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Growth characteristics of micropropagated, regenerated and transgenic *Gladiolus* plants

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Abstract

The growth characteristics of transgenic *Gladiolus* plants cvs. 'Peter Pears' and 'Jenny Lee' were compared to non-transformed plants either regenerated from embryogenic callus or micropropagated *in vitro*. Micropropagated and regenerated plants of 'Peter Pears' showed similar sprouting percentage of corms *in vitro* and daughter corm production after one season in the greenhouse. Differences were found in the weight of corms produced *in vitro* and the length of leaves with the regenerated corms weighing less and having shorter leaves than those of micropropagated plants. Transgenic plants of 'Peter Pears' had similar corm weights to those from regenerated plants, but the greenhouse sprouting percentage, leaf length, and daughter corm production was less than that of regenerated plants. Micropropagated plants of 'Jenny Lee' were similar to regenerated plants in weight of corms grown *in vitro*, sprouting efficiencies, and the length of leaves. Transgenic plants of 'Jenny Lee' produced larger corms *in vitro* than regenerated plants, and both the final weight of transgenic corms and leaf length after one season in the greenhouse were comparable to that of regenerated plants of 'Jenny Lee'. 'Jenny Lee' plants were less affected by the regeneration and transformation conditions than 'Peter Pears'.

Key words: Flower bulbs, biolistics, transgenes, regeneration, tissue culture, corms, ornamentals, gene gun bombardment, callus

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; CMV, *Cucumber mosaic virus*; CMV CP, *Cucumber mosaic virus* coat protein; CMV AB, *Cucumber mosaic virus* antibody; CPO, chloroperoxidase; FW, fresh weight; GUS, β-glucuronidase; MS, Murashige and Skoog's medium; NAA, α-naphthaleneacetic acid; PAT, phosphinothricin acetyltransferase

Introduction

Because floral crops are valued for their appearance, it is important that genetically engineered plants appear phenotypically normal. Somaclonal variation that results when plants are regenerated from callus has been documented previously (Larkin and Scowcroft, 1981; Lee and Phillips, 1987; Phillips *et al.*, 1994; Park *et al.*, 2009). Flowers of regenerated *Gladiolus* plants have been reported to be both phenotypically normal when callus was induced from ovaries (Kasumi *et al.*, 1998) or variable when regenerated from stem tips of cormels (Kasumi *et al.*, 1999). These variants were smaller and had a shorter flower spike and fewer, smaller florets as compared to control plants. The frequency of flower color variation differed for each cultivar. The original color of the flower was retained, but the hue changed to either darker or lighter. *Gladiolus* plants of cvs. Blue Isle, Jenny Lee, Peter Pears, and Rosa Supreme regenerated from callus induced with NAA were phenotypically normal (Stefaniak, 1994). Remotti *et al*. (1997) screened suspension cells of *Gladiolus* cv. Peter Pears for resistance to fusaric acid, and two plants regenerated from resistant cell lines had a lower DNA content than control plants. These suspension cells were grown in 2,4-D, and a few albino plants were amongst the regenerated plants indicating the occurrence of somaclonal variation (Remotti, 1995).

Somaclonal variations were assessed in the progeny of transgenic barley plants, and it appeared that the transformation process induced more somaclonal variation than the tissue culture process of regenerating barley plants from callus (Choi *et al.*, 2000). A high frequency (50%) of chromosomal variation has been found in oat plants transformed using the gene gun. Somaclonal variation resulting from transformation could not be attributed to either transgene insertion or expression (Bregitzer *et al.*, 1998). Fortunately a single backcross eliminated the phenotypic abnormalities observed in the transgenic barley plants (Bregitzer *et al.*, 2008). Many floral crops are propagated vegetatively, and often backcrossing is not an option in order to maintain the desired characteristics of an ornamental cultivar.

This study was conducted to assess the variation in growth and development that has been observed with *Gladiolus* plants transformed with either an antiviral gene to *Cucumber mosaic virus*, a GUS reporter gene, or an antifungal gene to evaluate the factors affecting long term growth of these transgenic plants.

