Planta (2006) 223: 1219–1230
DOI 10.1007/s00425-005-0170-3

ORIGINAL ARTICLE

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High-efficiency transformation of the diploid strawberry (*Fragaria vesca*) for functional genomics

Received: 31 August 2005 / Accepted: 31 October 2005 / Published online: 1 December 2005

Abstract *Fragaria vesca* L., a diploid ($2n = 2x = 14$) relative of the commercial octoploid strawberry, is an attractive model for functional genomics research in Rosaceae. Its small genome size, short reproductive cycle, and facile vegetative and seed propagation make *F. vesca* a promising candidate for forward and reverse genetics experiments. However, the lack of a high-efficiency transformation protocol required for systematic production of thousands of T-DNA insertional mutant lines and high-throughput gene validation is a major bottleneck. We describe a new transformation procedure that uses leaf explants from newly unfolded trifoliate leaves obtained from stock plants 6–7 weeks after seed germination, co-cultivation with *Agrobacterium* strain GV3101, and stringent selection on MS medium containing 4 mg l^{-1} hygromycin. Using this protocol we achieved 100% transformation efficiency for 6 of 14 *F. vesca* accessions tested. Accession PI 551572 was determined to be the best candidate for a model in *F. vesca* functional genomics research, as it showed the greatest propensity for callus formation, transformation, shoot regeneration, ex vitro establishment, and plant growth, requiring only 14–15 weeks to complete its life cycle in different seasons in the greenhouse.

Keywords Rosaceae · T-DNA tagging · Insertional mutagenesis · *Agrobacterium tumefaciens* · Woodland strawberry

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Abbreviations BA: Benzyladenine · GFP: Green fluorescent protein · IBA: Indole-3-butyric acid · *hpt*: Hygromycin phosphotransferase · Kn: Kanamycin

Introduction

The Rosaceae ranks third among families of economically important cultivated crops in temperate regions. Genetic improvement of most rosaceous crops is hampered by cumbersome genome size, intolerance to inbreeding, and lengthy life cycle. The woodland or alpine strawberry, *Fragaria vesca* L., however, does not have these limitations and thus offers a model within Rosaceae for genomic investigation. *F. vesca*, the most closely related diploid ($2n = 2x = 14$) to the cultivated octoploid strawberry, *Fragaria* × *ananassa* ($2n = 8x = 56$) (Ontivero et al. 2000; Potter et al. 2000), has a small genome size [estimated at 164 Mbp (Akiyama et al. 2001), only 1.3 times larger than that of *Arabidopsis thaliana* (125 Mbp)], short generation time (about 4 months), and small plant size (Battey et al. 1998). Genetic linkage maps have already been constructed for *F. vesca* based on both RAPD (Davis and Yu 1997) and microsatellite markers (Sargent et al. 2004). The diploid strawberry has been used to study genetic and environmental control of development and flowering in perennial plants (Brown and Wareing 1965; Galletta and Maas 1990; Battey et al. 1998). Its fleshy fruit has unique secondary metabolism including synthesis of diverse aroma and flavor compounds (Manning 1993). Furthermore, the fruit can be used to study molecular mechanisms of development and ripening of non-climacteric fruit (Given et al. 1988; Manning 1998; Nam et al. 1999). An inbred line of cultivated *F. vesca* has recently become available (J. Slovin, personal communication). There is a growing number of ESTs being deposited in the Genbank for both *F. vesca* and *Fragaria* × *ananassa* (currently 4,076 and 5,377, respectively). These characteristics make diploid strawberry a promising model species for functional genomics research in Rosaceae.

Two fundamentally different genetic approaches are currently used in functional genomics research. A forward genetics approach begins with observation of a mutant phenotype and focuses on identifying the gene(s) responsible. Conversely, reverse genetics begins with a candidate gene and describes the mutant phenotypes that result from disruption of that gene (Mathur et al. 1998). One way to generate mutant phenotypes in plants is to inactivate genes by insertion of foreign DNA via *Agrobacterium*-mediated transformation. Despite the growing use of *F. vesca* as a model plant in genetic studies, forward and reverse genetic platforms have not yet been developed for strawberry or any other rosaceous crop. To use T-DNA mutagenesis effectively as a tool for functional genomics, it is necessary to produce a population of mutants with a high probability for disruption of every gene in the genome. Based on the 164 Mbp genome size and assuming random T-DNA integration and an average gene length of 2 kb, we calculate that at least 255,000 independent T-DNA-transformed lines are required to mutate any single gene in *F. vesca* with a probability of 95%. In strawberry, *Agrobacterium tumefaciens*-mediated transformation has been developed using tissue culture systems with varying rates of success, optimally reported at 68% by Zhao et al. (2004). However, a more robust and efficient protocol for *Agrobacterium*-mediated transformation of *F. vesca* is essential for high-throughput generation of T-DNA insertional mutant lines.

