Development of an efficient transformation method by *Agrobacterium tumefaciens* and high throughput spray assay to identify transgenic plants for woodland strawberry (*Fragaria vesca*) using NPTII selection

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Abstract

**Key message** We developed an efficient *Agrobacterium*-mediated transformation method using an Ac/Ds transposon tagging construct for *F. vesca* and high throughput paromomycin spray assay to identify its transformants for strawberry functional genomics.

**Abstract** Genomic resources for Rosaceae species are now readily available, including the *Fragaria vesca* genome, EST sequences, markers, linkage maps, and physical maps. The Rosaceae Genomic Executive Committee has promoted strawberry as a translational genomics model due to its unique biological features and transformability for fruit trait improvement. Our overall research goal is to use functional genomic and metabolic approaches to pursue high throughput gene discovery in the diploid woodland strawberry. *F. vesca* offers several advantages of a fleshy fruit typical of most fruit crops, short life cycle (seed to seed in 12–16 weeks), small genome size (206 Mbp/C), small plant size, self-compatibility, and many seeds per plant. We have developed an efficient *Agrobacterium tumefaciens*-mediated strawberry transformation method using kanamycin selection, and high throughput paromomycin spray assay to efficiently identify transgenic strawberry plants. Using our kanamycin transformation method, we were able to produce up to 98 independent kanamycin resistant insertional mutant lines using a T-DNA construct carrying an Ac/Ds transposon Launchpad system from a single transformation experiment involving inoculation of 22 leaf explants of *F. vesca* accession 551572 within approx. 11 weeks (from inoculation to soil). Transgenic plants with 1–2 copies of a transgene were confirmed by Southern blot analysis. Using our paromomycin spray assay, transgenic *F. vesca* plants were rapidly identified within 10 days after spraying.

**Keywords** *Agrobacterium tumefaciens*-mediated transformation · Woodland strawberry · Kanamycin selection · Paromomycin spray assay

Introduction

Strawberry is a member of the family Rosaceae, which also includes apples, pears, peaches, raspberries, cherries, and roses. Rosaceae represent the third most economically important crop family grown in temperate regions, with an annual value of approximately $7 billion. Despite its importance, genetic research is difficult because of the relative large genome, long life cycle, and self-incompatibility of most rosaceous plants. *Fragaria vesca*, the woodland strawberry, is an ideal candidate for a model Rosaceae functional genomics because it has a small genome (2n = 2x = 14, 240 Mbp), short life cycle...
(3–4 months), abundant seed set on self-pollination, ease of vegetative propagation, and its genome sequence is publicly available (Shulaev et al. 2011). Recent advances in functional genomics now make it possible to identify the genes responsible for valuable economic traits by insertional mutagenesis.

*A. tumefaciens* mediated-transformation of octoploid strawberry (*Fragaria × ananassa* Duch.) has been reported with transformation frequencies ranging from 0.91 to 10 % except for 20 to 65 % obtained in cv. Chandler by Cordero de Mesa et al. (2000, 2004) and Abdal-Aziz et al. (2006) using leaf disks and stems, stipules, and petioles as explants (Qin et al. 2008). Several studies reported that genotypes of *F. vesca* Alpine accessions (FRA197, 198, and PI), “Alpine Wonder;” *F. v. semperflorens* were transformed using *A. tumefaciens*-mediated method and kanamycin selection with a frequency of approx. 15 % for producing transgenic plants evidenced by Southern blot analysis (Folta and Dhingra 2006). An efficient *A. tumefaciens* mediated-transformation method of *F. vesca* using hygromycin selection was developed with a transformation cycle of approx. 14–15 weeks, and used to produce insertional mutants for strawberry functional genomics (Oosumi et al. 2006, 2010). Therefore, efficient transformation methods for *F. vesca* using kanamycin and other selection systems are still needed to efficiently produce large number of transgenic plants.