Materials and methods

Plants *in vitro*: *Gladiolus* plants of cvs. Peter Pears and Jenny Lee were grown *in vitro* in Magenta jars containing Murashige and Skoog's medium (MS; Murashige and Skoog, 1962) solidified with 0.2% Phytagel (Sigma Aldrich Chemical Company, St. Louis, MO). Cultures were maintained at 25°C under a 12 h photoperiod at 40-60 μmol/m^2/s using cool white fluorescent lights. "Micropropagated" plants refers to non-transformed plants grown *in vitro*.

Corms were stored at 4 °C in the dark for 6-9 months and then cultured in the light on MS medium for sprouting. Plants were initiated from corms each year.

Sprouting of the corms *in vitro* was assessed by placing 10 corms/Petri plate on MS medium in the light. Three Petri plates of corms were grown for each plant line, if available, to determine the sprouting percentage.

Regenerated plants of 'Peter Pears' and 'Jenny Lee' were obtained by culturing plants growing *in vitro* on MS medium supplemented with 2.3 μM 2,4-D for 6 months to induce embryogenic callus (Kamo *et al.*, 1990). Embryogenic callus formed after two months from the base of the plant, and it was maintained in the dark at 25 °C on MS medium with 2.3 μM 2,4-D. Subcultures to fresh medium were performed monthly. Six-month-old callus was subcultured to MS medium lacking plant growth regulators for plant regeneration. Small plants approximately 1 cm in height were transferred to light conditions for further growth. "Regenerated" plants refers to non-transformed plants regenerated from embryogenic callus.

Plants in the greenhouse: Corms collected from plants grown *in vitro* were exposed for 6-9 months to 4 °C before planting in Metromix 510 formulated with bark, peat moss, vermiculite, slow release nitrogen, dolomitic limestone, bark ash "starter nutrient charge" (Sun Gro Horticulture, Agawam, MA) in clay pots in the greenhouse. Plants were grown in the greenhouse from April through November. The greenhouse temperature was maintained at 24-25 °C during the day and 21-23 °C at night.

The length of the longest leaf was measured from ten plants for each plant line.

Transformation: Suspension cells were initiated from embryogenic callus induced from *in vitro*-grown plantlets as described under "Plants *in vitro*". Suspension cells were cultured in the dark at 25 °C in liquid MS medium supplemented with 2.3 μM 2,4-D. Each 125 mL flask contained 30 mL of liquid medium for the cells, and flasks were kept on a gyratory shaker at 100 rpm. Every two weeks half of the *Gladiolus* suspension cells were transferred to a new flask of medium.

Suspension cells were cultured in MS medium containing 2.3 μM 2,4-D and 0.125 M mannitol for 2 h prior to collecting them on a Whatman no. 4 filter paper for bombardment using the gene gun. The Whatman filter paper with cells was placed in a Petri plate containing MS medium with 2.3 μM 2,4-D, 0.125 M mannitol, and 1.4% Phytoblend (Caisson Laboratories, www.caissonlabs.com) for bombardment at 8.4 MPa (1200 psi) using the PDS-1000/He system (BioRad, Richmond, CA). Gold particles (1 μm) were coated with plasmid DNA according to Sanford *et al.* (1993) and used to introduce the DNA. The gene gun was set with a 1 cm gap and 1 cm flying membrane distance, and the Petri plate with the cells was at a 12 cm target distance. Each plate of cells was bombarded once. Following bombardment, the cells were transferred to MS medium lacking osmoticum immediately after bombardment.

