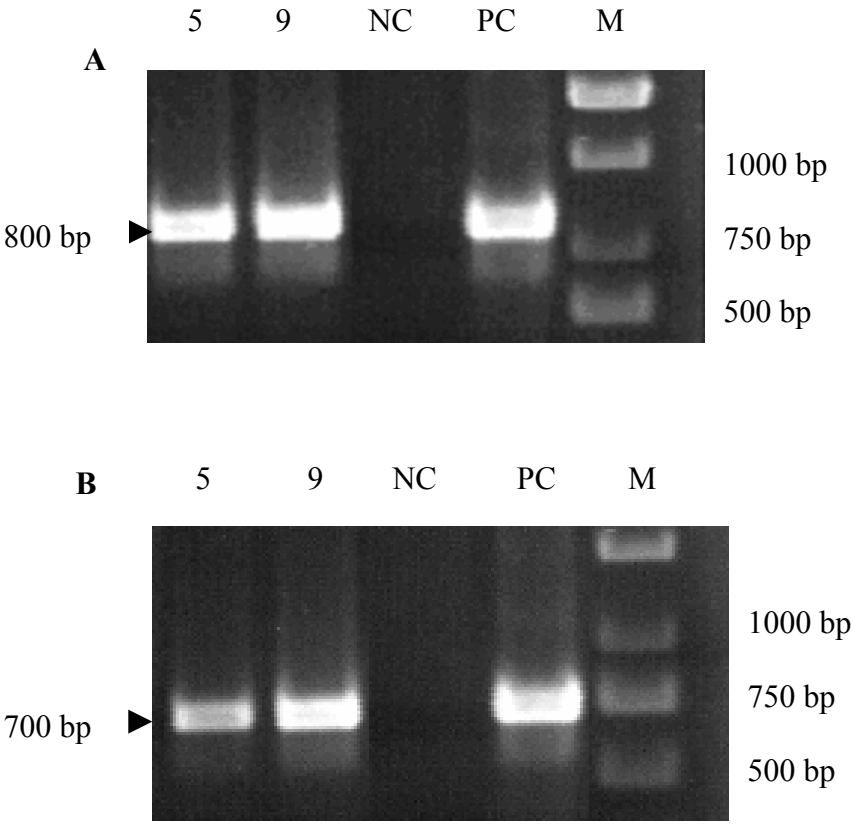


Figure 3.2. PCR analysis of DNA amplified with *Arabidopsis PHYB* primers (A) and *NPTII* primers (B).

Lanes: 5 and 9: DNA from transgenic plants T5(1) and T9(1), NC: DNA from an untransformed plant (negative control), PC: DNA from plasmid p35SA.thPhyB (positive control) M: DNA size marker

3.3.3 Leaf characteristics of T1 transgenic plants

Average number of leaves per plant at bolting was significantly higher in transgenic plants. However, the size of leaves was significantly smaller than those of controls (Table 3.3). Fresh leaf weight and thickness of leaves for transgenic plants was significantly higher than controls, while no significant difference was observed between the two transgenic lines for the same traits.

Table 3.3. Leaf characteristics of transgenic (T1) and non-transgenic plants at the time of bolting

Parameter	T5(1)	T9(1)	Control	n/n [a]
Average number of leaves	9.3*	9.6*	8	10
	(1.2)	(1.4)	(1.1)	
Leaf length (*cm*)	11.4*	10.8*	13.0	10
	(1.3)	(0.7)	(1.2)	
Leaf width (*cm*)	9.4*	8.6*	11.5	
	(0.6)	(0.3)	(1.6)	
Chlorophyll content	45.9*	45.2*	42.9	100/10
	(3.9)	(2.2)	(1.7)	
Fresh leaf weight(*mg/cm2*) [b]	28.8*	28.3*	26.6	40/10
	(1.5)	(1.5)	(1.9)	
Leaf thickness (μ*m*) [b]	277*	274*	263	40/10
	(10)	(9)	(7)	

Data are means and standard deviations given in brackets

All data are recorded at the time of bolting, [a]Number of samples/ number of individual plants, [b]data are from the 3rd and 4th leaves from the apex, *denotes a statistically significant difference from the control at P=0.05

Chlorophyll content was measured at different stages of development to study the course of maturation and senescence of transgenic plants. Leaf chlorophyll content of transgenic plants increased sharply during/from the second and/to fourth week and reached a maximum at around bolting. Immediately after first flowering (approx. 40 days), senescence started simultaneously for transgenic and non-transgenic plants. Thereafter, leaf chlorophyll content of control plants decreased almost linearly while chlorophyll degradation in transgenic plants was at a decelerated rate (Fig. 3.3).

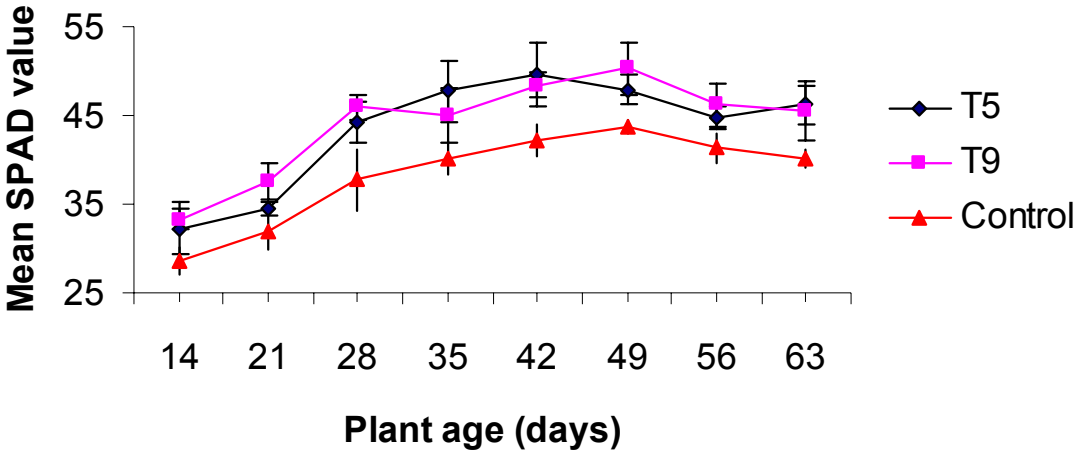


Figure 3.3. Average chlorophyll content during the course of plant maturation for transgenic plants (T5(1) and T9(1)) and non-transgenic controls. Each data point represents the mean chlorophyll content of three randomly selected plants. Vertical bars represent SD.

3.3.4 Morphological, yield and quality traits of T1 transgenic plants

No distinct difference in plant height was noted between the transgenic and non-transgenic plants during *in vitro* culture phase. However, under greenhouse conditions, a significant height reduction (~20%) was observed with transgenic plants at bolting, first flowering and at maturity (Table 3.4).

