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# A study of different (CaMV 35S and *mas*) promoter activities and risk assessment of field use in transgenic rapeseed plants

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*Key words: Agrobacterium, Brassica napus,* CaMV 35S promoter, *mas* promoter, gene expression, risk assessment

## Summary

Gene fusions between the $\beta$-glucuronidase (GUS) reporter gene and the promoters of the cauliflower mosaic virus 35S RNA transcript (CaMV 35S) and the mannopine synthase (*mas*) genes were introduced into rapeseed varieties via *Agrobacterium*-mediated transformation. Fluorometric assay of $\beta$-glucuronidase activity indicated different expression patterns for the two promoters.

In seedlings, the CaMV 35S promoter had maximum activity in the primary roots, while the *mas* promoter was most active in the cotyledons. Etiolated seedlings cultured in the dark showed reduced activity of the *mas* promoter.

Before vernalization at the rosette stage, both promoters were more active in older plant parts than in younger ones. At this stage the highest activity was recorded in cotyledons.

After the plants had bolted reduced promoter function was detected in the upper parts of the transformed plants. Both promoters were found to be functional in the majority of the studied organs of transgenic rapeseed plants, but the promoter activity varied considerably between the organs at different developmental stages.

The ability of pollen to transfer the introduced genes to other varieties and related species (e.g. *Brassica napus* and *Diplotaxus muralis*) by cross-pollination was studied in greenhouse experiments, and field trials were carried out to estimate the distance for biologically – relevant gene dispersal. In artificial crossing, the introduced marker gene was transferable into other varieties of *Brassica napus*. In field trials, at a distance of 1 metre from the source of transgenic plants, the frequency of an outcrossing event was relatively high ($10^{-3}$). Resistant individuals were found at 16 and 32 metres from the transgenic pollen donors, but the frequency of an outcrossing event dropped to $10^{-5}$.

## Introduction

Genetic modifications and the introduction of new characters into crop plants have opened up new possibilities for analysis of the activity of different transcriptional control elements during the life time of the plant. The use of gene fusion constructs carrying a defined promoter sequence from the gene of interest and a reporter gene has contributed significantly to the understanding of the molecular components involved in the regulation of gene expression (Jefferson, 1987; Benfey et al., 1989). Currently one of the most widely used reporter genes is the *uid* A gene from *E. coli*, genetically modified to make it suitable for expression experiments in plants. It encodes for the protein $\beta$-glucuronidase. This enzyme catalyses the cleavage of several $\beta$-glucuronides, including commercially-available substrates. Fluorometric and colorimetric quantitative assays and procedures for tissue-specific detection by histochemical staining have been developed. It has been demonstrated that the expression pattern of a promoter may vary with the plant species (Harpster et al., 1988; Benfey et al., 1989; Blaich, 1992); thus, a comprehensive analysis of expression pattern is presumed to be based on a broader set of host plants. It has also been found that the

use of alternative reporter genes may result in different expression patterns (Jefferson et al., 1987; Schneider et al., 1990).

The promoter of gene IV of the cauliflower mosaic virus (CaMV) encodes the 35S RNA transcript. It drives the synthesis of an RNA serving as a non-reusable template for CaMV DNA synthesis. Although the natural host range of CaMV is limited to species in the Cruciferae, the promoter is active in the genome of the large variety of monocotyledonous and dicotyledonous plants (Harpster et al., 1988; Boulter et al., 1990; Blaich, 1992). This promoter has been considered to be constitutive (Odell et al., 1985; Benfey et al., 1989), but highly-sensitive methods have shown some tissue-specificity (Jefferson et al., 1987; Battraw & Hall, 1990) and cell cycle stage-dependent expression (Nagata et al., 1987). The dual bi-directional promoter TR1′-2′ is derived from the genes for mannopine synthase (*mas*) on the TR-DNA of an octopine-type Ti plasmid (Velten & Schell, 1985). This promoter has been preferably used for studies on the expression of chimeric genes both in transient experiments and in transgenic plants (Velten & Schell, 1985; Debleare et al., 1987; Saito et al., 1991; Stefanov et al., 1991). It has been assumed that its transcriptional activity is constitutive (Debleare et al., 1987; Rogers et al., 1987). Recent results, however, demonstrated the tissue-specific, organ-dependent and auxin-enhanced expression of the *mas* promoter (Langridge et al., 1989; Leung et al., 1991).