Bombarded cells were maintained in the dark at 25 °C and transferred to MS medium supplemented with 2.3 μM 2,4-D and 0.1 mgL^{-1} bialaphos (Meiji Seika Kaisha, Tokyo, Japan, www.meiji.co.jp) one week following bombardment. One month later cells were transferred to MS medium with 2.3 μM 2,4-D and 1 mgL^{-1} bialaphos. Callus was transferred monthly to fresh medium for 3-6 months. If plants regenerated from the callus, the plants were transferred to MS medium lacking hormones and containing either 1 mgL^{-1} phosphinothricin (AgrEvo, Somerville, NJ) for 'Peter Pears' or 2 mgL^{-1} phosphinothricin for 'Jenny Lee'. After six months, all remaining callus that was still alive was transferred to MS medium containing 9.3 μM kinetin and 1 mgL^{-1} bialaphos. Regenerated plants were grown under the same light/dark conditions as described under "Plants *in vitro*".

The plasmids used for transformation were either the D4E1 gene under control of the CaMV 35S promoter (Rajasekaran *et al.*, 2005), the chloroperoxidase gene under control of the CaMV 35S promoter (Rajasekaran *et al.*, 2000), the *Cucumber mosaic virus* coat protein II gene under control of the *Arabidopsis UBQ3* promoter (Kamo *et al.*, 2010), the *Cucumber mosaic virus* replicase gene under control of a duplicated CaMV 35S promoter (Kamo *et al.*, 2010), the antibody gene to CMV under control of the duplicated CaMV 35S promoter (Kamo *et al.*, 2012a), the *uidA* gene coding for β-glucuronidase (GUS) expression under either the *GUBQ2*, *GUBQ4*, or *GUBQ1* promoter (Kamo *et al.*, 2009, 2012b). Cells were co-bombarded with one of the plasmid DNAs and p35SAc that codes for the phosphinothricin acetyltransferase (PAT) gene under the control of the CaMV 35S promoter (received from Dr. P. Eckes, AgroEvo, Somerville, NJ). Plasmids were isolated from *E. coli* DH5α by alkaline lysis and then purified on a cesium chloride gradient (Maniatis *et al.*, 1982).

Data collection and statistical analysis: Thirty corms were collected from each line of *in vitro*-grown plants, and ten of these corms were weighed to obtain an initial fresh weight value for each plant line. These 30 corms were planted in Metro-Mix 510 in the greenhouse. If corms did not sprout, replacement corms were planted in an effort to obtain data on leaf lengths and daughter corm production.

The percent of corms that sprouted in the greenhouse was determined for each plant line. Each plant line was an independent transformation event. For 'Peter Pears' a total of 355 corms were planted for ten regenerated lines, 878 corms for eight transgenic lines containing the chloroperoxidase gene, 801 corms for six lines with the D4E1 gene, 457 corms for five lines with a CMV replicase gene, and 183 corms for four lines with a CMV antibody gene. For 'Jenny Lee', 386 corms were planted for 10 regenerated plant lines, 880 corms for 18 lines with the GUS gene, 141 corms for two lines with the CMV coat protein gene, and 233 corms for five lines with the CMV antibody gene. A One Way Anova followed by the Holm-Sidak Method indicated statistical differences in sprouting at $P \leq 0.05$.

The percent of corms that sprouted *in vitro* was also determined for *in vitro*-grown corms. For each plant line, ten corms were placed in each of the three Petri plates. Corms (47-50) from micropropagated plants of either 'Peter Pears' or 'Jenny Lee' and corms (287-300) from ten regenerated plant lines of each cultivar were grown *in vitro*. For 'Peter Pears' 202 corms were available for seven lines with the chloroperoxidase gene, 176 corms for six lines with the D4E1 gene, 13 corms for four lines with the CMV replicase gene, and 120 corms for four lines with the CMV antibody gene. For 'Jenny Lee there were 577 corms for 24 lines with the GUS gene, 167 corms from four lines with the CMV coat protein gene, and 210 corms from four lines

with the CMV antibody gene. A One Way Anova followed by the Holm-Sidak Method indicated statistically differences in sprouting at $P \leq 0.05$.

The length of the longest leaf was measured for ten plants, if available, of each line growing in the greenhouse. Measurements were taken from plants that had completed their full fan development of their leaves. The length of the longest leaf for micropropagated, regenerated, and transgenic plants was compared using a One Way Anova followed by the Dunn's Method at $P \leq 0.001$.

