

Regeneration and Transformation of Oilseed (*Brassica napus*) Using CaMV 35S Promoter- β -glucuronidase Gene

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ABSTRACT

CaMV 35S promoter activity has not been analysed in different stages of growth in oilseed (*B. napus*). Higher plants lack intrinsic β -glucuronidase activity, thus enhancing the sensitivity with which measurements can be made. The binary transformation vector pVW432 (Buchanan-Wollaston, unpublished), carrying the GUS gene fused to the CaMV 35S promoter, was used to test transformation efficiencies and promoter activity in *B. napus*. The effect of different components of culture media was tested. Having studied different antibiotics, two selectable markers, and *Agrobacterium* strains, the combination of 500 mg/l carbenicillin with 25 mg/l kanamycin was the optimum level for inhibition of *Agrobacterium* strain C58pGV3101 and the selection of transformants, respectively. Different developmental stages of plants were analysed by protein assay. DNA extraction and PCR amplification confirmed the presence of the gene. High level of β -glucuronidase activity, hence CaMV 35S promoter activity, was observed for one of the transgenic plants. The β -glucuronidase activity altered from maturity to senescence indicating that the CaMV 35S promoter has different activity at different developmental stages in *B. napus*. Therefore, the growth stage of *B. napus* may influence the CaMV 35S promoter activity.

Keywords: CaMV 35S Promoter, β -glucuronidase (GUS) activity, *B. napus*, Growth stages, Transformation.

INTRODUCTION

The 35S RNA promoter is a strong promoter, and necessarily so, because it drives the synthesis of an RNA that serves as a nonreusable template for CaMV synthesis [21]. It drives high levels of RNA production in a wide variety of monocotyledonous and dicotyledonous plants [6, 7, 14]. This promoter has been considered to be constitutive [5], but highly-sensitive methods have shown some tissue-specificity [4] and cell cycle stage-dependent expression [20, 22].

The GUS gene has been used to determine the efficiency of transformation in many plants including *B. napus* [23, 27, 28]. Higher plants lack intrinsic β -glucuronidase

activity, thus enhancing the sensitivity with which measurements can be made. Histochemical analysis demonstrates the localisation of gene activity in cells and tissues of transformed plants [2, 16, 26, 28]. Using the histochemical stain, one may detect transformed cells soon after infection and examine parameters such as the effectiveness of the *Agrobacterium tumefaciens* strain [15, 23]. Plants expressing GUS are normal, healthy, and fertile. GUS is very stable, and tissue extracts continue to show high levels of GUS activity after prolonged storage [16].

The functional analysis of the CaMV 35S promoter is important to determine the activity of the promoter that is often used to drive gene expression in many transgenic plants.

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When the regeneration and transformation efficiencies are optimised using the GUS gene, it is then possible to introduce other constructs using similar methods. The 35S-GUS fusion was used to identify the best *Agrobacterium* strain and transformation method for *B. napus*, to test and optimise the levels of appropriate antibiotics, selectable markers and some other components involved in the transformation, and finally to determine the promoter activity.

MATERIALS AND METHODS

Three different protocols [3, 19, 25 described as MoGen, Moloney, and Schroder, respectively] were tested for the *in vitro* regeneration and transformation of *B. napus*. The hypocotyls of 10 day-old seedlings were used in both the MoGen and Schroder methods, while the cotyledon petioles of 4-5 day-old seedlings were used in the Moloney method.

The binary transformation vector, pVW 432 (Buchanan-Wollaston, unpublished) carrying (a) the GUS gene fused to the CaMV 35S promoter, (b) the neomycin phosphotransferase (NPTII) gene which confers resistance to aminoglycoside antibiotics such as kanamycin and neomycin (Figure 1), and (c) a tetracycline resistance gene that was used to select for the *Agrobacterium* carrying the plasmid, was used to test transformation efficiencies. Three different strains of *Agrobacterium* were employed in

the transformation experiments with the GUS gene: C58pGV2260, C58pGV3101, and EHA101. Conjugation by triparental mating was used to insert the pVW432 plasmid into each *Agrobacterium* strain.

