**GROWTH CHARACTERISTICS OF TRANSGENIC *GLADIOLUS* PLANTS**

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Running title: Growth of transgenic Gladiolus

**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. In vitro-grown corms from regenerated plants of ‘Peter Pears’ and ‘Peter Pears’ plants transformed with the chloroperoxidase or D4E1 gene weighed less than corms from micropropagated plants whereas corms from ‘Peter Pears’ plants transformed with the *Cucumber mosaic virus* replicase or *Cucumber mosaic virus* antibody gene were comparable in weight to corms from micropropagated plants. All in vitro-grown corms from regenerated and transgenic plants of ‘Jenny Lee’ were comparable in weight to those from micropropagated plants of ‘Jenny Lee’. Only a low percent (10-38%) of corms from transgenic plants of ‘Peter Pears’ and ‘Jenny Lee’ sprouted in the greenhouse. In comparison, a high percentage (70-73%) of corms produced by both micropropagated and regenerated ‘Peter Pears’ plants sprouted. Sprouting was moderate (35-52%) for regenerated and micropropagated corms of ‘Jenny Lee’. The length of the longest leaf was measured as an indicator of the plant’s size. Both regenerated and transgenic plants of ‘Peter Pears’ and ‘Jenny Lee’ had shorter leaves than micropropagated plants of the same cultivar. All ‘Jenny Lee’ plants, non-transformed and transgenic, did not increase their number of corms after one season. Non-transformed plants of ‘Peter Pears, increased their number of corms 4.8-5 fold, but transgenic lines with the chloroperoxidase, D4E1, and CMV replicase genes showed a drastic decline in the number of corms harvested.

*Keywords:* flower bulbs, gene gun, transgenes

*Abbreviations*: 2,4-D, 2,4-dichlorophenoxyacetic acid; BYMV, *Bean yellow mosaic virus*; CMV, *Cucumber mosaic* *virus*; 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 on 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 done 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 hr photoperiod at 40-60 µmol m-2 s-1 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 being planted in Metromix 200 (Scotts Company, Marysville, OH) 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 two hr 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 mg l-1 bialaphos (Meiji Seika Kaisha, Tokyo, Japan, [www.meiji.co.jp](http://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 mg l-1 bialaphos. Callus was transferred monthly to fresh medium for three-six months. If plants regenerated from the callus, the plants were transferred to MS medium lacking hormones and containing either 1 mg l-1 phosphinothricin (AgrEvo, Somerville, NJ) for ‘Peter Pears’ or 2 mg l-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 mg l-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, NY). Plasmids were isolated from *E. coli* DH5α by alkaline lysis and then purified on a cesium chloride gradient (Maniatis et al. 1993).

**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 Metromix 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. 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.

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.

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.

Plants were grown approximately 5 months in the greenhouse before 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 difference between a micropropagated plant was significant as compared to regenerated or transgenic plant lines that contained each gene at P<0.05.

**RESULTS**

**Weight of corms**

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 equivalent to or significantly higher than corms of the same cultivar produced by micropropagated plants (Fig. 1, Initial FW). Corms from in vitro-grown plants of regenerated ‘Peter Pears’ and transgenic ‘Peter Pears’ plants containing the chloroperoxidase or D4E1 genes weighed less than corms from micropropagated plants of ‘Peter Pears’. Although the initial corm weight for transgenic plants containing five of the seven transgenes was equivalent or higher than corms produced by micropropagated plants, the final fresh weight for corms from all ‘Peter Pears’ and ‘Jenny Lee’ transgenic and regenerated plants grown one season in the greenhouse was significantly less than that produced by micropropagated corms (Fig. 1).

These results indicate that corms from regenerated and transgenic plants of both ‘Peter Pears’ and ‘Jenny Lee’ are not significantly larger than those from micropropagated plants of the same cultivar.

**Sprouting of corms**

Micropropagated and regenerated plants of ‘Peter Pears’ produced corms that sprouted at high percentages (70-73%) in the greenhouse and in vitro (98-100%) (Fig. 2). In comparison, the percent of corms from transgenic ‘Peter Pears’ plants sprouting in the greenhouse was only 10-38%. These transgenic corms were capable of sprouting as indicated by a 62-82% sprouting percentage in vitro.