Plants were grown approximately 5 months in the greenhouse untill their leaves turned brown and fell off. At this time approximately ten corms, if available, were weighed for each plant line to obtain a final fresh weight of the corms. A student's t test was performed using SigmaPlot (Systat Software, Inc., San Jose, CA, www.sigmaplot.com) to compare if the weight of corms from micropropagated plants was significantly different from corms of regenerated or transgenic plant lines that contained each gene at $P \leq 0.05$.

Results

Weight of corms: Corms from *in vitro*-grown plants of regenerated 'Peter Pears' weighed less than corms from micropropagated plants of 'Peter Pears' whereas the weight was comparable between corms from regenerated and micropropagated plants of 'Jenny Lee' (Fig. 1, Initial FW). Transgenic 'Peter Pears' plants containing either the CMV replicase or CMV antibody gene, and transgenic 'Jenny Lee' plants containing either the GUS, CMV coat protein, or CMV antibody gene produced corms *in vitro* with a fresh weight significantly higher than corms from regenerated plants of the corresponding cultivar.

Corms from regenerated plants of both cultivars grown for one season in the greenhouse did not weigh as much as corms from micropropagated plants (Fig. 1, Final FW). The final weight of transgenic corms harvested from 'Peter Pears' containing either the chloroperoxidase, D4E1, or CMV antibody gene was similar to, and corms with a CMV replicase gene weighed more, than corms from regenerated plants of 'Peter Pears. After one season in the greenhouse all transgenic corms of 'Jenny Lee' were similar in weight to corms from regenerated plants of 'Jenny Lee'.

Corms from micropropagated plants of both cultivars had a significantly greater fresh weight than regenerated and transgenic corms after one season of growth in the greenhouse.

Sprouting of corms: Corms from *in vitro*-grown plants of both micropropagated and regenerated plants of 'Peter Pears' sprouted at similar percentages *in vitro* (98-100%) (Fig. 2). The sprouting percentage *in vitro* was also similar (79-82%) for corms from micropropagated and regenerated 'Jenny Lee' plants grown *in vitro*. Transgenic corms of 'Peter Pears' containing the CPO, D4E1, CMV replicase, and CMV antibody genes were capable of sprouting *in vitro* at a 62-63% sprouting percentage *in vitro* (Fig. 2). Transgenic corms of 'Jenny Lee' containing the GUS gene, but not the CMV coat protein or CMV antibody genes, sprouted *in vitro* at a percentage (70%) similar to that of regenerated 'Jenny Lee' corms (82%).

A similar percent (70-73%) of corms produced by both micropropagated and regenerated 'Peter Pears' plants sprouted in the greenhouse (Fig. 2). Only a small percent (10-38%) of corms from transgenic plants of 'Peter Pears' sprouted in the greenhouse as compared to 'Peter Pears' corms from regenerated plants (73%). Sprouting in the greenhouse was higher (53%) for corms from micropropagated plants of 'Jenny Lee' as compared to corms from regenerated 'Jenny Lee' plants (35%). Corms from transgenic plants of 'Jenny Lee' sprouted in the greenhouse at percentages (19-38%) comparable to that of corms from regenerated plants of 'Jenny Lee' (38%).

Corms from micropropagated, regenerated, and transgenic plants of 'Peter Pears' sprouted at 62-100% *in vitro*, but sprouting of transgenic 'Peter Pears' was much lower (10-38%) in the greenhouse. Sprouting of 'Jenny Lee' corms from micropropagated, regenerated, and transgenic corms was 19-50% in the greenhouse.

