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Inherited Transgene Expression of the *uidA* and *bar* Genes in *Lilium longiflorum* cv. 'Nellie White'

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ABSTRACT

The expression of two transgenes, *bar* and *uidA*, was studied in *Lilium longiflorum* cv. 'Nellie White' plants. 'Nellie White' had been transformed using the gene gun to bombard with pDM327 that contains the *bar-uidA* fusion gene under control of the CaMV 35S promoter. PCR analysis verified that eight T_0 plant lines were probably not chimeric. Crosses using the eight T_0 plants were made to *L. longiflorum* cvs. 'Sakai', 'Yin tung', 'Snow Queen', 'White Europe', and 'Flavo'. The *bar* gene was transmitted to 15% of the 151 T_1 seedlings analyzed with transmission success rates ranging from 0-100% depending on the T_0 plant line. Only 13 of the 22 T_1 seedlings with the *bar* gene also contained the *uidA* gene. Expression of the *bar* gene as determined by immunological detection of phosphinothricin acetyltransferase occurred in all six T_1 plant lines that contained the *bar* gene. One T_0 parent line had a notably high level of both *bar* and GUS expression, and this high level expression continued in the T_1 plants. This study indicates that transmission of these transgenes to progeny occurred with low frequency, and expression of both transgenes occurred in the T_1 generation.

Keywords: biolistics, Easter lily, floral monocot, flower bulb crops
Abbreviations: MS, Murashige and Skoog's medium; **PCR**, polymerase chain reaction; **PPT**, phosphinothricin

INTRODUCTION

Lilies have been transformed by several researchers using the gene gun and *Agrobacterium tumefaciens*, but no one has examined transmission of the transgenes to progeny (Watad *et al*. 1998; Irifune *et al*. 2003; Mercuri *et al*. 2003; Ahn *et al*. 2004; Cohen *et al*. 2004; Hoshi *et al*. 2004; Kamo and Han 2008; Ogaki *et al*. 2008; Krens *et al*. 2009; Azadi *et al*. 2010). It is of interest for future applications of genetic engineering in lilies to know if the transgene is stably integrated in the genome of a non-chimeric plant and will be transmitted and expressed in the progeny.

In our study, the extent of transgene silencing in lilies was determined in T_0 and T_1 plants since silencing frequently occurs in the progeny of monocots transformed using the gene gun. Bregitzer and Tonks (2003) studied barley and found that the failure to transmit transgenes to progeny occurred more often than transgene silencing. Transgenes were possibly not transmitted to the progeny because the parent plant was chimeric or the gene was not stably integrated into the plant's genome. Corn transformed with the *bar* and *uidA* genes using the gene gun resulted in transformants that generally had multicopy transgenes at a single locus (Register III *et al*. 1994). Some of these corn lines exhibited gene silencing, and some showed a low frequency of transgene transmission to the progeny. Zhang *et al.* (1996) reported that with corn 30% of the T_2 lines expressed *bar*, and GUS was expressed at a much lower level of 2%. The low frequency expression of the non-selected gene, GUS, in comparison to the higher frequency expression of the selected gene, *bar*, has been observed by others in corn (Spencer *et al.* 1992). Transgene silencing appears to occur more often in wheat than corn. Six wheat lines transformed with the *bar* and *uidA* genes showed transmission of both transgenes in the T_1 and T_2 generations, but one line was unstable and lost both transgenes in the T_3 generation (Srivastava *et al.* 1996). GUS expression was lost in five T_2 lines of wheat and maintained in only one line. Wheat lines transformed with PR (pathogen-resistance) genes showed transgene silencing in 20 of 24 lines in the T_1 or T_2 generations (Anand *et al.* 2003).

Progeny of *Lilium longiflorum* were obtained by crossing eight transgenic plant lines previously transformed with the *bar* and *uidA* genes using the gene gun (Kamo and Han 2008). Both T_0 and T_1 plants were analyzed for *bar* and GUS expression in this study.