Many crop transformation methods that use NPTII selection have a common problem of regeneration of non-transgenic shoots while imposing kanamycin selection at the shoot formation stage. These non-transgenic shoots occur in high frequencies in many crop species although the exact reason for its occurrence is unknown. For instance, the occurrence of ranging from 40 to 90 % have been reported for apple (James et al. 1989), pear (Mourgues et al. 1996), banana (Murkute et al. 2003), grapevine (Perl et al. 1996), citrange (Moore et al. 1992; Pena et al. 1995a), sweet orange (Pena et al. 1995b; Cervera et al. 1998), and lime (Pena et al. 1997). In cauliflower, Stipic et al. (2000) found that approximately 95 % of shoots regenerated from selective media were non-transgenic shoots. Therefore, it is critical to develop an efficient selective assay to screen transgenic plants containing a functional nptII gene, particularly for large scale screening of transgenic plants. The gene codes for the aminoglycoside 3′-phosphotransferase (denoted NPTII) enzyme, which inactivates by phosphorylation a range of aminoglycoside antibiotics such as kanamycin, gentamicin (G418), and paromomycin. Paromomycin spray was successfully used to identify transgenic wheat plants and it is more effective and less expensive than G418 for these assays (Cheng et al. 1997; Zale et al. 2009).

In *F. vesca* strawberry, the generation of a mutant population large enough to disrupt all of the genes in the genome has been estimated to comprise at least 255,000 independent transformation events (Oosumi et al. 2006), which would take years. An Ac/Δs transposon tagging system, used successfully in *Arabidopsis* (Woody et al. 2007), carrot (Ipek et al. 2006), rice (Kolesnik et al. 2004), barley (Ayliffe et al. 2007), and wheat (Pastori et al. 2007), could potentially create a nearly unlimited number of transposon insertion mutants from a relatively small number of initial transformants. This would reduce the time and labor inputs needed for the transformation system, and establish a streamlined system by which hundreds of thousands of insertional mutants could be efficiently created in subsequent generations, facilitating functional genomics studies of the Rosaceae family.

In this study, our objectives were to develop an efficient *A. tumefaciens* mediated-transformation method using kanamycin selection and an Ac/Δs transposon tagging construct for *F. vesca* PI 551572 and to develop a high throughput paromomycin spray assay to identify *F. vesca* transgenic plants. Accession PI 551572 was the best candidate selected from 14 *F. vesca* accessions for a model in *F. vesca* functional genomics research, as it had the greatest transformation efficiency in *A. tumefaciens* mediated-transformation using hygromycin selection (Oosumi et al. 2006).

### Materials and methods

#### Stock plant growth

*F. vesca* PI 551572 was obtained from the temperate fruit National Clonal Germplasm Repository (NCGR, Corvallis, OR, USA) or their derivatives from self-pollination in our facility. Fully ripe self-pollinated fruit was dried, placed in a 15 ml conical tube and shaken vigorously for 2 min. The seeds that detached were collected and stored at 4 °C.

To improve germination and growth conditions of *F. vesca* seeds for efficient production of explant leaf materials for *A. tumefaciens* mediated-transformation experiments, we tested four different medium compositions: sterile water-moistened sand, sterile water-moistened filter paper, sterile Fafard 3-B (Fafard, Agawam, MA, USA) medium (soil) and MS media (MS salts supplemented with B5 vitamins and 2 % sucrose, pH 5.8). Seedlings were plated either in the dark or under an 11 h photoperiod with light intensity of 260 μM m−2 s−1. Day and night temperatures were set at 22 and 16 °C, respectively. Two independent experiments were conducted (total 80 seeds/treatment). Data were collected 6 weeks after plating.
After the first true leaf appeared, the seedlings were transferred to soil-less Fafard Mix #3-B.