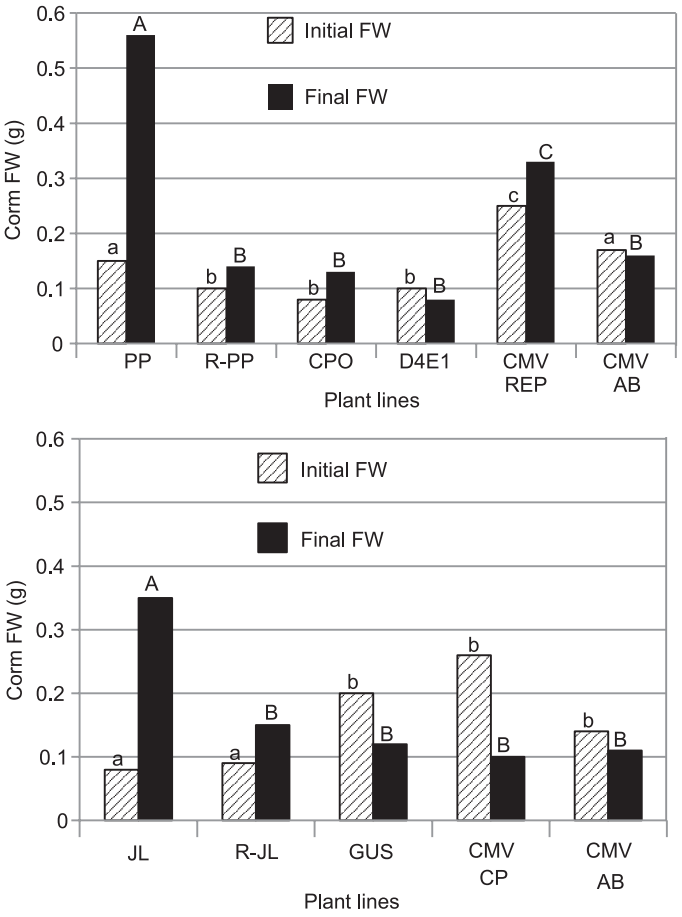

Fig. 1. Fresh weight of *Gladiolus* corms from *in vitro*-grown plants (initial FW) and corms harvested after one season of growth in the greenhouse (final FW). Ten corms from *in vitro*-grown plants were weighed as the initial fresh weight (FW), and 30 corms were planted in the greenhouse. All transgenic plants contained the PAT selectable marker gene. Top graph: Transgenic 'Peter Pears' (PP) plants contained either the chloroperoxidase (CPO), D4E1 peptide, the CMV replicase (CMV REP), or CMV antibody gene (CMV AB). Bottom graph: Transgenic Jenny Lee (JL) plants contained either the GUS, CMV coat protein subgroup II (CMV CP), or CMV antibody gene (CMV AB). A Student's t test was performed comparing corms from regenerated corms (R-PP or R-JL) and transgenic plants to those of micropropagated plants (PP or JL). A different lower case letter above the bar indicates that the initial weight was statistically different between the micropropagated corms and either the transgenic or regenerated corms, and a capital letter indicates a significant difference for the final weights ($P \leq 0.05$).