MATERIALS AND METHODS

All chemicals used for plant tissue culture, transformation, and biochemical analysis were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Transformation and transgenic plants

Lilium longiflorum cv. 'Nellie White' had previously been transformed using the gene gun to propel plasmid DNA, pDM327 (received from David McElroy, Verdia, Redwood City, CA) (Kamo and Han 2008). Briefly, callus bombarded with pDM327 was selected on Murashige and Skoog's (MS) medium supplemented with 2 mg/L dicamba and phosphinothricin (PPT) (AgroEvo, Somerville, NJ) using a step selection of 0.1 mg/L PPT for one month, then 0.2 mg/L PPT for one month, and followed by at least four transfers, once per month, onto 1 mg/L PPT. Putatively transormed plants were grown on MS medium with 0.1 mg/L PPT followed by 1 mg/L PPT with a 12 h light photoperiod provided by cool white fluorescent bulbs (40-60 μmol m^2 s^1).

T_0 generation plants were planted in Metromix 200 (Scotts Company, Marysville, OH), grown at 4°C in the dark for 6 weeks before being planted in the greenhouse. Each T_0 plant line resulted from an independent transformation event. Plants to be pollinated were emasculated to prevent self and cross contamination with pollen. Each T_0 plant line was crossed with either *L. longiflorum* 'Sakai', 'Yin tung', 'Snow Queen', 'White Europe', or 'Flavo' (gift from Lee Riddle, Easter Lily Foundation). T_0 plants were not selfed because both Lee Riddle, manager of the Easter Lily Foun-

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dation, and our lab have found that viable seed are not produced when 'Nellie White' is self pollinated. The greenhouse was maintained at 24-26°C during the day and 21-23°C at night. Usually the T_0 plant was the pollen parent in the crosses.

Seed pods from the crosses were surface-sterilized by dipping in 70% ethanol and then flaming them. Seeds were cultured on solid MS medium at 25°C in the dark and then placed in the light for germination. All T_1 progeny were maintained *in vitro* in the light.

Polymerase chain reaction (PCR) analysis

Three vegetatively propagated plants for each T_0 line were analyzed for both the *bar* and *uidA* genes using PCR to verify that the plants were not chimeric. Genomic DNA was isolated using the FastPrep® system (Qbiogene, Carlsbad, CA). Leaf tissue was collecting in Lysing Matrix tubes containing one ceramic ball and then pulverized with the FastPrep system machine. Following centrifugation at 13,000 × *g*, the cell extract was used to isolate genomic DNA according to the instructions in the FastDNA kit. The *bar* gene was amplified using the forward primer 5′-GTCAAC TTCCGTACCGAGCCGCAG-3′ and reverse primer 5′-CATGCC AGTTCCCGTGCTTGAAG-3′ to produce a 379-bp band (Ahn *et al.* 2004). Amplification of the *uidA* gene was done using the forward primer 5′-TAACCTTCACCCGGTTGCCAGAGG-3′ and reverse primer 5′-CTTTAACTATGCCGGAATCCATCG-3′ to produce a 253-bp band (Kamo and Han 2008). The program for amplification was 94°C for one min followed by 36 cycles of 94°C for 20 s, 67°C for 30 s, 72°C for 2 min, and then one cycle of 72°C for 10 min using an MJ Research PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). PCR products were run on a 0.9% agarose gel. T_1 progeny plants were analyzed by PCR first for the presence of the *bar* gene, and if the *bar* gene was present, the plant was analyzed for the *uidA* gene.

