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Q. Liu · S. Salih · F. Hammerschlag

Etiolation of 'Royal Gala' apple (*Malus* × *domestica* Borkh.) shoots promotes high-frequency shoot organogenesis and enhanced β-glucuronidase expression from stem internodes

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Abstract Internodal explants from etiolated 'Royal Gala' apple shoots were compared with those from non-etiolated shoots for frequency of shoot organogenesis and for efficiency of β-glucuronidase (GUS) expression after cocultivation of explants with *Agrobacterium tumefaciens* strain EHA105 (p35SGUSint). First (youngest) internodal explants from etiolated shoots produced 2-, 8- and 73-fold numbers of shoots compared to second, third, and fourth internodal explants, respectively. Moreover, these explants produced sevenfold the number of shoots as similar explants from non-etiolated shoots. All first internodes from etiolated shoots exhibited GUS-expressing zones and yielded fourfold as many GUS-expressing zones as commonly used leaf explants from non-etiolated shoots, which exhibited GUS-expressing zones in only 63% of the explants. An average of 9.8 GUS expressing calli per explant were observed on first internodes from etiolated shoots 2 weeks after cocultivation with *A. tumefaciens*.

Key words *Agrobacterium tumefaciens* · *gusA* expression · Transformation

Abbreviations *GUS* β-glucuronidase · *gusA* β-glucuronidase gene from *Escherichia coli* · *gusAint* β-glucuronidase gene containing an intron in the coding region · *kan* kanamycin · *NAA* α-naphthaleneacetic acid · *TDZ* thidiazuron

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Q. Liu[1] · S. Salih · F. Hammerschlag (✉)
U.S. Department of Agriculture, Agricultural Research Service, Fruit Laboratory, Beltsville, MD 20705 USA
Fax: 301-504-5062
e-mail: fhammers@asrr.arsusda.gov

Present address:
[1] Shandong Research Institute of Pomology, 64 Longtan Road, Taian, Shandong 271000, China

Introduction

Among the temperate fruit tree species, apple (*Malus* × *domestica* Borkh.) is an attractive candidate for gene transfer studies because of its economic importance (Korban and Chen 1992), long juvenile period, high level of self-incompatibility, and high heterozygosity (Brown 1975). Improvement of apple or any other plant species via genetic engineering depends on an efficient gene delivery system, a system for selection, and reliable, high-frequency shoot regeneration. Although regeneration has been reported for a wide range of apple cultivars (Korban and Chen 1992), the number of shoots per regenerating explant has generally not exceeded 20. In most cases, leaves have been selected as explants for regeneration experiments (Fasolo et al. 1989; Welander 1988; Predieri et al. 1989; Swartz et al. 1990; Korban et al. 1992; Welander and Maheswaran 1992; Yepes and Aldwinckle 1994; Gercheva et al. 1994), and factors such as antibiotics, dark treatment, gelling agent, growth regulators, leaf-explant origin, maturity, position on the stem and orientation on the medium, nitrogen source and concentration, and particle bombardment can have profound effects on the efficiency of shoot regeneration. There are only a few reports on shoot regeneration from stem explants (James et al. 1984; Welander 1988; Belaizi et al. 1991), but the morphogenetic response from these explants was very low. Recently, etiolation has been shown to enhance shoot regeneration (Mohamed et al. 1992; Kiss et al. 1995) and gene expression (Kamo 1997). Here we report a high frequency of shoot organogenesis and enhanced GUS expression from internodes of etiolated shoots after cocultivation with *Agrobacterium tumefaciens*.