Leaf lengths: The length of the longest leaf was measured in the

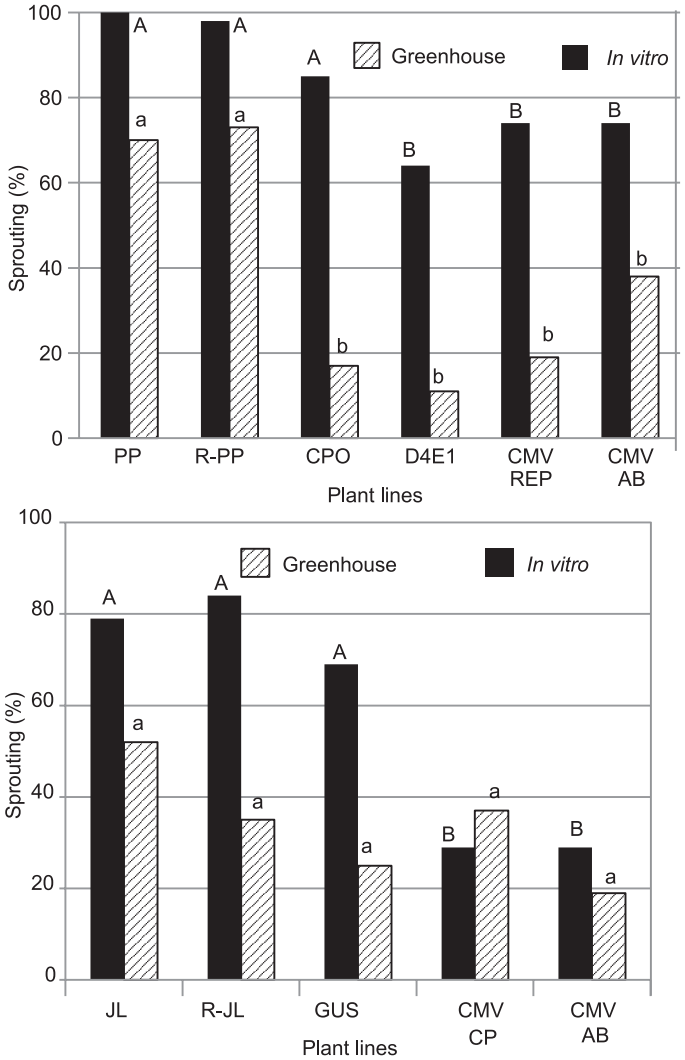

Fig. 2. Sprouting of *Gladiolus* corms from *in vitro*-grown plants of either 'Peter Pears' (top) or 'Jenny Lee' (bottom) planted either in the greenhouse or *in vitro*. Thirty corms were planted for each line. A One Way Anova followed by the Holm-Sidak Method was performed for comparing the sprouting from micropropagated plants to either regenerated or transgenic plants. A different capital letter above the bar indicates that the sprouting was statistically different between the micropropagated plants and either the regenerated or transgenic leaves at $P \leq 0.05$ for corms grown *in vitro*, and a different lower case letter indicates differences for sprouting in the greenhouse.