Using suggested antibiotics, the bacterial growth was not successfully inhibited in the primary experiments. To develop a rapid and efficient *Agrobacterium*-mediated transformation method, different concentrations and combinations of antibiotics (carbenicillin 200-500, cefotaxime 100-200, spectinomycin 10, ticarcillin 200-250, and vancomycin 200, all mg/l) were tested to investigate their effects on bacterial survival after transformation. Hypocotyls were co-cultivated with each *Agrobacterium* strain containing the GUS scorable marker and cultured on appropriate media. The effect of kanamycin on regeneration was studied using six different concentrations between 20 and 75 mg/l in two independent experiments. In addition, three different levels of neomycin from 50 to 200 mg/l were studied in three independent experiments.

PCR was carried out in a total volume of 25 μ l, containing 3 μ l template DNA and 22 μ l reaction mixture (final concentration of 1 \times Taq buffer, 2 mM MgCl₂, 200 μ M dNTP mix, 0.4 μ M primer, 0.2 Unit Taq polymerase). The cycling parameters consisted of a preliminary 5 min denaturation at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds 60°C (using the primers of GAGGCTATTCGGCTATGACT and CCA-

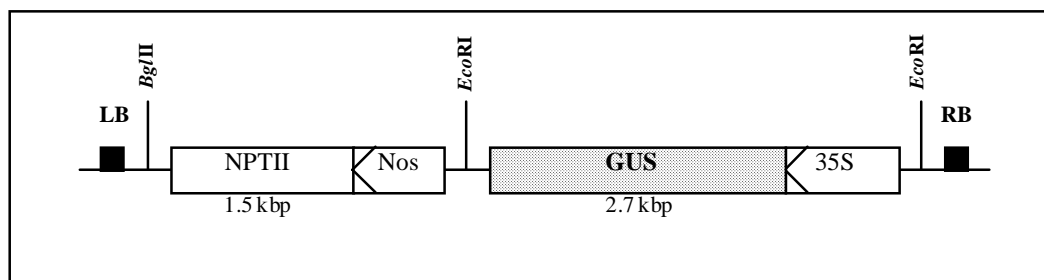


Figure 1. The GUS gene CaMV 35S promoter fusion in the vector T-DNA pVW432 plasmid. The T-DNA region of the pVW432 carries the tetracycline resistance gene, the NPTII gene under the control of the *nos* promoter and the GUS gene as a scorable marker.

ACGCTATGTCCTGATAG), and 120 seconds at 72°C. The amplification was completed with an incubation at 72°C for ten minutes. An aliquot of the amplification was analysed by agarose gel electrophoresis.

A Bio-Rad protein assay kit was used to determine protein concentration [8]. Histochemical localisation of the GUS reaction in transgenic plants was carried out by immersing a small section of leaf tissue in staining buffer (50 mM sodium phosphate buffer pH 7, 1 mM X-Gluc (5-bromo-4-chloro-3-indolylglucuronide), 0.1% Triton X-100, 4 mM potassium ferricyanide, 100 µg/ml chloramphenicol) for four hours at 37°C. The sample was cleared in several changes of 70% ethanol to remove the chlorophyll.

To extract GUS protein, leaf tissue was ground in 500 µl GUS lysis buffer (50 mM sodium phosphate buffer pH 7, 10 mM EDTA, 0.1% Sarkosyl, 0.1% Triton X-100, 10 mM 13-mercaptoethanol) and micro-fused for 15 minutes at room temperature. The supernatant was removed into a fresh tube and kept on ice until assayed.

The GUS activity was determined by enzymatic conversion of 4-methylumbelliferyl glucuronide (MUG) to 4-methylumbelliferone (MU) measured using a fluorimeter (Perkin Elmer LS30) as described by Jefferson *et al.* [16]. Ten µl of the protein extract in GUS lysis buffer was added to 440 µl of GUS lysis buffer and incubated at 37°C. The reaction was started by the addition of 50 µl of 10 mM MUG (1 mM final concentration) at regular 30 second intervals between each sample. At the time points of 15, 30, 60 minutes, 100 µl of the reaction mixture was removed and added to 2.9 ml of 0.2 M Na₂CO₃ buffer to stop the reaction. The fluorimeter was set with an excitation setting of 365 nm and emission setting of 455 nm, and calibrated with MU standards of 5 to 250 nM in the same buffer. The standard curve was read for each extract. The GUS activity of each extract was determined in nM MU produced/ min/ mg protein. Three independent extracts from the same leaf of the transformant were assayed three times and GUS enzyme activity was

measured. Data were analysed by analysis of variance using the ANOVA procedure in SAS (Statistical Analysis System) package.

