Adventitious Shoot Regeneration and *Agrobacterium tumefaciens*-mediated Transient Transformation of Almond × Peach Hybrid Rootstock ‘Hansen 536’

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Abstract. ‘Hansen 536’ (*Prunus dulcis* × *Prunus persica*) is an important commercial rootstock for peach and almond. However, susceptibility to wet soil and bacterial canker has limited its use primarily to areas with less annual rainfall. Genetic engineering techniques offer an attractive approach to improve effectively the current problems with this cultivar. To develop an efficient shoot regeneration system from leaf explants, 10 culture media containing Murashige and Skoog (MS) or woody plant medium (WPM) supplemented with different plant growth regulators were evaluated, and adventitious shoot regeneration occurred at frequencies ranging from 0% to 36.1%. Optimal regeneration with a frequency of 32.3% to 36.1% occurred with WPM medium containing 8.88 μM 6-benzylamino-purine (BAP) and 0.98 to 3.94 μM indole-3-butyric acid (IBA). The regenerated shoots had a high rooting ability, and 80% of the in vitro shoots tested rooted and survived after being transplanted to substrate directly. Transient transformation showed an efficient delivery of the β -glucuronidase (GUS) reporter gene (*gusA*) using all three *Agrobacterium tumefaciens* strains tested with a concentration of OD₆₀₀ 0.5 to 1.0 for 4 days of cocultivation. The protocols described provide a foundation for further studies to improve shoot regeneration and stable transformation of the important peach and almond rootstock ‘Hansen 536’.

The almond × peach hybrid rootstock ‘Hansen 536’ was introduced from the Hesse-Hansen program (Kester and Asay,

1986). It is an important commercial rootstock for peach, almond, prune, and plum in California. ‘Hansen 536’ rootstocks show medium size and short dormancy, and have a relatively high adventitious root system that provides excellent anchorage in orchards (Kester and Asay, 1986). However, because of its high susceptibility to root rot fungus infection and wet soils, this rootstock cultivar is not suitable for areas with high annual rainfall (Kester and Asay, 1986). Therefore, further improvement of this rootstock is de-

sirable. Because the ‘Hansen 536’ rootstock is propagated clonally, genetic engineering is an effective approach to incorporate target genes of interest for its further improvement. To achieve this, an efficient in vitro shoot regeneration system is needed.

Successful in vitro shoot regeneration from seed-derived or leaf explants has been reported for several *Prunus* species, such as *P. persica* (Druart, 1999; Gentile et al., 2002; Hammerschlag et al., 1985; Mante et al., 1989; Pooler and Scorza, 1995; Scorza et al., 1990), *P. canescens* (Antonelli and Druart, 1989), *P. padus* (Hammatt, 1993), *P. domestica* (Bassi and Cossio, 1991, 1994; Yancheva, 1993), *P. dulcis* (Ainsley et al., 2000; Miguel and Oliveira, 1999; Miguel et al., 1996), *P. armeniaca* (Pérez-Tornero et al., 2000), *P. serotina* (Espinosa et al., 2006; Hammatt and Grant, 1998), *P. avium* (Bhagwat and Lane, 2004; Canli and Tian, 2008; Feeney et al., 2007; Hammatt and Grant, 1998; Matt and Jehle, 2005; Tang et al., 2002; Zong et al., 2019), *P. cerasus* (Mante et al., 1989; Sarropoulou et al., 2012; Song and Sink, 2006; Tang et al., 2002), and several hybrid rootstocks (Hasan et al., 2010; Ochatt et al., 1988; Pérez-Jiménez et al., 2012, 2014; Pooler and Scorza, 1995; San et al., 2015; Sarropoulou et al., 2012; Zhou et al., 2010). For most cultivars of *Prunus* species, leaf explants are preferable to seed-derived explants for maintaining an identical genetic background of the cultivars because of their heterozygosity and self-incompatibility. To date, plant regeneration from leaf explants of the ‘Hansen 536’ rootstock has not been reported.

Peach and peach rootstocks are among the most recalcitrant plants for genetic transformation, although some efforts using particle bombardment or *Agrobacterium*-mediated transformation in peach have been made (Escalettes et al., 1997; Padilla et al., 2006; Pérez-Clemente et al., 2005; Scorza et al., 1995; Ye et al., 1994). Only one study reported the success of obtaining stable transgenic plants from an *Agrobacterium*-mediated transformation using embryo sections of mature seeds (Pérez-Clemente et al., 2005). Lack of an efficient in vitro shoot regeneration system remains the main obstacle for stable transformation of peach and peach rootstocks.

