Plant Cell Rep (2016) 35:1955–1962
DOI 10.1007/s00299-016-2010-2

ORIGINAL ARTICLE

Biolistic transformation of Carrizo citrange (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.)

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Received: 31 March 2016 / Accepted: 1 June 2016 / Published online: 8 June 2016

Abstract
Key message **The development of transgenic citrus plants by the biolistic method.**
Abstract A protocol for the biolistic transformation of epicotyl explants and transgenic shoot regeneration of immature citrange rootstock, cv. Carrizo (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) and plant regeneration is described. Immature epicotyl explants were bombarded with a vector containing the *npt*II selectable marker and the *gfp* reporter. The number of independent, stably transformed tissues/total number of explants, recorded by monitoring GFP fluorescence 4 weeks after bombardment was substantial at 18.4 %, and some fluorescing tissues regenerated into shoots. Fluorescing GFP, putative transgenic shoots were micro-grafted onto immature Carrizo rootstocks in vitro, confirmed by PCR amplification of *npt*II and *gfp* coding regions, followed by secondary grafting onto older rootstocks grown in soil. Southern blot analysis indicated that all the fluorescing shoots were transgenic. Multiple and single copies of *npt*II integrations were confirmed in five regenerated transgenic lines. There is potential to develop a higher throughput biolistics transformation system by optimizing the tissue culture medium to improve shoot regeneration and narrowing the window for plant sampling. This system will be appropriate for transformation with minimal cassettes.

Keywords Citrus transgenics · Carrizo citrange · Biolistics · Particle bombardment · Genetic transformation

Introduction

The *Citrus* genus is composed of several important species and hybrids grown in the subtropical and tropical regions of the world. The multi-billion dollar citrus industry in Florida is under assault from huanglongbing (HLB) disease thought to be caused by the gram negative, phloem restricted bacterium *Candidatus* Liberibacter asiaticus, which has still not been cultured in vitro. Although some tolerant genotypes exist, primarily in rootstock (Albrecht and Bowman 2011, 2012; Lopes et al. 2009), tolerance in sweet orange scion cultivars has yet to be identified. The selection of citrus genotypes grown from seed is a lengthy endeavor due to the long juvenile phase, in which no fruit is produced, and juvenility can last from 5 to 20 years depending on the cultivar. The American National Academy of Sciences recommended that disease tolerant, stable citrus transgenics be produced to combat HLB (National Research Council 2010), because biotechnology is potentially faster than traditional breeding. Moreover, the insertion of a single gene cassette does not substantially alter the plant genome.

Agrobacterium-mediated transformation (AMT) and biolistics are the two most widely used methods for plant genetic transformation (Gao and Nielsen 2013). Moore et al. (1992) first reported AMT in citrus rootstocks and key lime, and subsequently, AMT has been the predominant transformation method for over two decades. AMT utilizes

Communicated by V. Srivastava.

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the natural mechanism of a soil borne bacterium to transfer the DNA into plant genome. In biolistics, DNA is coated onto gold or tungsten micro-particles and bombarded at high velocity in a stream of helium into intact cells or tissues (Fu et al. 2000; Sanford 1990; Sanford et al. 1987). Traditionally, AMT was thought to be superior over biolistics, because it normally produces transgenic plants with single or low copy number of transgenes. However, these advantages have been challenged with minimal cassette technology in biolistic transformation (Fu et al. 2000). Biolistic transformation has several advantages over AMT in that it might be species and genotype independent, and plasmid construction is simplified, since DNA sequences essential for T-DNA replication and transfer in *Agrobacterium* are not required. Also, the co-transformation of multiple plasmids or cassettes can be accomplished with particle bombardment (Zale et al. 2015). Furthermore, false positives resulting from growth of *Agrobacterium* in plant tissue are eliminated during screening, and shoot regeneration from tissue culture might be improved due to the elimination of potentially toxic antibiotics (Wu et al. 2015a). In essence, transformation protocols are simplified, because complex bacteria/plant interrelationships that vary with each plant genotype are removed (Gray and Finer 1993).