Table 3.4. Morphological, yield and quality traits of T1 transgenic plants

Parameter		T5 (1)	T9(1)	Control
Bolting (d*ays*)		23.3*	24.7*	27.6
		(1.9)	(2.4)	(3.4)
1st flowering (*days*)		35.3*	37.2*	43.7
		(1.7)	(2.8)	(3.6)
Duration of flowering (*days*)		26.5	25.91	24.9
		(3.9)	(3.7)	(2.7)
Plant height (*cm*)	At bolting	21.6	19.9*	24.9
		(2.4)	(4.3)	(5.1)
	1st flowering	38.7*	38.1*	50.3
		(6.0)	(4.6)	(2.2)
	Maturity	90.4*	95.0*	113.9
		(16.1)	(15.3)	(2.8)
No. siliques in main raceme		30.8	28.7	24.6
		(8.5)	(7.8)	(6.2)
Mean length of siliques (*cm*) [a]		7.1*	6.8*	6.2
		(1.1)	(0.9)	(0.6)
No. of seeds per siliques [a]		25*	24*	20
		(5.2)	(5.7)	(6.3)
Mean seed yield per plant (g)		1.7	1.4	1.8
		(0.9)	(0.7)	(0.6)
1000 kernel weight (g)		2.5*	2.4*	2.7
		(0.21)	(0.17)	(0.19)

Data are means and standard deviation given in brackets

* denotes a statistically significant difference from the control at P=0.05, [a] mean of five randomly selected siliques from the main raceme

There was no significant difference in number of siliques in the main raceme. Length of siliques and number of seeds per silique (mean value of five randomly selected siliques from the main raceme, for each plant) was significantly higher for transgenic plants than those of controls. No statistically significant difference was observed in seed yield per

plant. Thousand kernel weight was significantly higher in control plants than in the two transgenic lines (Table 3.4). Analysis of mature seeds obtained from primary transformants (or T1 plants) showed no significant differences in major storage components, such as oil and protein content, when compared with non-transgenic controls. Seed glucosinolate content, oleic acid and linolenic acid content was significantly lower in transgenic plants (Table 3.5).

Table 3.5. NIRS analysis of T2 seeds (T1 plants)

Line	Fatty acid profile (%)				
	Oil	Protein	GSL	C18:1	C18:3
T5(1) (n=10)	45.6	26.6	33.0*	76.7*	11.9*
T9(1) (n=10)	46.9	25.5	35.6*	76.8*	11.9*
Control(n=10)	46.5	25.2	36.9	74.0	12.8

n= number of individual plants measured, GSL= Glucosinolates, C18:1= Oleic acid, C18:3= Linolenic acid,

* denotes a statistically significant difference from the control at P=0.05

3.3.5 Hypocotyl elongation in T2 seedlings

T2 seedlings of transgenic clones (T5 and T9) grown *in vitro* under complete darkness were indistinguishable from those corresponding non-transgenic control seedlings (Fig. 3.4). However, a significant inhibition of hypocotyl elongation was observed with *in vitro* transgenic seedlings when grown under 16/8 hours day/night light condition. This inhibition caused a pronounced short hypocotyl phenotype amongst transgenic seedlings (Fig.3.5).

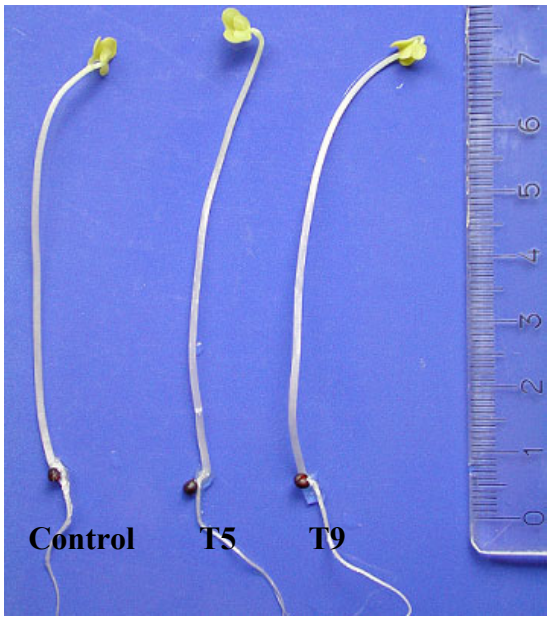


Figure 3.4. Appearance of transgenic and non-transgenic seedlings grown under continuous darkness for 7 days. C: non-transgenic control, T5(2) and T9(2): representative seedlings from two transgenic lines.

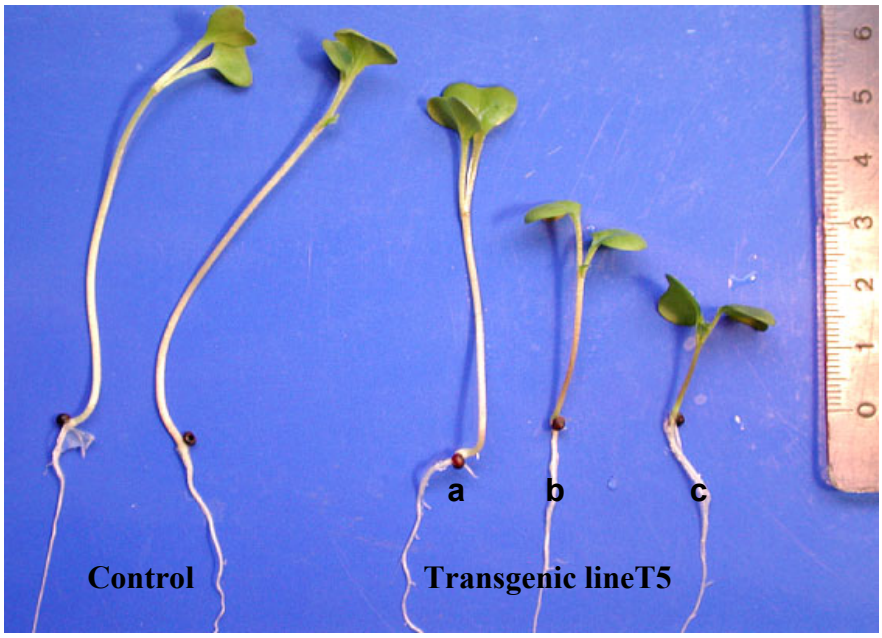


Figure 3.5. Appearance of non-transgenic and transgenic seedlings following 7 days of growth under white light regime of 16/8 hours day/night. From left to right, representative seedlings from non-transgenic control and seedlings from transgenic line T5(2) showing different degree of hypocotyl elongations (a: long, b: intermediate and c: short).

Transgenic seedlings had three height groups; long seedlings which were indistinguishable from non-transgenic control seedlings (Fig. 3.5a), intermediate tall seedlings (Fig. 3.5b) and short seedlings (Fig. 3.5c). Short hypocotyls seedlings were characterized by fully expanded cotyledons and increased anthocyanine pigmentation at hypocotyls and at lower surfaces of cotyledons. Intermediate tall and short seedlings were grouped together and termed as short hypocotyl seedlings.

