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Promoter Tagging for Designer Transgenic Plants

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Transgenic plants conferring agronomically and industrially useful traits become a reality in these days. Furthermore, appropriate control of introduced traits may be critical to make transgenic plants more desirable and marketable. A key component for achieving this goal is a promoter that can control temporal and spatial expression of a specific gene in transgenic plants.

A traditional approach to the isolation of differentially expressed genes involves the use of cDNA libraries. Genes expressed in particular tissues or organs can be identified by hybridization to labeled cDNA probes representing mRNA pools of the different tissues (Maniatis *et al.* 1982). Another important method involves gene tagging by transposable genetic elements (Transposons) (Saedler and Nevers 1985). Transposons are randomly inserted in the genome, resulting in altered phenotypes. The transposon can then be used as a probe to facilitate the molecular cloning of the target sequences whether they are promoters or genes from the phenotypically altered target material. By using plant transposons as mobile tag elements, it has been possible to isolate regulatory genes (Vollbrecht *et al.* 1991).

A powerful alternative method to transposon tagging is to use T-DNA as an efficient gene tag (Koncz *et al.* 1989). T-DNA tagging is based on the natural process of *Agrobacterium*-mediated transformation in which the single-stranded T-DNA is carried from the bacterium to a wounded dicotyledonous plant cell and inserted into the genomic DNA (Zambryski *et al.* 1988). When transferred to the plant genome, T-DNA has been shown to target frequently into transcriptionally-active sequences (Koncz *et al.* 1989: Herman *et al.* 1990). An advantage of T-DNA is that it can be manipulated inside of its border sequences while maintaining its transfer functions. For example promoterless reporter genes which can be activated by a promoter in the target plant genome, have been engineered into T-DNA (Teeri *et al.* 1986: Koncz *et al.* 1989: Kerbundit *et al.* 1991: Topping *et al.* 1991, 1994: Lindsey *et al.* 1993: Topping and Lindsey 1995). The gene *npt*II, which codes for neomycin phosphotransferase II (NPTII) and which gives resistance to aminoglycoside antibiotics, has been used in experiments to tag plant promoters. Since *npt*II is a selectable marker gene, it is possible to select directly for activation of the gene. Also the activity of the *npt*II gene can be enzymatically assayed in several ways. Another gene used for promoter tagging is *gus* (Fobert *et al.* 1991, 1994: Kerbundit *et al.* 1991: Lindsey *et al.* 1993: Topping *et al.* 1994), which has the advantage that it can be histochemically assayed to determine the developmental stage and tissue(s) in which a tagged promoter is expressing (Jefferson 1987: Jefferson *et al.* 1987). However the *gus* gene can not be used as a selectable marker (Hodal *et al.* 1992).

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

The aim of this study was to evaluate the efficiency of a

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

MATERIALS and METHODS

Plant materials

'Rapid cycling' *Brassica oleracea*, broccoli (*B. oleracea* var. *italica*) cultivar 'Green Valiant', cauliflower (*B. oleracea* var. *botrytis*) cultivar 'Snow Crown' and *B. napus* cultivar 'Westar' were used in this study.

Culture media and explant preparation

The MSB5 (MS salts with B5 vitamins, Cat. No. M0404, Sigma, USA) medium was used as a basal medium. Modifications were made to this medium and other media used in this study and the details are summarized in Table 1.

Table 1. Media used in brassica transformation.

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

Antibiotics were added after the medium was autoclaved.

Bacterial strains and gene construct

For transformation, *Agrobacterium tumefaciens* strain GV3101/pMP90 (Koncz and Schell 1986) harboring the promoterless binary vector pΔGUS::NPTII was used. The promoterless binary vector pΔGUS::NPTII was kindly provided by Dr. R. Datla (National Research Council Canada, Saskatoon, SK, Canada S7N 0W9). This promoterless construct contains a chimeric *gus*::*npt*II gene (Datla *et al.* 1991) and nopaline synthase terminator in modified pBin19 with synthetic T-DNA borders. The 5' end of the *gus* sequence is close to the T-DNA right border sequence. A translation enhancing leader sequence from alfalfa mosaic virus (AMV) was placed between the right border and 5' end of the *gus* sequence to facilitate gene expression through improved translation when this cassette is inserted adjacent to a plant promoter during transformation (Figure 1).