In citrus, there were two published attempts to produce transgenics with biolistics. The first attempt was by Yao et al. (1996) in tangelo (*Citrus reticulata* Blanco × *C. paradisi* Macf) using embryogenic cells from suspension cultures. Southern blots confirmed that foreign genes were integrated into eight transgenic callus lines. Some of these lines produced embryos and tiny plantlets in vitro, but no transgenic plants were established. Bespalhok Filho et al. (2003) optimized bombardment parameters by transient GUS expression in thin, transverse epicotyl sections of Carrizo citrange and most GUS foci were concentrated in the cambial layer, but no transgenic plants were regenerated. There are no other published reports of biolistic transformation and regeneration of transgenic plants in citrus (Donmez et al. 2013; Febres et al. 2011).

The objective of this research was to develop a biolistic transformation system for citrus that provides an alternative to AMT. This protocol has the potential to transfer minimal cassettes with no pest sequences.

Materials and methods

Plasmid DNA

The plasmid tested was an intermediate binary vector originally designed for *Agrobacterium*-mediated transformation. The *gfp* cassette was PCR amplified using forward primer CaMV35S-P1-*Hin*dIII (5′-AGGTAAGCTTGCATGCCTGCAGGTCCCCAGATTA-3′) and reverse primer NosT-P2-*Spe*I (5′-AGGTACTAGTCCGATCTAGTAACATAGATGACAC-3′) from p1380-35S-GFP (Jia and Wang 2014). After *Hin*dIII and *Spe*I digestion, the CaMV35S-*gfp*-NosT fragment was inserted into the *Hin*dIII-*Spe*I-digested p1380N-Cas9 vector, which was previously described (Jia and Wang 2014), to produce the intermediate vector p1380N-Cas9-35S-GFP (Fig. 1), which is 15.66 kb in size with 6.72-kb backbone and 8.94-kb gene construct. In the current study, the *gfp* reporter and *npt*II selectable markers were used for transformation purposes, but the Cas9 cassette did not contribute to this biolistics research (Fig. 1). A colony of *E. coli* (DH5α) harboring p1380N-Cas9-35S-GFP was grown in LB broth with 100-mg l^{-1} kanamycin at 37 °C with shaking (260 rpm) overnight. Midi-preps were performed on a 100-ml aliquot using Qiagen Plasmid Midi kits (Qiagen, Valencia, CA) to obtain high-quality plasmid DNA for use in biolistics.

Plant materials and tissue culture

Carrizo seeds were obtained from Lyn Citrus Nursery, Arvin CA. The two seed coats were removed, seeds were surface sterilized in 20 % commercial bleach (1.6 % sodium hypochlorite) with a few drops of Tween-20 for 20 min, and washed 5 times in sterile distilled water, 5 min per wash. Seeds were plated onto sterile seed germination medium (Cervera et al. 2004; Orbović et al. 2015; Wu et al. 2015a) in culture tubes. The seeds were geminated and grown in vitro for 4–6 weeks at 28 °C in the dark and then moved to low light (30 μE^{-1} m^2 s^{-1}) with a 16-h photoperiod (Percival Scientific, IA) for 4–5 days (Fig. 2a). Four days prior to bombardment, the epicotyls were aseptically cut into 1-cm explants (Fig. 2b), exaggerating the diagonal cut on the terminal ends to increase surface area, and plated onto Murashige and Skoog (1962) medium supplemented with 22.2-μM (5 mg l^{-1}) BAP, 5 % sucrose, and 2.6-g l^{-1} Phytogel (Sigma) (MSB). After biolistics, the explants were plated onto MSBK selection media, which is MSB with 100-mg l^{-1} kanamycin for selection of transgenic shoots, and placed in low light (30 μE^{-1} m^{-2} s^{-1}) with a 16-h photoperiod (Percival Scientific, IA) for 4 days at 26 ± 1 °C. Afterward, the explants were transferred to a higher light intensity of 150 μE^{-1} m^{-2} s^{-1} with the same photoperiod at 28 ± 1 °C, and subcultured onto the same fresh selection media every 2 weeks until shoots developed.