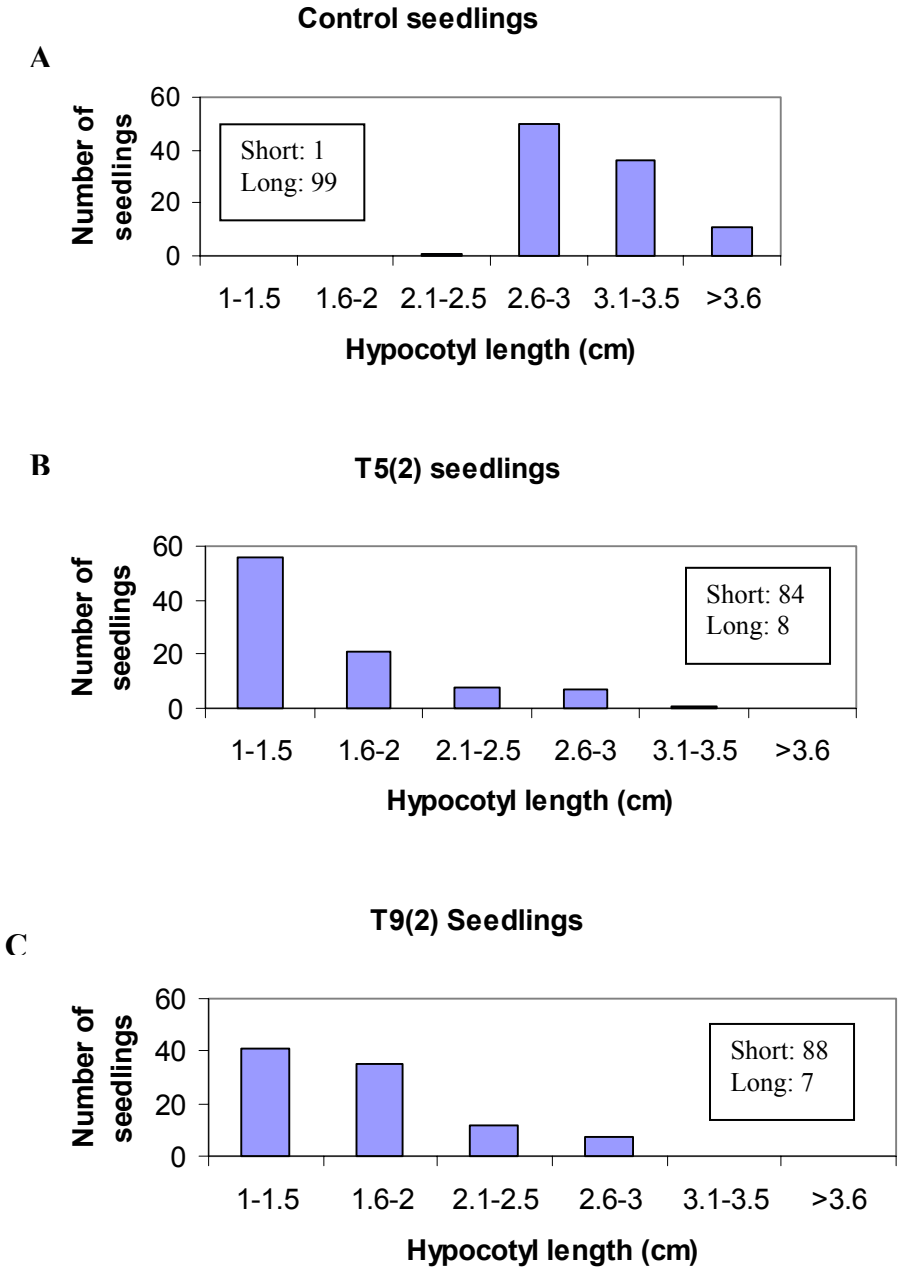


Figure 3.6. Hypocotyl length distribution of 7 days old *in vitro* grown seedlings of two transgenic lines; T5 (B) and T9 (C), and non-transgenic control seedlings (A). Absolute numbers of short and long hypocotyl seedlings are in the inserted box

99% of non transformed seedlings had a hypocotyl length of >2.5 cm (Fig. 3.6A). A bimodal distribution of hypocotyl length was observed in transgenic line T5(2), with approximately 91% (84) of seedlings having hypocotyl length of < 2.5 cm and 9% (8) > 2.5 cm (Fig. 3.6B). The same tendency was observed with seedlings of transgenic line T9(2) in which 93% (88) showing short hypocotyls (< 2.5 cm) and 7% (8) showing long hypocotyls (> 2.5 cm) (Fig. 3.6C).

3.3.6 Kanamycin sensitivity of T2 seedlings

In vitro grown transgenic seedlings segregating for a short hypocotyl phenotype also segregated for kanamycin resistance. Upon transfer into MS medium supplemented with 100 mg/l kanamycin, short hypocotyls seedlings showed resistance and grew further while those tall seedlings were kanamycin sensitive and died without further growth. Inheritance of kanamycin resistance gene amongst T2 seedlings indicated the presence of two transgenic copies in each of the transgenic line. As calculated chi-square (χ^2) values were below the tabulated value at P=0.05, the null hypothesis of 15:1 segregation ratio could not be rejected (Table 3.5).

Table 3.6. Transgenic T2 seedlings segregating for kanamycin resistance

Line	Kan.^{S}	Kan.^{R}	Segregation(χ^2)	Copy number
T5(2) (n=92)	5	87	15:1 (χ^2=0.5)	2
T9(2) (n=95)	9	88	15:1 (χ^2=0.1)	2
Control (n=100)	100	0	-	-

χ^2 = 3.84 (5%, 1 DF)

Amplification of total DNA extracted from ten shortest seedlings and ten intermediate tall seedlings (they were selected out from a seven days old, *in vitro* grown seedling population of 100 seedlings derived from transgenic line T5) with *phyB* primers resulted in an expected 800 bp band with all the seedlings tested. Amplification of total DNA extracted from ten tallest seedlings from the same population with *phyB* primers resulted in no amplification product with seven seedlings while three (shortest among the selected ten) showed the 800 bp band (Fig 3.7).

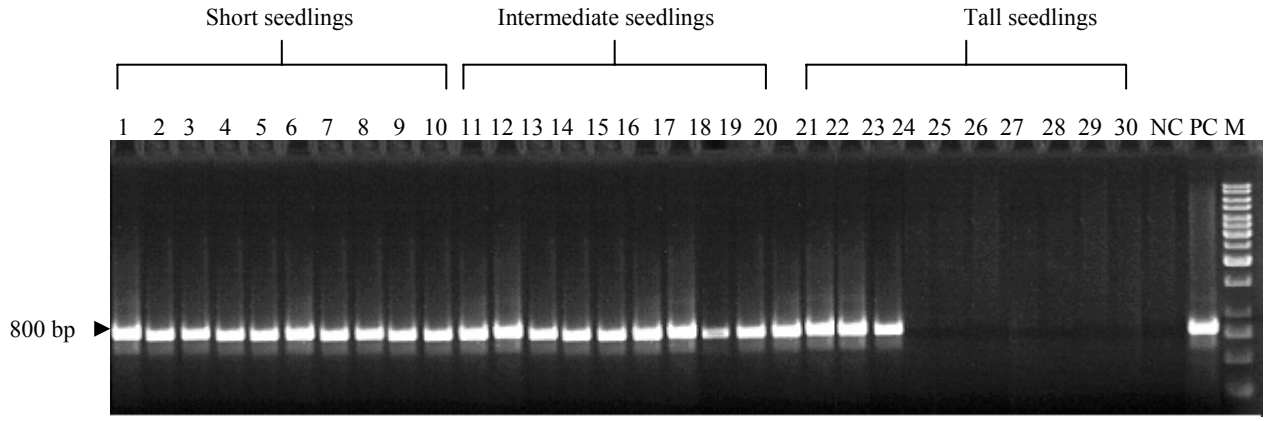


Figure 3.7. PCR analysis of total DNA amplified with *Arabidopsis PHYB* primers.
Lanes: 1 to 30 DNA extracted from selected *in vitro* grown seedlings of transgenic line T5(2). Seedlings are arranged in the ascending order, the shortest (lane 1) to the tallest (lane 30). NC: DNA from a untransformed seedling (negative control), PC: DNA from plasmid p35SA.thPhyB (positive control), M: DNA size marker