Shoot and root killing curve using kanamycin

To determine an optimal concentration of kanamycin for selection stringency at the shoot induction stage a killing curve experiment was conducted using leaf explants of *F. vesca*. Leaves from 7-week-old seedlings were excised and surface-sterilized as described below. These leaves were sliced into 1 mm thick strips perpendicular to the midvein of the leaf and placed on Shoot Induction Media (SIM). SIM contained MS salts, B5 vitamins, 3 mg/l benzyladenine (BA), 0.2 mg/l indole-3-butyric acid (IBA), 500 mg/L carbenicillin (Phytotechnology Laboratories), and 0.2 % Phytagel (Sigma). After 1 week of culture, the strips were cut into square secondary explants and placed on SIM with 0, 10, 20, 30, 40, or 50 mg/l kanamycin. Three independent experiments were conducted with 51 explants per treatment. The explants were placed under a 16 h photoperiod, 24 μM m⁻² s⁻¹ light intensity at 22 °C. The explants were transferred to fresh media every 2 weeks and scored for growth of calli and/or shoots 5 weeks after culture.

To determine an optimal concentration of kanamycin stringency at the rooting stage shoots regenerated from wild-type *F. vesca* calli were excised and placed on root induction media (RIM) containing 0, 10, 20, 30, 40, or 50 mg/l kanamycin. RIM had MS salts supplemented with B5 vitamins (Phytotechnology Laboratories, Overland Park, KS), 2 % sucrose (Sigma), 100 mg/l carbenicillin (Phytotechnology Laboratories), 0.7 % agar (Sigma), pH 5.8. Three independent experiments were conducted with 36 shoots for each treatment and the culture conditions were the same as described above. The explants were scored for growth of roots 5 weeks after culture.

A. tumefaciens mediated-transformation

Plasmid pCAMBIA-1304 was used to develop *F. vesca* transformation method using hygromycin (Oosumi et al. 2006). In this study, we first used pCAMBIA-1304 to develop *F. vesca* transformation method using kanamycin. *A. tumefaciens* strain GV3101 housing binary vector pCAMBIA-1304 with mgfp5-gusA-HisG6 fusion (Center for the Application of Molecular Biology to International Agriculture, Black Mountain, Australia) was used for *F. vesca* transformation. In addition, the plasmid pSKI074 (Veilleux et al. 2012) containing an Ac/Ds transposon tagging system was introduced into GV3101 and EHA105 and used for the transformation (Fig. 1).

A. tumefaciens-mediated-transformation experiments were conducted using a modified method of Oosumi et al. (2006). A newly opened trifoliate leaf was selected from 10- to 15-week-old plants. The leaves were sterilized in 70 % ethanol for 1 min followed by 1 % sodium hypochlorite for 8 min and then rinsed five times with sterile deionized water. The leaves were then cut into approx. 1 mm wide pieces by removing the petiole and midvein and cutting perpendicular to the secondary veins. The leaf strips were inoculated with *A. tumefaciens* suspension in a liquid medium containing MS Basal salts, B5 vitamins, 2 % sucrose at pH of 5.5 for 25 min with gentle agitation every 5 min. The leaf strips were placed abaxial side up on a co-cultivation medium and co-cultured in the dark for 2 days at 25 °C. The co-culture medium contained MS salts, B5 vitamins, 2 % sucrose, 3 mg/l BA, 0.2 mg/l IBA, 7 g/l agar, pH 5.5. After 2 days, the leaf strips were transferred to shoot induction selection media I (SIMI) under 16 h photoperiod and 24 μM m⁻² s⁻¹ light intensity at 22 °C. SIMI medium had MS salts, B5 vitamins, 2 g/l sucrose, 3 mg/l BA, 0.2 mg/l IBA, 500 mg/l carbenicillin, 2 g/l Phytagel at pH 5.8. After 6 days on SIM, the explants were cut into smaller leaf strips between the veins as secondary explants, each bisected by a secondary vein. These secondary explants were then cultured on SIMI for another 7 days. The explants were then transferred to shoot induction selection II medium (SIMII). The SIMII medium contained MS salts, B5 vitamins, 2 g/l sucrose, 3 mg/l BA, 0.2 mg/l IBA, 30 mg/l kanamycin, 250 mg/l carbenicillin, 2 g/l Phytagel at pH 5.8. These explants were subcultured every 2 weeks. When the shoots were more than 3 mm in length, they were excised from the explants and placed on root induction medium (RIM) in 100 × 200 mm Petri dishes at 22 °C under 16 h photoperiod and light intensity of 90 μM m⁻² s⁻¹. RIM medium was supplemented with MS salts and B5 vitamins, 3 g/l sucrose, 100 mg/l carbenicillin, 30 mg/l kanamycin, and 2 g/l phytagel at pH 5.8. When the plants were well-rooted, the putative transgenic plants were transferred into Magenta boxes (Phytotechnology Laboratories, Overland Park, KS) containing 50 ml of MS medium supplemented with 30 g/l sucrose, 100 mg/l carbenicillin and 2 g/l phytagel at pH 5.8 and