ELISA analysis of *bar* gene expression

Leaf tissue was analyzed for *bar* gene expression using LibertyLink® PAT/bar ELISA plates (Envirologix, Portland, ME) according to the manufacturer's instructions. Leaf tissue was collected in a 1.5 ml microfuge tube placed on ice. Extraction buffer provided in the kit (300 µl) was added to each sample and ground with a Kontes pestle followed by vortexing for 10 sec. The sample was centrifuged at 5,000 × *g* for 5 min. The supernatant was placed in a new tube, frozen in liquid nitrogen and then stored at -75°C until use. The PAT-enzyme conjugate (50 µl) was added to each well of the ELISA plate followed immediately by 50 µl of Wash/extraction buffer, and 50 µl of each plant extract. Contents of the plate were mixed by rotating the plate by hand for 30 sec and then the plate was covered with Parafilm and incubated at 25°C for 2 h. Wells were flooded with the Wash/extraction buffer and then emptied completely. Substrate was added to each well (100 µl) followed by incubation for 30 min at 25°C. Stop solution (100 µl of 1 N HCl) was added, and the plate was read at 450 nm.

Specific activity determination and histochemical determination of GUS gene expression

Specific activity of the *uidA* gene and histochemical expression of GUS were analyzed according to Jefferson *et al.* (1987). Approximately 300 mg fresh weight of either leaves or roots were ground in a Lysing Matrix tube with one ceramic ball and 500 µl of extraction buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.1% sarkosyl, and 10 mM β-mercaptoethanol) using the FastPrep system (QBiogene, Carlsbad, CA), and then centrifuged at 16,000 × *g* in a microcentrifuge. Supernatant was added to assay buffer consisting of 1 mM methyl umbelliferyl-β-D-glucuronide in extraction buffer (Molecular Probes, Eugene, OR), and incubated at 37°C. The reaction was carried out for various lengths of time, and terminated using 0.2 M sodium carbonate. Fluorescence of each sample was then determined using a BioRad Versa Fluor Fluorometer (BioRad, Richmond, CA) that had a 365 nm excitation and 455 nm emission. Protein concentrations were measured using the bicinchoninic protein assay reagent (Pierce Co., Rockford, IL) according to the directions provided with the reagent. Non-transformed plants served as the negative control.

The buffer used for histochemical detection was modified by adding 0.1% (v/v) Triton X-100, 20% (v/v) methanol, 0.5 mM potassium ferricyanide, and 0.5 mM ferrocyanide to Jefferson's original GUS buffer to prevent non-specific background that commonly occurs when staining lily tissue. Leaves and roots were collected, GUS staining buffer added, and samples were placed on a gyratory shaker at 25 rpm for 16 h at 37°C. The buffer was then removed and 70% ethanol added to remove chlorophyll. Incubation in 70% ethanol was at 25°C.

Ethics

All transgenic plants were grown in a greenhouse and all experiments were conducted in a laboratory approved for recombinant DNA research by the Animal Plant Health Inspection Service of the US Department of Agriculture and the USDA Beltsville Area Research Biosafety Committee under recombinant DNA projects #061 and #064.

RESULTS AND DISCUSSION

PCR analysis of T_0 and T_1 plants

Stable integration of the transgenes in four of the T_0 lines was previously confirmed by Southern hybridization and in six lines by genetic inheritance (Kamo and Han 2008). Three plants of each T_0 transgenic plant line were analyzed by PCR for the presence of both the *bar* and *uidA* genes to verify that the original plant was probably not chimeric. The