The aim of the present work was a detailed analysis of the expression pattern of these nominally constitutive promoters in *Brassica napus*, with GUS being used as the reporter gene. Results on the risk assessment of the field use of transgenic rapeseed are also described.

## Materials and methods

### *Plant material*

'Arabella' and 'Santana' rapeseed (*Brassica napus*) cultivars were used in the experiments. Transformation was achieved by *Agrobacterium* infection (Stefanov et al., 1994) using the following plasmids: plasmid pPCV701 GUS (a kind gift from Dr. A. Zilberstain, Tel-Aviv University, Tel-Aviv, Israel, and Dr. Cs. Koncz, Max-Planck-Institut für Züchtungsforschung, Köln, Germany) contains the *uid* A gene coding sequence under the control of the CaMV 35S promoter and linked to the *nos* termination signal (Koncz & Schell, 1986); plasmid pIS 412 was constructed by replacing an *Eco* RI/*Hind* III fragment of pGA 482 (An, 1987) with an *Eco* RI/*Hind* III fragment of the transient expression vector pIDS 411 (Stefanov et al., 1991), which contains the GUS coding sequence under the control of the *mas* promoter and the *nos* termination signal.

As hosts for the binary vectors, *A. tumefaciens* strains LBA 4404 (Ooms et al., 1982) and GV3101 (pMP90RK) (Koncz & Schell, 1986) were used. Before plant transformation, the structures of the binary vectors were checked by restriction endonuclease digestion of plasmid DNA.

Conditions of transformation, selection and plant regeneration were as detailed previously (Stefanov et al., 1994). Transformation was confirmed by Southern hybridization of genomic DNA from primary transformants and PCR analysis of DNA from second generation seedlings.

### *Analysis of rapeseed transformants and their progeny*

The NPT II tests were performed as described by Reiss et al. (1984). Transformation was confirmed by Southern hybridization of genomic DNA from primary transformants and PCR analysis of DNA from offspring (Stefanov et al., 1994).

Assays for GUS activity and histochemical staining of plant tissues were carried out according to Jefferson (1987) with minor modifications. Plant tissues were ground with quartz sand in 200 ml of ice-cold extraction buffer in an Eppendorf tube, using a glass pestle connected to a mixer head. After centrifugation in an Eppendorf centrifuge for 5 min, the cell debris was precipitated and 25 $\mu$l samples were taken from the clear phase. One sample was used for protein determination, according to Bradford (1976), while the others were incubated for 0 min, 60 min or 120 min with 75 $\mu$l of assay buffer containing 1 mM methyl-umbelliferyl $\beta$-glucuronide (MUG) in microtitre plate wells. The reaction was stopped with 25 $\mu$l of 1 M $Na_2CO_3$ and the fluorescence of methylumbelliferone (MU), the product of the reaction catalysed by GUS, was measured with a Perkin-Elmer fluorometer. The specific activity of the reporter enzyme was expressed in nmoles mg$^{-1}$ protein min$^{-1}$.

Cross-pollination experiments were carried out in the greenhouse in winter to avoid natural pollination by insects. In the field trial, six genetically-modified plants were grown at the centre of the field (1 ha) of non-modified rapeseed plants. The field trial was carried out in isolation, surrounded by forest, meadow and cereal experiments. An attempt was made to minimize the genetic-environmental interaction. In the field trial, the ratio of transformed/non-transformed plants was $1{:}2\times10^5$. Seed of transformants was harvested and seed samples were collected from 1-, 2-, 4-, 8-, 16-, 32- and 50-m sectors at the four cardinal points.



