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GUS Expression in *Gladiolus* Plants Controlled by Two *Gladiolus* Ubiquitin Promoters

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ABSTRACT

Ubiquitin represents a conserved family of genes that is involved in many metabolic processes. The most commonly used promoter for genetic engineering of cereal monocots is the maize ubiquitin promoter because it directs high levels of expression in most plants' tissues, but this promoter results in low levels of expression in *Gladiolus.* Several ubiquitin promoters were isolated from *Gladiolus* to find one that directs higher levels of expression than the maize ubiquitin promoter in *Gladiolus*. Two ubiquitin promoters isolated from *Gladiolus*, GUBQ2 and GUBQ4, are characterized here for their levels of expression and tissue-specific location of expression when transformed into *Gladiolus*. *Gladiolus* cv. 'Jenny Lee' plants were transformed with the *uidA* gene coding for β-glucuronidase (GUS) expression under control of either the GUBQ2 or GUBQ4 ubiquitin promoters. Five plant lines with either the GUBQ2 or GUBQ4 promoter were confirmed to be independently transformed by Southern hybridization. Two plant lines each contained one copy of pGUBQ2, and the other lines with either promoter were multicopy. There was a range in the levels of GUS expression. One of the GUBQ4 lines appeared to be silenced as GUS was not expressed in their young leaves, young roots, and callus derived from the plants. Levels of GUS expression were higher in young roots than in young shoots and callus with the GUBQ2 promoter. Three of the four expressing lines with GUBQ4 showed the highest levels of GUS expression in callus followed by roots. Histochemical staining showed that GUS was expressed throughout the leaves and roots of *Gladiolus* plants transformed with either GUBQ2 or GUBQ4.

Keywords: flower bulb, intron, non-cereal monocot, polyubiquitin
Abbreviations: 2,4-D, 2,4-dichorophenoxyacetic acid; **GUS**, β-glucuronidase; **MS** medium (Murashige and Skoog medium); **SDS**, sodium dodecyl sulfate; **SSC**, sodium chloride/sodium citrate

INTRODUCTION

Several of the flower bulb crops have been successfully transformed, usually with a reporter gene to demonstrate transformation. These crops include *Lilium* sp. (Watad *et al*. 1998; Irifune *et al.* 2003; Mercuri *et al.* 2003; Ahn *et al*. 2004; Cohen *et al.* 2004; Hoshi *et al.* 2004; Ogaki *et al.* 2008; Kamo and Han 2008), *Iris germanica* (Jeknic *et al*. 1999), *Zantedeschia elliottiana* (Yip *et al.* 2997), *Narcissus tazzeta* (Lu *et al.* 2007), *Ornithogalum* (De Villiers *et al*. 2000; Cohen *et al*. 2004), *Anthurium* (Chen *et al.* 1997), *Tricyrtis hirta* (Adachi *et al*. 2005), and *Alstroemeria* (Akutsu *et al.* 2004; Kim *et al.* 2007). Successful genetic engineering of flower bulb crops with various genes of interest will require the availability of promoters that express at useable levels and that have been characterized as to where they direct expression in the plant. There have been only a few studies comparing the expression as controlled by various promoters in flower bulb crops (Wilmink *et al.* 1995; De Villiers *et al.* 2000).

Only two promoters, a cytokinin oxidase from orchids and a polyubiquitin from *Gladiolus*, have been isolated from flower bulb crops and their expression characterized (Yang *et al.* 2003; Joung and Kamo 2006). Two ubiquitin promoters, GUBQ2 and GUBQ4, have been isolated from multiple phage genomic DNA libraries (Kamo *et al.* 2009). We chose to isolate and characterize ubiquitin promoters from *Gladiolus* because the ubiquitin gene is highly conserved in all eukaryotes making it possible to screen a genomic DNA phage library with a heterologous probe, and ubiquitin promoters have been demonstrated to result in high levels of expression in other plant species. High levels of gene expression have been reported for ubiquitin promoters isolated from *Arabidopsis thaliana*, *Solanum tuberosum*, *Saccharum officinarum*, *Oryza sativa*, *Zea mays* (Norris *et al*. 1993; Garbarino and Belknap 1994; Christensen and Quail 1996; Wang *et al*. 2003; Wang and Oard 2003; Wei

Fig. 1 ***Gladiolus*** **cv. 'Jenny Lee' plants.**

Received: 11 September, 2009. Accepted: 25 September, 2009.