In the greenhouse, ‘Jenny Lee’ corms from micropropagated plants sprouted at a higher percent (52%) than corms from regenerated (35%) and transgenic plants (18-38%) of ‘Jenny Lee’ (Fig. 2). The sprouting percentage was much higher in vitro (79-84%) than in the greenhouse for micropropagated and regenerated ‘Jenny Lee’ plants, respectively.

Only a small percent (10-38%) of corms from transgenic plants of ‘Peter Pears’ and ‘Jenny Lee’ sprouted in the greenhouse. A high percent (70-73%) of corms produced by both micropropagated and regenerated ’Peter Pears’ plants sprouted in the greenhouse. Sprouting in the greenhouse was a moderate 35-53%, respectively, for regenerated and micropropagated corms of ‘Jenny Lee’.

**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 both ‘Peter Pears’ and ‘Jenny Lee’ were significantly less (22 and 12 cm, respectively) than leaves from micropropagated plants of the same cultivars (52 and 26 cm, respectively) (Fig. 3). Leaves from transgenic ‘Peter Pears’ plants with the CMV antibody gene and transgenic ‘Jenny Lee’ plants with the GUS, CMV coat protein, or CMV antibody genes were comparable in length to leaves from regenerated plants of the same cultivar. Leaves from ‘Peter Pears’ plants transformed with either the CPO, D4E1, or CMV replicase genes were shorter than those from regenerated ‘Peter Pears’ plants.

Both regenerated and transgenic plants of cultivars ‘Peter Pears’ and ‘Jenny Lee’ had significantly shorter leaves than micropropagated plants of the same cultivar.

**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 similar to the 5 fold increase in corms by micropropagated plants (Fig. 4). 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’ with the CMV antibody gene maintained its original number of corms.

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

All ‘Jenny Lee’ plants, both non-transformed and transgenic, did not increase their number of corms after one season. Non-transformed plants of ‘Peter Pears’, both micropropagated and regenerated, increased their 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 both regenerated plants and transgenic plants transformed with a variety of genes grew relatively poorly compared to micropropagated plants. Both cultivars, ‘Peter Pears’ and ‘Jenny Lee’, showed this effect. The low sprouting percent (10-38%) of corms combined with the decreasing number of daughter corms produced after one season of growth in the greenhouse are factors that have made it very laborious to grow the transgenic ‘Jenny Lee’ and ‘Peter Pears’ for more than one season in the greenhouse. Half of the ‘Peter Pears’ lines containing either the D4E1, chloroperoxidase, or CMV replicase gene produced only a few or no corms.

The sprouting percentage of corms was significantly higher in vitro than in the greenhouse for all corms, indicating that the corms have the ability to germinate. Typically corms from plants grown in vitro are softer and do not have the thick, papery outer layer found on corms harvested from greenhouse and field-grown plants. The absence of a thick outer layer to protect the corm from changes in moisture and from rotting when there is too much moisture as found under greenhouse growing conditions may have contributed to the lower sprouting percentages found for corms grown in the greenhouse as compared to in vitro. We have not had much success transplanting young *Gladiolus* plants grown in vitro to the greenhouse which is the reason corms are planted.

Regenerated plants of *Gladiolus* were not as robust as micropropagated plants indicating the need to alter regeneration protocols for developing plants that grow as well as micropropagated plants. In addition, 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. 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. The effect on growth of a transgenic plant due to the expression of a transgene needs to be investigated for *Gladiolus*. All seven transgenes used in this study appeared to have a negative effect on growth of *Gladiolus* 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 should be done to improve the quality of regenerated plants of *Gladiolus* so that they will produce daughter corms that sprout at a high frequency.

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**FIGURE LEGENDS**

**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 < 0.05).

**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.

**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 Student’s t test was performed 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<0.05.

**Fig. 4**. Corms from in vitro-grown plants were planted in the greenhouse and harvested after one season of growth. The number of corms planted and harvested is shown for micropropagated plants (PP or JL), regenerated plants (R-PP or R-JL), and transgenic PP lines (top) or transgenic JL lines (bottom).