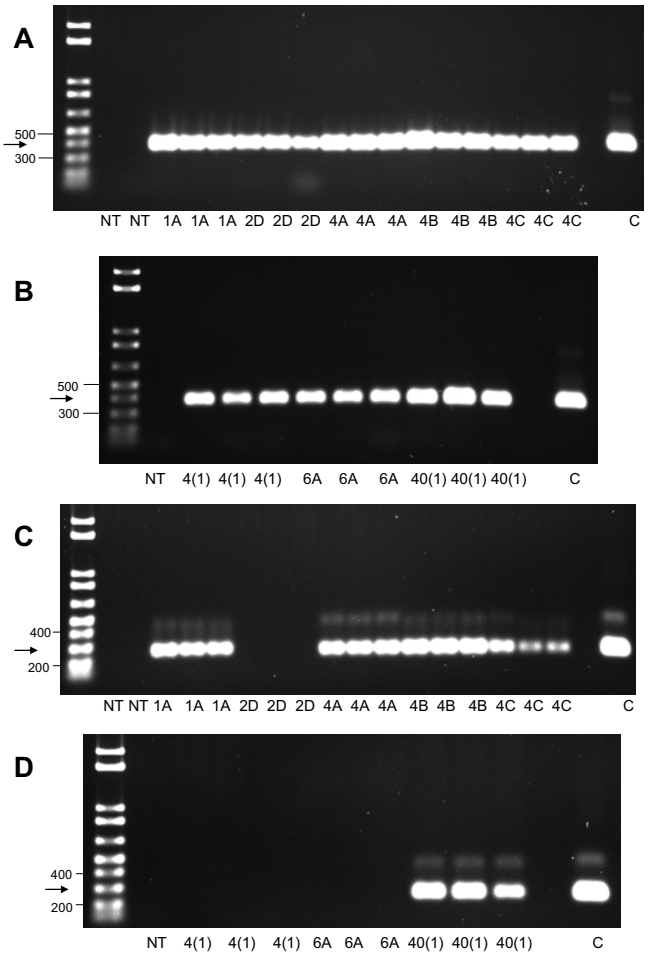

Fig. 1 PCR analysis to verify the presence of the *bar* (A, B) and *uidA* (C, D) genes. Three plants were analyzed for each T_0 plant line. Plant line numbers are indicated below each lane. Molecular weight markers (Invitrogen's 1 kb Plus DNA ladder) are shown in the left lane. Non-transformed plant DNA (NT) was the negative control, and pDM327 was the positive control (C).

Table 1 Eight transgenic *L. longiflorum* 'Nellie White' lines (T_0) were crossed with non-transformed plants resulting in T_1 plants that were analyzed by PCR for the *bar* and *uidA* genes.

Cross	T_1 line no.	Number seed pods (No. plants)	Total number of T_1 plants		
			***bar* positive**	***uidA* positive**	***bar* negative**
Flavo × T_0-1A	F-1A	5 (7)	7	7	0
Flavo × T_0-2D	F-2D	2 (3)	1	0	2
Sakai × T_0-2D	S-2D	2 (1)	1	0	0
Yin tung × T_0-2D	Yt-2D	2 (2)	1	0	1
T_0-2D × Yin tung	2D-Yt	1 (1)	1	0	0
Yin tung × T_0-4A	Yt-4A	1 (8)	0	ND[a]	8
Flavo × T_0-4A	F-4A	3 (15)	0	ND	15
Snow Queen × T_0-4A	SQ-4A	1 (9)	1	0	8
White Europe × T_0-4B	WE-4B	1 (3)	0	ND	3
Flavo × T0-4B	F-4B	1 (13)	0	ND	13
Snow Queen × T_0-4B	SQ-4B	1 (16)	5	5	11
Yin tung × T_0-4C	Yt-4C	1 (11)	0	ND	11
Flavo × T_0-4C	F-4C	1 (11)	0	ND	11
Yin tung × T_0-4C	Yt-4C	1 (10)	0	ND	10
Flavo × T_0-4(1)	F-4(1)	1(1)	1	0	0
Snow Queen × T_0-4(1)	SQ-4(1)	1 (2)	1	0	1
T0-4(1) × Yin tung	4(1)-Yt	1 (2)	1	0	1
Snow Queen × T_0-6A	SQ-6A	1 (2)	1	1	1
Flavo × T_0-6A	F-6A	1 (5)	1	0	4
Flavo × T_0-40(1)	F-40(1)	1 (9)	0	ND	9
Snow Queen × T_0-40(1)	SQ-40(1)	3 (20)	0	ND	20
Total for all crosses		32(151)	22	13	129