Original Research Paper

et al. 2003). This report describes the levels of gene expression conferred in the transgenic plant lines of *Gladiolus* and callus derived from them with the GUBQ2 and GUBQ4 promoters.

MATERIALS AND METHODS

Isolation of two ubiquitin promoters from *Gladiolus*

Two ubiquitin promoters (GenBank accession numbers EU563360 and EU563361) were isolated by screening phage genomic DNA libraries made from *Gladiolus* cv. 'Jenny Lee' (**Fig. 1**) with a heterologous probe, RUBQ2, the ubiquitin gene from rice (Wang *et al*. 2000) (Kamo *et al*. 2009). The *uidA* gene that codes for GUS expression was subcloned under control of either the GUBQ2 or GUBQ4 promoters into a pUC-based vector with *nos* as the terminator.

Transformation of *Gladiolus*

Embryogenic callus was initiated from *in vitro*-grown plants and cormels and bombarded as previously described (Kamo *et al.* 1995). Delivery of plasmid DNA coated onto 0.8 μm gold particles was accomplished using the PDS-1000/Helium gene gun (Sanford *et al*. 1993). Both the selectable marker plasmid p35SAc (received from AgrEvo, Sommerville, NY) that contains the phosphinothricin acetyltransferase gene under control of the CaMV 35S promoter and a plasmid containing either the GUBQ2 or GUBQ4 promoter-*uidA*-*nos* were used to co-bombard callus.

Bombarded callus was grown on MS medium (Murashige and Skoog 1962) supplemented with 0.5 mg/L (2.2 μM) 2,4-D (2,4-dichlorophenoxyacetic acid) and 1 mg/L bialaphos (Meiji Seika Kaisha, www.meiji.co.jp) for selection (Kamo *et al*. 1995). Plants regenerated from the callus were initially selected by their ability to grow on MS medium containing 1 mg/L phosphinothricin and their GUS expression shown by histochemical staining of the callus and small regenerating plants. Histochemical staining was performed according to Jefferson *et al.* (1987).

Southern hybridization and PCR

Genomic DNA isolated according to the method of Dellaporta *et al*. (1993) was digested with either *Eco*RI or *Hind*III and then separated by electrophoresis on a 0.9% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Each lane on the gel contained 30 μg of DNA as determined by measuring the optical density at 260 nm. The DNA was transferred to a Nytran membrane (Schleicher-Schuell, Keene, NH, www.schleicher-schuell.com), and the DNA blot was hybridized according to Maniatis *et al.* (1982).

A probe specific to either the G2 and G4 promoter was prepared by PCR (**Fig. 2**), purified by QIAquik PCR Purification Kit (Qiagen, Valencia, CA, www.qiagen.com), then labeled with [α-^{32}P]dCTP using a DECAprime Kit (Ambion, Austin, TX, www.ambion.com). The PCR probe used for hybriziding GUBQ2 DNA blots consisted of the forward primer 5′-GAGTAGGCATTTAGC TCCCCC-3′ and reverse primer 5′-ACACCAACATGCGCAAAT AA-3′ (**Fig. 2**). The GUBQ4 probe consisted of the forward primer 5′-TTCCTCCAACATTTTCTGGC-3′ and reverse primer 5′-CAC ATGTGCCCGTTTAGTTG-3′.