Co-cultivation

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

Selection strategy

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

AMV: Alfalfa mosaic virus translation enhancing leader, 45bp: GUS::NPTII: fused gene of β-glucuronidase and neomycin phosphotransferase II genes, 2.8Kb: Tnos: Nopaline

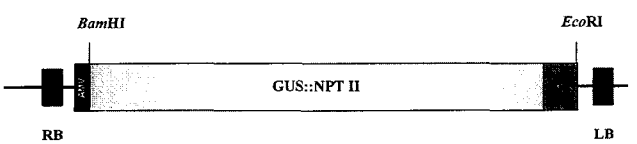

Figure 1. Schematic map of transcriptional fusion cassette of vector pΔGUS::NPTII.

synthase terminator, 250bp: RB: right border of the T-DNA, LB: left border of the T-DNA (Dr. R. Datla, unpublished data).

Cultivation of transgenic plants

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

RESULTS

Selection strategy and transformation frequency

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

Seven putative promoter tagged transgenic plants were identified out of 18,911 *Agro*-infected cotyledonary petiole explants. The seven promoter tagged lines recovered included one plant of broccoli and six plants of *B. napus*. The efficiency of recovering promoter tagged lines in broccoli was about 0.012% while in *B. napus* it was 0.056% (Table 2). None of the transgenic lines showed phenotypic abnormalities.

Table 2. Results of transformation of *B. oleracea* and *B. napus* with the promoterless construct pΔGUS::NPTII.

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

Table 3. Histochemical localization of GUS activity in transgenic plants transformed with a promoterless construct pΔGUS::NPTII.

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

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

Expression pattern analysis by GUS localization

GUS expression patterns were analyzed in various tissues of one broccoli (*B. oleracea* var. *italica*) line (PT10-2) and six *B. napus* lines (PT4-1, 6-1, 6-2, 7-1, 12-1 and 12-2). These tissues included: root, stem, leaf, filament, anther, pollen, style, stigma, sepal, petal and seed tissues. In most cases, primary transformants (R_0) were used for GUS staining. Results are summarized in Table 3. Although transgenic lines PT4-1, 6-1 and 6-2 failed to show GUS expression in anther tissue, expression in PT4-1, 6-1, 6-2 and 7-1 was considered to be constitutive while PT10-2 showed expression in all plant parts except the root system. Transgenic line PT12-1 showed GUS

Table 4. Expression patterns of promoter tagged transgenic plants.

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

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

expression predominantly in the root tissue, whereas PT12-2 exhibited GUS expression in the phloem tissues of the stem but not in other parts of the plant (Table 4).

PT4-1, PT6-1, PT6-2

Interestingly, these three lines of *B. napus* cv. Westar showed similar GUS expression patterns. GUS staining was observed in the root, stem and leaf tissue and most parts of the flower with the exception of pollen grains. Staining at specific stages of microsporogenesis was not attempted. R_1 seeds showed GUS activity and R_1 seedlings also showed GUS activity in root, hypocotyl and cotyledons similar to the expression patterns observed in the primary transgenic lines (Figure 2). The expression patterns observed in these lines suggest that the tagged promoters have a pattern of expression similar to CaMV 35S which is often considered to be a constitutive promoter but in fact is not usually active in pollen grains.

PT7-1

This line was derived from *B. napus* cv. Westar. GUS activity was expressed in all tested tissues or organs including pollen. R_1 seeds and seedlings also showed strong intensities of GUS staining as in the primary transgenic plant (Figures 3). These observations suggest that the GUS::NPTII insert was fused to a strong constitutive promoter in the transgenic plant.

PT10-2

This line was derived from the broccoli cultivar 'Green Valiant'. Strong GUS staining was observed throughout the primary transgenic plant except the root system when grown *in vitro*. A mature, greenhouse grown plant also showed