![Fig. 1 Map of the T-DNA region of the transposon tagging Ac/Ds construct used for transformation of F. vesca. RB Right border, TPase maize transposase, Ds dissociator part of the element, p35S cauliflower mosaic virus promoter, nptII kanamycin resistance gene, Pnas manropine synthase promoter, EGFP-enhanced green fluorescent protein gene, LB left border. Triangular borders of the Ds element represent inverted repeats. The T-DNA was inserted into plasmid pSKI074.](image-url)
cultured at the growth conditions described above except under the light intensity of 160 μM m\(^{-2}\) s\(^{-1}\) for about 2 weeks. The putative transgenic plants were transferred to soil in pots.

Effects of concentration and acetosyringone on transformation efficiency

From our initial experiments successful transformations were obtained when using pCAMBIA 1304 at bacterial concentrations of optical density (OD\(_{600}\)) ranging from 0.028 to 0.042. However, these concentrations failed to produce any transformants when using plasmid pSKI074 housing the AcDs system. Therefore, bacterial concentrations at optical density (OD\(_{600}\)) of 0.035, 0.1, 0.4, 0.7, and 1 were tested using plasmid pSKI074 containing AcDs. Also acetosyringone at 200 mM was added to the inoculation medium and explants co-cultured with A. tumefaciens for 20 min. The transformation frequency was determined as the percentage of secondary explants producing kanamycin resistant plants.

Southern blot analysis

Genomic DNA was isolated according to Oosumi et al. (2010). Southern blot analysis was performed by Lofstrand Labs Ltd. (Gaithersburg, MD 20879). Approx. 15 μg genomic DNA of strawberry plants were digested with HindIII. The digested DNA samples were loaded onto a 350 ml 0.7 % TBE agarose gel and the gel was electrophoresed at 50 V for 18 h. It was transferred to a Nytran Supercharge nylon membrane (Whatman/Schleicher and Schuell). Each membrane was UV cross-linked and air-dried. The membrane was prehybridized using 6× SSC, 5× Denhardt’s solution, and 0.5 % SDS at 68 °C for 6 h. They were separately hybridized using the random primed probes of gfp template DNA that were labeled with P\(^{32}\). The hybridizations were carried out at 68 °C for 27 h. The membranes were washed in 2× SSC + 0.1 % SDS at 68 °C with three buffer changes over a period of 60 min; and 20 min at 68 °C with 0.1× SSC + 0.1 % SDS. The membranes were autoradiographed for approx. 41 h and 4 days using an intensifier screen at −80 °C.