Here we report the development of a highly efficient transformation protocol for *F. vesca* based on stringent hygromycin selection and green fluorescent protein (GFP) as a facile selectable marker. This transformation protocol allows efficient production of the T-DNA mutagenized collections for forward and reverse genetic studies and high-throughput gene validation in *F. vesca*.

Materials and methods

Plant growth

Fragaria vesca accessions used in this study are summarized in Table 1. Seeds of *F. vesca* accessions were obtained from the temperate fruit National Clonal Germplasm Repository (NCGR, Corvallis, OR, USA). Seeds of *F. vesca* 'Alpine' were obtained from Seedlings.com (Fairfield, ME, USA). Seeds were disinfected in 70% ethanol for 5 min and in 1% sodium hypochlorite for 5 min and then rinsed in sterile water six times before germination in Petri dishes containing sterile sand. Seedlings were transferred to soil-less medium (Fafard Mix #3-B, Fafard, Agawam, MA, USA) or Magenta GA7 vessels (Magenta, Chicago, IL, USA) containing 50 ml MS basal medium (Murashige and Skoog 1962) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) with 0.7% agar (Sigma-Aldrich, St. Louis, MO, USA) after appearance of the first true leaf. The seedlings were transferred to soil-less medium and cultivated in a growth chamber under an 11 h photoperiod with a light intensity of 500 μM m^{-2} s^{-1} at 22°C (day)/16°C (night). The seedlings in Magenta vessels were placed in an incubator under a 16 h photoperiod with a light intensity of 170 μM m^{-2} s^{-1}at 22 ± 1°C.

Leaf explants from in vitro 'Alpine' plants were used to develop the transformation method. Fourteen *F. vesca* accessions were screened to select those with optimal transformation efficiency. Plants grown in soil-less medium were used for *Agrobacterium*-mediated transformation after 6–7 weeks of cultivation.

Preparation of plant material for transformation

A newly unfolded trifoliate leaf was excised from each plant. Leaves from growth chamber-grown plants were surface-sterilized immediately after harvesting. The leaflets were separated, thoroughly washed under running tap water for 30 min, disinfected in 70% ethanol for 1 min, and then gently agitated in 1% sodium hypochlorite for 8 min. After sterilization the leaflets were rinsed five times in sterile water. The sterilized leaflets were placed abaxial side up on three to four layers of sterile filter paper (Whatman 1, Whatman, Brentford, UK) in the lid of a Petri dish. The filter paper was moistened with sterile water. After petiole and midvein tissue was removed, the leaf blades were cut diagonally across the secondary veins into 1.0–1.5 mm wide pieces. For one transformation experiment, two in vivo leaflets or four in vitro leaves were used.

Agrobacterium culture

A binary vector pCAMBIA-1304 [GenBank accession: AF234300, Center for the Application of Molecular Biology to International Agriculture (CAMBIA), Black Mountain, Australia], containing *mgfp5-gus*A-His6 fusion, was introduced into two *A. tumefaciens* strains, GV3101 (pMP90) (Koncz and Schell 1986) and LBA4404 carrying the ternary plasmid pBBR1MCS-5.virGN54D containing constitutive *virG* mutant gene (van der Fits et al. 2000). The *Agrobacterium* strains were cultured on Luria broth (LB) agar plates containing 20 μg ml^{-1} rifampicin (Rifampin, Fisher Scientific, Pittsburgh, PA, USA), 50 μg ml^{-1} gentamycin sulfate (Cellgro, Herndon, VA, USA), and 100 μg ml^{-1} kanamycin (Kn) monosulfate (Fisher Scientific) for 2 (GV3101) or 3 days (LBA4404) at 28–30°C. For one transformation experiment, a single colony was incubated in 2 ml LB containing antibiotics for 20–22 (GV3101) or 24 h (LBA4404) at 30°C on a shaker (240 rpm).