PCR analysis of selected greenhouse grown T2 plants (selection criteria was seedling height after one week) showed that *PHYB* gene was present in majority of plants. Contrary to the results obtained with *in vitro* grown seedlings, selected shortest and tallest plants from T5(2) and T9(2) populations grown under greenhouse conditions were shown to be equally transgenic (Table 3.7). Out of 20 selected tallest plants, 19 from T5(2) and all twenty from T9(2) 19 showed the presence of *PHYB* gene after PCR analysis.

Table 3.7. PCR detection of *PHYB* gene in greenhouse grown selected tall and short T2 plants

Line	$PHYB^+$ seedlings	$PHYB^-$ seedlings
T5(2) selected tallest (n=20)	18	2
T5(2) selected shortest (n=20)	19	1
T9(2) selected tallest (n=20)	20	0
T9(2) selected shortest (n=20)	19	1

n= number of plants analysed

3.3.7 Plant height of T2 transgenic plants

Nineteen plants out of the 206 plant population of transgenic line T5(2) showed a phenotype and development distinct from the rest of the plants in the population. These 19 plants attained flowering much later than the rest of the 187 plants and were thus termed 'late-flowering' and described separately in comparisons. Nevertheless, these plants grew within the 206 plant population from the time of sowing until plants attained full maturity. After two weeks of growth under greenhouse conditions, seedlings of transgenic line T5(2) showed a significant height reduction than controls (Table 3.8).

Table 3.8. Comparison of plant height of T2 plant population at different developmental stages

Line	Seedling height after two weeks (*cm*)	Plant height (*cm*)		
		At bolting	At flowering	At maturity
T5(2) population	1.8*	18.4*	37.3*	107.0*
(n=187)	(0.5)	(8.2)	(9.1)	(23.4)
Late-flowering plants of	1.9*	18.7*	35.1*	81.4*
T5(2) population (n=19)	(0.6)	(5.3)	(6.2)	(15.3)
Control	3.2	23.0	46.4	110.9
(n=30)	(0.5)	(3.1)	(5.3)	(11.2)
T9(2)selected (tallest)	2.4	21.7	45.5	113.0
(n=50)	(0.4)	(8)	(11.4)	(17.5)
T9(2)selected (shortest)	1.1*	16.0*	33.7*	92.8*
(n=50)	(0.2)	(16.3)	(9.6)	(21.4)
Control	2.7	24.6	50.2	110.7
(n=10)	(0.3)	(4.3)	(5.1)	(10.7)

Values are means and standard deviations given in brackets, n=number of individual plants
* denotes a statistically significant difference from the control at P=0.05

At the time of bolting (emergence of the main raceme) and at first flowering (appearance of first open flower), transgenic plants, except those selected tallest plants of the T9(2) population (selection criteria was seedling height after one week), displayed a significantly reduced plant height. For T5(2) population of 187 plants, reduction in plant height at first

flowering was 20% when compared with non-transgenic control plants. Selected shortest T9(2) plants displayed a marked reduction in plant height at first flowering than corresponding control plants and the difference was 33% (Table 3.8). No significant difference was observed between selected tallest T9(2) plants and the control plants. The difference in plant height between transgenic and control plants became narrow when plants attained maturity. Selected tallest plants grew even taller than the control plants. Nevertheless, those selected shortest plants of T9(2) population and 19 late-flowering plants of the T5(2) population remained significantly shorter than the controls after plants attained full maturity. Reduction in plant height of late-flowering plants at maturity was 27% than the controls (Table 3.8)

3.3.8 Bolting, first flowering and duration of flowering in T2 plants

Bolting occurred about one week earlier for control plants than transgenic plants, in both lines, T5 and T9. Consequently, first flowering also took place about one week later for transgenic plants. There was no significant difference in the duration of flowering between transgenic and control plants (Table 3.9). However, the above mentioned 19 late-flowering plants out of the population of 206 T5(2) plants, displayed an extremely delayed bolting and first flowering. Bolting in these plants occurred at a mean of 114 days from sowing, at such time contemporaries from the same population attained full maturity (marked by siliques turning yellowish-brown), and first flowering occurred at a mean of 127 days (Table 3.9).

Table 3.9. Comparison of days taken for bolting, first flowering and duration of flowering, in T2 transgenic plants

Line	Bolting (*days*)	First flowering (*days*)	Duration of flowering (*days*)
T5(2) population (n=187)	42.6* (8.6)	52.6 (8.9)	25.8 (3.6)
T5(2) Late-flowering (n=19)	114.2* (19.6)	126.6* (18.3)	25.5 (1.7)
Control (n=30)	34.5 (2.4)	48.9 (3.4)	23.9 (2.8)
T9(2) selected (tallest) (n=50)	44.2* (10.2)	58.0* (11.1)	24.6 (1.9)
T9(2) selected (shortest) (n=50)	43.2* (8.4)	51.2 (8.4)	24.6 (3.6)
Control (n=10)	36.8 (1.8)	48.1 (1.6)	25.5 (3.5)

Values are means and standard deviations given in brackets, n= number of individual plants

* denotes a statistically significant difference from the control at P=0.05

3.3.9 Phenotypic characteristics of late-flowering plants

Apart from the unusual delayed flowering, the 19 late-flowering plants exhibited distinct dissimilarities in basic plant architecture when compared with other 187 plants in the same plant population and with those non-transgenic control plants. Average plant height, as measured at the 90th day after sowing, was 17.5 cm. At this stage the surrounding plants had reached a plant height of about 100 cm or more, and already were in a mature stage of plant development. All late-flowering plants showed reduced apical dominance with excessive branching where 2-3 parallel branches originated from the basal part of main stem and grew concurrently with the main stem. When these plants attained flowering, 2-3 blooming racemes could be seen (Fig. 3.8).

Figure 3.8. Two late-flowering plants of T9(2) (left) and T5(2) (right) transgenic lines showing excessive branching and higher number of leaves. *Scale bar =2 cm*

Late-flowering plants had comparatively smaller leaves, but the average number of leaves per plant at bolting and at first flowering was significantly higher than controls (Table 3.9). After neighbouring plants reached full maturity (at about 90 days from sowing) and their shading effects were diminished, late-flowering plants gained a thrust in plant growth, with increased branches, leaves and eventually entered from vegetative to reproductive state. Amplification (with *PHYB* primers) of total DNA extracted from these late-flowering plants revealed that *Arabidopsis PHYB* gene is present in all 19 plants tested.