In the investigation to optimise the concentrations of the selective antibiotics and other media components of interest, the MoGen method was used in all experiments, unless otherwise stated.

RESULTS

Among three studied protocols, the frequency of regeneration using the method of Schroder was 1.53 times that obtained with the MoGen method (49 regenerated shoots/ 49 hypocotyl explants =100% for MoGen, 49 regenerated shoots/ 32 hypocotyl explants =153% for Schroder). The regeneration efficiency of the Moloney method was not estimated in this study. Moloney *et al.* [19] reported 80% regeneration efficiency for non-cocultivated explants.

The methods of MoGen and Schroder were used in this part of the study. *Agrobacterium* strains C58pGV2260 and EHA101 survived in most of the antibiotic combinations tested. Culture on media with combinations of vancomycin and cefotaxime caused faster browning and enhanced vitrification of the callus in the Schroder method, while just the browning effect was detected in the MoGen method. Growth of *Agrobacterium tumefaciens* C58pGV3101 was inhibited by 500 mg/l carbenicillin in both methods (Figure 2-A). A combination of 500 mg/l carbenicillin and 200 mg/l cefotaxime successfully controlled the *Agrobacterium* C58pGV2260 in the MoGen method. A similar concentration of carbenicillin (500 mg/l) was used in the Moloney method and this successfully inhibited *Agrobacterium* C58pGV3101. The regeneration efficiency of untransformed hypocotyl segments was higher in medium containing carbenicillin and cefotaxime than in normal medium.

The optimum level of kanamycin for selection of transformants was 25 mg/l in the presence of *Agrobacterium* C58pGV3101 carrying pVW432, in which transformed