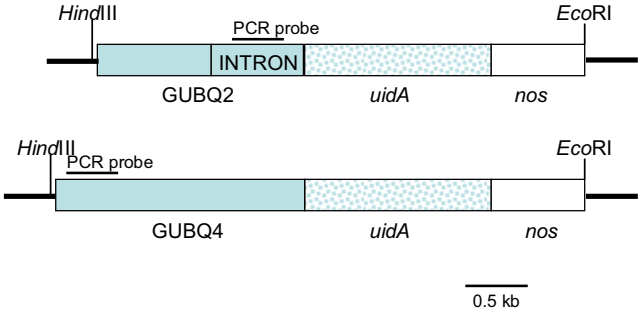

Fig. 2 Diagram of the GUBQ2 (top) and GUBQ4 (bottom) plasmids used to transform *Gladiolus*. Location of the PCR probes and restriction sites used for the Southern hybridization are indicated.

Presence of the *uidA* gene in plants transformed with either the GUBQ2 or GUBQ4 promoters and was confirmed by PCR amplication of genomic DNA using the forward primer 5′-TAA CCTTCACCCGGTTGCCAGAGG-3′ and reverse primer 5′-CTT TAACTATGCCGGAATCCATCG-3′. Plasmid DNA was amplified using a MJ Research Microcycler PTC-200 programmed for 94°C for 2 min, 30 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min), 72°C for 10 min.

DNA blots were hybridized at 58°C followed by washing for 15 min each wash at 55°C in 2X SSC/0.2% SDS, 1X SSC/0.2% SDS, and lastly 0.1X SSC/0.2% SDS. Blots were exposed to BioMax X-ray film at -70°C for 2-5 days.

GUS expression

Levels of GUS expression were determined in leaves, roots, and callus. Young leaves and young roots used for GUS expression analysis were collected from plants that had been grown one month *in vitro* on MS medium. Plants were grown at 25°C under a 16-hr photoperiod with light from cool-white fluorescent bulbs (General Electric F34CWRS ECO) providing an intensity of 40-60 μmol $m^{-2}s^{-1}$. *In vitro*-grown plants were cultured for 6 months in the light on MS with 0.5 mg/L 2,4-D to induce callus from the basal meristem of the plant. Callus was grown in the dark on MS with 0.5 mg/L 2,4-D and collected 21 days after subculture for analysis. The fluorescent assay was used for determination of the specific activity of β-glucuronidase according to Jefferson *et al.* (1987). Specific activity is expressed as the nmol 4-MU (methylumbelliferone)/mg protein/min. Protein content was determined using the bicinchoninic reagent (BCA) according to the manufacturer's instructions (Pierce, www.piercenet.com).

Statistics

The specific activity of GUS was determined for each independently transformed plant line, and a non-transformed plant was the control. Three samples taken from different plants or callus pieces were analyzed for GUS for each independently transformed plant line. An analysis of variance followed by Dunn's multiple comparison with a 95% confidence interval ($P \leq 0.05$) was performed using Sigmastat (www.systat.com) to compare the means of relative GUS activity for each type of plant tissue (callus, leaves, or roots) transformed with either the GUBQ2 or GUBQ4 promoter.

Ethics

All work was conducted in a lab that has been approved by both the USDA Beltsville Area Biotechnology committee and the USDA's Animal Plant Health Inspection Agency that follows the National Institute of Health guidelines.

RESULTS AND DISCUSSION

Transformation of *Gladiolus*

Integration of the GUBQ2 and GUBQ4 promoters was confirmed by Southern hybridization in all transformed lines of *Gladiolus* plants selected for analysis, and each line was an independently transformed plant line (**Fig. 3**). Presence of the *uidA* gene was shown in all 10 plant lines by PCR (**Fig. 4**). T1 plants were used for characterization of GUS expression under control of the GUBQ2 and GUBQ4 promoters because cv. 'Jenny Lee' is apparently sterile. Seed was not obtained following efforts by the authors and two breeders to either self or outcross 'Jenny Lee' for two seasons. Genomic DNA was digested with either *Eco*RI or *Hind*III both of which cut once within the transgene (*Eco*RI) or very close to it (*Hind*III) (**Figs. 2, 3**). Hybridization of the GUBQ2 and GUBQ4 probes occurred in genomic DNA from non-transformed plants because this promoter occurs in wild type plants. Two plant lines, numbers 3 and 4, transformed with GUBQ2 contained one copy, and the other GUBQ2 lines