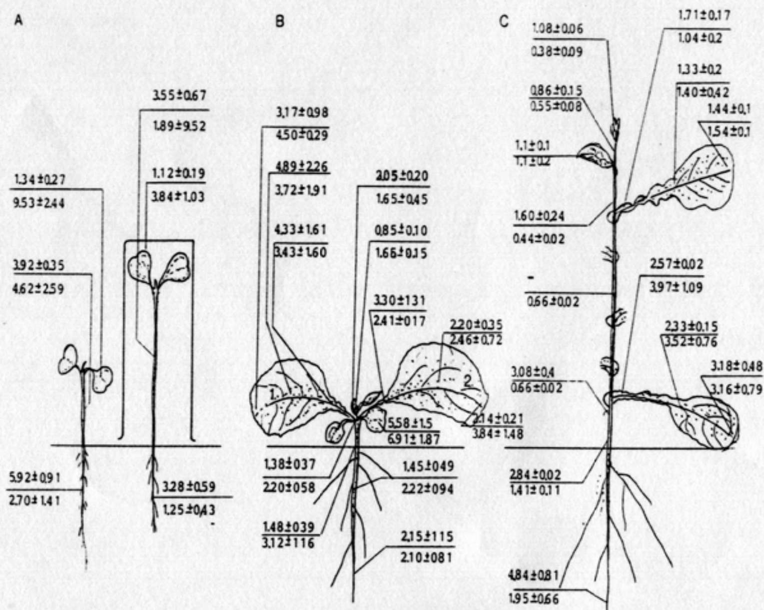


*Fig. 1.* GUS activity in second generation ($R_1$) transgenic *Brassica napus* plants at different stages of development A: Seedlings grown under light (left) or darkness (right). B: Young plant at rosette stage. C: Mature plant before flowering. The row indicates the GUS activity (nmoles mg$^{-1}$ protein min$^{-1}$, ± standard error) detected in transformants with the CaMV 35S (upper), or *mas* (lower) promoter.

## Results and discussion

### *Expression pattern of the chimeric $\beta$-glucuronidase gene in transgenic rapeseed plants*

The expression of the transgene was tested in the offspring of $R_0$ transformants. After self-pollination of independent transformants, seedlings were grown on medium containing 100 mg l$^{-1}$ Kanamycin and the resistant segregants were used for analysis of the expression of the *gus* reporter gene.

Transgenic seedlings grown either in the dark or under conditions of 16/8 hours light/dark period exhibited significant differences. In seedlings, the CaMV 35S promoter had maximum activity in the primary roots, while the *mas* promoter was most active in the cotyledons. Etiolated seedlings cultured in the dark showed a reduced activity of the *mas* promoter (Fig. 1A).

The CaMV 35S promoter activity was affected by illumination only in the root. Roots grown in the dark displayed a reduced growth rate and lower CaMV 35S promoter activity.

Activity of the *mas* promoter was strongly influenced by the light conditions. The GUS activity in etiolated seedlings was 2–2.5 times lower than that in non-etiolated ones. The differences could be observed in all parts of the seedlings. Down-regulation of the *mas* promoter in etiolated seedlings may be caused by the presence of inhibitory factors.

GUS activity was also analysed in plants at different stages of development. When 6 to 8-week-old plants grown in the greenhouse without vernalization were tested, the activity of both promoters was highest in the cotyledons and both promoters were more active in older than in younger plant parts (Fig. 1B). The fully-expanded leaves (first leaf) exhibited a substantially higher promoter activity than developing ones. At this developmental stage, the activity of the CaMV 35S promoter was found to be weak in the meristematic buds. The leaves surrounding the apical bud had an activity similar to that of the growing leaves.

The plants were vernalized to induce the generative phase. After transfer to the greenhouse, the plants reached a height of 70–90 cm in 3–4 weeks. This process involves an increased production of growth hormones, especially gibberellins (Trewavas, 1981). Accordingly, considerable changes may be expected in the activities of the promoters analysed (Fig. 1C). After the plants had bolted, reduced promoter activity was detected in the upper parts of the vernalized plants. A decrease in activity of both promoters was seen in the upper leaves. An even greater decrease in promoter activity was detected in the leaves beneath the flower. In the stem and the root, the CaMV 35S promoter displayed increased activity in comparison with the *mas* promoter. We detected a sharp decrease in activity of the *mas* promoter especially in the upper part of the shoots. This is a condition similar to that found in etiolated seedlings. Since gibberellic acids are the main hormones involved in etiolated growth and bolting (Trewavas, 1981), it may be speculated that the lower activity of the *mas* promoter in these two cases was caused by the increased level of gibberellic acids.