Table 1 *Fragaria vesca* L. accessions used for transformation experiments

Accession ID	Subspecies (ssp), forma (f), cultivar (cv)	Day-neutral flowering[a]	Runnering[a]
PI 551552	ssp. *bracteata*	No	Yes
PI 551572	Hawaii-4	Yes	Yes
PI 551782		No	Yes
PI 551791		No	Yes
PI 551792	ssp. *vesca*	No	Yes
PI 551833		No	Yes
PI 551834	ssp. *vesca f. semperflorens*, cv. Reugen	Yes	No
PI 551890	ssp. *vesca*	Yes	Yes
PI 551892		No	Yes
PI 602578	ssp. *vesca f. alba*	Yes	No
PI 602923	ssp. *vesca*, cv. Alexandria	Yes	No
PI 602924	ssp. *vesca*	No	Yes
PI 602931		No	Yes
PI 616513		No	Yes

[a]Plants grown in a growth chamber (Experimental procedure) for 2–3 months were transferred to a greenhouse between early May and early June

Fragaria vesca transformation

The *Agrobacterium* culture (0.7 ml) was centrifuged at 13,000 rpm for 3 min and the pellet suspended in 20 ml MS + B5 medium containing MS basal salts (Phyto-Technology Laboratories), B5 vitamins (Gamborg et al. 1968) (Sigma-Aldrich), 2% sucrose, pH 5.5 (GV3101) or 5.3 (LBA4404) in a 50 ml centrifuge tube.

A regeneration protocol was designed by combining and optimizing the techniques reported by Nehra et al. (1990a), El Mansouri et al. (1996), and Mathews et al. (1998). The explants were placed into the *Agrobacterium* suspension immediately after cutting. The tube was incubated for 20 min at room temperature and shaken gently every 5 min. After incubation, the explants were blotted dry on sterile filter paper (Whatman 1) and transferred to a deep Petri dish (100×25 mm^2, Nalge Nunc International, Rochester, NY, USA) containing co-cultivation medium (CCM): MS salts, B5 vitamins, 2% sucrose, 3 mg l^{-1} N^6-benzyladenine (BA) (Sigma-Aldrich), 0.2 mg l^{-1} indole-3-butyric acid (IBA) (Sigma-Aldrich), 0.7% agar (Sigma-Aldrich), pH 5.5. The explants were placed abaxial side up on CCM. The plate was sealed with Micropore surgical tape (3 M, St. Paul, MN, USA) and incubated at 25°C in the dark for 2 (GV3101) or 3 days (LBA4404). The explants were rinsed twice in washing-off medium (WOM) [20 ml MS + B5 supplemented with 500 mg l^{-1} carbenicillin (Fisher Science)] in a 50 ml centrifuge tube with vigorous shaking to remove *Agrobacterium*. The WOM was decanted and replaced with 30 ml fresh WOM solution. The tube was incubated at room temperature for 30 min and shaken gently every 5 min. The WOM was again decanted and the explants were rinsed with 10 ml WOM briefly, then blotted dry on sterile filter paper. After washing, the explants were transferred to non-selective shoot induction medium (SIM): [MS salts, B5 vitamins, 2% sucrose, pH 5.8, 3 mg l^{-1} BA, 0.2 mg l^{-1} IBA, 500 mg l^{-1} carbenicillin, 0.2% Phytagel (Sigma-Aldrich)].

To select the most effective concentration of antibiotic selection, we incubated explants at 22 ± 1°C under 16 h photoperiod at light intensity of 20–30 μM m^{-2} s^{-1}. After 6 days, the explants were transferred to selective SIM containing hygromycin B (MP Biomedicals, Irvine, CA, USA) at 0, 0.5, 1, 2, or 4 mg l^{-1} and 250 mg l^{-1} carbenicillin. Before transfer to SIM medium, the primary explants were divided into four to six secondary explants by cutting them between the veins so that each secondary explant was bisected by a secondary vein. After another 7 days of culture, the explants were transferred to fresh selective SIM and subcultured every 2 weeks. Transgenic calli and shoots were screened for GFP expression 6 weeks after infection. Explants with GFP^+ calli or shoots were scored and transferred to non-selective SIM containing 100 mg l^{-1} carbenicillin. The selected explants were incubated under the same conditions and subcultured every 2 weeks until GFP^+ shoots grew large enough for excision. Well-developed GFP^+ shoots were cut from calli and placed in deep Petri dishes containing 40 ml root induction medium (RIM): MS salts and vitamins, 3% sucrose, pH 5.8, 0.2% phytagel. Rooted plants were transferred to Magenta vessels containing 50 ml RIM. After 2 weeks of incubation, the plants were transplanted to soil-less medium in a growth chamber as described above.