We describe a protocol for in vitro shoot regeneration from leaf explants of the ‘Hansen 536’ rootstock. Several factors affecting in vitro shoot regeneration were optimized. Under optimal in vitro shoot regeneration conditions, two key factors for *A. tumefaciens*-mediated gene delivery—*Agrobacterium* strains and time of cocultivation—were evaluated. The overall results provide guidelines for stable transformation of the ‘Hansen 536’ rootstock.

Materials and Methods

Plant materials. The ‘Hansen 536’ rootstock cultures were provided by North

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American Plants, Inc (McMinnville, OR). The stock cultures, four to five internode segments each with one to three nodes, were cultured on 50-mL shoot proliferation medium (SPM) in a Magenta® GA7 box (Sigma-Aldrich, St. Louis, MO). All basal media, hormones, and antibiotics used in this study were purchased from PhytoTechnology Laboratories (Shawnee Mission, KS). The SPM contained MS medium (Murashige and Skoog, 1962), 4.44 μM BAP and 0.49 μM IBA. All cultures were maintained at 25 °C with a 16-h photoperiod (40 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) and cool-white fluorescent lighting unless otherwise noted, and they were subcultured every 4 weeks using the same culture medium. The fully expanded leaves of 5- to 6-week-old stock cultures were used for regeneration studies.

Optimizing shoot regeneration from leaf explants. Young, fully expanded leaves (length, 1.5–2.0 cm) that included the petiole from in vitro regenerated shoots were wounded by several cuts transversely along the midrib while leaving the sections intact. Ten regeneration media [woody plant regeneration medium (WPRM) 1–WPRM10] were designed to evaluate the effects of the combination and concentration of hormones (Table 1). Leaf explants were cultured with the abaxial surfaces either up or down on WPRM1 to WPRM10 media in each 100 × 20-mm petri dish (Fisher Scientific, Wal-

tham, MA). In vitro shoot induction was conducted in the dark at 25 °C for 2 weeks and then was transferred to a 16-h photoperiod. Subcultures to the same fresh regeneration media were performed every 3 weeks. The number of explants that produced at least one shoot (length, >0.5 cm) was recorded at 4, 8, and 12 weeks. The number of shoots (length, >0.5 cm) per leaf were counted after 8 or 12 weeks.

Three types of basal media WPM (Lloyd and McCown, 1980), MS, and ½ MS, each containing 8.88 μM thidiazuron (TDZ) and 1.97 μM IBA, were evaluated for in vitro shoot induction from leaf explants (Table 2). The leaf explants were placed with the abaxial side either up or down on the media to evaluate the effects of leaf orientation on in vitro shoot regeneration.

Rooting and acclimatization. The in vitro regenerated shoots were transferred to 50 mL SPM in each Magenta® GA7 box (Sigma-Aldrich) to increase the number of shoots. After 6 weeks of culture in SPM, the elongated shoots (length, >3 cm) were excised and transferred directly to a 4-inch plastic pot (10.2 × 10.2 × 12.7 cm) containing water-saturated planting medium (Michigan Grower Products, Inc., Galesburg, MI) that was autoclaved at 121 °C for 10 min before use. Each container was sealed with a clear 1-gal zippered polyethylene bag (SC Johnson, Bay City, MI) and was watered

every 3 d to keep the humidity inside the bag. After 4 weeks, the bags were opened gradually for a couple of hours daily for 1 week and subsequently removed completely. The total number of surviving plants was recorded at the end of the 4-week acclimation.

Optimizing *A. tumefaciens*-mediated transformation factors via transient expression experiments. The experimental design was based on previous studies of cherry rootstocks and sour cherry (Song, 2015; Song and Sink, 2005, 2006). Briefly, *A. tumefaciens* strains EHA105, LBA4404, and GV3101, each harboring the binary vector pBISN1 (Narasimhulu et al., 1996), were used. pBISN1 contains the neomycin phosphotransferase gene (*nptII*) directed by the nos promoter and a plant intron interrupted GUS directed by the chimeric super promoter (Aocs)₃AmasPmas (Ni et al., 1995). Single colonies of the three strains were each cultured in 10 mL liquid YEB + 50 mg·L⁻¹ kanamycin monosulfate at 28 °C in the dark for 48 h. Bacterial cells were collected by a 2-min centrifuge at 2500 g_n and resuspended in liquid coculture medium (WPM + 8.88 μM TDZ + 1.97 μM IBA + 100 μM acetosyringone) to an optical density, with a wavelength of 600 nm (OD₆₀₀), of 0.1, 0.5, and 1.0 separately. The explants, 30 for each treatment, were cut and incubated in 30 mL suspension cells for 30 min at 28 °C. After

Table 1. Effect of plant growth regulators on shoot regeneration frequency and number of shoots per regenerated leaf of peach rootstock ‘Hansen 536’.