To monitor changes in promoter function during morphogenesis *in vitro*, callus and shoots were induced from hypocotyl explants of R2 plants. Morphogenic and non-morphogenic calli, and shootlets were sampled. The CaMV 35S promoter had significantly higher activity in the morphogenic callus. The first organized structure to emerge from the callus is often a leaf, and the process of regeneration is similar to the development of a lateral bud. The first leaflet had a lower *mas* promoter activity that of the shoot. The trend to lower activity of this promoter is organized structures has already been seen in tobacco (Langridge et al., 1989).

The transformation and regeneration system of diverse *Brassica napus* cultivars was thus established by induction of callus formation from transformed cells before plant regeneration. Use of the *gus* reporter gene permitted a detailed analysis of the activity of the CaMV 35S and *mas* promoters in the transformed rapeseed plants studied. The results demonstrated consistent differences in the expression of the promoters studied. Both regulatory elements can be considered constitutive in the sense that they are expressed in all plant parts, but the level of expression was strongly dependent on the developmental stage of the plants and on the specific organ. We also detected differences between the two promoters in tissue-specificity. The growth conditions of the individual plants also influenced promoter function. Since actual GUS enzyme activity can be modified by some factors, we propose that the promoters analysed are the key factors generating the observed differences.

### *Study of transferred gene dispersal*

#### *Cross-pollination*

In the greenhouse, artificial crosses were made to study the frequency and extent of cross-pollination between transformed crop plants containing the marker gene, and other plants of related species (e.g. weeds). When we used genetically-transformed 'Arabella' and 'Santana' rapeseed plants as pollen donors, incompatibility was not found with commercial, non-transgenic rapeseed varieties. The pollinated flowers (85 samples) produced healthy fruits in 83.5% of the crossed flowers. In the hybrid seeds (4220), the introduced marker gene was expressed in 3:1 and 1:4 ratio for resistant/sensitive plantlets, depending on the pollen donor parent, 'Arabella' and 'Santana', respectively. The 1:4 segregation ratio means that the transgene is not efficiently transmitted. Gene transfer from rapeseed to relatives (*Brassica rapa, Diplotaxus muralis* etc.) – using embryo rescue in *D. muralis* – has not been confirmed so far. The experiments of sexual transfer of the transgene are continuing.

#### *Field trials*

Field trials were carried out to examine the interaction of genetically-modified plants with the cultivation environment. Six modified plants were grown at the centre of the plot, surrounded by 1 ha of non-modified rapeseed plants (Fig. 2A). Pollen dispersal was achieved by natural pollination with bees (Fig. 2B). Marker gene dispersal from the transformed plants was monitored by screening progeny from the non-modified plants for the presence of the introduced character (*nptII* gene). Selection for $Kan^R$ was made in each case after a second exposure (selective liquid and agar-solidified shoot culture) to kanamycin and the resistance was tested by *npt*II assay. So far, kanamycin-resistant individuals have been found in the immediate area (within 1 m of the test plants) in $10^{-3}$ frequency, but resistant individuals were found at 16 and 32 metres from the transgenic pollen donors in very low frequencies ($10^{-5}$). Screening for very low frequencies still continues. The harvested yield of the 1 ha field surrounding the trial was burnt.