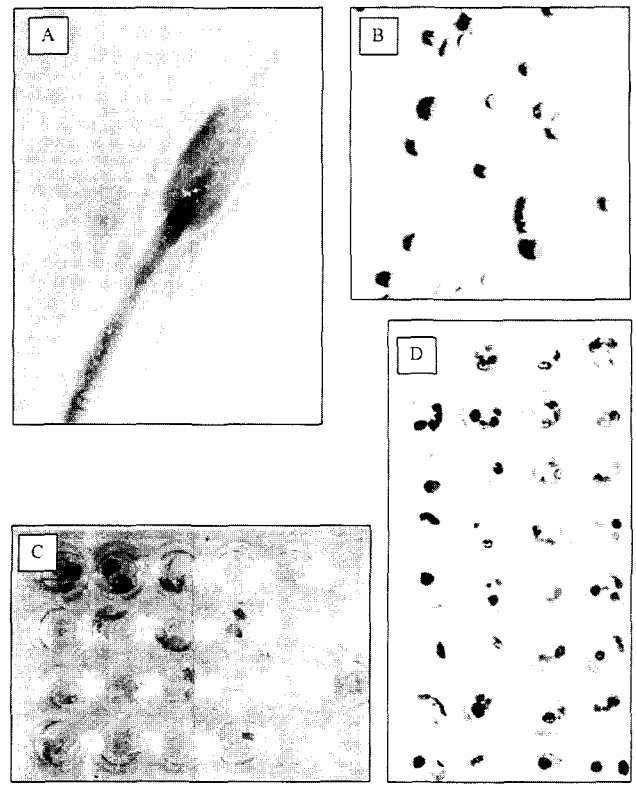

Figure 2. GUS activity in various tissues of the promoter-tagged transgenic line PT6-2 (*B. napus* cv. Westar).
A: The filament of the primary transgenic line shows GUS activity but the anther does not: B: Pollen grains from A, GUS activity was not detected: C: Cotyledon, hypocotyl and root of R_1 seedlings show GUS activity and segregate in a 3:1 ratio: D: Seeds from the R_1 plant show GUS activity and also segregate in a 3:1 ratio.

strong expression patterns in all parts of the plant including pollen grains with the exception of the root system. Seed staining revealed that all tissues including the seed coat, cotyledons and hypocotyls stained GUS-positive with the exception of the region of the hypocotyl from which the root would develop. The R_1 seedlings showed GUS activities in hypocotyl and cotyledons but not in the root system (Figure 4). This tagged promoter was therefore considered to exhibit shoot specific expression.

PT12-1

This line was derived from *B. napus* and exhibited GUS activity predominantly in the root system including root hairs. Expression was also observed in auxiliary bud regions on stem. No GUS activity was observed in R_1 seeds. In R_1 seedlings, GUS activity was observed only in the root system. This expression pattern was similar to the primary transgenic plant. GUS staining of the roots indicated that the staining

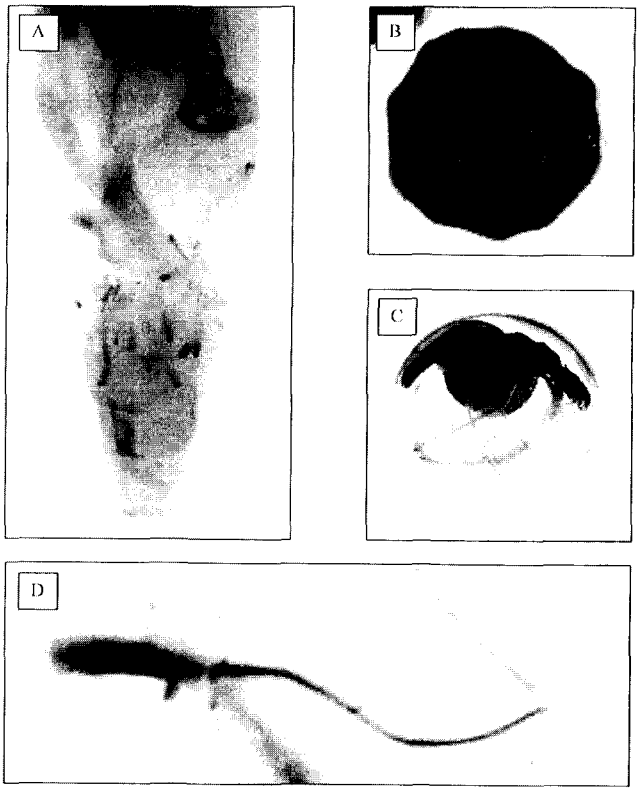

Figure 3. GUS activity in various tissues of the promoter-tagged transgenic line PT7-1 (*B. napus* cv. Westar). A: Primary transgenic plantlet grown on rooting medium shows GUS activity in roots, root tips, stem and leaf; B: Primary transgenic plant in greenhouse shows GUS activity in all stem tissues; C: GUS-positive R1 seedling; D: Root and root tips of a GUS-positive R1 seedling.