Development of paromomycin spray assay

Approximately, 6-week-old wild-type and transgenic T\(_1\) F. vesca plants were grown in 4 in. pots and were used for all experiments. The T\(_1\) transgenic plants were from self-pollination of eight independent T\(_0\) parental transgenic events generated from A. tumefaciens mediated-transformation using the plasmid pSKI074 and confirmed by our routine in vitro kanamycin screening assay and multiplex PCR (Veilleux et al. 2012). The T\(_1\) transgenic plants were identified by the kanamycin screening assay. Five concentrations of paromomycin solutions (1, 2, 3, 4 and 5 %) were tested against a control solution without paromomycin. The wild-type and transgenic strawberry plants each were divided into six groups based on their size and appearance to have a relatively uniform representation for each group. Also, the transgenic plants from each of the eight independent events were equally assigned to each group. Each group was randomly assigned to one of the five concentrations and the control (no paromomycin) to insure an unbiased representation of each group for each concentration to test. Experiments were conducted three and four times for the transgenic and wild-type strawberry plants, respectively, with approximately 30–40 plants for each concentration. All plants were placed under a fume hood and sprayed until all leaves had been well-saturated to runoff with the solution (average of 9–12 sprays). The sprayed plants were grown in growth chamber under 16 h photoperiod and approx. 15 μM m\(^{-2}\) s\(^{-1}\) light intensity at 22 °C. Because the leaf lesion in wild-type plants started to develop 4 days after paromomycin spray, but the leaf lesion in transgenic plants did not develop until approx. 3 weeks after the spray, the wild-type plants were rated for leaf necrotic lesion symptom severity 10 days after the spray, but 4 weeks for transgenic plants. A five-point rating scale was used to rate leaf lesion symptom severity: 0 = no leaf lesions; 1 = >1 to ≤25 % of leaf area having necrotic lesions; 2 = >25 to ≤50 % of leaf area having necrotic lesions; 3 = >50 to ≤75 % of leaf area having necrotic lesions; and 4 = >75 to ≤100 % of leaf area having necrotic lesions.

Data analysis

All data were statistically analyzed using ANOVA and Tukey HSD All-Pairwise Comparisons Test (Statistix 9, Analytical Software Tallahassee, FL, USA).

Results and discussion

Seed germination improvement

Among the eight treatments of the four medium compositions combined with two light conditions Fafard 3-B medium under the dark generated significantly (P < 0.05) higher percentage (85 %) of seed germination compared with the rest of the treatments except the treatment of MS medium under the light condition (Fig. 2). Also, there was significant (P < 0.05) 2.4-fold increase in the percentages
of seeds germinated in the dark (85 %) compared with under the light (35 %) when using sterile Fafard 3-B medium (Fig. 2). There was a tendency for seeds of *F. vesca* germinated on Fafard 3-B medium in the dark to produce many more plants than *F. vesca* seeds germinated on sterile water-moistened sand in the light. We improved the protocol of Oosumi et al. (2006) by germinating seeds on sterile Fafard 3-B medium in the dark rather than germinating seeds on sterile water-moistened sand in the dark to efficiently produce stock plants for *F. vesca* transformations.

### Determination of kanamycin killing curve

A killing curve was determined by testing different concentrations of kanamycin on shoot and root growth (Fig. 3). With 50 mg/l kanamycin in MS media, both shoots and roots were killed. The 40 mg/l of kanamycin in MS media inhibited the growth of all shoots and inhibited most shoots from growing roots (2.8 % of shoots having roots), respectively. The 30, 20, and 10 mg/l of kanamycin in MS media inhibited the growth of 96.1, 72.6, and 56.9 % of shoots and 97.2, 66.7 % of shoots from growing roots, respectively. When no kanamycin was placed in MS media, all shoots had new growth and produced roots. Therefore, 30 mg/l kanamycin was selected for shoot and root selection.

Following the killing curve experiments, we examined the effect of 50 mg/l kanamycin in SIMII medium compared to 30 mg/l kanamycin in SIMII for production of calli and shoots after transformation. After the primary explants were transformed, each explant was cut into 4 or 5 pieces and distributed evenly on a plate of SIMII containing either 50 mg/l of kanamycin or 30 mg/l of kanamycin. Six experiments were conducted using approximately 100 secondary explants per treatment. The SIMII containing 30 mg/l of kanamycin produced significantly (*P* < 0.01) more calli (37.2 %) than the explants on SIMII containing 50 mg/l of kanamycin (4.7 %).

