Leaf Wounding Increases Efficiency of *Agrobacterium*-mediated Transformation of Apple

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Abstract. In vitro-grown leaves of *Malus ×domestica* Borkh. cv. Royal Gala were either crush-wounded with forceps, cut, or left whole, and then inoculated with *A. tumefaciens* strain EHA105 (p35SGUS_INT), with and without vacuum infiltration. Transformation was quantified 13 days after inoculation by determining the rate of β -glucuronidase (GUS) activity. Leaf wounding by crushing with nontraumatic forceps significantly increased transformation when compared with cutting leaves. Vacuum infiltration of inoculum had no effect on transformation.

Genetic transformation of apple typically involves the inoculation of cut leaf blades with disarmed *Agrobacterium tumefaciens* strains containing plasmid binary vectors (De Bondt et al., 1994; James et al., 1989; Maheswaran et al., 1992; Sriskandarajah et al., 1994; Yao et al., 1995). Histochemical observation of apple leaf pieces 3 to 14 days after inoculation with *Agrobacterium* plasmid binary vectors containing the *uidA* gene, encoding β -glucuronidase (GUS) (Jefferson et al., 1987), indicated that the majority of GUS expression appeared to be associated with wound sites caused by forceps during leaf manipulation, rather than with cut leaf edges (Norelli, unpublished). Vacuum infiltration of *Agrobacterium* inoculum is a useful aid in the transformation of *Arabidopsis thaliana* (L.) Heyn. (Bechtold et al., 1993). Based on these observations, we evaluated the effect of leaf wounding vs. cutting, and the effect of vacuum infiltration of inoculum on the ability of *A. tumefaciens* to transform apple leaf cells. Here we report that leaf wounding by crushing the leaf blade with nontraumatic forceps significantly increased transformation of apple leaf cells when compared with cutting leaf blades. Vacuum infiltration of inoculum did not have a significant effect on transformation.

Leaf wounding treatments consisted of 1) a control (whole, uncut leaves), 2) leaf blades cut transversely into 3- to 5-mm sections, and 3) whole, uncut leaf blades uniformly crushed with nontraumatic forceps, a type of surgical

instrument (Aesculap #BD-157, Burlingame, Calif.). They were 200-mm-long dissecting forceps containing a carbide surface on the inner gripping area of the forceps tip that was worked into a fine grid surface, rather than the usual steel ridge gripping surface of typical dissecting forceps. The amount of pressure used to crush leaf blades was a learned skill and varied depending on several variables, including leaf age, size, and succulence. In general, proper crushing resulted in immediate water-soaking of tissue that could be detected by holding leaves up to a light source but did not result in maceration, piercing, or cutting of leaf tissue. Properly crushed leaves examined 13 days after inoculation showed either no evidence of wounding or small necrotic dots (<1.0 mm²) dispersed over the leaf surface. Confluent necrosis of tissue 13 days after inoculation was indicative of excess force applied during crushing.

Vacuum treatments consisted of subjecting leaves to 1) 2 min of vacuum infiltration, in inoculum, at 138 kPa or 2) 2 min in inoculum without vacuum infiltration. The three wounding treatments were done with and without vacuum infiltration in a factorial design, with each treatment consisting of 12 leaves. The experiment was repeated. The experiments were conducted using the plasmid binary vector p35SGUS_INT (Vancanney et al., 1990) with a chimeric GUS gene containing a plant intron. Since this GUS_INT reporter gene cannot be expressed in *A. tumefaciens*, it has been a useful tool for studying the early events of transformation in apple (Norelli and Aldwinckle, 1993).

Inoculum consisted of *Agrobacterium tumefaciens* strain EHA105 (p35SGUS_INT) grown for 48 h on Luria Bertani solid medium (Sambrook et al., 1989) containing kanamycin at 50 μ g·ml⁻¹. Inoculum was resuspended in a *vir* gene-induction medium containing acetosyringone (James et al., 1993), and incubated at room temperature (24–27°C) with gentle agitation for 4.5 h. The youngest unfolded, but still expanding, leaves were harvested from in vitro axillary shoot cultures of

the *Malus* cultivar Royal Gala. Leaves were treated as described above. Noninoculated control leaves were also prepared for each wounding/vacuum combination. Leaf explants were blotted and placed adaxial surface up on a cocultivation medium consisting of regeneration medium (Liu, 1987) containing (in μ g·ml⁻¹) 2.0 thidiazuron and 0.3 indole-butyric acid, with the addition of 100 μ M acetosyringone and 1.0 mM betaine phosphate. Plates were incubated in darkness at 25°C for 64 h. Explants were then transferred, abaxial surface up, to selective medium consisting of a Gelrite-based regeneration medium containing (in μ g·ml⁻¹) 350 cefotaxime and 100 kanamycin and incubated in darkness at room temperature. Leaves that were not wounded, and those that were crush-wounded, were cut transversely into 3- to 5-mm sections before transfer to the selective medium.