For rootstock seedlings to be used in micro-grafting, Carrizo seeds were plated onto sterile seed medium in culture tubes and grown in the dark for 4–6 weeks. Transgenic Carrizo shoots were micro-grafted onto decapitated in vitro grown rootstocks as previously

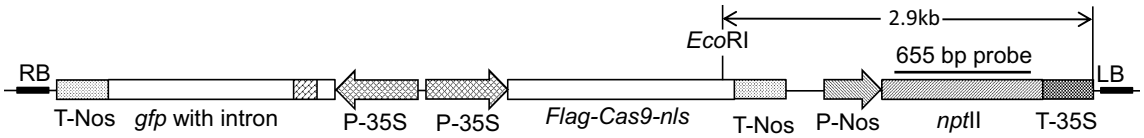

Fig. 1 The expression cassettes of plasmid p1380N-Cas9-35S-GFP showing *P-35S* the cauliflower mosaic virus 35S promoter, *gfp* green fluorescence protein gene, *T-Nos* the nopaline synthase gene terminator, *Flag-Cas9-nls* the Cas9 endonuclease containing Flag tag at its N-terminal and nuclear location signal at its C-terminal, *P-Nos* the promoter of nopaline synthase gene, *nptII* neomycin phosphotransferase II gene, *T-35S* the cauliflower mosaic virus 35S terminator, *LB* T-DNA left border, *RB* T-DNA right border, *EcoRI* restriction site. The 655-bp probe in the *npt*II coding region was used for Southern blot analysis

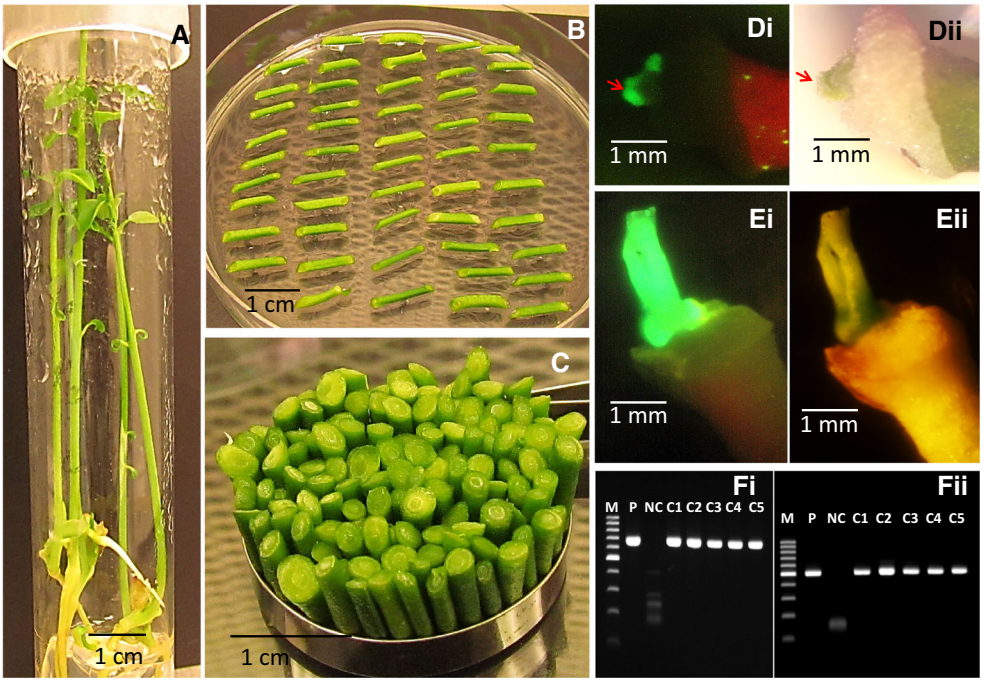

Fig. 2 **a** In vitro seedlings used for transformation; **b** epicotyl explants on 22.2-μM (5 mg l^{-1}) BAP for 5 days prior to biolistics; **c** explants bundled together for biolistics; **di** GFP positive tissue (*arrow*) under UV light; **dii** GFP positive tissue (*arrow*) under white light; **ei** GFP positive shoots under UV light; **eii** GFP positive shoots under white light; **fi** *npt*II PCR: *M* molecular markers (100 bp ladders), *P* positive control (plasmid), *NC* negative control (non-transgenic shoot), *C1–C5* transgenic lines; **fii** *gfp* PCR: *M* molecular markers (100 bp ladders), *P* positive control (plasmid), *NC* negative control (non-transgenic shoot), *C1–C5* transgenic lines

described (Cervera et al. 2004; Orbović et al. 2015; Wu et al. 2015a).