Qin et al. (2011) reported that kanamycin significantly inhibited callus induction, bud differentiation and root morphogenesis when culturing leaf explants of *F. ananassa* Duch. cv. Toyonaka. Kanamycin, even at 2.5 mg/l, significantly inhibited callus induction, shoot regeneration and root formation; while no shoots regenerated at concentrations above 15 mg/l. Rooting was completely inhibited in the presence of 50 mg/l kanamycin. Biochemical analysis of the tissues showed that kanamycin caused electrolyte leakage and triggered active enzymatic processes and metabolism. This suggested a possible mechanism for the inhibition of strawberry growth caused by kanamycin.
Effect of *Agrobacterium tumefaciens* concentration and acetosyringone on transformation efficiency and regeneration of transgenic plants

Among the concentrations of *A. tumefaciens* tested (OD$_{600}$ = 0.035, 0.1, 0.4, 0.7 and 1) a concentration of OD$_{600}$ = 0.1 significantly (P < 0.05) increased the frequency of kanamycin resistant plants from 0 to 14.8 % compared with the rest of bacterial concentrations when using GV3101 harboring the Ac/Ds system in *F. vesca* (Table 1). Also, increasing the OD from 0.04 (control) to 0.85 had significantly (P < 0.05) 21-fold higher percentage (45.5 vs. 2.1 %) of explants producing kanamycin resistant plants using EHA 105 harboring the Ac/Ds system in *F. vesca*.

The addition of acetosyringone during co-culture significantly (P < 0.05) increased the transformation frequency from 0 to 25.5 % using EHA 105 harboring the Ac/Ds system in *F. vesca*. Calli formed approx. 5–7 days after selection, buds expressing green fluorescent protein (GFP) developed after 2–4 weeks and shoots emerged 4–6 weeks after selection; 79 % of kanamycin resistant shoots generated roots on medium RIM 2–4 weeks after culture.

An average of transformation frequency was 89.7 % under the optimal conditions using *A. tumefaciens* concentration at OD$_{600}$ = 0.85, 200 mM acetosyringone, 30 mg/l of kanamycin in selection medium and EHA 105 harboring the Ac/Ds system. Transgenic plants expressing GFP were transplanted into soil. The transformation cycle from inoculation to soil took approx. 11–15 weeks depending on the construct. Transgenic plants confirmed by Southern blot analysis contained 1–2 copies of the *gfp* gene (Fig. 4).

The *A. tumefaciens* concentration used by Oosumi et al. (2006), which efficiently produced transgenic *F. vesca* plants when using pCAMBIA 1304 and hygromycin selection, was initially adapted to our transformation system and represented for a measured OD$_{600}$ of approx. 0.028. However, with this concentration, we could not regenerate any transformatant when using plasmid pSKI074 containing Ac/Ds and kanamycin selection. Zhao et al. (2004) reported that an *A. tumefaciens* density of 2 × 10$^8$ cfu ml$^{-1}$ in MS liquid medium supplemented with 20 mM acetosyringone (pH 5.4) efficiently produced transgenic octoploid strawberry plants. Strawberry transformation frequency relies on many factors, including antibiotic type and concentration, inoculation and co-culture period and the presence or absence of acetosyringone (Qin et al. 2008). Zhang and Wang (2005) reported that the optimal conditions for *A. tumefaciens*-mediated transformation of “Allstar” strawberry leaf disks were: 10–15 min inoculation, 3-day coculture with 50 µM acetosyringone, followed by transfer to a selection medium containing 25 mg/L kanamycin plus 450 mg/L carbenicillin. Under these conditions a transformation frequency of 1.1 % was achieved based on kanamycin resistance assay. Acetosyringone for inducing vir gene has been used in many transformation protocols to enhance transformation frequency (Sheikholeslam and Weeks 1987; Wu et al. 2003; Cheng et al. 2004; Kuta and Tripathi 2005).