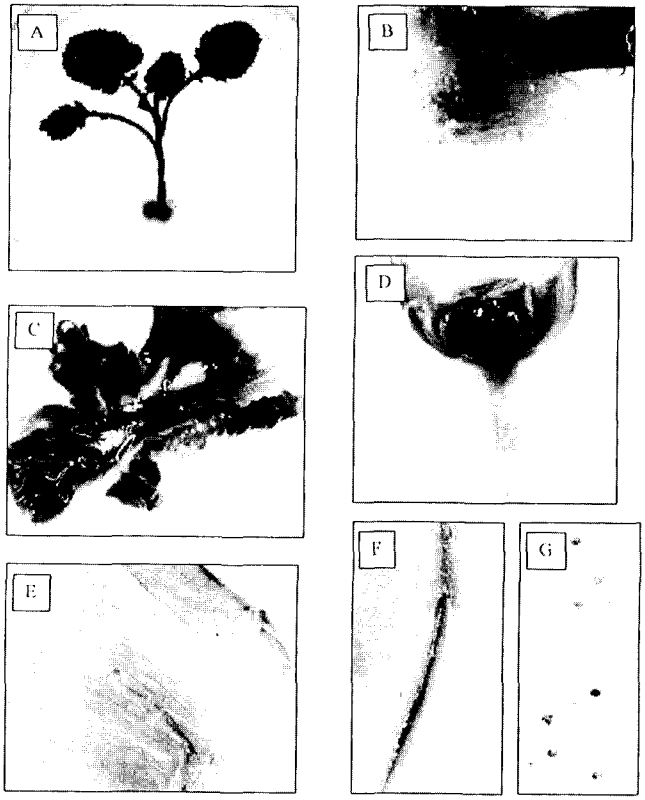

Figure 4. GUS activity in various tissues of the primary promoter-tagged transgenic line PT10-2 (*B. oleracea* var. *italica* cv. Green Valiant). A, B: Plantlet grown on rooting medium; GUS was not detected in the roots; C: Leaf; D: Pedicel and receptacle; E: Filament and anther.

intensity and pattern were irregular (Figure 5).

PT12-2

This line was derived from *B. napus* and exhibited GUS activity predominantly in the stem. Staining of cross and longitudinal stem section revealed that GUS activity was only localized in the phloem. GUS activity was not detected in the petiole, leaf and root (Figure 6). This line was transferred to soil and further detailed analyses are in progress.

Inheritance of GUS activity and Southern analysis

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

Table 5. Segregation for GUS staining in R1 progeny of promoter tagged transgenic lines.

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

[a]: significant level was 5%, [b]: results from Southern analysis, N/D: not determined.

R1 progeny from five selfed primary transformants (PT4-1, 6-1, 6-2, 7-1, 10-2) showed segregation ratios of 3:1 (GUS-