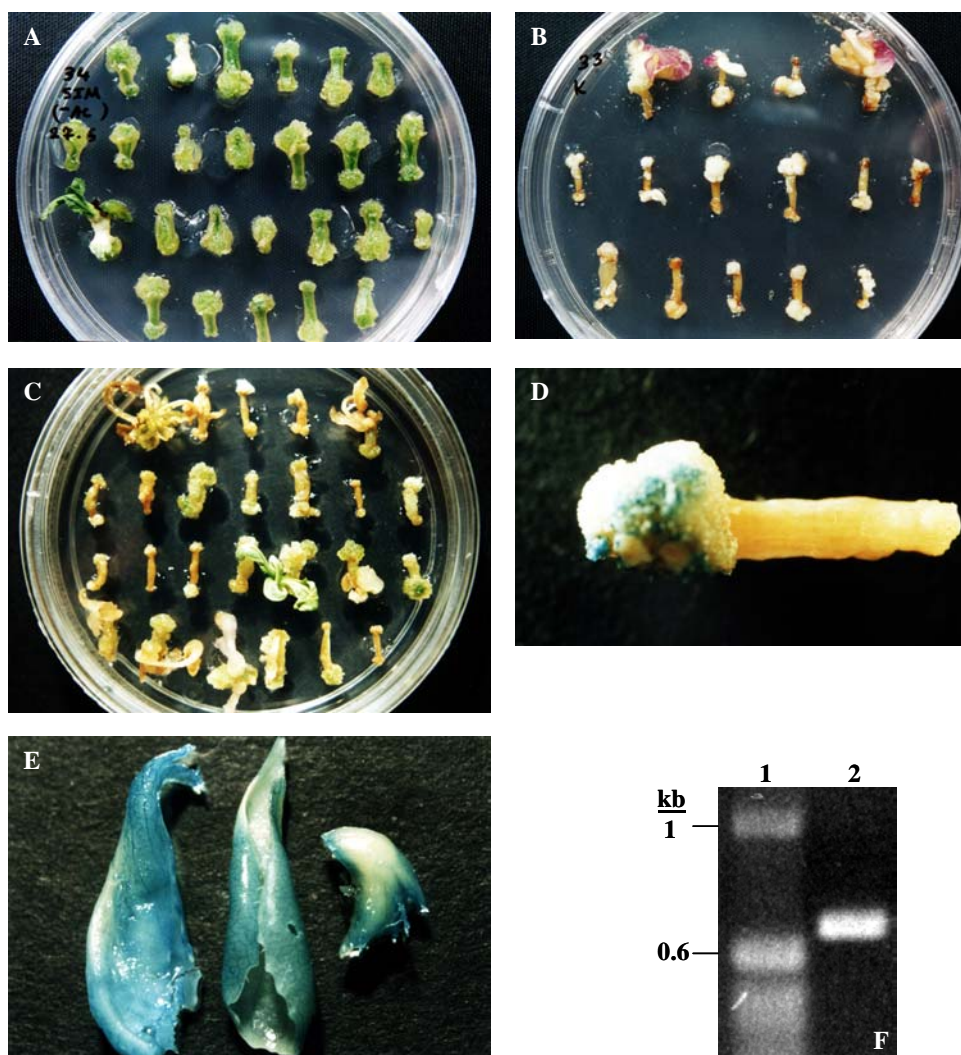


Figure 2. *B. napus* hypocotyls explants were regenerated in culture media to test different components for transformation as follows:

A: Co-cultivated hypocotyls explants on SIM with 500 mg/l carbenicillin to inhibit *Agrobacterium* C58pGV3101.

B: Non-cocultivated hypocotyls explants on SIM with 25 mg/l kanamycin to study efficiency of selectable marker.

C: Co-cultivated hypocotyls explants with *Agrobacterium* C58pGV3101 on SIM with 500 mg/l carbenicillin and 25 mg/l kanamycin showing shoot regeneration from transformed callus.

D: Co-cultivated explant exhibiting presence of β -glucuronidase in the transformed callus carrying GUS gene.

E: *B. napus* leaf showing a blue colour in the GUS staining, revealing the presence of β -glucuronidase. This test confirms a successful transformation.

F: Analysis of PCR product from DNA from transgenic GUS plant. The lanes No. 1 and 2 of plate represent, respectively, marker and PCR amplification of transformed *B. napus* plant.

shoots were confirmed by the β -glucuronidase assay. This level of kanamycin affected untransformed explants during a period 45-50 days after co-cultivation, after which explants were completely yellow with no green callus or shoot (Figure 2-B). Culture on media containing 200 mg/l of neomycin resulted in vigorously growing green

technical No. 3, when the same culture media of Schroder was used. Hence, the agar technical No. 3 was used for all methods. An optimum combination of method was selected to achieve successful transformation of *B. napus* cv Westar and this is summarised in Table 1.

For all methods, explants co-cultivated

Table 1. Components tested in regeneration experiments with *B. napus*. Regeneration rates were measured to select the method and components to optimise the *B. napus* regeneration

No.	Object of Test	Characteristics	Best Result
1	Experimental Methods	MoGen, Schroder, Moloney	1-Schroder 2-Moloney 3-MoGen
2	<i>Agrobacteria</i>	C58pGV2260, C58pGV3101, EHA101 (GUS gene)	C58pGV3101
3	Antibiotics	Carbenicillin, Cefotaxime, Vancomycin, Augmentin, Ticarcillin, Kanamycin, Neomycin	500 mg/l Carbenicillin 25 mg/l Kanamycin
4	Scorable Markers	Kanamycin, Neomycin	25 mg/l Kanamycin
5	Gelling Agents	Technical Agar No. 3, Phytigel	Technical Agar No. 3

Table 2. Efficiency of transformation using the different methods. The table shows the results from three different transformation methods used to introduce the GUS gene into *B. napus* plant.