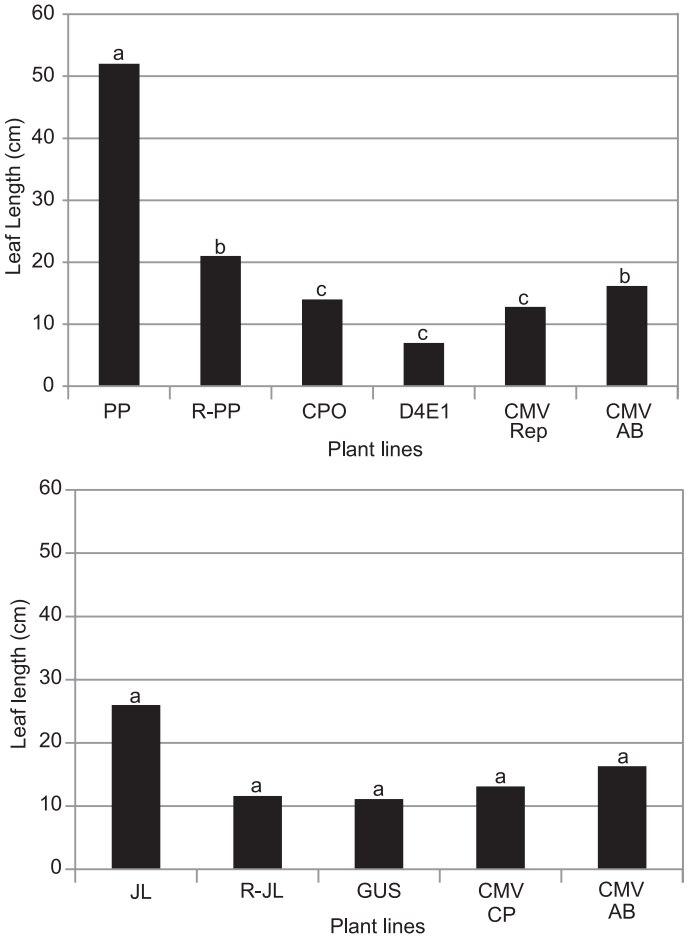

Fig. 3. Length of the longest leaf from either micropropagated (PP or JL), regenerated (R-PP or R-JL), or transformed plants of *Gladiolus* 'Peter Pears' (top) or 'Jenny Lee' (bottom) grown in the greenhouse. Means are shown for each group of plant lines. A One Way Anova followed by the Dunns Method was performed for comparing leaves from micropropagated plants to either regenerated or transgenic plants, and a different letter above the bar indicates that the leaf lengths were statistically different between the micropropagated plants and either the regenerated or transgenic leaves at $P \leq 0.001$.

greenhouse to assess size of the young *Gladiolus* plants. Leaf lengths from regenerated plants of 'Peter Pears' were significantly less (21 cm) than leaves from micropropagated plants of the same cultivar (52 cm) (Fig. 3). Leaves from 'Peter Pears' plants transformed with either the chloroperoxidase, D4E1, or CMV replicase genes were shorter and leaves from CMV antibody plants were similar to regenerated 'Peter Pears' plants.

The leaf length was decreased for regenerated and transgenic plants of 'Peter Pears' as compared to micropropagated plants. In comparison, the leaf length was comparable for micropropagated, regenerated, and transgenic plants of 'Jenny Lee'.

Number of corms harvested from the greenhouse: Regenerated plants of 'Peter Pears' produced daughter corms resulting in a 4.8 fold increase in the number of corms harvested after one season of growth in the greenhouse, comparable to the 5 fold increase in corms by micropropagated plants of 'Peter Pears' (Table 1). In comparison, transgenic lines of 'Peter Pears' containing the chloroperoxidase, D4E1, or CMV replicase gene showed a drastic decrease in the number of corms after one season. 'Peter Pears' plants with the CMV antibody gene maintained their original number of corms.

Table 1. The number of corms planted in the greenhouse and the number harvested after one season of growth for *Gladiolus* cvs. Peter Pears and Jenny Lee indicates multiplication of corms

Plant cultivar-line	Number of corms		Multiplication
	Planted	Harvested	(fold increase)
Propagated Peter Pears	30	150	5X
Regenerated Peter Pears	355	1,708	4.8X
Peter Pears-CPO	878	262	0.3X
Peter Pears-D4E1	801	49	0.06
Peter Pears-CMV rep	457	58	0.13
Peter Pears-CMV Ab	183	219	1.2
Propagated Jenny Lee	109	52	0.48
Regenerated Jenny Lee	386	158	0.41
Jenny Lee-GUS	880	212	0.24
Jenny Lee-CMV CP	141	20	0.14
Jenny Lee-CMV Ab	233	116	0.50

Corms from *in vitro*-grown plants were planted in the greenhouse, and the number of corms harvested following one season of growth in the greenhouse indicates multiplication of corms by daughter corm production or shoots that formed and produced corms.

There were only 41% of the original number of corms planted after one season of growth for both regenerated and micropropagated 'Jenny Lee' plants. Transgenic lines of 'Jenny Lee' with the GUS, CMV coat protein, and CMV antibody genes also did not show an increase in the number of corms after one season of growth in the greenhouse.

All 'Jenny Lee' plants, both non-transformed and transgenic, did not recorded increase in number of corms after one season. Non-transformed plants of 'Peter Pears', both micropropagated and regenerated, exhibited increased number of corms 4.8-5 fold, but the transgenic 'Peter Pears' lines with the chloroperoxidase, D4E1, and CMV replicase genes showed a drastic decline in the number of corms harvested.