The amount of transformation in leaf explants was quantified 13 days after inoculation by determining the rate of GUS activity using a fluorometric assay based on the cleavage of 4-methylumbelliferyl- β -D-glucuronide (MUG) to 4-methylumbelliferone (MU) (Norelli and Aldwinckle, 1993). Twelve leaf pieces were selected at random from non-necrotic leaf pieces of each of the six wounding/vacuum combinations for use in the GUS assay, as well as 12 noninoculated controls. GUS activity data were normalized by a *ln* transformation before analysis of variance based on the results of a Box–Cox transformation analysis (Box and Cox, 1964). Reported means were calculated from *ln* transformed data and were then converted back to a nontransformed value.

Histochemical staining for localization of GUS in leaf tissue was performed 9 days after inoculation. The histochemical assay used the 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) substrate with which GUS reacts to produce an intensely blue precipitate (Jefferson et al., 1987). Twelve leaf pieces from each wounding/vacuum combination were selected at random, and qualitative notes were recorded based on the appearance and distribution of histochemical staining.

Crushing apple leaves before inoculation resulted in a 10-fold increase in the amount of transformation, expressed as GUS activity, when compared with the nonwounded control and a 4.4-fold increase over the cut leaves (Table 1). The rate of GUS activity in cut leaves also was significantly higher than in nonwounded *Agrobacterium*-treated leaves (Table 1). The rate of GUS activity in noninoculated leaves was 0.1 nmol·min⁻¹·mg⁻¹ tissue fresh weight. In a repetition of the experiment, the mean rate of GUS activity 13 days after inoculation was (nmol·min⁻¹·mg⁻¹, fresh-weight basis) 0.3 MU for nonwounded, 0.3 for cut, and 1.4 for crushed leaf pieces. Although the rate of GUS activity in crushed leaves was significantly higher than that in nonwounded and cut leaves in the repeat experiment, there was no significant difference in the rates for nonwounded and cut leaves.

Vacuum infiltration of *Agrobacterium* inoculum during the transformation procedure

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had no effect on the rate of GUS activity (Table 1). Likewise, in a repetition of the experiment, the effect of vacuum infiltration was not significant, with absolute values of 0.6 nmol·min⁻¹·mg⁻¹.

When treated leaf pieces were observed for the localization of GUS by histochemical staining, vacuum infiltration did not alter the distribution of blue pigmentation. In cut leaves, most of the staining occurred adjacent to the cut edges, with randomly distributed stains in the center of the leaf piece, possibly due to forcep manipulation. Nonwounded leaf pieces stained sparsely in isolated dots and adjacent to the petiole. Forcep wounds in the petiole area were frequently stained. Crush-wounded leaf blade pieces were more uniformly stained, with consistently greater overall staining. Staining at wound sites was intensely blue surrounded by areas of paler blue.

These results suggest that crushing apple leaf blades before inoculation produces infec-

tion sites for *Agrobacterium* that results in a higher level of transformation. Wounding had no negative effects on regeneration of meristems from leaves. Leaf blade crushing before inoculation with *Agrobacterium* has been incorporated into our standard apple transformation protocol and has resulted in the reliable recovery of transgenic plants. In general, we are able to recover transgenics from 5% to 20% of *Agrobacterium*-inoculated leaves of 'Royal Gala' and 'Marshall McIntosh' apple scions and the M.7 apple rootstock (Norelli, unpublished). A transformation rate of 0.4% using cut leaves of 'Royal Gala' has been described by Yao et al. (1995).

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Table 1. Effect of leaf blade wounding and vacuum infiltration of *Agrobacterium tumefaciens* (p35SGUS_INT) inoculum on GUS (β-glucuronidase) activity^a in apple leaf tissue 13 days following inoculation.

Wounding	GUS activity ^a		
	Vacuum infiltration		Mean
	+ ^y	–	
None ^w	0.3 ^s	0.5	0.4
Cut ^v	0.9	0.9	0.9
Crushed ^u	3.5	4.7	4.0
Mean	1.1	1.3	1.2
Analysis of variance ^d			
Source of variation	df	MS	F
Wounding	2	31.738	39.97***
Vacuum infiltration	1	0.733	0.92 ^{NS}
Interaction	2	0.305	0.38 ^{NS}
Error	66	0.794	

^aRate of MUG (4-methylumbelliferyl-β-D-glucuronide) to MU (4-methylumbelliferone) conversion (nmol·min⁻¹·mg⁻¹, fresh-weight basis) determined by fluorometric assay (Jefferson et al., 1987) after inoculation with *Agrobacterium*, cocultivation for 3 days on permissive regeneration medium, and growth on regeneration medium containing (in μg·ml⁻¹) 350 cefotaxime and 100 kanamycin for 10 days.

^yVacuum infiltration for 2 min, in inoculum, at 138 kPa.

^sMean of 12 replications calculated from *ln* transformed data and converted back to a nontransformed value. The rate of GUS activity in nontransformed leaf pieces was (nmol·min⁻¹·mg⁻¹, fresh-weight basis) 0.1.

^wWhole, uncut leaves.

^vLeaf blades transversely cut into 3- to 5-mm sections.

^uWhole, uncut leaf blades uniformly crushed with nontraumatic forceps.

^dCalculated from *ln* transformed data.

^{NS}, ***Nonsignificant at *P* ≤ 0.001.

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