Fluorescence microscopy

Visualization of GFP fluorescence in plant tissue was done using an Olympus fluorescence microscope SZXZ-RFL3 (Olympus America, Melville, NY, USA) with a 100 W mercury burner (Olympus BH2-RFL-T3) and filter sets for GFP longpass, narrow-band GFP, and FITC/TRITC (filter sets no. 41018, 41020, and 52004V2, respectively, Chroma Technology Corp., Rockingham, VT, USA).

PCR amplification analysis of transgenic strawberry plants

DNA was isolated from leaves of GFP^+ and untransformed plants using a modified rapid CTAB method (Doyle and Doyle 1990; Lodhi et al. 1994; Porebski et al.

1997). Single folded trifoliate leaves were harvested from each plant and ground in a 1.5 ml centrifuge tube containing 50 µl extraction buffer with a disposable grinder. Subsequently, 150 µl extraction buffer supplemented with polyvinylpyrrolidone (20 mg ml^{-1}) was added and mixed. The extraction mix was heated at 65°C for 15 min. DNA solution was extracted twice with equal volumes of chloroform:isoamyl alcohol (24:1). DNA was precipitated with two volumes of ethanol and 0.5 volumes of 5 M NaCl, and dissolved in 50 µl TE buffer containing 20 µg ml^{-1} RNase.

Primers for PCR amplification of hygromycin phosphotransferase (*hpt*) were: *hpt* forward, 5′-GAACTTCGTGCTTTCAGC-3′ and *hpt* reverse, 5′-CATCACAGTTTGCCAGTG-3′. For each 20 µl PCR reaction mix, 0.5 units of Ex Taq polymerase (Takara Bio Inc., Shiga, Japan), 1× Ex Taq polymerase buffer, 200 µM of each dNTP, 2 µM of each primer, and 1 µl DNA solution were used. The positive control was 20 ng plasmid DNA of pCAMBIA-1304. A GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) was used for PCR amplifications using the following step cycle program: 94°C, 30 s; 54°C, 30 s, and 72°C, 30 s for 30 cycles. A 5 µl aliquot of each PCR reaction was analyzed by 2% agarose gel electrophoresis using 1× TAE buffer. The expected product size of amplification was 402 bp.

Southern blot hybridization analysis

DNA was extracted from leaf tissue of transgenic plants of PI 551572 as described above with some minor alterations. Leaf buds were ground in liquid nitrogen in a 1.5 ml tube using a disposable grinder prior to homogenization in the extraction buffer. DNA dissolved in TE buffer was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. About 15 µg genomic DNA was digested with restriction enzyme *Hin*dIII (Promega, Madison, WI, USA) and separated on a 0.7% agarose gel in 0.5× TBE buffer. DNA was transferred to a positively charged nylon membrane (Zeta-Probe Blotting Membrane, Bio-Rad Laboratories, Hercules, CA, USA) by capillary blotting with Alkaline transfer buffer. An mGFP5-specific probe was amplified from pCAMBIA-1304 by PCR with the primers 5′-ACGCACAATCCCACTATC-3′ and 5′-GTAATCCCAGCAGCTGTT-3′ and labeled using the AlkPhos direct kit (Amersham Biosciences, Little Chalfont, UK). Hybridization and signal generation were performed using the AlkPhos direct kit. Chemiluminescent signal was exposed to Hyperfilm-ECL (Amersham).

Screening T_1 plants for morphological mutants

Progeny (T_1) seeds of transgenic plants were disinfected as described above and sown on rockwool cubes (1×1×1 cm^3, Grodan Rockwool Growcubes, Grodan, Hedehusene, Denmark) in deep Petri dishes (100×25 mm^2, Nalge Nunc International) under an 11 h photoperiod with a light intensity of about 140 µM m^{-2} s^{-1} at 22°C. After the first true leaf opened, seedlings were screened for GFP expression and then transferred to soil-less medium in pots (5×7 cm^2). T_1 plants were grown under the same conditions until mature fruits were produced.