[a] ND=not done. Seedlings that were negative for the *bar* gene by PCR were not checked for the *uidA* gene.

plants have been vegetatively propagated for six years which allows many opportunities for a chimeric plant to segregate into transformed and non-transformed plants. Each T_0 line represents an independent transformation event. All vegetatively propagated plants originating from eight T_0 transgenic plant lines were found to have the *bar* gene (**Fig. 1A, 1B**). Only five of the eight T_0 lines, 1A, 4A, 4B, 4C, and 40(1), had the *uidA* gene; an unexpected result since the *bar* and *uidA* genes were fused. Possibly the plasmid DNA used for bombardment was quite fragmented. Three plants originating from each of the five T_0 plant lines with the *uidA* gene were analyzed by PCR, and all were confirmed to contain the *uidA* gene again indicating that the eight T_0 plants did not appear to be chimeric (**Fig. 1C, 1D**).

Crosses were made between the eight T_0 plant lines and *L. longiflorum* 'Sakai' (S), 'Yin tung' (Yt), 'White Europe' (WE), 'Flavo' (F), or 'Snow Queen' (SQ) using the transgenic lines as pollen parent in all but the following two crosses: 4(1) × Yt and 2D × Yt. T_1 seedlings resulting from the crosses were analyzed first for the presence of the *bar* gene, and if the *bar* gene was present, they were then analyzed for the *uidA* gene (**Table 1** and **Fig. 2**). Inheritance of a transgene to the progeny is genetic evidence for stable integration of the transgene. Transmission of the *bar* gene occurred in 15% of the 151 seedlings analyzed. There was no transmission in crosses using T_0-4C and T_0-40(1). Only one of three SQ-4A plants showed a faint PCR band for the *bar* gene, and the other two SQ-4A plants lacked the *bar* gene indicating that the *bar* gene was transmitted to only 20% of the T_1 progeny (**Fig. 2A**). At least 40% transmission occurred in progeny from crosses using T_0-1A, T_0-2D, and T_0-4(1). Thirteen of the 22 T_1 seedlings (59%) found to contain the *bar* gene also contained the *uidA* gene.

Low transmission rates of transgenes have been reported for both barley and corn. The level of transmission was significantly lower when the transgenic plant was the pollen donor rather than the female parent in crosses with non-transformed plants (Register III *et al.* 1994; Zhang *et al.* 1996; Bregitzer and Tonks 2003). There is evidence for the deletion of sequences between repeated sequences following homologous recombination (Peterhans *et al.* 1990; Assaad and Signer 1992; Puchta *et al.* 1995). Loss of a transgene reported for both tobacco cells cultured *in vitro* and poplar plants grown in the greenhouse is thought to result from homologous recombination that occurs during mitosis when multiple copies of a transgene are present