### Table 1 Effect of *Agrobacterium tumefaciens* concentrations on the frequency of kanamycin resistant plants

<table>
<thead>
<tr>
<th><em>A. tumefaciens</em> concentration (OD$_{600}$)</th>
<th>No. of explant</th>
<th>Frequency of kanamycin resistant plants (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.035</td>
<td>220</td>
<td>0 B</td>
</tr>
<tr>
<td>0.1</td>
<td>183</td>
<td>14.8 A</td>
</tr>
<tr>
<td>0.4</td>
<td>155</td>
<td>0 B</td>
</tr>
<tr>
<td>0.7</td>
<td>161</td>
<td>0 B</td>
</tr>
<tr>
<td>1.0</td>
<td>165</td>
<td>0 B</td>
</tr>
</tbody>
</table>

$^a$ The treatment with different letters was significant (P < 0.05)

Fig. 4 Southern blot hybridization of *F. vesca* PI 551572 T$_0$ transgenic plants transformed with plasmid pSKI074 containing an Ac/Ds transposon tagging system (15 µg genomic DNA was digested with *Hind*III and hybridized with an *mGFP5*-specific probe). Lane 1 1 kb ladder, lane 2 a wild-type *F. vesca* plant, lanes 3–20 T$_0$ transgenic *F. vesca* plants expressing GFP
Development of high throughput paromomycin spray assay to identify transgenic plants

All five concentrations of paromomycin tested on wild-type plants induced significantly ($P < 0.05$) greater percentages of leaf area having necrotic lesions than the control (without paromomycin spray) 10 days after spray application (Fig. 5, panel A). The 4 and 5 % paromomycin had significantly ($P < 0.05$) greater average percentage of leaf area with necrotic lesions compared to the 3, 2, and 1 % treatments (Fig. 5, panel A). There was no significant difference in the average percentages of leaf area having necrotic lesions among the five concentrations and control treatment in transgenic plants tested even 4 weeks after spraying wild-type plants and transgenic plants. Treatments with different letters were significantly ($P < 0.05$) different using Tukey HSD All-Pairwise Comparisons Test (Statistix 9, Analytical Software, Tallahassee, FL, USA) for the wild-type plants and transgenic plants, respectively. b Wild-type and transgenic F. vesca plants sprayed with 3 % paromomycin compared with control (without spray) 7 and 14 days, respectively after spray

Fig. 5 a Experiments were conducted three and four times for transgenic and wild-type strawberry plants, respectively, with approximately 30 to 40 plants for each concentration. Y axis represented the mean percentage of leaves having necrotic lesions collected 10 days and 4 weeks after paromomycin spray for wild-type and transgenic F. vesca plants, respectively. Pictures of F. vesca plants in each treatment were taken 7 days after the spray for (Fig. 5, panel A). The leaf lesion of wild-type plants started to develop 4 days after paromomycin spray, but the leaf lesion in transgenic plants did not develop until approx. 2 weeks after the spray, and there were only a few necrotic lesions 4 weeks after the spray. These results indicated that all wild-type plants were susceptible to paromomycin and all transgenic plants were resistant to paromomycin at all the concentrations tested. Paromomycin at 1–5 % concentrations can be used to identify transgenic strawberry plants with a functional nptII transgene. A dramatically visible difference was observed between the transgenic and wild-type strawberry plants
Criteria | Paromomycin spray assay | PCR
--- | --- | ---
Number of putative transgenic plants to screen | 2,000 plants/person/day (spraying plants and identifying symptom) | 100 plants/person/day (DNA isolation and PCR assay)
Material cost$ | $6.15 per 100 plants | $300 per 100 plants

$ Material cost included paromomycin (PhytoTechnology Laboratories, Shawnee Mission, KS 66282) for paromomycin spray assay at the concentration of 3% and PCR reagents