Preparation of gold particles

Gold particles (1.0 μm) were purchased from Crescent Chemical Company (Islandia, NY). Thirty milligrams of gold particles were prepared with 300 ng of whole plasmid according to Altpeter and Sandhu (2010) resulting that a total volume of 100-μl DNA-coated gold particle suspension and 5-μl aliquots (~15-ng plasmid DNA) were spread onto each macrocarrier (Biorad, Hercules, CA) for every shot.

Particle bombardments

Prior to biolistics, the explants were transferred to a high osmotic pretreatment of MSB with 0.4-M sorbitol for 4 h (Kadir and Parveez 2000). Afterward, the explants were bundled together by an autoclaved stainless steel ring (Fig. 2c), and the bundle was positioned in the center of an empty petri dish. The explants were positioned on their terminal ends, so that the cut ends were a target for bombardment. The explants were bombarded using the Biorad PDS 100/He^{TM} with 1550 psi Biorad rupture disks, 6-cm stage height and a chamber vacuum of 27.5 in Hg. After bombardment of one end, the bundle of explants was

flipped vertically 180° and the opposite end bombarded, so that each explant was shot twice. Double bombardments to each explant were designated a paired shot (PS). After releasing the explants from the ring, the explants were transferred directly to selection medium.

Fluorescence microscopy and data collection

Four weeks after bombardment, independent stably transformed tissues (ISTT) and developing shoots were examined for GFP fluorescence. Fluorescence was visualized using a Zeiss Stemi SVII—HB0100 fluorescence stereoscope (New York, NY) with a blue filter, which induces green light transmission from the GFP protein. Transgenic tissues or shoots were screened by comparison with the non-fluorescing neighbor tissues or shoots, and tissues or shoots from non-transgenic controls. Data were collected for the number of explants, the number of independently stably transformed tissues (ISTT), and transgenic shoots (TS).

Primary and secondary micro-grafting

GFP positive shoots were micro-grafted onto immature Carrizo rootstock in vitro following the protocol described in Wu et al. (2015a). Transgenic shoots were maintained by clipping back the faster growing rootstock shoots. After the shoots healed onto the immature Carrizo rootstocks, secondary grafts were performed onto 6-month-old, soil grown rootstocks in the growth facility set to temperature of 86 ± 2 °C, 60 % relative humidity, with a 16-h light cycle (Wu et al. 2015a). These conditions encourage rapid tissue growth to enable molecular analyses.

Molecular analyses

PCR screening was used as a rapid method to confirm transgenesis. Plant DNA was extracted from leaves using the CTAB protocol (Porebski et al. 1997) and used in PCR with *gfp* primers: *gfp*F, 5′-aggtgatgcaacatacgga-3′ and *gfp*R, 5′-gcagattgtgtggacaggta-3′ with one cycle of 2 min at 95 °C, 35 cycles of 15 s 94 °C, 15 s 60 °C, 25 s 72 °C, and a final extension of 1 min 72 °C to yield a 516-bp product. PCR was also conducted with *npt*II primers: *npt*IIF, 5′-gcttgggtggagaggctattc-3′, and *npt*IIR, 5′-gctcttcagcaat atcacggg-3′ using a PCR cycle with the same condition as above to yield a 655-bp product. PCR products were size fractionated in 1.5 % agarose gels containing Gel Red (VWR, Atlanta, GA).

Transgene copy number was determined with a Southern blot hybridized to the digoxigenin (DIG) labeled *npt*II probe. Briefly, genomic DNA was extracted using the CTAB protocol for leaves (Porebski et al. 1997), digested to completion using *Eco*RI enzyme, which cuts once within the T-DNA but outside of the probe region. The digested DNA was size fractionated in 0.8 % agarose gel electrophoresis, blotted onto Hybond N+ (Roche, Indianapolis, IN) membrane according to the instructions, and the membrane was UV cross-linked. The *npt*II probe was synthesized in PCR using digoxigenin (DIG) labeling (Roche) with primers *npt*IIF, 5′-gcttgggtggagaggctattc-3′, and *npt*IIR, 5′-gctcttcagcaatatcacggg-3′ with one cycle of 2 min at 95 °C; 9 cycles of 30 s at 94 °C, 30 s at 60 °C, 40 s at 72 °C; 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 40 s at 72 °C for the first cycle, and increase 20 s per cycle for the remainder of PCR, with a final extension of 10 min at 72 °C as recommended in the DIG labeling PCR protocol to yield a 655-bp product. The DIG probe was hybridized to the membrane, and washed according to the manufacturer's instructions. The blot was exposed to Kodak X-ray film for 5 min prior to image analyses.