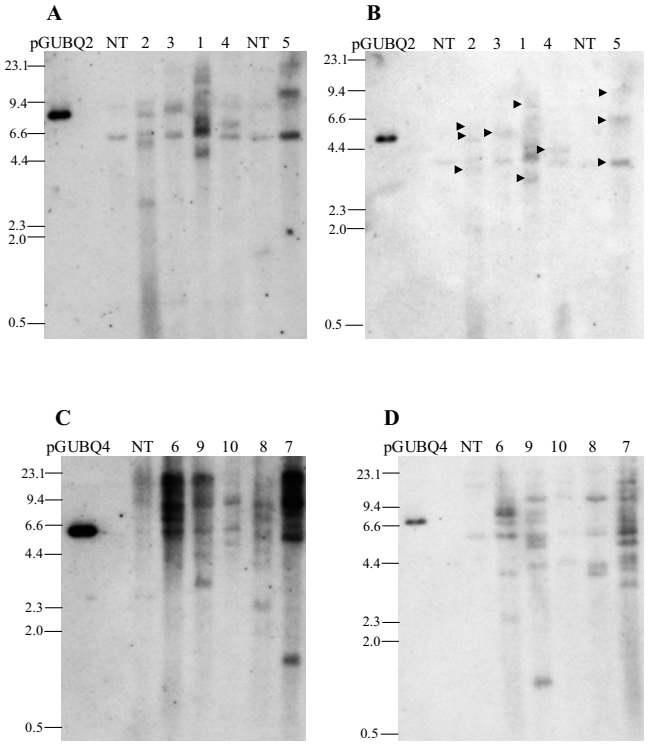

Fig. 3 Southern hybridization of genomic DNA from either GUBQ2 plant lines digested with *Eco*RI (A) or *Hind*III (B) and GUBQ4 plant lines digested with either *Eco*RI (C) or *Hind*III (D). DNA blots were hybridized with GUBQ2 or GUBQ4 probes labeled with [α-^{32}P]dCTP. Each lanes contains 30 µg of genomic DNA as determined by optical density at 260 nm. The plant line numbers are indicated above each lane, and non-transformed (NT) DNA was used as a negative control. Molecular weight markers are shown in kb.

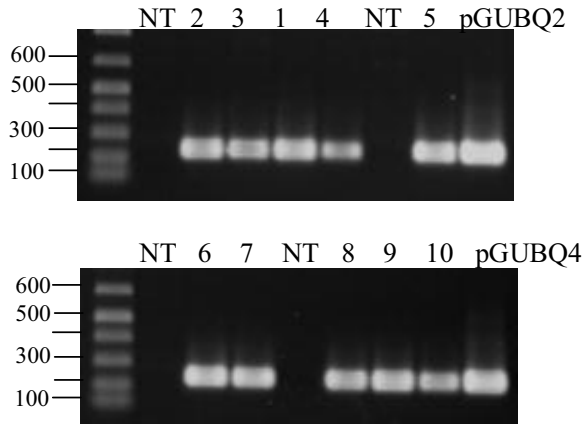

Fig. 4 Presence of the *uidA* gene in GUBQ2 (upper gel) and GUBQ4 (lower gel) plant lines is confirmed by PCR amplification. The plant line numbers are indicated above each lane. Non-transformed (NT) plants and plasmid DNA, pGUBQ2 or pGUBQ4, are the negative and positive controls, respectively. Molecular weight markers are shown on the left in bp.

had 2-4 copies. Plant lines transformed with GUBQ4 had 3-8 copies. The one line with GUBQ4 that appeared to be silenced had a relatively low copy number, 3 copies.

Levels of GUS expression

Moderate GUS expression was visualized by histochemical staining throughout the leaves and roots of transgenic plants with either the GUBQ2 or GUBQ4 promoters (**Fig. 5**).