No.	Objective	Conclusion (%)		
		MoGen	Schroder	Moloney
1	Frequency of green explants in kanamycin (green co-cultivated explants/ total co-cultivated explants)	4/389=1%	12/150=8%	8/50=16%
2	Frequency of transformed callus (transformed callus/ regenerated hypocotyl explants)	2/4=50%	7/12=58%	6/8=75%
3	Total frequency of transformed callus (transformed callus/ total co-cultivated explants)	2/389=0.51%	7/150=4.6%	6/50=12%

callus in up to 57% of cases. Therefore, a higher concentration of neomycin was required to select for real transformants.

Two different gel agents, phytigel and technical agar No. 3, were tested for their effects on regeneration. The explants exhibited increased vitrification and were also faster growing on phytigel than on agar

with C58pGV3101: pVW432 (GUS) were regenerated in relevant media containing 500 mg/l carbenicillin and 25 mg/l kanamycin (Figure 2-C). Histochemical GUS assays showed positive results for all methods, as determined by the presence of a blue precipitate in the transformed cells after staining in X-Gluc. Although, it was possible to

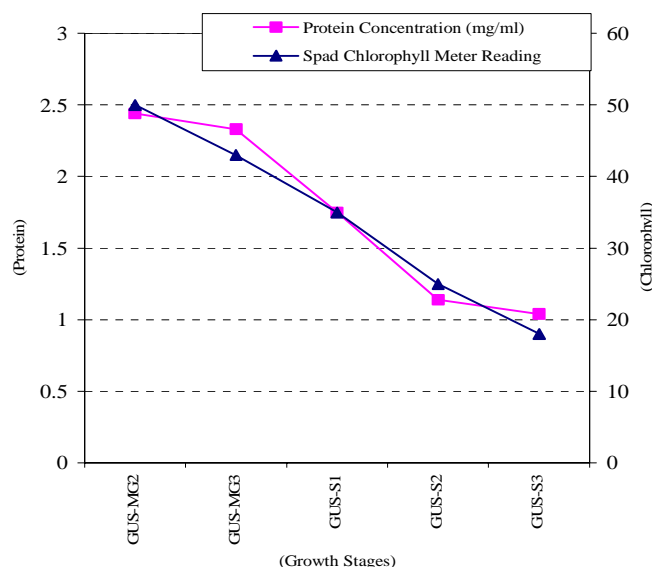


Figure 3. Analysis of the growth rate of transformed *B. napus*. Figure shows Spad chlorophyll meter reading and total protein extracted from different developmental stages of transformed plant. Spad chlorophyll meter reading and protein concentration were 39 and 1.92 mg/ml for wt-MG3, respectively. (S:senescence, MG:mature green, wt:wild type).

detect transformed areas of explants as early as 10 days after infection, but the areas were often very small and difficult to recognise (Figure 2-D). Routinely, GUS staining was measured 4-8 weeks post infection, by which time the transformed areas were easily detectable (Figure 2-E). The frequency of transformation using the Moloney method was higher than for the MoGen and Schroder methods (Table 2). Schroder *et al.* [25] and Moloney *et al.* [19] reported higher frequencies of regeneration of transformed plants, 22.3% and 60%, respectively, than were obtained in this study. Most of the shoots that regenerated from the explants on selection medium survived while a few bleached and died. Some of the differentiated shoots were vitrified on the SIM (shoot induction medium) in the Moloney method. Collectively, all methods tested were successful for both regeneration and transformation.

PCR experiments were performed to confirm the presence of the transforming T-DNA in the putative transgenic plants that

had been selected on kanamycin. DNA was isolated from the plants and, following PCR amplification using primers for the NPTII gene, a DNA fragment of the expected 0.68 kbp size was seen, when the amplified DNA samples were fractionated by electrophoresis on a 1% agarose gel (Figure 2-F).

To analyse plant developmental stages, the transformed plant were characterised by measurement of chlorophyll and total protein (Figure 3). A high level of GUS activity was observed in the transgenic *B. napus* plant expressing the β -glucuronidase gene. The level of GUS activity per mg protein in extracts from the different developmental stages was significantly different, when the transgenic and wild-type *B. napus* plants were statistically analysed (Table 3). GUS activity per extract was higher in the MG2 stage than in the other developmental stages, then it gradually decreased as approached to the last stage of plant growth (Figure 4, top line). This indicates that the CaMV 35S promoter activity decreases with the progress of senescence. However, protein lev-

Table 3. Brief results of analysis of variance for different developmental stages of transgenic plant carrying 35S-GUS fusion. Activities were significantly different at the level of %1. Comparison of treatment means was done in LSD method.