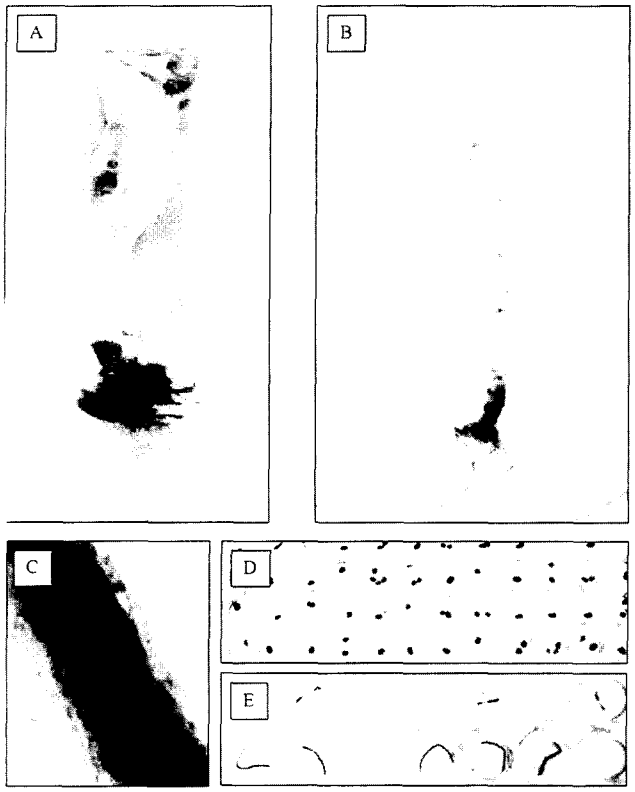

Figure 5. GUS activity in various tissues of the promoter-tagged transgenic line PT12-1 (*B. napus* cv. Westar). A: Primary transgenic plantlet grown in the rooting medium shows GUS activity predominantly in the root system. B: Primary transgenic plantlet grown in the rooting medium shows GUS activity predominantly in the root area and at the nodal junctions. C: Magnified picture of B, strong GUS activity in the root meristem and root hairs: D: GUS activity is absent in R1 seeds. E: GUS activity is revealed in seedling roots.

positive : GUS-negative) (Table 5). These lines were therefore expected to contain a single T-DNA insert and these were further characterized by Southern analysis to confirm T-DNA copy number.

Genomic DNA from each of the seven transgenic lines was digested with the restriction enzyme *Bam*HI to generate T-DNA border fragments and hybridized with a *npt*II probe. These results suggested that most of the transgenic lines (6 out of 7) contained a single copy of the full-length T-DNA insert however PT12-1 appears to have five copies. All transgenic plants analyzed to date have had different sizes of flanking junction fragments, suggesting that the T-DNA integrated into different sites in the plant genome. These results also suggest that although transgenic lines PT4-1, 6-1 and 6-2 exhibit similar constitutive GUS expression patterns, their tagged promoters are likely different.

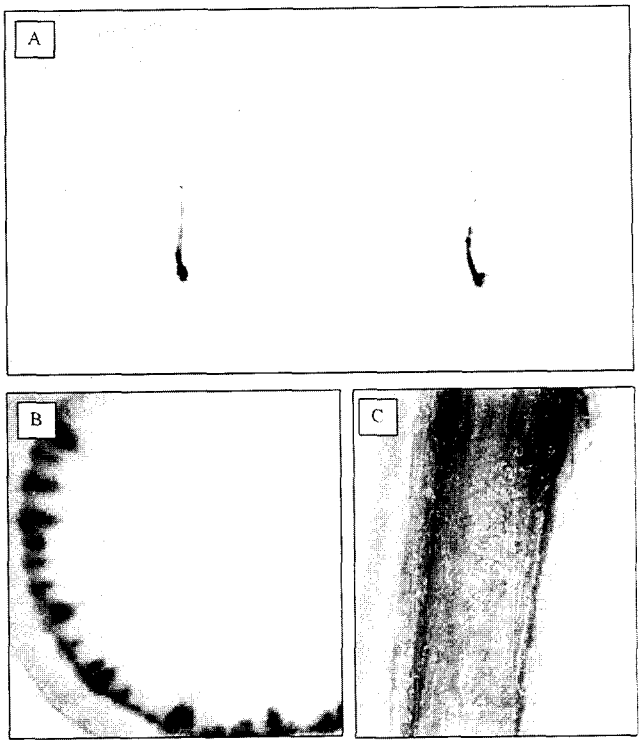

Figure 6. GUS activity in various tissues of the primary promoter-tagged transgenic line PT12-2 (*B. napus* cv. Westar). A: GUS activity is exhibited predominantly in stems but not in roots, petioles and leaves (grown in the rooting medium). B: Cross-section of stem: only the phloem tissues show GUS activity in the stem (grown in the greenhouse) C: Longitudinal-section of stem (grown in the rooting medium).

DISCUSSION

Promoter tagging represents an effective method of identifying and cloning plant genes and may be particularly useful for identifying genes that are expressed in restricted cell types or that are expressed during short periods of development (Koncz *et al.* 1989, 1990: Kertbundit *et al.* 1991: Topping *et al.* 1991: Walden *et al.* 1991, 1994: Lindsey *et al.* 1993). A strategy based on T-DNA insertional mutagenesis may be exploited to identify plant promoters using specially designed T-DNA vectors which contain a reporter gene without transcriptional elements (i.e. promoterless constructs) located close to the border sequences (Teeri *et al.* 1986: Koncz *et al.* 1989: Kertbundit *et al.* 1991: Lindsey *et al.* 1993: Suntio and Teeri 1994: Topping *et al* 1994: Fobert *et al.* 1991:1994). If the coding sequence of the promoterless reporter gene is integrated downstream of a native gene's promoter elements, the reporter gene will be transcribed and expressed. The resulting hybrid genes produced by T-DNA-mediated promoter tagging, consist of unknown plant