Levels of GUS expression were higher in callus, young shoots, and young roots with the GUBQ4 promoter as compared to the GUBQ2 promoter although there was a wide

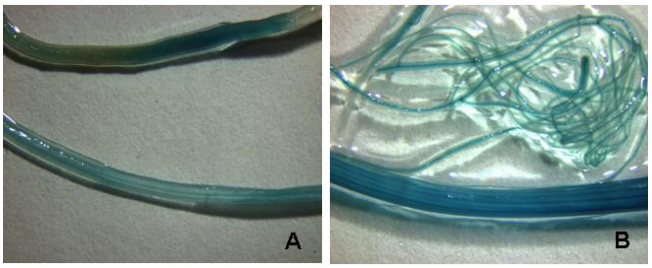

Fig. 5 Histochemical staining of *Gladiolus* plants transformed with either GUBQ2-*uidA* (A) or GUBQ4-*uidA* (B) showing roots (top) and leaves (bottom) in each photo. Plants were grown *in vitro*.

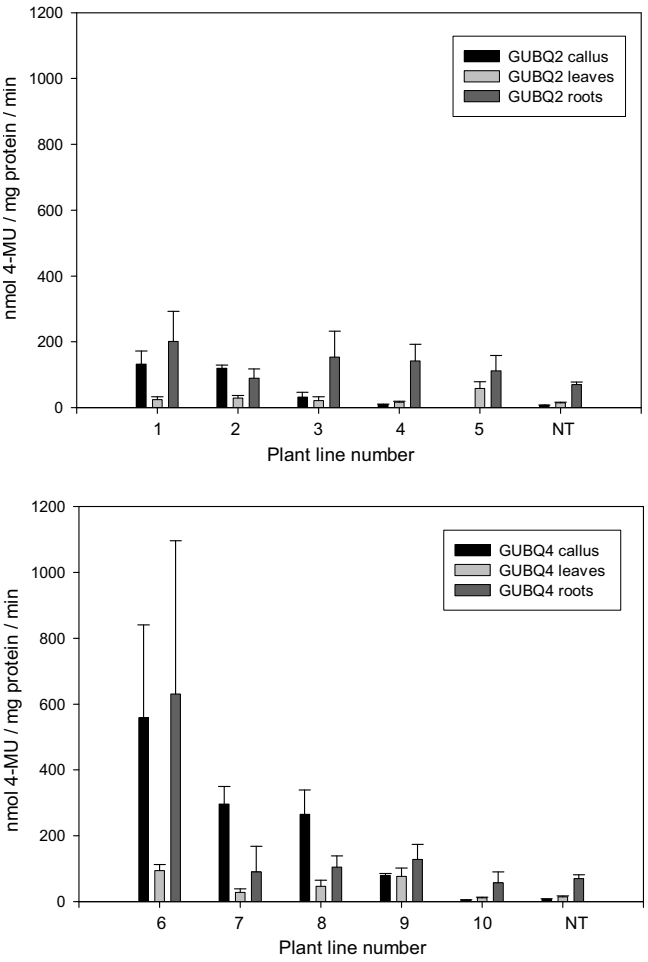

Fig. 6 Levels of GUS expression for callus, young leaves, and young roots of *Gladiolus* plants transformed with either the GUBQ2 (top) or GUBQ4 promoter (bottom). Plant line numbers correspond to numbers on the DNA blots (**Fig. 2**). Each line analyzed consists of three plant samples, and standard error bars are shown.

Table 1 GUS expression in callus, young leaves, and young roots under control of either the GUBQ2 or GUBQ4 promoters. Five transformed plant lines were analyzed for GUBQ2. Four plant lines were analyzed for GUBQ4 because one appeared to be silenced, and it was not included below. Each line, including non-transformed plants, consisted of three samples for each type of tissue.