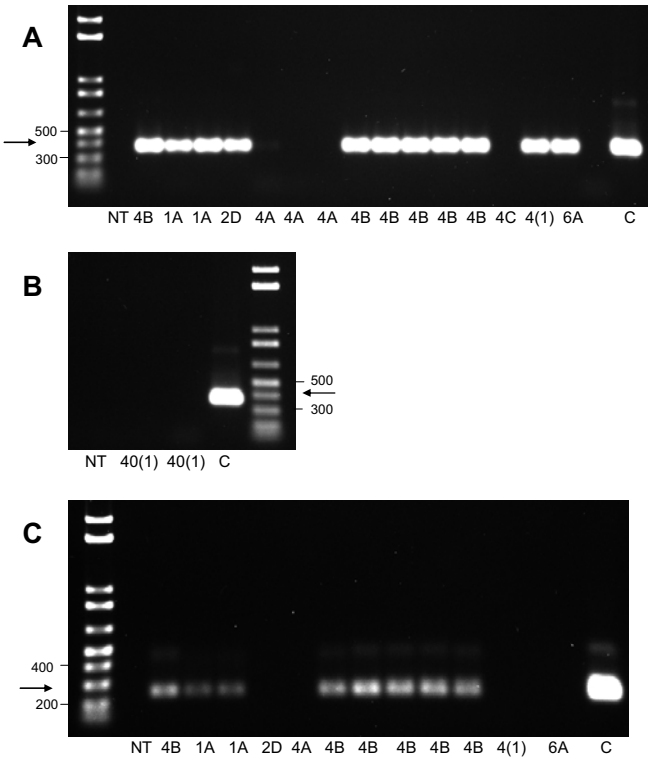

Fig. 2 PCR analysis to verify the presence of the *bar* (A, B) and *uidA* (C) genes in T_1 plant lines. Molecular weight markers are shown on the left (**A**, **C**) or right (**B**). Plant line numbers are shown below each lane. Non-transformed plant DNA (NT) was the negative control, and pDM327 was the positive control (C).

(Risseeuw *et al.* 1997; Fladung 1999). Our lilies were transformed using the gene gun, and this technique typically results in multiple copies of the transgene that could be deleted following homologous recombination during meiosis and mitosis that occurs during formation of the pollen grains. Possibly only a few pollen grains received the transgene resulting in only a few progeny plants that received the transgene.

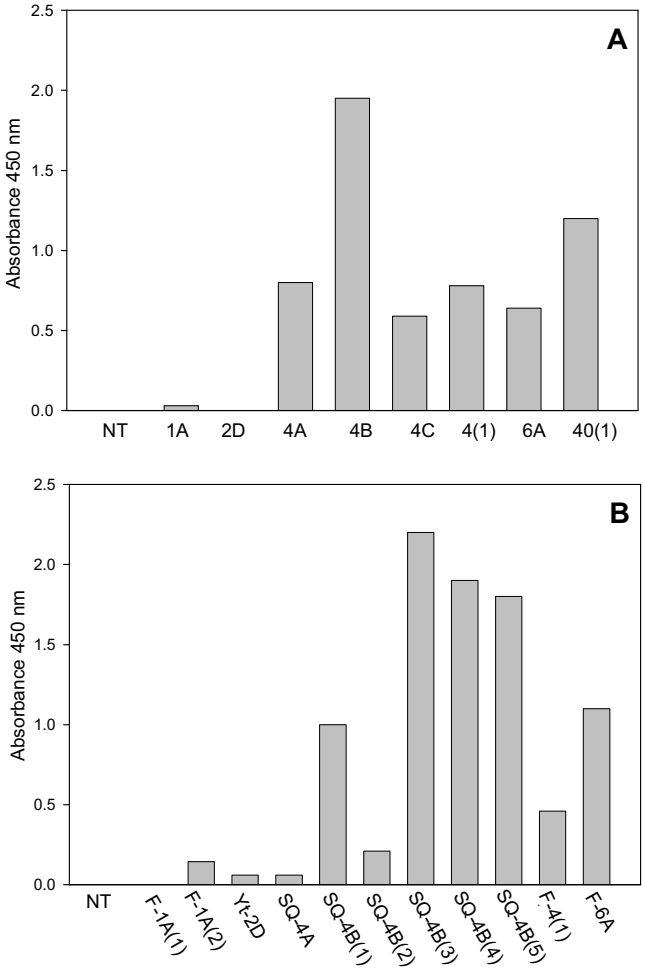

Fig. 3 Levels of *bar* gene expression in the T_0 (A) and T_1 (B) plants using the LibertyLink® PAT/bar ELISA plate assay. Two leaves were analyzed from each plant line, and the mean with standard deviation are shown on the graph.