Treatment	PGR (μM)				No. of explants	Regeneration rate (100%) ^z	Mean no. of shoots/explant ^y
	BAP	IBA	TDZ	NAA			
WPRM1	8.88	0.49			33	12.1 ac ^x	1.3 ± 0.6 ab
WPRM2	8.88	0.98			31	32.3 b	1.9 ± 0.2 b
WPRM3	8.88	1.97			33	33.3 b	1.5 ± 0.5 b
WPRM4	8.88	3.94			36	36.1 b	1.8 ± 0.5 b
WPRM5		0.49	4.54		39	2.6 c	1.0 ± 1.7 ac
WPRM6		0.98	4.54		37	2.7 c	2.0 ± 1.2 bc
WPRM7		2.46	4.54		37	10.8 ac	2.0 ± 1.3 bc
WPRM8			4.54	0.54	41	2.4 c	1.0 ± 1.7 ab
WPRM9			4.54	2.69	36	0 c	0.0 ± 0.0 a
WPRM10	8.88		2.69		37	21.6 ab	2.1 ± 1.7 bc

^zPercentage of leaves that regenerated shoots and SE of replicates between petri dishes (No. of explants with at least one shoot/Total no. of explants × 100).

^yNumber of shoots per regenerating leaf (mean ± SE) = Total number of shoots/Total no. of explants with at least one shoot.

^xNumbers in each group with completely different letters are significantly different at *P* = 0.05 according to the least significant difference test.

Table 2. Effects of basic media and explant orientation on shoot regeneration frequency and number of shoots per regenerated leaf of peach rootstock ‘Hansen 536’.

Groups	Treatment			Regeneration rate (100%) ^z	Mean no. of shoots/explant ^y
	Basic media	PGR (μM)	Orientation		
A	WPM	BAP 8.88 IBA 1.97	Abaxial up	29.0 a ^x	3.2 ± 1.6 a
B	MS	BAP 8.88 IBA 1.97	Abaxial up	11.4 b	1.8 ± 1.1 b
C	½ MS	BAP 8.88 IBA 1.97	Abaxial up	0.0 c	0.0 ± 0.0 c
D	WPM	BAP 8.88 IBA 1.97	Abaxial down	13.9 b	1.6 ± 1.0 b
E	MS	BAP 8.88 IBA 1.97	Abaxial down	10.9 b	1.3 ± 0.7 b
F	½ MS	BAP 8.88 IBA 1.97	Abaxial down	0.0 c	0.0 ± 0.0 c

^zPercentage of leaves that regenerated shoots and SE of replicates between petri dishes (No. of explants with at least one shoot/Total no. of explants × 100).

^yNumber of shoots per regenerating leaf (mean ± SE) = Total number of shoots/Total no. of explants with at least one shoot.

^xNumbers in each group with different letters are significantly different at *P* = 0.05 according to the least significant difference test.

blotting dry on sterile filter paper, the explants were placed on solidified coculture medium [WPM + 8.88 μM TDZ + 1.97 μM IBA + 100 μM acetosyringone + 0.6% (w/v) Bacto-agar]. Transient *gusA* expression was determined after 2 or 4 d cocultivation in the dark at 25 °C. Three dishes each with 10 explants were used as replications for each treatment. The number of blue foci was counted under a dissecting microscope after removal of chlorophyll by 80% (v/v) ethanol rinses.

Statistical analysis. All experiments were arranged in completely randomized designs. For the experiments of regeneration optimization, three petri dishes (replicates) with 10 leaf explants each were used. The experiment was conducted twice. Regeneration rate is expressed as the average percentage of leaves producing shoots. Shoot number was calculated as the total number of shoots divided by the total number of explants with at least one shoot. For the transient expression experiments, 30 explants were used for *Agrobacterium* infection in each treatment and were then divided into three dishes each with 10 explants for cocultivation. Transient transformation frequency refers to the percentage of the infected leaf explants that showed blue staining. Data were analyzed statistically using analysis of variance and are presented as the mean $\pm$ SD. The means were separated using the least significant difference test, and significance was determined at 5% using the SPSS 20.0 program (IBM Corporation, Armonk, NY).