Discussion

This study showed that regenerated plants of *Gladiolus* were not as robust as micropropagated plants indicating the need to alter regeneration protocols for developing plants comparable to growth with micropropagated plants. Both cultivars, 'Peter Pears' and 'Jenny Lee', showed this effect, but regeneration was more detrimental to 'Peter Pears' than 'Jenny Lee'. Corms from regenerated plants of 'Peter Pears', not 'Jenny Lee', grown *in vitro* weighed significantly less than corms from micropropagated plants of 'Peter Pears' (Fig. 1, Initial FW). The *in vitro* sprouting percentage was similar for corms from both regenerated and micropropagated plants of 'Peter Pears' and 'Jenny Lee' indicating that regenerated plants had a functional basal meristem (Fig. 2). Regenerated plants of 'Peter Pears' grown in the greenhouse were smaller than micropropagated plants as indicated by their shorter leaf lengths (Fig. 3). After one season of growth in the greenhouse, the number of daughter corms produced by regenerated plants of 'Peter Pears' increased five fold, similar to that for micropropagated plants (Table 1). Daughter corm production was reduced for both regenerated and micropropagated 'Jenny Lee' plants. Daughter corms from regenerated plants of both cultivars weighed less than those from micropropagated plants (Fig. 1, Final FW).

Regenerated plants were compared with transgenic plants to determine the effect of the transformation process on plant growth. Corms from *in vitro*-grown plants of transgenic 'Peter Pears' were either similar in weight (CPO and D4E1 corms) or weighed more (CMV replicase and CMV antibody) than those from regenerated 'Peter Pears' plants (Fig. 1, Initial FW). All corms from *in vitro*-grown plants of transgenic 'Jenny Lee' weighed more than those from regenerated plants. Transgenic 'Peter Pears' corms sprouted *in vitro* at a comparable percentage (62-82%) to that of regenerated 'Peter Pears' corms (98%) (Fig. 2). *In vitro* sprouting of transgenic 'Jenny Lee' corms with the GUS gene (70%) was similar to that of regenerated 'Jenny Lee' corms (82%). The basal meristems of transgenic 'Peter Pears' and 'Jenny Lee' corms were functional as indicated by sprouting efficiencies in the range of that for micropropagated plants.

Transgenic 'Peter Pears' corms and plants did not grow as well in the more stressful greenhouse conditions as compared to regenerated plants. Corms from transgenic 'Peter Pears' plants sprouted at a lower percentage (10-39%) than regenerated plant corms of 'Peter Pears' (73%) in the greenhouse (Fig. 2). Leaves of the transgenic 'Peter Pears' plants with the CPO, D4E1, and CMV replicase genes, not the CMV antibody gene, were shorter than those of the regenerated 'Peter Pears' leaves (Fig. 3). Transgenic 'Peter Pears' plants containing the CPO, D4E1, and CMV replicase genes showed a drastic decline in the daughter corm production as compared to the increased number of daughter corms from regenerated plants after one season of growth in the greenhouse (Table 1). In comparison, transgenic plants and corms of 'Jenny Lee' performed better than 'Peter Pears' in the greenhouse. 'Jenny Lee' corms from transgenic plants sprouted at a similar percentage (19-38%) to that of regenerated 'Jenny Lee' corms (35%) (Fig. 2). Leaves of transgenic 'Jenny Lee' plants were comparable in length to those of regenerated 'Jenny Lee' plants (Fig. 3). The weight of corms harvested after one season in the greenhouse was similar for transgenic and regenerated 'Jenny Lee' plants (Fig. 1, Final FW).

Studies should also be done to determine the specific factors used in gene gun-mediated transformation of *Gladiolus* that may affect the growth of the transformed plant, particularly the cultivar 'Peter Pears' that was more sensitive than 'Jenny Lee' to the transformation process. For example, Choi *et al.* (2001) showed that the osmoticum used in the medium for bombardment contributed to cytological abnormalities of the transformed barley plants. A decrease in fertility, height, and a deficiency in chlorophyll were observed in rice plants transformed with a Bt gene (Shu *et al.*, 2002).

Originally experiments were done to optimize transformation. In the future, research is needed to improve the quality of regenerated plants of *Gladiolus* and to determine the specific conditions during gene gun bombardment that adversely affect *Gladiolus* plant development. A comparison of cultivars may be useful as this study showed that 'Jenny Lee' was less affected by the regeneration and transformation conditions than 'Peter Pears'.

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