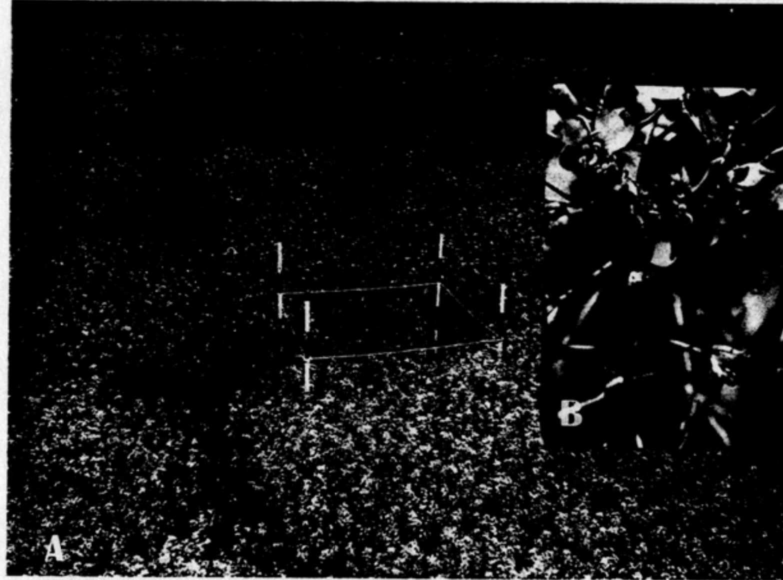


*Fig. 2.* Rapeseed field trial (A) to examine the interaction of genetically-modified plants with non-modified ones, by natural pollination by bees (B).

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# Assessing the risks of wind pollination from fields of genetically modified *Brassica napus* ssp. *oleifera*

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*Key words:* gene flow, transgene, pollen movement, genetically modified oilseed rape, wind pollination, risk assessment, feral populations

## Summary

Intensive research over the past 10 years has produced many genetically-modified lines of oilseed rape with market potential. Assessment of these lines in statutory trials prior to their release as cultivars is necessary, owing to concern over the likelihood of transgene escape from such crops. Here, we examine the movement of airborne pollen grains from oilseed rape fields and assess their capacity for long-range geneflow.

Pollen dispersal from isolated rape fields was monitored over two seasons and related to the distribution of fields and 'feral' (domesticated plants growing outside cultivations) populations of the crop in Tayside and North East Fife regions of Scotland. Airborne pollen density declined with distance and at 360 m was 10% of that at the field margin. Pollen counts of 0–22 pollen grains $m^3$ were observed 1.5 km from source fields and apparently were sufficient in number to allow seed set on emasculated bait plants. Oilseed rape pollen has greater capacity for long-range dispersal than had been suggested by small-scale field trials. Mean separation of oilseed rape fields in the survey area was 410 m and the mean distance from 'feral' populations to commercial fields was 700 m. Sixty percent of 'feral' populations with more than 10 plants occurred downwind and within 2 km of an oilseed rape field. Provided that the flowering biology of genetically-modified oilseed rape does not differ from the conventional crop, these data suggest that transgene movement to non genetically-modified fields or 'feral' populations is likely following commercial release.

## Introduction

Annual production of oilseed rape (*Brassica napus* ssp. *oleifera*) has grown dramatically in recent years and 232,190 million tonnes were produced worldwide in 1992 (Anon, 1993). Development of a transformation system for the crop (Ooms et al., 1985) has enabled the production of modified lines containing transgenes for herbicide resistance (Miki et al., 1990; Mariani et al., 1991), increased methionine in seed meal (Altenbach, 1992), male sterility and restorer genes (Mariani et al., 1990, 1992, respectively), heavy metal tolerance (Misra & Gedamu, 1989) and antibiotic resistance (Arnoldo et al., 1992).

The performance of genetically-modified oilseed rape under agricultural regimes has been assessed in numerous small-scale experimental field trials (e.g. Gressel & Ben-Sinai, 1985; De Greef et al., 1989; Arnoldo et al., 1992). Eighty-two such field releases were approved worldwide by 1992 (Chasseray & Duesing, 1992; Dale et al., 1993), and the number submitted for approval is rising annually. Attention is now focused on the prospects for commercial release of genetically-modified oilseed rape. Crawley et al. (1993) identified three areas of concern associated with the release of genetically-modified crops:

i. genetically-modified plants themselves may become weeds and/or invade natural habitats

ii. the release of genetically-modified crops may enable the sexual transfer of the inserted genes to neighbouring commercial or natural populations whose offspring may then become more weedy or invasive