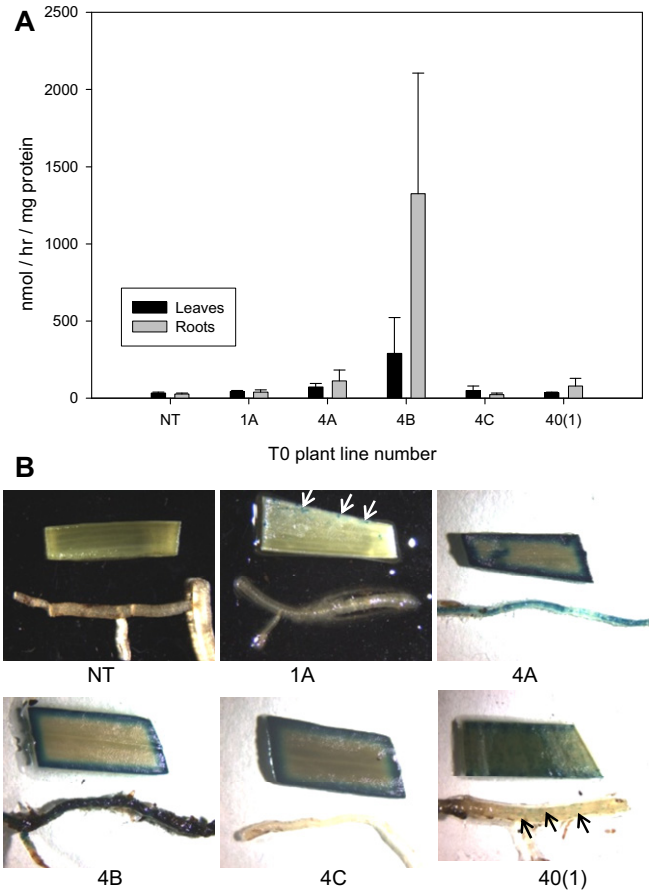

Fig. 4 GUS expression for each T_0 plant, except lines 2D, 4(1), and 6A, because they lacked the *uidA* gene. (**A**) The specific activity of β-glucuronidase was determined using three samples for each T_0 line. (**B**) Histochemical staining of a leaf (top) and root (bottom) for each T_0 line. Arrows point to small Gus-positive spots.

Transgene expression in T_0 and T_1 plants

Putatively transformed plants were selected by growing them on MS medium with 1 mg/L PPT. All eight T_0 plant lines continue to grow on this concentration of PPT indicating expression of the *bar* gene. Immunological detection of phosphinothricin acetyltransferase showed that all T_0 plant lines expressed this protein, except for T_0-2D, and the level was close to background in T_0-1A (**Fig. 3**). Phosphinothricin acetyltransferase expression was detected in all six T_1 lines analyzed, except for one of the two F-1A plants analyzed which was not surprising considering the level of expression was close to background in the T_0-1A plant. *Bar* was expressed at varying levels in all T_1 plants that came from a single seed pod produced by a 4B plant crossed with 'Snow Queen'. In comparison, *bar* was not expressed in T_1 plants resulting from crosses made between T_0-4B and either 'White Europe' or 'Flavo'. The highest level of phosphinothricin acetyltransferase expression was detected in T_0-4B and four of the five SQ-4B tested indicating inheritance of transgene expression.