Results

Adventitious shoot regeneration. To evaluate the effects of exogenous hormones on adventitious shoot regeneration, 10 regeneration media (WPRM1–WPRM10) were designed to reveal the optimal combination and concentration of plant growth regulators (Table 1). Among the media, the combination of BAP at 8.88 μM and IBA at 0.98 to 3.94 μM induced greater adventitious shoot regeneration rates at 32.3% to 36.1% than the other media, and the greatest mean number of shoots was 1.9 per regenerated explant. TDZ at 4.54 μM combined with IBA (0.49, 0.98, and 2.46 μM) or 1-naphthylacetic acid (NAA) at 0.54 and 2.69 μM also induced shoot regeneration at low frequencies (0% to 10.8%). Higher concentrations of TDZ (9.08 μM and 13.62 μM) induced calluses at the wound sites that turned brown 4 weeks after moving from dark to light conditions, but no shoot regeneration was observed. The combination of BAP at 8.88 μM and NAA at 2.69 μM (WPRM10) induced adventitious shoots from 21.6% of the explants, less than in media WPRM2 to WPRM4, but the difference was not statistically significant. Although regenerated shoots occurred occasionally from the calli at the wounded midrib, 90% of them were observed at the petiole on the media containing BAP and IBA (Fig. 1B).

Of the three basal media tested, no significant difference in shoot regeneration was

observed between the WPM and the MS medium, although the WPM resulted in the greatest regeneration rate (29.0%) and the most shoots (3.2) per regenerated leaf explant. The $\frac{1}{2}$ MS medium did not lead to any shoot regeneration. The orientation of the explants in WPM affected shoot regeneration, and the leaf explants cultured abaxial side up showed greater regeneration rates than those cultured adaxial side up (Table 2).

In vitro rooting and acclimatization. Shoots induced from in vitro leaf explants of ‘Hansen536’ had high rooting ability. Eighty percent of shoots (length, >3 cm) tested rooted directly in substrate and survived after 4 weeks of acclimatization (Fig. 1C and D).

Transient *gusA* expression in leaf explants. As indicated by GUS staining in Fig. 2, the ‘Hansen536’ leaf explants were susceptible to all three *A. tumefaciens* strains

Fig. 1. Adventitious shoot regeneration from leaf explants for peach rootstock ‘Hansen 536’. (A) Callus regenerated from the leaf explant media after 2 weeks of dark cultivation. (B) Adventitious shoots produced from leaf explants, which are ready to be transferred to the shoot proliferation medium. (C) Root formation on a regenerated shoot. (D) Rooted plants transferred to 4-inch pots.

Fig. 2. Effects of *Agrobacterium* strains, concentration of cell suspensions, and duration of cocultivation on the transient expression of the *gusA* gene. Leaf explants were infected by soaking them in three *Agrobacterium* strain suspensions (EHA105, GV3101, and LBA4404), each with three concentrations ($\text{OD}_{600} = 0.1, 0.5, 1.0$) for 5 min at room temperature (25 °C) in the dark. Transient expression of the *GusA* gene was analyzed after 2 and 4 d of cocultivation separately.

Table 3. Transient transformation frequencies of peach rootstock ‘Hansen 536’ leaf explants infected with different *Agrobacterium* strains at different concentrations.

Transient transformation frequency (%)	OD ₆₀₀ = 0.1		OD ₆₀₀ = 0.5		OD ₆₀₀ = 1.0	
	2 d	4 d	2 d	4 d	2 d	4 d
EHA105	13.3 ± 9.0 a ²	57.9 ± 20.8a	81.0 ± 8.3 a	94.7 ± 8.3 a	84.2 ± 12.0 a	100 a
GV3101	18.8 ± 20.1 a	36.4 ± 12.6 b	22.2 ± 19.3 b	69.2 ± 8.7 b	42.9 ± 9.6 b	72.7 ± 25.0 b
LBA4404	0 b	25.0 ± 2.5 b	9.1 ± 1.4 c	42.1 ± 8.4 c	27.2 ± 4.8 c	31.6 ± 12.5 c

²Numbers in each group with different letters are significantly different at *P* = 0.05 according to the least significant difference test.

OD₆₀₀ = optical density at a wavelength of 600 nm.