promoters residing at their natural location within the chromosome, and the coding sequence of a marker gene located on the inserted T-DNA (Fobert *et al.* 1991). The plant promoter sequences controlling the expression of these gene fusions can subsequently be isolated and further analyzed by standard molecular genetic techniques. This T-DNA based promoter tagging system requires *Agrobacterium*-mediated transformation as a prerequisite and therefore, application of this system is limited to few species in spite of its powerful potential for promoter studies. The most commonly used target plants represent species such as *Arabidopsis thaliana* (Koncz *et al.* 1989: Lindsey *et al.* 1993), *Nicotiana tabacum* (Teeri *et al.* 1986: Fobert 1991: 1994) and *Solanum tuberosum* (Lindsey *et al.* 1993) in which an *in vitro* culture system was well established with a high transformation frequency by *Agrobacterium*. The objective of this study was to evaluate the feasibility of the application of a T-DNA based promoter tagging strategy with the genus *Brassica*.

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

More than 18,000 cotyledonary petiole explants were infected with *Agrobacterium* strain GV3101/pMP90 harboring the promoterless construct pΔGUS::NPTII. A chimeric kanamycin-resistance gene present in this vector allowed for the selection of transgenic plants. As soon as green shoot buds appeared on explants, they were transferred to a kanamycin-free shoot elongation medium. Because kanamycin resistance is expressed at the early stage of cell division and callus formation (even though the tagged promoters posses tissue specific regulatory properties), it is critical to make selections during the early stage of shoot differentiation in order to ensure recovery of tagged lines. Although this promoterless bifunctional gene system requires more attention during the selection stage, the system require less labor than the two step selection system used by others in previous studies (Koncz *et al.* 1989: Kertbundit *et al.* 1991: Lindsey *et al.* 1993).

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

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

Based on Southern analysis and segregation data, six out of seven transgenic lines had single copy insertions while one had five inserts. These results suggest that this tagging system using a GUS::NPTII bifunctional promoterless construct provides a high frequency of single copy insertions (85%). This is preferred for cloning the tagged promoters. The observation in this study contrast that of oher studies that have reported a high frequency of multiple insertions. More than 67% of multiple insertions in *Arabidopsis* and *Nicotiana* (Koncz *et al.* 1989), 40-50% in *Arabidopsis* (Topping *et al.* 1994) and 67% in *Nicotiana* and 48% in *Arabidopsis*

(Lindsey *et al.* 1993) have been reported with various types of promoterless constructs. Multiple insertion events in some of these promoter tagging experiments may be due to the specific construct, the transformation method, the target plant or combinations of these factors (Walden *et al.* 1991: Topping and Lindsey 1995).

Constitutive expression in the tagged lines (PT6-1 and PT7-1) was further investigated to find their relative strength in comparison with the most widely used CaMV 35S constructs. Based on a shoot regeneration test on 200 mg/L kanamycin, the transgenic line PT6-2 showed almost the same regenerability as transgenic plants containing a single CaMV 35S promoter. Another transgenic line, PT7-1 exhibited levels of regenerability comparable to transgenic lines with a tandem repeated CaMV 35S promoter enhanced by AMV. In tests with transgenic line PT7-1 on 100 mg/L kanamycin, shoot regeneration was virtually unaffected. This suggests that the insert in the transgenic line PT7-1 may be downstream of a strong constitutive promoter that can be used as an alternative to the widely used CaMV 35S promoter.

Most of the promoters so far identified by T-DNA tagging methods exhibit organ or tissue specific expression (Teeri *et al.* 1986: Kertbundit *et al.* 1991: Lindsey *et al.* 1993: Suntio and Teeri 1994: Babic *et al.* 1994) with limited information on the identification of constitutive promoters available. Promoter tagged line investigated in this study could potentially provide new constitutive promoter for crop genetic engineering and other tissue specific promoters could also be useful for better control of genes of interest in transgenic plants.

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