Results

Biolistics, transgenic shoot identification, and transformation efficiency

Green fluorescing, transgenic tissues (Fig. 2di) and shoots (Fig. 2ei) were examined by fluorescence microscopy and scored as positive for the GFP protein. In contrast, GFP negative tissues or shoots of the non-transgenic controls did not fluoresce (Fig. 2dii, eii, respectively). After approximately 1–1.5 months, 5 GFP expressing shoots were identified from eight experiments after subculturing immature explants on shoot regeneration medium with 100-mg l^{-1} kanamycin. Transgenic shoots were micro-grafted in vitro onto decapitated Carrizo rootstocks to reduce the possibility of losing the transgenics due to failure of rooting. Once the micro-grafts healed, the transgenic micro-grafted shoots were secondarily grafted onto rootstocks growing in the soil to encourage rapid shoot growth. Figure 3 shows a representative shoot of Carrizo2 (line C2) after secondary grafting.

Eight independent experiments (IEs) were conducted totaling 15 paired shots (PS). The number of paired shots (NPS), explants, independent stably transformed tissues (ISTT), transgenic shoots (TS), the total number of shoots regenerated (SR), the regeneration rate (RR), the transformation efficiency of independent stably transformed tissues (TEISTT), and the transformation efficiency of transgenic shoots (TETS) were recorded (Table 1). Experiments 1– 3 have only 1 PS; experiments 4–7 have 2 PS; and experiment 8 had 4 PS. The number of PS was based on the availability of plant material at the time of bombardment;

Fig. 3 Secondarily grafted transgenic Carrizo shoot (line C2) attached to macrophylla rootstock

therefore, some experiments had only 1 PS, while others had 2 or 4 PS. There were 398 independent stably transformed tissues (ISTT) out of 2163 bombarded explants recorded 4 weeks after biolistics from all eight experiments. This is a transformation efficiency of 19 ± 2 %, which is an impressive rate of transformation, and it demonstrates that the citrus tissues are transgenic, since there is minimal auto-fluorescence in citrus. The TESTs were calculated by TS/SR and the values varied from 0 to 2.9 % in eight experiments with an overall mean transformation efficiency of 0.7 ± 0.3 % (Table 1).

Molecular and phenotypic analyses of the transgenics

PCR was used as a rapid method to confirm the presence of the *npt*II or *gfp* coding sequences in the GFP fluorescing shoots. Non-transgenic shoots served as negative controls and plasmid was the positive control. The amplicon of *npt*II was 655 bp and that of *gfp* was 516 bp (Fig. 2fi, fii). The amplified products from transgenics and plasmid were the expected sizes indicating all five putative transgenic plants contain both *npt*II and *gfp* coding sequences, while the negative control did not amplify. Southern blot analysis was conducted with *npt*II probe to investigate the integration patterns. Three transgenics (Carrizo1, Carrizo3, and Carrizo5) had multiple integrations of the transgenes, while two transgenics (Carrizo2 and Carrizo4) had single integrations of the *npt*II transgene (Fig. 4). All the bands in the Southern blot were larger than 2.9 kb, which is the expected size from the *Eco*RI restriction site to the end of the cauliflower mosaic virus 35S terminator in the *npt*II expression cassette (Fig. 1), indicating that the *npt*II cassettes were not truncated. All 5 transgenics looked similar to wild-type and Carrizo2 (line C2) (Fig. 5).

Discussion

In this protocol, 1.0-μm gold particles were used, because large gold particle sizes (1.5–3.0 μm) resulted in poor transient GUS expression in Carrizo (Bespalhok Filho et al.