(EHA105, LBA4404, and GV3101) regardless of the difference in susceptibility. Using a bacterial density of 0.1 (OD₆₀₀) for infection, the percentage of explants with at least one blue foci (used to indicate the frequency of transient *gusA* expression) was only 13.3% after 2 d of cocultivation (Table 3). No blue focus was observed in the LBA4404 infection groups. After 4 d of cocultivation, the frequency of GUS staining induced by EHA105, GV3101, and LBA4404 increased to 57.9%, 36.4%, and 25%, respectively (Table 3). Four days of cocultivation with a bacterial density of OD₆₀₀ 0.5 increased GUS staining for all three *A. tumefaciens* strains. For example, 94.7% of the leaf explants infected by EHA105 had blue foci, which was significantly greater than the other two strains. When using an OD₆₀₀ of 1.0 cultures for infection, the frequency of blue foci induced by EHA105 approached 84.2% after 2 d of cocultivation, and all the explants expressed the *gusA* gene at the wounded sites after 4 days of cocultivation. In addition, the blue areas were larger than those using GV3101 infection (Fig. 2). Only 31.6% of leaf explants showed GUS staining when using LBA4404 with a density of OD₆₀₀ 1.0 for 4 d of cocultivation, indicating a lower efficiency of this strain in transgene delivery (Table 3). Taken together, an infection using EHA105 cultures with OD₆₀₀ of 0.5 and 1.0 followed by cocultivation for 2 to 4 d was efficient for gene delivery to the leaf explants of ‘Hansen 536’.

Discussion

In vitro shoot regeneration from leaf explants of peach rootstocks is genotype dependent. For example, rootstock ‘Guardian’ showed a 16% regeneration rate (San et al., 2015). Rootstocks ‘Garnem’ and ‘GF677’ had relatively high regeneration rates (Pérez-Jiménez et al., 2012). Our study demonstrated that ‘Hansen 536’ leaf explants produced a 32.3% to 36.1% regeneration rate under optimal in vitro shoot regeneration conditions.

In general, the most important factors for in vitro shoot regeneration include explant type, basal medium, and composition and concentration of hormones. The best in vitro shoot regeneration rates for peach, plum, and apricot were achieved by using MS or ½ MS as basal media (Feeney et al., 2007; Pérez-Jiménez et al., 2012; Petri and Scorza, 2010; Tian et al., 2007; Wang et al., 2011; Zhou et al., 2010). In our study, the greatest

regeneration efficiency was obtained in WPM for ‘Hansen 536,’ despite some regeneration in MS media. In terms of hormones, some studies have indicated that TDZ is more effective in inducing in vitro shoot regeneration from leaf explants of fruit crops (Ainsley et al., 2001; Zhou et al., 2010). In contrast, our study demonstrated that TDZ in combination with either IBA or NAA resulted in much lower regeneration rates than BAP and IBA, suggesting that optimal hormones for in vitro shoot regeneration are species and genotype dependent.

In addition to the two factors analyzed, explant type also affected in vitro shoot regeneration. The calli at petiole parts were amenable to shoot production (Fig. 1A and B). Media containing TDZ only induced adventitious shoots from the callus at petioles. Similarly, in another study of adventitious shoot regeneration from peach rootstock ‘Guardian’, all the regenerated shoots were from petioles of the leaf explants (San et al., 2015). Similar results have been reported for other *Prunus* spp. leaves (Antonelli and Druart, 1989; Escalettes and Dosba, 1993; Miguel et al., 1996). Therefore, the petiole presence is another essential factor for obtaining morphogenesis for ‘Hansen 536’ leaf explants. Our results provide a guide to develop shoot regeneration systems from leaf explants of other peach genotypes.

Efficient gene delivery is essential for genetic transformation. *A. tumefaciens* C58 was reported to be more efficient than EHA105 (Pérez-Clemente et al., 2005). In another report, EHA105 appeared to give the greatest rate of gene delivery in peach epicotyl internodes, cotyledons, leaves, and embryonic axes (Padilla et al., 2006). To date, EHA105 has been used for a successful transformation of several other *Prunus* spp., including choke cherry (*P. virginiana* L.) (Dai et al., 2007), Montmorency sour cherry (*P. cerasus* L.), Gisela 6 (*P. cerasus* × *P. canescens*) cherry rootstock (Song, 2015), and black cherry (*P. serotina*) (Liu and Pijut, 2010). In our study, we compared three *A. tumefaciens* strains and found EHA105 was the most efficient strain for gene delivery. The result was similar to those observed in other plants, such as tomato (*Solanum lycopersicum* L.) (Chetty et al., 2013) and blueberry (*Vaccinium corymbosum* L.) (Song and Sink, 2004), suggesting that the supervirulence of the EHA105 strain was likely responsible for the increased efficiency of gene delivery (Hood et al., 1993).

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