Table 3.10. Leaf chlorophyll content and average number of leaves per plant at bolting and at first flowering, in T2 transgenic plants

Line	Leaf chlorophyll content [a]		Average leaves per plant	
	at bolting	at flowering	at bolting	at flowering
T5(2) population (n=187)	44.2*	43.2*	6.6*	8.7*
	(3.4)	(4.6)	(1.3)	(1.1)
T5(2) Late-flowering (n=19)	47*	46*	9.6*	12.5*
	(2.7)	(2.3)	(2.1)	(1.3)
Control (n=30)	40.1	38.6	5.8	7.5
	(2.4)	(2.2)	(1.2)	(1.4)
T9(2)selected (tallest) (n=50)	44.6*	43.8*	6.8	9.5*
	(3.6)	(2.3)	(1.2)	(1.1)
T9(2)selected (shortest) (n=50)	45.3*	45.0*	6.8	8.9*
	(2.2)	(2.5)	(1.3)	(1.2)
Control (n=10)	41.5	37.8	6.4	7.9
	(2.3)	(2.1)	(1.4)	(1.2)

Values are means and standard deviations given in brackets, n= number of individual plants. [a]Chlorophyll content represented by the measured SPAD value

* denotes a statistically significant difference from the control at P=0.05

Leaf chlorophyll content was significantly higher in transgenic plants than non-transgenic controls, which reached a maximum value at the time of bolting and started to decrease instantaneously when plants attended flowering. The highest average chlorophyll content was observed with late-flowering plants (Table 3.10). Chlorophyll degradation was fairly a decelerated process in the transgenic plants when compared with non-transgenic control plants, a phenomenon also observed with greenhouse grown primary transformants (T1plants).

3.4 Discussion

The pronounced inhibition of hypocotyl elongation observed with *in vitro* seedlings (under light conditions) in this study resembles the short hypocotyl phenotype described for light-grown transgenic tobacco seedlings (McCormac et al. 1993a, Halliday at al. 1997) and with transgenic *Arabidopsis* seedlings (Wagner et al. 1991), overexpressing either an *Arabidopsis* or a rice *PHYB* gene under the control of CaMV35S promoter. The short hypocotyl phenotype is strictly light-dependent as no significant difference in hypocotyl length was observed between transgenic and non-transgenic seedlings, when grown in complete darkness (see Fig 3.4). This observation is in agreement with Wagner et al. (1991) and Halliday et al. (1997). Exhibition of a light-dependent short hypocotyl phenotype in *B. napus* seedlings in this study could therefore be considered as an indirect proof of a successful integration of the *Arabidopsis PHYB* gene into the *B. napus* genome and production of biologically active phyB. Overexpression of oat *PHYA* cDNA in *Arabidopsis* (Bagnall et al. 1995) and in tobacco (Halliday et al. 1997) has also resulted in exaggerated shorting of hypocotyls, indicating both phytochromes, A and B, are capable of regulating hypocotyl elongation. However, more detailed physiological analysis of overexpressers and mutants have revealed distinct photosensory roles for these two phytochromes. Transgenic seedlings of tobacco and *Arabidopsis* overexpressing an oat *PHYA* cDNA displayed an enhanced sensitivity to prolonged exposure to FR with respect to the inhibition of hypocotyl elongation (McCormac et al. 1992, Whitelam et al. 1992). In contrast, transgenic *Arabidopsis* seedlings overexpressing either *Arabidopsis* or rice *PHYB* cDNA displayed wild-type responsiveness to prolonged FR, as do seedlings of the *phyB* mutant (McCormac et al. 1993a). Hence, responsiveness to prolonged FR would appear to be an intrinsic property of phyA which is not shared by phyB. The photophysiology of transgenic seedlings overexpressing a particular type of phytochrome appears to be the exact opposite of the photophysiology of mutants that are null for the same phytochrome. For instance, etiolated *Arabidopsis* seedlings overexpressing *PHYB* cDNAs displayed increased sensitivity to red light, with respect to the inhibition of hypocotyl elongation, which is in contrast with the selective lack of responsiveness to red light displayed by the phyB deficient *phyB* mutants (McCormac et al. 1993a). This complementary effect is not limited for hypocotyl elongation, but also for some other important photoresponses such as seed germination, chloroplast and leaf development, stem elongation and flowering (Reed et al.1993, McCormac et al. 1993b, Bagnall et al. 1995).

The *ein* mutant in *B. rapa* was found to be partially dominant, where heterozygous *EIN/ein* seedlings displayed an elongation response which was intermediate between *EIN/EIN* (wild type) and *ein/ein* (mutant homozygous), under white light culture conditions (Devlin et al. 1997). *EIN/ein* was found to contain about 50% of the level of immunochemically detectable phyB of equivalent wild type *EIN/EIN* seedlings. These observations provide evidence that the level of functional phyB produced is proportional to the number of copies of the *PHYB* gene present in the plant. In the same way, the photoresponses of the plant are proportional to the amount of biologically functional phyB. It appears that the amount of phyB produced by wild type *PHYB* allele is poised at a concentration that allows a plant the maximum sensitivity to changes in the light environment. Thus, any change to this steady state level of phyB could result in large elongation growth response under low R:FR ratio in which phyB is active.

Transgenic seedlings segregating for kanamycin resistance indicated the presence of two copies of the transgene (*Arabidopsis PHYB*) in each transgenic line, T5 and T9 (Table 3.6). Therefore, in the segregating plant population, there would be one non-transgenic plant for every 15 transgenic plants and one homozygous (*PHYB*/*PHYB*) transgenic plant for every fourteen heterozygous (*PHYB*/-) transgenic plants. In this study, transgenic *B. napus* seedlings (grown *in vitro* under white light) segregating for short hypocotyls phenotype consisted of very short, intermediate and long hypocotyl seedlings (Fig. 3.5). The PCR analysis confirmed that all short and intermediate tall seedlings germinated *in vitro* were carrying the transgene while those displaying a hypocotyl elongation similar to non-transgenic control seedlings, did not (Fig. 3.7). Those intermediate seedlings could therefore be the heterozygous seedlings whilst those shortest could be homozygous. The amount of hypocotyl elongation (in this study under *in vitro* conditions) could be a true reflection of the genotypic state of transgenic seedlings, thus could be used as a phenotypic marker to identify potential transgenic plants. These observations are in agreement with findings of those of Devlin et al. (1997), where hypocotyl elongation was proportional to the number of *PHYB* gene copies present in the plant and the amount of phyB produced. However, success with such a selection criterion seems reliant on certain optimal culture conditions, such as intensity of light provided, duration of the light regime and culture temperature, since selected short and long hypocotyl seedlings in this study, under

greenhouse conditions without controlled environment, proved to be equally transgenic (Table 3.7).

Contrary to greenhouse grown plants, T1 transgenic plants and corresponding non-transgenic plants did not show a noteworthy difference in plant height under *in vitro* culture conditions (however this was not studied *in vitro* in a replicated trial). The same observation has been made with transgenic potato (overexpressing *Arabidopsis PHYB* cDNA) grown under *in vitro* conditions where no significant difference in plant height was noted between transgenic and wild type plants (Thiele et al. 1999).