Expression of the *uidA* gene was determined by specific activity of the β-glucuronidase enzyme that codes for GUS gene expression and histochemical staining. Leaves of all five T_0 lines that contained the *uidA* gene expressed GUS (**Fig. 4**). Three T_0 lines, 4A, 4B, and 40(1), expressed GUS in their roots (**Fig. 4**). Only one of the two T_1 lines that inherited the *uidA* gene, SQ-4B, showed GUS gene expression (**Fig. 5**). It was not surprising that both T_1 crosses of 1A, F-1A(1) and F-1A(2), showed no GUS activity because the level of activity was close to background level in the T_0 parent. GUS activity was high for the T_0 line 4B and some of its progeny. The level of activity was higher in the leaves than roots for four of the five *uidA*-positive plants of SQ-4B, and comparable levels of gus activity were expressed in leaves and roots of SQ-4B(5). Histochemical staining was performed because many of the GUS activity levels were close to background, and it was hoped that histochemical staining would clarify the GUS activity result. Low levels of GUS activity for leaves of T_0 lines 1A, 4A, 4C, and 40(1) were confirmed by histochemical staining (**Fig. 4**). Leaves showed a deep blue color where they had been cut. Leaves of both T_1 crosses F-1A(1) and F-1A(2) were negative by GUS staining indicating that the low levels of GUS activity were background levels (**Fig. 5**).

In conclusion, transgenes *bar* and *uidA* were inherited in only 15% and 9%, respectively, of the *L. longiflorum* progeny. *Bar* expression occurred in seven of the eight T_0 lines and in all six T_1 lines that contained the *bar* gene. GUS expression occurred in the five T_0 lines that contained the *uidA* gene and in only one of the two lines resulting from T_1 crosses. One line, 4B, showed high levels of *bar* and GUS expression, and this high level of expression was inherited in its progeny.

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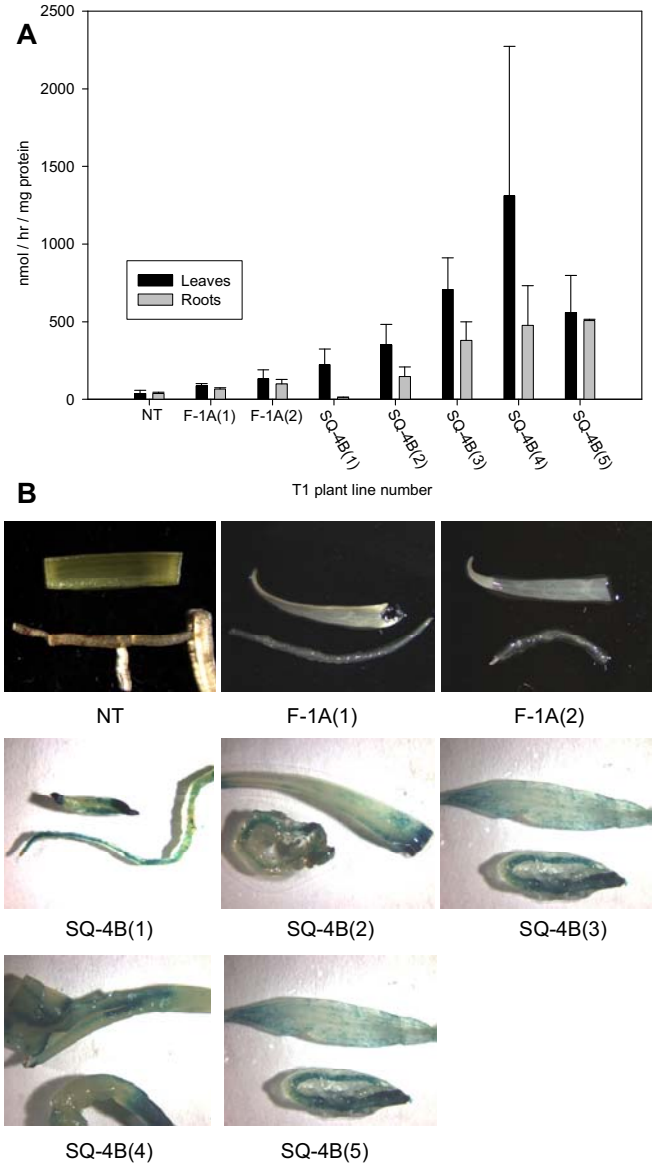

Fig. 5 GUS expression for T_1 plant lines as determined by (A) the specific activity of β-glucuronidase using three plants for each line and (B) histochemical staining.

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