Promoter	**GUS expression (nmol 4-MU/mg protein/min ± SE)**		
	Callus	**Leaves**	**Roots**
GUBQ2	59 ± 31 a*	30 ± 8 c	140 ± 24 e
GUBQ4	300 ± 99 b	61 ± 15 d	238 ± 131 e
Non-transformed	8 ± 0.3 a	15 ± 2 c	70 ± 8 e

*Values with different letters are significantly different at P≤0.05 according to Dunn's Method used to compare GUS activity for each type of tissue transformed with either the GUBQ2 or GUBQ4 promoter.

variation in expression for the GUBQ4 plant lines (**Table 1, Fig. 6**). Line 6 with the GUBQ4 promoter showed high levels of GUS expression in the roots, 631 nmol 4-MU/mg protein/min, as compared to the other five GUBQ4 lines that showed only a maximum 128 nmol 4-MU/mg protein/min. Young roots showed comparable levels of GUS expression with the GUBQ2 promoter (90-140 nmol 4-MU/mg protein/min) as compared to the GUBQ4 promoter (57-128 nmol 4-MU/mg protein/min), except for the highly-expressing GUBQ4 line 6 (**Fig. 6**). The GUBQ4 promoter construct used for transforming *Gladiolus* was 2011 bp in length. The GUBQ2 construct was shorter, 1614 bp, of which 680 bp is its intron with 934 bp upstream from it. Possibly there was an upstream enhancer that was not included in the GUBQ2 construct. An enhancer that increased transient GUS expression 2.4X was found 739 bases upstream from the TATA box of the rice RUBQ2 promoter (Wang and Oard 2003).

In all lines with either the GUBQ2 or GUBQ4 promoter, the level of GUS expression was low in leaves (30-61 nmol 4-MU/mg protein/min) as compared to the highest levels of expression attainable with the CaMV 35S promoter (480 nmol 4-MU/mg protein/min) (unpublished). Levels of transient GUS expression in *Gladiolus* suspension cells were over 3X times higher with the CaMV 35S promoter than for the maize Ubi1 promoter even though the maize Ubi1 promoter is the most commonly used promoter for monocots because of the high levels of expression that it directs (Joung and Kamo 2006). Plants of all lines transformed with GUBQ2 and GUBQ4 were phenotypically normal in tissue culture and grew well whereas the plants with the CaMV 35S promoter and *uidA* gene were typically difficult to propagate because they did not grow vigorously. Frequently plants with the CaMV 35S promoter had short, 1-2 cm long, leaves, and there was not much active proliferation of side shoots whereas the leaves of plants transformed with the GUBQ2 and GUBQ4 promoters typically had longer leaves, about 5 cm long, accompanied with active proliferation of side shoots. Possibly plant growth was affected by the level of GUS expression, and protein synthesis for growth was competing for that of GUS protein production.

One of the five GUBQ4 plant lines, number 10, appeared to be silenced as there was no GUS expression in their young leaves, young roots, or callus. Possibly there was homology-dependent silencing because GUBQ4 is an endogenous promoter in *Gladiolus.* The RUBQ1 and RUBQ2 promoters from rice expressed very well in rice as did the maize ubi1 promoter in maize (Wang *et al*. 2000). In sugarcane transformed with a sugarcane polyubiquitin promoter, ubi9, silencing occurs in plants but not callus (Wei *et al*. 2003). It remains to be determined if homology-based co-suppression is a problem with GUBQ4 when used to transform *Gladiolus*.

CONCLUSION

Two ubiquitin promoters, one confirmed to be a polyubiquitin promoter, have been characterized for the levels of *uidA* expression that they control in *Gladiolus*. Levels of GUS expression were relatively low-moderate in leaves transformed with the GUBQ2 and GUBQ4 promoters and higher in roots and callus, particularly with GUBQ4. Although the levels of expression with these two promoters was lower than that with the highly expressing CaMV 35S promoter, transgenic plants with the moderately expressing GUBQ2 and GUBQ4 promoters were phenotypically normal whereas transgenic plants with CaMV 35S were much slower growing. In developing transgenic *Gladiolus* plants it may be necessary to use promoters such as GUBQ2 and GUBQ4 for normal plant growth.

ACKNOWLEDGEMENTS

The authors thank Anne O'Connor for excellent technical assistance, Dr. Jim Oard (Louisiana State University, Baton Rouge, LA) for providing the RUBQ2 plasmid, and AgrEvo (Sommerville, NJ) for providing p35SAc. Mention of a trademark, proprietary product or vendor does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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