In the greenhouse, primary transformants (T1 plants) showed 24% height reduction at flowering when compared with non-transgenic controls, but it was reduced to 20% at maturity (Table 3.4). In the T5(2) population, there was 22% and 20% height reduction at bolting and first flowering, respectively, but this dwarf nature was indeed provisional since at maturity transgenic plants averaged 96% of the non-transgenic control plants (Table 3.4). Similar results have been observed with transgenic potato overexpressing *Arabidopsis PHYB*, under greenhouse conditions where the early semidwarf nature of transgenic plants diminished when plants attained maturity (Thiele et al. 1999, Schittenhelm et al. 2004). Observed increment in average plant height in the T5(2) plant population could be to some extent, due to the contribution by some elongated plants in the population. Since T5(2) represents a segregating plant population for transgene *PHYB*, it is composed of heterozygous, homozygous as well as non-transgenic plants. It is reasonable to assume that, non-transgenic plants, under dense plant population, would resort to typical shade avoidance responses, such as stem elongation and early flowering, to gain an upper hand over the other plants, thus creating an asymmetrical type of competition within the population. This asymmetrical competition may have stimulated transgenic plants to 'forage for light' by resorting to shade avoidance responses. However, it is obvious that transgenic plants responded to shade much later than those non-transgenic plants. This late response to shade is evident by the fact that transition from vegetative to reproductive state in transgenic plants occurred much later than in non-transgenic plants. The reason could be that increased phyB level in transgenic plants (as a results of overexpression) is capable of alleviating shading effects until the R:FR ratio drops under a certain threshold. This means increased phyB pool neither completely disables nor eliminates shade avoidance responses of transgenic plants, rather it restrains or delays shade avoidance responses. 19 late-flowering plants observed with T5(2) plants population could represent the homozygous

fraction of the segregating population. Late-flowering plants may possess more *PHYB* gene copies and consequently could produce more functional phyB than those heterozygous counterparts. Increased concentration of phyB in late-flowering plants, could efficiently restrain shade avoidance responses much longer, even under a very low R:FR ratio. This is evident by the fact that, late-flowering plants remained in the vegetative state much longer than rest of the plants and resort to flowering only after the shade by the neighbouring plants is considerably reduced. Late-flowering plants remained significantly shorter than control plants at all developmental stages (Table 3.8). Similarly selected shortest plants of T9(2) population also remained significantly shorter than control plants at all times and reached only 83% of the non-transgenic plants at maturity. This may be a consequence of initial selection (even though it was found to be unsuccessful under the greenhouse conditions, Table 3.7), which may have allocated more potential homozygous plants into this group which remained significantly shorter reducing the average plant height at maturity.

Most of phenotypical and phenological changes which could be directly linked to *PHYB* overexpression were confined mainly to leaves, as it was observed in this study as well as in previous studies with transgenic potatoes (Thiele et al. 1999, Schittenhelm et al. 2004) and tobacco (Robson et al. 1996). Leaves of T1 plants were significantly thicker, heavier and yet comparatively smaller than those from non-transgenic control plants. Anatomical studies of leaves in transgenic potatoes overexpressing *PHYB* have revealed that increased leaf thickness was basically due to the increased length of palisade cells in leaf mesophyll, which were 19-30% longer than the wild type palisade mesophyll cells (Thiele et al. 1999). Increased fresh leaf weight per unit area in transgenic plants in this study may be a direct consequence of thicker leaves and increased production of assimilates in leaves. Further studies, preferably with homozygous plants, would be necessary to establish whether increased thickness and fresh leaf weight is contributing positively to increase the total biomass of transgenic *B. napus* plants. In such a study, dry leaf weight would be a more reliable index than fresh leaf weight. A close relationship was observed between flowering time and number of leaves per plant. Plants that flowered late had a higher number of leaves than those plants flowered earlier. For instance, late-flowering plants had significantly a higher number of leaves than non-transgenic plants at bolting or at first flowering (Table 3.10). A similar observation has been made with transgenic phyB overexpressing *Arabidopsis* plants in which late-flowering plants (due to a low-fluence end

of the day light treatment) were having significantly higher number of rosette leaves than those early flowering plants (Bagnall et al. 1995). Hence, increased leaf number could be a direct consequence of the prolonged vegetative phase experienced by transgenic plants.

Leaf chlorophyll content of transgenic plants remained significantly higher than non-transgenic plants, a trait that was observed in all transgenic plants in common. The highest concentration of leaf chlorophyll was registered by those late-flowering plants, which was 18% higher than the controls (Table 3.10). Chlorophyll degradation (initiated when plants shifted from vegetative to reproductive state) occurred at a much decelerated rate in transgenic plants than controls (Fig. 3.3). In transgenic potato, a decreased rate of chlorophyll degradation and 35% more leaf chlorophylls production have been reported, when *PHYB* is overexpressed (Thiele et al. 1999). Transgenic tobacco plants overexpressing *PHYA*, has yielded 30% more chlorophyll per unit leaf area than controls (Robson and Smith 1997). It has been well established that, synthesis of chlorophyll *a* and *b* is closely related with the far red absorbing form of phytochromes (Pfr) (Oelze-Karow and Mohr 1981). It is reasonable to suppose that, increased production of Pr due to phyB (or phyA) overexpression could elevate the total Pfr pool after absorbing red light during the day time. Pfr is involved in accumulation of an important upstream component (5-aminolevulinate) of chlorophyll biosynthesis pathway which stimulates a higher production of chlorophyll (Kasemir and Mohr 1981). A higher photosynthesis rate in leaves of transgenic potatoes (overexpressing phyB) has been reported, where increased photosynthesis rate is proportional to the amount of chlorophyll produced (Thiele et al. 1999). However, the benefits of excess chlorophyll production would depend largely on the characteristics of chloroplast produced, since aberrant chloroplasts produced in transgenic tobacco overexpressing phyA, has hampered the photosynthesis performance of transgenic plants (Sharkey et al. 1991). How this increased chlorophyll pools effects photosynthesis performance in transgenic *B. napus* plants merits further investigations.