Materials and methods

Plant material

Stem internode and leaf-blade explants were dissected from in vitro-cultured shoots of 'Royal Gala' (a red-fruited sport of 'Gala') apple

(Gercheva et al. 1994). Donor shoot cultures were maintained by transferring 1- to 2-cm-long axillary shoots at 4-week intervals onto a shoot multiplication (J3) medium consisting of MS salts (Murashige and Skoog 1962) supplemented with 0.56 m*M* myo-inositol, 1.2 μ*M* thiamine-HCL, 4.4 μ*M* 6-benzyladenine, 0.5 μ*M* indole-3-butyric acid, 1.3 μ*M* gibberellic acid, 87.6 m*M* sucrose, and Difco Bacto-agar (7 g l^{-1}). The pH was adjusted to 5.6 before autoclaving at 121 °C and 131 kPa for 15 min. Shoot cultures were incubated at 25 °C with a 16-h photoperiod provided by cool-white fluorescent lights at 40 μmol · m^{-2} · s^{-1}. Etiolation was promoted by placing shoots in the light for 2 weeks followed by 2 weeks in the dark.

Shoot organogenesis

Stem internode segments (the first through the fourth, beginning at the apex, and each approximately 4 mm in length) were removed from 4-week-old green (non-etiolated) and etiolated shoots, wounded (two transverse cuts), and transferred, cut side up, to a regeneration medium. The regeneration (N6) medium (Fasolo et al. 1989) consisted of N6 macro salts (Chu et al. 1975), LS micro salts (Linsmaier and Skoog 1965), 0.56 μ*M* myo-inositol, 3 μ*M* thiamine-HCl, 87.6 m*M* sucrose, casein hydrolysate (300 mg l^{-1}), Difco Bacto-agar (7 g l^{-1}), and was supplemented with either 1, 5, or 10 μ*M* TDZ. The medium was adjusted to pH 5.4 before autoclaving. All explants were incubated in the dark for 10 days before transfer to light conditions (as above). Explants were subcultured onto fresh medium once every 2 weeks. After 42 days, all explants were transferred to elongation (J3-h) medium consisiting of J3 medium (see above) without growth regulators. The number of adventitious shoots per explant and percentage of regeneration were determined after 52 days of culture. Each treatment was replicated three times with a minimum of 10 explants per treatment per replication.

Agrobacterium strain and cocultivation

Agrobacterium tumefaciens strain EHA105 (Hood et al. 1993) containing the binary vector p35SGUSint (Vancanneyt et al. 1990) was used in all experiments. Since p35SGUSint contains a chimeric *gusA* with a plant intron, the *gusAint* reporter gene cannot be expressed in *A. tumefaciens*, thus making it a useful tool for studying early events in apple transformation (De Bondt et al. 1994; Norelli et al. 1996). This strain was maintained at 28 °C on yeast extract broth (YEB) medium, pH 7.2 (Van Larebeke et al. 1977), containing 5.0 g Difco Bacto-beef extract, 5.0 g peptone, 5.0 g sucrose, and 1.0 g yeast extract per liter of media, 1.5% Difco Bacto agar, and supplemented with 50 mg kan (Sigma). For cocultivation, bacteria were grown overnight (200 rpm, 28 °C) in the same medium without agar, pelleted, and resuspended to a density of 2×10^8 cfu/ml (by measuring absorbance at 550 nm) in N6 liquid medium containing 20 μ*M* acetosyringone, 5 μ*M* TDZ, and 1.1 μ*M* NAA. Stem internodes (described above) and leaf explants (Gercheva et al. 1994) were dipped in *A. tumefaciens*, blotted on sterile filter paper, transferred to sterile filter paper saturated with the same medium as that used for resuspending *A. tumefaciens*, and incubated in the dark at 28 °C for 48 h. Following cocultivation, explants were washed in N6 liquid medium without growth regulators, blotted with sterile filter paper and either assayed for expression of the *gusAint* gene or transferred, abaxial side up, to N6 medium containing 5 μ*M* TDZ, 1.1 μ*M* NAA, cefoxitin (100 mg l^{-1}) (Sigma), carbenicillin (100 mg l^{-1}) (Sigma) and kan (100 mg l^{-1}) (selection medium) for 2 weeks and then assayed.