No.	Plant Developmental Stages	GUS Activity (pico moles MU/ min/ mg protein)	Result of Mean Comparison
1	GUS-MG2	585426±17769	bc
2	GUS-MG3	500020±15384	c
3	GUS-S1	522737±19293	c
4	GUS-S2	730668±37695	a
5	GUS-S3	684923±31759	ab
6	Buffer	301003±10698	d
7	wt-MG3	5956±2228	e

els are falling during senescence, therefore, the actual GUS activities per mg protein showed an increase. The activity per mg protein was lower in mature green leaves than in senescence (Figure 4, bottom line). It steady decreased from MG2 to MG3 and then increased with the initiation of senescence (51). The activity was highest in the S2 stage compared to the other stages.

DISCUSSION

Various methods were compared for the regeneration and transformation of *B. napus*, and they showed a wide variation in efficiency. The most efficient shoot regeneration system was achieved through the use of hypocotyl explants and the MoGen and Schroder methods. According to some stud-

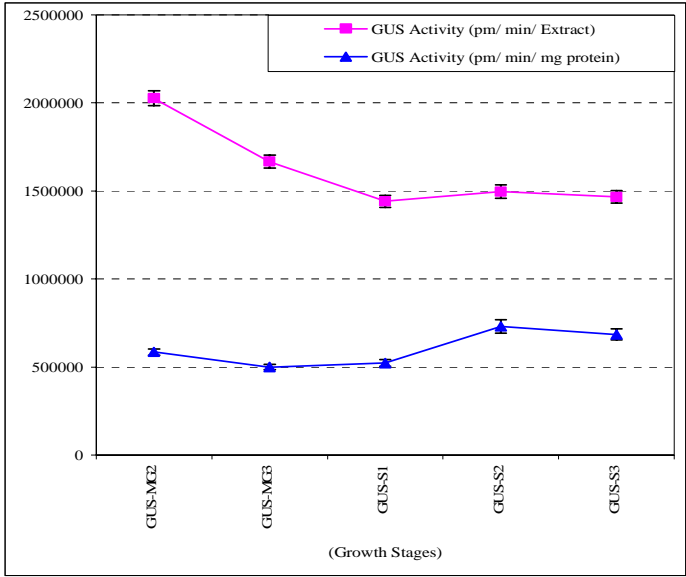


Figure 4. GUS activity in transgenic *B. napus*. GUS activity and standard error (shown as an error bar) were calculated for plant with three independent extracts from the same leaf. GUS activity of the control wild type plant (wt-MG3) was 5956±2228 pm/ min/ mg protein and 11445±4281 pm/ min/ extract. (pm: picomoles

ies [23,25] the shoot regeneration efficiency from hypocotyl explants is improved up to ten times by the addition of AgNO_3 to the medium. Moloney *et al.* [19] did not add AgNO_3 to the media composition (data are not shown). Although AgNO_3 improves shoot regeneration, it is not essential for achieving transformed shoots [9, 23]. However, AgNO_3 eliminates negative effects on shoot regeneration from certain antibiotics used to inhibit the growth of *Agrobacterium* [25]. This might be one of the reasons for the lower regeneration frequency of Moloney compared with the other two methods used in our study.

The *Agrobacterium* contamination problem was examined using hypocotyl explants with combinations of 5 different antibiotics and 3 *Agrobacterium* strains carrying the GUS gene. Carbenicillin inhibited the growth of *Agrobacterium tumefaciens* C58pGV3101 [2, 24] in all stages of subculture for all methods used in this study. Unlike *Agrobacterium* C58pGV2260, the *Agrobacterium* C58pGV3101 has no carbenicillin resistance gene in its disarmed Ti plasmid [11]. This could be one of the reasons why a combination of at least two different antibiotics was required to obtain inhibition of *Agrobacterium* C58pGV2260. Mixtures of different antibiotics have also been used to inhibit *Agrobacterium* EHA101 growth in some experiments [1,10].