In *Arabidopsis,* it has been found that phyA and phyB regulate-flowering time in antagonistic manner. PhyA, which discriminates short days from long days, weakly promotes flowering in long day conditions whereas phyB acts to delay flowering under both long and short day conditions (Mouradov et al. 2002, Cerdán and Chory 2003). It has been suggested that phyB could have an inhibitory effect on PHYTOCHROME AND FLOWERING TIME1 or PFT1, a protein that supposed to act downstream of phyB, which

could otherwise activate its downstream target FT (Flowering Locus T) (Cerdán and Chory 2003). In *Arabidopsi*s *phyB* mutants, flowering has been accelerated due to the lack of functional phyB (Reed et al. 1993, Devlin et al. 1997). If the effects of overexpression are the antithesis of null-mutants (which was the case for other photomorphogenic responses such as, stem elongation and chlorophyll development) a late-flowering could be anticipated in phyB overexpressers. However, this was not clearly established in other studies carried out with phyB overexpressers and response to flowering may be species-specific. No significant difference in flowering has been observed between transgenic and wild type potato plants overexpressing phyB (Thiele et al. 1999). In transgenic *Arabidopsis*, phyA overexpressers flowered earlier under a high R:FR ratio, while phyB overexpressers exhibited a wild type response to flowering (Bagnall et al. 1995). Contrary to these observations, phyA and phyB overexpressors of day sensitive tobacco (short-day) have demonstrated an exaggerated delay in flowering (up to 8 months) under long day conditions than the wild type plants while under sort day conditions phyB overexpressors showed no significant difference than corresponding wild type plants. In response to a 30 min night break, phyB overexpressors caused a significant delay in flowering (Halliday et al. 1997). These observations demonstrate that responses to flowering depend mainly on the type of radiation provided and photoperiod to which plants was exposed. Apart from light signals, temperature is found to be the other most important environmental factor that regulates flowering time (Simpson and Dean 2002). It has been found that early flowering phenotype of *phyB* mutant in *Arabidopsis* was temperature dependent. When grown under constant temperature of 22ºC *phyB* showed an early flowering, while those *phyB* mutant plants grown under 16 ºC were late-flowering under both SD and LD conditions (Halliday et al. 1997). In this study flowering was significantly delayed in supposed homozygous *B. napus* plants (described as late-flowering plants), presumably with high phyB concentrations. Delayed flowering in these plants could be due to increased phyB pools, which would have down-regulated FT in a similar biological pathway described by Cerdán and Chory (2003). However, the effect of phyB overexpression seems mostly on flowering time but not on the duration of flowering, as no significant difference in duration of flowering was observed between late-flowering and corresponding controls plants (Table 3.9). In this study T1 transgenic plants flowered about one week earlier than controls (Table 3.4). The reason here could simply be a maturity or environmental effect rather than any detrimental effect by phyB to cause an early flowering. On the other hand the origin of T1 and T2 plants is also different, since T1 plants were vegetatively propagated while T2 were seed raised.

Therefore flowering times of T1 and T2 plants could not be directly compared. Whether late-flowering phenotype observed in this study was a true manifestation of phyB overexpression or whether it is due to any other environmental factor, such as light intensity or temperature could be well established under controlled environmental conditions.

Nineteen late-flowering *B napus* plants in this study, displayed the most of classical pleiotropic effects described previously for potato (Thiele et al. 1999) and tobacco (Halliday at al. 1997) after phyB overexpression. These included decreased apical dominance, excessive branching, small but large number of leaves and increased chlorophyll production which are the antithesis of null mutants of *PHYB*. However, these pleiotropic effects were not straightforward in T1 plants and 187 transgenic plants in the T2 segregating population. The main reason for this could be that, the endogenous production of phyB in these transgenic plants would not have been sufficient enough to make a big impact on biological and physiological processors of the plant to make drastic changes to the basic plant architecture. In transgenic potato, pleiotropic effects were found to be predominant in strong phyB overexpressing line Dara-12 but not in the moderately expressing line Dara-5 (Thiele et al. 1999). In a similar observation with *Arabidopsis*, photomorphogenic responses were closely related to the number of *PHYB* copies which in turn is proportional to the immunochemically detectable phyB level in the transgenic plants (Wester et al. 1994). These observations suggest a strong correlation between number of *PHYB* copies, amount of phyB produced and expression of pleiotropic effects.

Shade avoidance responses could be beneficial for individual plants in their natural environment, but could be deleterious for the growth of the crop in monoculture in modern agricultural setup. Despite strong selection for better yield, modern crops still react to a drop in photon fluence rate resorting to shade avoidance responses such as stem and petiole elongation (Ballaré et al. 1997). It is assumed that these elongation responses might tap assimilates that the plant would otherwise allocate to agronomically important storage organs. Therefore it has been proposed that disablement of shade avoidance responses or producing plants which are less sensitive to a low R:FR ratio would pay off to increase agricultural productivity, by reducing the wasteful resources allocation for stem elongation (Smith 1995, Ballaré et al. 1997). Transgenic manipulation of relative levels of phyA and phyB can markedly modify the allocation of assimilates in crop plants in the field has often

been suggested; however experimental support for this is still emerging. Transgenic potatoes overexpressing phyB has resulted in higher photosynthesis performance, greater biomass production and extended underground organs with increased tuber yields (Thiele at al. 1999). In a separate study using the same transgenic plant materials Schittenhelm et al. (2004) reported most of the observations of Thiele at al. (1999) but found no significant difference in tuber and total biomass between transgenic and non-transgenic plants. In the present study, transgenic *B. napus* plants overexpressing phyB resulted in a higher number of relatively thicker leaves with higher chlorophyll content (Table 3.3). Number of seeds per silique in two transgenic lines; T5(1) and T9(1) was significantly higher than corresponding control plants (Table 3.4). However, there was no significant difference in seed yield per plant between transgenic and control plants. Thousand kernel weight was significantly lower in transgenic plants than in the controls (Table 3.4). These observations suggest that, even though there was an increase in number of seeds produced by transgenic plants, the actual size of a single seed is becoming smaller than those of controls. In other words, seed number in transgenic plants increased more than the seed yield with the consequent reduction in weight per seed. A similar observation has been reported with phyB overexpressing transgenic potatoes where transgenic plants resulted in higher number of small tubers with reduced weight (Boccalandro et al. 2003). It seems that phyB overexpression results in an increase number of sinks in transgenic plants. It has been suggested that increased photosynthesis observed with transgenic potato phyB overexpressers could be to meet the higher demand for assimilates by increased number of tubers (Boccalandro et al. 2003).

PhyB overexpression in *B. napus* had no significant effect on major seed storage components such as oil and protein content. Results obtained in this study also do not provide conclusive evidences to prove the hypothesis that phyB overexpression allocate more resources to reproductive and storage organs. The observed low response to shade displayed by transgenic *B. napus* plant in this study could pay off to improve total biomass of individual plants under increased planting densities, as a consequence of increased production of photosynthetically active leaves. PhyB overexpressing potatoes has resulted in increased tuber yield under high planting densities (Boccalando et al. 2003). When grown at low planting densities, transgenic tobacco expressing an oat *PHYA* cDNA and wild type had similar architecture, but when planting density was increased the transgenic *PHYA* expressers became shorter displaying less shade avoidance responses than wild type,

which showed a greater response to shade at the expense of leaf development (Smith 1995). These observations suggest that best performance of phytochrome overexpressers could only be achieved at higher planting densities. Phytochrome overexpressers could therefore be used in breeding programs to shift optimum crop densities to higher values and to achieve an increase in number of individual plants per unit area, which may possibly result in proportional increment in crop yield.

In spite of possible advantages of disturbed photomorphogenic response by phytochrome overexpression, several negative effects have also been outlined. It has been found that plants lacking normal responses to R:FR ratio could simultaneously fail to optimize the positioning of light harvesting organs (Ballaré et al. 1997). Disabled shade avoidance of plants may be disadvantageous when facing intraspecific competition at high planting densities where FR insensitive plants undergo size structuring, which results in an asymmetrical type of competition within the plants population. This increased size structuring has shown to reduce the harvest index and crop yield (Ballaré and Scopel 1997). Even though, *phyB* overexpressing *B. napus* plants in this study resulted in reduced plant height which could reduce lodging under field conditions, presence of unfavourable traits such as small seed size and extremely delayed flowering make these plants difficult to fit in to a rapeseed breeding program.