GUS assays

Explants were assayed for expression of the *gusAint* gene following the histochemical staining procedure described by Jefferson et al. (1987) with some modifications. Explants were stained overnight at 37 °C in 100 m*M* sodium phosphate buffer (pH 7) containing 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, 10 m*M* Na_2EDTA, 0.5% (v/v) Triton X-100, and 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) at 0.5 mg l^{-1}. Following overnight staining, explants were cleared and fixed in 95% (v/v) ethanol: 1% (v/v) acetic acid. Transient *gusAint* expression was measured immediately after cocultivation by counting the number of GUS-expressing zones appearing as blue spots on a white background following the staining procedure. The number of GUS-expressing calli developed on kan selection medium after 2 weeks was determined by counting the number of blue calli after X-Gluc staining. GUS assays were replicated three times with a minimum of 10 explants per treatment per replication.

Table 1 Response of the first four internodes from etiolated and non-etiolated stems of 'Royal Gala' apple on medium containing 10 μ*M* TDZ [a]

Internode position	Percentage organogenesis		Mean number of shoots per internode	
	Etiolated	Non-etiolated	Etiolated	Non-etiolated
1	93 a	63 a	21.8 a*	3.3 a
2	87 a*	43 a	11.9 b*	1.7 b
3	37 b	10 b	2.8 c*	0.1 b
4	17 b	2 c	0.3 d*	0.0 b

[a] Values represent the mean of three replications with 10 explants per treatment per replication. Percentage data were subjected to arcin transformation before analysis. Mean separation within each column by LSD at $P \leq 0.05$. Etiolated stem means followed by * are significantly different from those of non-etiolated stem means by LSD at $P \leq 0.05$

Statistical analysis

Data were analyzed using the general Linear Models Procedure of SAS (SAS Institute 1997). Percentage data were subjected to arcin transformation before analysis. Variation among treatment means was analyzed using the LSD test at $P \leq 0.05$.

Results and discussion

Effect of etiolation, internodal position, and level of TDZ in the medium on shoot organogenesis

First (youngest) internodes from etiolated shoots generated almost twofold the percentage of shoot regeneration and sevenfold the number of shoots per explant as similar explants from non-etiolated shoots (Table 1). These explants also produced 2-, 8-, and 73-fold the number of shoots per explant compared to second, third and fourth internodal explants, respectively (Table 1). The frequency of organogenesis (93%) reported in our study is considerably higher than that previously reported for apple internodal stems, i.e., less than 5% for 'McIntosh Wijcik' (Welander 1988), 5% for rootstock M26 (James et al. 1984), and 23% for 'Golden Delicious' (Belaizi et al. 1991). The low efficiency of organogenesis from stem internodes has been a deterrent to using these explants in gene transfer studies.

Both etiolation and internodal position are key factors in promoting high efficiency shoot organogenesis from

Table 2 Response of first internodes from etiolated and non-etiolated stems of 'Royal Gala' apple on media with 1, 5, or 10 µ*M* TDZ[a]

TDZ (µ*M*)	Percentage organogenesis		Mean number of shoots per internode	
	Etiolated	Non-etiolated	Etiolated	Non-etiolated
1	100 a	97 a	68.8 a*	32.5 a
5	100 a*	93 a	53.6 b*	23.3 a
10	100 a*	83 a	28.6 c*	4.1 b

[a] Values represent the mean of three replications with a minimum of 10 explants per treatment per replication. Percentage data were subjected to arcin transformation before analysis. Mean separation within each column by LSD at $P \leq 0.05$. Etiolated stem means followed by * are significantly different from those of non-etiolated stem means by LSD at $P \leq 0.05$

Table 3 The effect of etiolation on GUS expression assayed immediately and 2 weeks after cocultivation of apples with *Agrobacterium tumefaciens*[a]

Explant type	Immediately after cocultivation		Two weeks after cocultivation	
	GUS+ explants (%)	Number of GUS+ zones per explant	Percentage explants with GUS+ calli	Number of GUS+ calli per explant
Etiolated internodes	100 a	35.9 a	100 a	9.8 a
Non-etiolated internodes	90 a	9.6 b	87 a	3.8 b
Non-etiolated leaves	63 b	8.8 b	80 a	3.4 b