Table 1 Biolistic transformation experiments

IE	NPS	Explants	ISTT	TS	SR	RR (%)	TEISTT (%)	TETS (%)
1	1	100	17	0	64	64	17	0.0
2	1	112	20	1	96	86	18	1.0
3	1	88	21	0	68	77	24	0.0
4	2	162	53	1	34	21	33	2.9
5	2	292	39	0	38	13	13	0.0
6	2	632	117	1	81	13	19	1.2
7	2	247	23	0	60	24	9	0.0
8	4	530	108	2	290	55	20	0.7
Sum	–	2163	398	5	731	–	–	–
–	–	270 ± 73	50 ± 14	0.6 ± 0.3	91 ± 29	44 ± 10	19 ± 2	0.7 ± 0.3

The mean ± SE are given in the last row. ISTT determined 4 weeks after biolistics

IE independent experiments, *NPS* No. paired shots, *Explants* no. explants, *ISTT* no. independent stably transformed tissues, *TS* no. transgenic shoots, *SR* No. shoots regenerated, *RR* regeneration rate shoots/no. explants), *TEISTT* transformation efficiency of independent stable transformed tissue (ISTT/no. explants), *TETS* transformation efficiency of transgenic shoots (TS/SR)

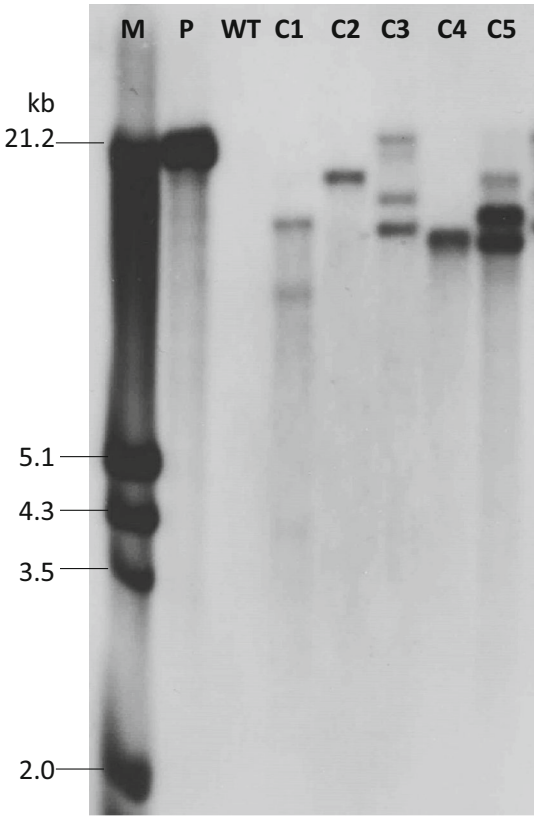

Fig. 4 Southern blot hybridized with *npt*II probe. *M* molecular markers, *P* plasmid, *WT* non-transgenic wild-type Carrizo plant, *C1–C5* 5 Carrizo transgenic lines. Plasmid and plant genomic DNA was digested with *Eco*RI

Fig. 5 Transgenic Carrizo (line C2, *left*) vs. non-transgenic wild-type Carrizo (*right*)

2003). Our preliminary experiments with GUS transient expression using different helium pressures (1100, 1350, 1550 psi) with a 6-cm stage height showed that 1550 psi was the best for transient expression (data not shown), which agrees with Bespalhok Filho et al. (2003) using thin epicotyl transverse sections of Carrizo. These pressures are relatively high compared with optimal pressures for other plant species (e.g., biolistics of indirect or direct embryos in sugarcane at 1100 psi (Altpeter and Sandhu 2010). Yao et al. (1996) did not observe significant differences among 1100, 1350, or 1550 psi in biolistics of tangelo embryogenic suspension cultures. These results suggest that different tissue types require different pressures in citrus.

Early researchers used embryogenic callus and transverse thin layers of citrus as targets in biolistics. Although transgenic callus lines or transient expression foci were produced (Yao et al. 1996; Bespalhok Filho et al. 2003), no transgenic plants were established in the soil or glasshouse, potentially due to difficulties of maintaining regenerated citrus shoots in vitro. Similarly, there is genotype dependency in the induction and maintenance of citrus callus cultures (Dutt and Grosser 2010). In the current protocol, we utilized epicotyl explants from which numerous reasonably sized, vigorous Carrizo shoots developed. Screening among large, vigorous shoots was critical to the survival of micro-grafts, because large shoots have a greater survival rate than smaller shoots.