3.5 Conclusions

Ectopic expression of *Arabidopsis PHYB* cDNA under the control of constitutive CaMV35S promoter in *Brassica napus* has resulted in photomorphogenic responses. A strictly light-dependent short hypocotyl phenotype was observed with transgenic seedlings under *in vitro* culture conditions. Exhibition of light-dependent short hypocotyl phenotype is considered to be the result of successful assembly of transgenically produced heterologous phytochrome apoproteins with endogenous chromophores to produce photoactive phyB. Photomorphogenic responses (such as hypocotyl elongation) seem closely associated with the transgene copy number and the level of phyB produced. Kanamycin sensitivity test and PCR analysis suggested the presence of two transgene copies in T5 and T9 transgenic lines. Supposed homozygous plants in the segregating population (T2) demonstrated a higher degree of pleiotropic effects under greenhouse conditions, such as reduced apical dominance, higher leaf chlorophyll content and extremely delayed flowering. There was no marked morphological difference between the

rest of 187 plants in the T5(2) population and non-transgenic control plants. Observed semi dwarfism with transgenic plants was mostly restricted to early developmental stages and gradually diminished when plants attained maturity. 19 late-flowering plants in the T5(2) population produced relatively smaller but a higher number of leaves which could be attributed to their prolonged vegetative phase than corresponding non-transgenic control plants. Number of seeds in transgenic plants increased more than the seed yield with the consequent reduction in weight per seed. Decreased plant height observed with late-flowering plants could reduce lodging under field conditions, however extremely delayed flowering makes these plants difficult to fit in to a crop rotation in the field conditions. How these plants behave in a controlled environmental condition or at higher planting densities merit further investigations. Such studies may help to expose the true consequence of phyB overexpression in *Brassica napus*.

Summary

Oilseed rape (*Brassica napus* L.) is a renowned crop worldwide for its edible oil as well as a source of protein in animal feed. Thus *Brassica napus* is an important target for crop improvements. *Agrobacterium*-mediated transformation has proved to work efficient for genetic engineering of this crop. However, transformation efficiency seems to vary among different genotypes, and the need to develop an improved transformation protocol still remains. The majority of successful transformation systems hitherto described for *B. napus* use diploid plant materials such as hypocotyls, cotyledons, and cotyledonary petioles. Owing to the diploid character of these materials, first generation transgenic plants (T_1) are hemizygous for the transformed gene(s). When multiple unlinked transgenic copies are present in the plants, much time and labour has to be invested to produce homozygous plants by repeated selfing or by applying the doubled haploid technology. The use of haploid cells and tissue explants could provide an alternative approach for efficient transformation of oilseed rape, since haploid transgenic plants will become homozygous in one step after colchicine treatment. This study reports the potential of using leaf and petiole explants of haploid plants propagated *in vitro* for an efficient *Agrobacterium*-mediated gene transfer. The haploid plants were obtained via microspore culture from *Brassica napus cv.* Drakkar.

Compared to a standard protocol for hypocotyl segments, significantly higher callus production (P=0.05) with leaf explants was achieved when cultured in callus induction medium *CIM-L* (MS basal medium supplemented with 1 mg/l BAP, 0.1 mg/l NAA, 0.1 mg/l GA_3, 5 mg/l AgNO3 and solidified with 5 g/l Agarose). For petiole explants *CIM-P* medium (MS basal supplemented with 2 mg/l BAP, 0.01 mg/l picloram, 5 mg/l AgNO3 and solidified with 5 g/l Agarose) had a significant positive effect (P=0.05) on callus production. Highest shoot regeneration was achieved in DKW medium supplemented with 1 mg/l BAP, 0.1 mg/l NAA, 0.1 mg/l GA_3, and 5 mg/l AgNO3 and solidified with 5 g/l Agarose. Analysis of the ploidy showed that 96% of plantlets regenerated from leaf and 92% from petiole explants remained haploid. Using *Agrobacterium tumefaciens* strain AGL0, which carried the binary plasmid pAK-UGI 9-1, a transformation efficiency of 1.8% and 2.9% was achieved for leaf and petiole explants, respectively. Presence of GUS gene in regenerated plantlets was confirmed by PCR. Results indicate that transformation of leaf and petiole explants derived from *in vitro* propagated haploid plants provides a suitable alternative for generation of homozygous transgenic plants in rapeseed.

Lodging could be a serious problem in oilseed *Brassica napus*. Many current *B. napus* cultivars are prone to lodging inflicting heavy yield losses. Lodging could be decreased by reducing plant height. This study investigated the possibility of reducing plant height by overexpression of *A. thaliana PHYB* gene in *B. napus* plants. Ectopic expression of *Arabidopsis PHYB* cDNA under the control of constitutive CaMV35S promoter in *Brassica napus* caused pleiotropic effects such as decreased apical dominance, a higher number of small leaves and increased leaf chlorophyll content. Most of physiological and phenotypical changes, which could be directly linked to *PHYB* overexpression, were confined mainly to leaves. Leaves of primary transformants (T1 plants) were significantly thicker and comparatively smaller than those from non-transgenic control plants. Leaf chlorophyll content was significantly higher in transgenic plants than in non-transgenic plants. However, no significant difference in seed yield per plant was observed with T1 plants when compared with non-transgenic control plants. A strictly light-dependent short hypocotyl phenotype was observed with transgenic seedlings under *in vitro* culture conditions. Supposed homozygous plants in the segregating population (T2) demonstrated a higher degree of pleiotropic effects under greenhouse conditions. These plants exhibited substantially modified plant architecture with reduced apical dominance and plant height. Beginning of flowering was delayed by 79 days when compared with control plants. Even though *PHYB* overexpressing plants resulted in reduced plant height, which could reduce lodging under field conditions, presence of unfavourable traits such as low seed yield and delayed flowering make these plants difficult to fit into a rapeseed breeding program.

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Curriculum Vitae

Personal data

Name	Kolitha Bandara Wijesekara
Date of birth	25th August 1970
Place of birth	Kandy, Sri Lanka
Nationality	Sri Lankan

Educational qualifications

2004 to date	Studying at the Department of Crop Science, Georg-August University, Göttingen, for the award of PhD. Degree in Plant Breeding
1999-2002	M.Phil. in Plant Reproductive Biology, at the Post Graduate Institute of Science, University of Peradeniya, Sri Lanka
1993-1997	B.Sc. degree in Biological Science from the University of Peradeniya, Sri Lanka
1977-1990	Elementary, secondary and high school education from Kingswood College, Kandy, Sri Lanka

Professional career

1998 to date	Working as a Research Assistant (Grade I) at the Department of Plant Biotechnology of Institute of Fundamental Studies, Hantana road, Kandy, Sri Lanka
1997-1998	Worked as an Assistant Lecturer in the Department of Botany, University of Peradeniya, Sri Lanka

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