[a] Values represent the mean of three replications with a minimum of 10 explants evaluated per treatment per replication. Percentage data were subjected to arcin transformation before analysis. Mean separation within each column by LSD at $P \leq 0.05$

Fig. 1 GUS staining in an etiolated internode (*left*), non-etiolated internode (*middle*), and non-etiolated leaf (*right*) that were cocultivated with *Agrobacterium tumefaciens* strain EHA105 (p35SGUSint) and assayed immediately after cocultivation (*upper photo*) and 2 weeks after kan selection (100 mg l^{-1}) (*lower photo*)

'Royal Gala' internodes. Etiolated stems have also been shown to promote shoot formation from nodal segments in pineapple (*Ananas comosus* L.) (Kiss et al. 1995), common bean (*Phaseolus vulgaris* L.) (Mohamed et al., 1992), and faba bean (*Vicia faba* L.) (Mohamed et al. 1992). This is the first report that etiolation increases shoot regeneration in a fruit tree species.

In previous apple regeneration studies, leaf-segment maturity (Welander 1988) and leaf maturity (Fasolo et al. 1989) were shown to be important factors with increasing regeneration from older to younger tissues. Yet in later studies with stem explants (Welander 1988; Belaizi et al. 1991), internodes were not separated based on maturity. This could explain the low efficiency of shoot regeneration observed in studies conducted by Welander (1988) and Belaizi et al. (1991). We did not observe any organogenesis from fourth internodes and only 10% organogenesis from third internodes of non-etiolated stems (Table 1).

Another important factor in apple shoot organogenesis from internodal explants was TDZ concentration, with 1 μ*M* TDZ promoting about two- and sevenfold the number of shoots per explant from etiolated stems and non-etiolated stems, respectively, compared to 10 μ*M* TDZ (Table 2). However, significantly more shoots per explant were produced from etiolated stems compared to non-etiolated stems at all TDZ concentrations (Table 2). Internodal explants from etiolated stems produced about 2-fold the number of shoots per explant on 10 μ*M* TDZ compared to 'Royal Gala' leaf explants (Table 2; Gercheva et al. 1994). In previous regeneration studies with apple leaf explants (Fasolo et al. 1989), optimum results were obtained with 10 μ*M* TDZ; however, Korban et al. (1992) demonstrated that optimum growth regulator levels for shoot organogenesis can be influenced by cultivar, amount of time in the dark, and whether auxins or not were included in the medium. Our study suggests that type of explant can also influence which concentration of TDZ will be most effective in promoting shoot organogenesis.

GUS expression in *Agrobacterium*-infected tissues

The evaluation of transformation efficiency was based on histochemical analysis of the number of GUS-expressing zones immediately after cocultivation, corresponding to zones of transient expression, and the number of GUS-expressing calli after kan selection, corresponding to zones of stable gene expression as GUS-expressing calli were probably derived from cells in which DNA integration occurred. Our experiments were conducted using the plasmid binary vector p35SGUSint (Vancanneyt et al. 1990) with a chimeric *gusA* gene containing a plant intron. Only plant cells expressing the *gusAint* reporter gene stained blue; whereas *A. tumefaciens* cells did not because the intron sequences inserted in the *gusA* gene cannot be processed in *A. tumefaciens*.

Our findings showed that the number of GUS-expressing zones on internodes from etiolated shoots (Table 3; Fig. 1) and on callus derived from these internodes (Table 3; Fig. 1) was significantly higher than that on internodes and leaves from non-etiolated shoots. Rapid stem elongation occurred within the 2 week period after subjecting shoot cultures to dark treatment. This observation may explain improved transformation after etiolation since other studies have demonstrated that the induction of cell division is an important process for efficient *Agrobacterium* infection (Sangwan et al. 1991; Akama et al. 1992).

In conclusion, the high frequency of shoot organogenesis combined with enhanced GUS expression on first internodes from etiolated apple shoots and on callus derived from these internodes suggest that these explants may be useful in gene transfer studies.

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