Growth characteristics of micropropagated, regenerated and transgenic Gladiolus plants

Kathryn Kamo1*, Kanniah Rajasekaran2 and Jeffrey Cary2

1Floral and Nursery Plants Research Unit, U.S. National Arboretum, U.S. Department of Agriculture, Bldg. 010A, Beltsville, MD 20705-2350, USA. 2Food and Feed Safety Research Unit, U.S. Department of Agriculture, Southern Regional Research Center, New Orleans, LA 70124, USA. *E-mail: Kathryn.Kamo@ars.usda.gov.

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 corn 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 corn 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, phosphinotricin 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/m²/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 phosphonitrin (AgrEvo, Somerville, NJ) for ‘Peter Pears’ or 2 mgL⁻¹ phosphonitrin 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 *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 phosphonitrin 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≤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 $P<0.05$.

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 $P<0.001$.

Plants were grown approximately 5 months in the greenhouse until 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 $P<0.05$.

### 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 spraying 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’ sprayed at 62-100% *in vitro*, but spraying 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.

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

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’.

**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

<table>
<thead>
<tr>
<th>Plant cultivar-line</th>
<th>Number of corms Multiplication (fold increase)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Planted</td>
</tr>
<tr>
<td>Propagated Peter Pears</td>
<td>30</td>
</tr>
<tr>
<td>Regenerated Peter Pears</td>
<td>355</td>
</tr>
<tr>
<td>Peter Pears-CPO</td>
<td>878</td>
</tr>
<tr>
<td>Peter Pears-D4E1</td>
<td>801</td>
</tr>
<tr>
<td>Peter Pears-CMV rep</td>
<td>457</td>
</tr>
<tr>
<td>Peter Pears-CMV Ab</td>
<td>183</td>
</tr>
<tr>
<td>Propagated Jenny Lee</td>
<td>109</td>
</tr>
<tr>
<td>Regenerated Jenny Lee</td>
<td>386</td>
</tr>
<tr>
<td>Jenny Lee-GUS</td>
<td>880</td>
</tr>
<tr>
<td>Jenny Lee-CMV CP</td>
<td>141</td>
</tr>
<tr>
<td>Jenny Lee-CMV Ab</td>
<td>233</td>
</tr>
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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.
Growth characteristics of micropropagated, regenerated and transgenic *Gladiolus* plants

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 record an 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’.

**Acknowledgements**

Anne O’Connor is thanked for providing excellent technical assistance throughout this study. Dr. Kaper (USDA ARS, Beltsville, MD) provided the CMV coat protein subgroup II gene and Dr. M. Zaitlin (Cornell University, Ithaca, NY) the CMV replicase gene. 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.

**References**


Received: June, 2014; Revised: July, 2014; Accepted: August, 2014