The timing of bombardment after cutting epicotyl explants and transferring to MSB is a factor deserving additional investigation. The cambial layer is composed of actively dividing meristematic cells which have been shown to regenerate shoots through direct and indirect organogenesis in citrus (de Almeida et al. 2006; Pena et al. 2004). In the present study, the timing of bombardment on day four was chosen, because histological changes to the vascular cambium were significant by the fifth day (de Almeida et al. 2006). We observed clusters of callus-like tissue by the fifth day, shoot primordia in 2 weeks, and whole shoots in 4 weeks (data not shown). No chimeras were produced from all eight experiments, which suggested that single meristematic cells were originally transformed. In biolistic transformation of sugarcane via direct embyogenesis, Snyman et al. (2006) did not obtain results from bombardments the day of explant cutting, and they

suggested that excised leaf rolls be targeted on the seventh day. If other citrus genotypes respond similarly, this protocol might be appropriate for consistent and reliable biolistic transformation of other immature citrus cultivars with minimal modifications regarding to the timing of bombardments and tissue culture conditions. However, optimal parameters might differ for each citrus genotype.

Another important aspect to success of biolistics in immature citrus epicotyl explants is the bombardment of the terminal ends of the explant bundles. However, it was tedious to bundle the immature epicotyl explants to form a bulls-eye for bombardment. Bundles of mature citrus stem explants are much more suited to biolistics, because the stems have significant girth (Wu and Zale, unpublished). Perhaps, one reason that citrus researchers have preferred AMT is because it is much easier to infiltrate immature epicotyl or mature stem explants in liquid *Agrobacterium* inoculation medium than position the explants in bundles for bombardments. Nevertheless, this biolistics method provides an alternative to AMT. The use of a ring to bundle explants together and target the terminal ends has never been previously reported, and it is an effective way of targeting explants.

Although the mean transformation efficiency based on the number of transgenic plants was low at 0.7 ± 0.3 % (no. of transgenic shoots/total no. shoots), transformation efficiencies calculated from independent stably transformed tissues represented 18.4 % of total explants (398 ISTT/2163 total explants), so if the tissue culture protocol can be improved to regenerate more shoots, the transformation efficiency could be increased. There was much variability in the mean number of shoots regenerated (mean ± SE 91 ± 29) and the mean regeneration rate (mean ± SE 44 ± 10), possibly due to the wide window (4–6 weeks) for seedling growth before sampling. To increase the shoot regeneration rate, Carrizo seedlings should be used in transformations at 4–4.5 weeks (Dutt, personal communication). Future research should adjust the tissue culture medium to promote shoot proliferation, and narrow the sampling window to promote shoot proliferation and the regeneration rate. Also, this protocol could be adapted for minimal cassettes which produce transgenics with low copy transgene insertions similar to *Agrobacterium* but without vector backbone sequence (Wu et al. 2015b; Fu et al. 2000).

Conclusions

This is the first report of biolistic transformation of immature citrus and establishment of transgenic citrus plants. Southern blot analysis confirmed that the plasmid DNA was integrated into citrus genome. Biolistics will become important to the genetic transformation of immature and mature citrus, particularly to stem the threat of HLB. However, the prevailing mood in the US regarding GM crops has been negative and attempts to generate a more consumer-friendly product are important. The development of cisgenic and intragenic citrus by biolistics might produce a more consumer-friendly product than using heterologous DNA sequences from bacteria. This approach also relies on the identification of HLB disease resistance genes, selectable markers, or reporter genes in citrus or related species. Future research will investigate biolistic transformation of minimal cassettes of disease resistance genes and anthocyanin reporters using all plants and no *Agrobacterium* plant pest sequences in immature and mature citrus.

Author contribution statement HW and JZ conceived of and designed the research, and wrote the manuscript. HW conducted biolistics, GFP microscopy, Southern blot analyses, tissue culture, and grafting. YA contributed to PCR and photography of GFP. NW and HJ designed and constructed the vector, respectively. All authors read and approved the manuscript.

Acknowledgments This research was supported by the Citrus Research and Development Foundation (CRDF), an arm of the Florida Citrus Growers, awards 583 and 15-045C to JZ. Facilities were provided by the CRDF and the University of Florida, IFAS, Citrus Research and Education Center, in Lake Alfred, FL.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

We complied with ethical standards.

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