7–10 days after the spray with the paromomycin solution of 3% and above. Therefore, we recommend paromomycin at 3% for *F. vesca* PI 551572 and other strawberry genotypes that may have similar tolerance to paromomycin as *F. vesca* PI 551572, and 4 and 5% for strawberry genotypes that may be more tolerant to paromomycin than *F. vesca* PI 551572 to identify transgenic diploid strawberry plants. However, it is recommended to determine an appropriate concentration for any specific genotype to effectively identify transgenic plants (Fig. 5, panel B). Paromomycin spray assay at 2% was successfully used to identify transgenic wheat plants when the plants reached the 3-leaf stage. Seven to ten days after the spray the plants were evaluated for paromomycin damage. The plants with a functional npt II gene showed no bleached spots, whereas the plants without a functional npt II gene exhibited bleached spots throughout (Cheng et al. 1997; Zale et al. 2009).

This paromomycin spray assay developed in this study possesses five elements of a high throughput system: (1) large-scale capability of screening approx. 2,000 plants per day each person in 2 in. square pot tray in growth chamber or greenhouse (vs. screening approx. 100 plants per day each person using PCR), (2) rapid screening cycle, which took 10 days to identify transgenic plants, (3) minimal screening complexity, which had a simple protocol with one step of spraying 36 plants in a tray in approx. 3–5 min, (4) inexpensive method (material cost of $6.15 for screening 100 plants) using paromomycin compared with PCR (material cost of $300 for screening 100 plants) (Table 2), G418 and hygromycin and (5) high quality method that 100% of the plants identified were transgenic plants. In most plant transformation, we need two levels of selections: (1) select transgenic shoots or transgenic plantlets at in vitro stage by adding selective agents such as antibiotics into selective medium and (2) select T_1 to T_X generation of transgenic plants in a segregating population that contain both transgenic and non-transgenic plants using destructive GUS assay, GFP expression under fluorescent microscope, PCR or Southern blot analysis. First, our paromomycin spray assay could be potentially explored as a selection tool at in vitro stage by spraying putative transgenic shoots or transgenic plantlets using sterile solution of paromomycin to select paromomycin resistant transgenic shoots or transgenic plantlets instead of using traditional method by adding antibiotics in selective medium in during transformation. This is important for transformation methods using kanamycin as selection because many crop transformation methods that use NPTII selection have a common problem of regeneration of non-transgenic shoots while imposing kanamycin selection at the shoot formation stage. Second, spraying of paromomycin can be used to select transgenic plants from a segregating population of transgenic and non-transgenic plants in growth chambers, greenhouse and field instead of using destructive GUS assay, GFP expression under fluorescent microscope, PCR or Southern blot analysis because spraying paromomycin is simple, non-destructive, efficient, rapid, and large-scale screening. Using this high throughput spray method to determine the expression of NPTII and identify a transgenic plant, the traditional method of growing explants in an antibiotic tissue culture medium and regeneration process could be eliminated. Tissue culture process is typically more expensive and takes more time to implement than the spray assay, particularly for a segregating population and large scale screening. This spray assay for determining gene expression and identifying transgenic plants can alleviate time and financial constraints as well as efficiently deliver transgenic plants. The method developed in this study could be used to identify transgenic plants for other plant species.

In summary, optimizing parameters for transformation led to a greater efficiency in producing transgenic plants of *F. vesca* using the Ac/Ds construct in *A. tumefaciens* strains EHA 105 and GV3101. *A. tumefaciens* concentrations of OD_{600} = 0.1 for GV3101 and 0.85 for EHA105, use of kanamycin at 30 mg/l as a selection agent in the shoot induction and root induction media, and the addition of 200 mM acetosyringone during inoculation increased the transformation efficiency. Paromomycin at 3% can be used to efficiently identify transgenic strawberry plants 10 days after spray. Using this high throughput spray method to identify transgenic plants, researchers can eliminate traditional method of growing plants in an antibiotic tissue culture medium for regeneration process. Tissue culture process is typically time consuming and
labor-intensive. This spray assay enabled high throughput screening and identifying transgenic *F. vesca* plants, which can alleviate time and financial constraints while increasing the efficiency of selection of transgenic plants.

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