Different concentrations of kanamycin (20-75 mg/l) and neomycin (50-200 mg/l) were tested to optimise the selection of transformants. The NPTII protein inactivates these two antibiotics. The regeneration of untransformed calli might increase with a lower concentration of kanamycin (less than 20 mg/l), since a similar result was observed in the *B. rapa* transformation experiments reported by Radke *et al.* [23]. Because a high level of kanamycin can inhibit the differentiation of shoot primordia, even in transgenic shoots, it could be difficult to obtain transformed shoots, although the inoculated explants produced calli carrying transgenic cells [12,18]. Hence, the calli were cultured in kanamycin free SIM for the first

two days after co-cultivation, and then transferred to kanamycin SIM. As well as inhibition of differentiation by kanamycin, other components such as *Agrobacterium* strain, plant variety, and transferred T-DNA may affect the regeneration frequency.

Analysis of GUS activity was carried out in *B. napus* transformants carrying the CaMV 35S-GUS fusion at five different stages of leaf development. The CaMV 35S promoter activity did show variation in the different developmental stages. It was found that the promoter activity varied considerably between organs and tissues at various developmental stages as previously showed by Stefanov *et al.* [26] and Stoger *et al.* [27] who found that the activity fell from the callus stage to the first leaf and later on fell again in the shoot during *in vitro* morphogenesis. The growth conditions of individual plant influences the promoter activity. For example, increases in activity of gibberellic acids or decrease in auxin concentration have been shown to cause a decrease in activity of the CaMV 35S and other promoters [17, 26].

Although the GUS activity did show some differences between the mature green and senescent stages, it was obviously high at both stages. Therefore, although reduction or increase of CaMV 35S promoter activity may occur with the other constructs, these activity differences will be unlikely to cause problems when analysing the transformants. Analysis of other general and also specific promoters upstream of the GUS gene might reveal more information about promoter activation in different tissues and plant growth phases of *B. napus* and these might be more useful for temporal or site specific control of gene expression.

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باززایی و تراریزش کلزا (*Brassica napus*) با استفاده از ژن GUS با پیش برنده CaMV 35S

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چکیده

میزان فعالیت پیش برنده CaMV 35S در مراحل مختلف رشد گیاه کلزا مشخص نمی باشد. گیاهان عالی فاقد آنزیم بتا گلوکورونیداز (β -glucuronidase) می باشند، بنابر این میزان فعالیت چنین آنزیمی در سلول گیاهی قابل اندازه گیری خواهد بود. پلاسمید مختص تراریزش گیاهی به نام pVW432 و حمل کننده ژن GUS تحت کنترل پیش برنده CaMV 35S برای اندازه گیری میزان باززایی و فعالیت پیش برنده در گیاه کلزا بکار گرفته شد. اثر مواد مختلف مرتبط با محیط کشت بافت مورد بررسی قرار گرفت. مطالعه ترکیبات مختلف آنتی بیوتیکها، مارکرهای انتخابی و آگروباکتریومها نشان داد که ترکیب ۵۰۰ و ۲۵ میلی گرم در لیتر به ترتیب برای کربنسیلین (Carbenicilin) و کانامایسین (Kanamycin) بهترین مقدار کنترل کننده رشد آگروباکتریوم سوش C58pGV3101 و انتخاب تراریزشها می باشد. مراحل مختلف رشد گیاه توسط میزان پروتئین مشخص گردید. حضور ژن بوسیله استخراج DNA و انجام PCR اثبات شد. فعالیت آنزیم بتاگلوکورونیداز، بنابر این فعالیت پیش برنده، در یکی از گیاهان تراریزش شده مشاهده گردید. نتایج نشان داد که میزان فعالیت ژن GUS از دوره بلوغ تا مرحله پیری گیاه متفاوت بوده که مبین فعالیت متفاوت پیش برنده CaMV 35S در دوره های مختلف رشد گیاه کلزا می باشد. در نتیجه شرایط رشد گیاه ممکن است بر میزان فعالیت پیش برنده CaMV 35S موثر باشد.