Results

Effect of hygromycin concentration on shoot regeneration and development of transformed cells in *F. vesca* ‘Alpine’

To optimize hygromycin selection of transformed strawberry leaf cells, we first had to determine the minimum concentration of the antibiotic. Hygromycin is generally used in the range of 10–200 mg l^{-1} for plant selection (Murthy et al. 2003; Hisano et al. 2004; Wang et al. 2004). Since earlier reports suggested that *F. vesca* is sensitive to antibiotics (El Mansouri et al. 1996; Alsheikh et al. 2002), we initially examined the effect of hygromycin at concentrations lower than 10 mg l^{-1}. Four leaves from 9-week-old ‘Alpine’ plants grown on MS medium were used in this experiment and were infected with *Agrobacterium* strains GV3101 (pMP90) or LBA4404.pBBR1MCS-5.virGN54D containing pCAMBIA-1304. Callus initiation on infected explants was observed 7 days after infection. The explants cultured on non-selective medium regenerated shoots 3 weeks after infection. The explants cultured on selective medium containing 0.5–2 mg l^{-1} hygromycin regenerated shoots after 4 weeks. However, the explants cultured on medium containing 4 mg l^{-1} hygromycin did not form shoots until 8 weeks after infection (Fig. 1).

GFP screening

We observed cells expressing GFP after 3 weeks of culture. At this time, GFP expression was detected in the calli that developed on SIM containing 2 or 4 mg l^{-1} hygromycin (Fig. 2). For leaf explants infected with *Agrobacterium* strain GV3101, the number of GFP^+ calli increased until 5 weeks after infection (Fig. 2). Most of the GFP^+ calli selected with 2 or 4 mg l^{-1} hygromycin had already developed meristematic nodules by that time (Fig. 3a–d). Once shoot formation had initiated, few new calli formed. The leaf explants selected with 4 mg l^{-1} hygromycin tended to form large, well-isolated calli with strong GFP expression, and GFP^- calli were rarely found (Fig. 3a, c). Moreover, some of the explants formed multiple calli that appeared to derive independently from different regions of the explant. Under selection with 2 mg l^{-1} hygromycin, some

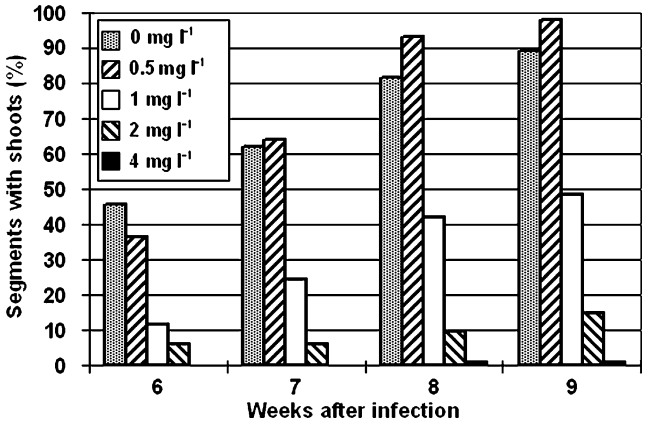

Fig. 1 Frequency of regenerating leaf explants as a function of incubation on medium with various concentrations of hygromycin from 6 to 9 weeks after infection of *F. vesca* cv. Alpine with *Agrobacterium tumefaciens* GV3101. Each value is the median of four independent experiments with about 50 segments per transformation

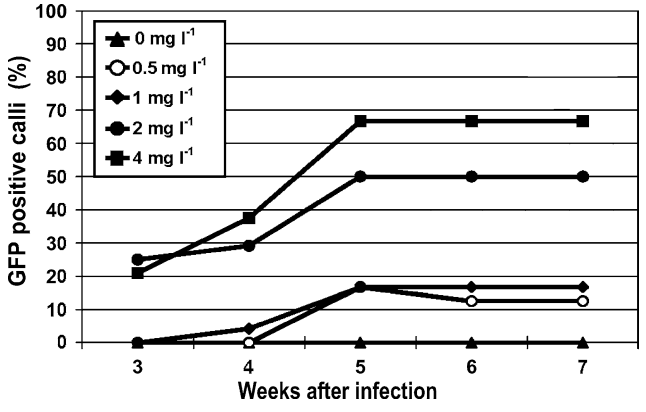

Fig. 2 Frequency of GFP$^+$ callus arising on leaf explants of *F. vesca* cv. Alpine as a function of hygromycin concentration in the incubation medium 3–7 weeks after transformation with *A. tumefaciens* GV3101

explants had one or two GFP$^+$ and many GFP$^-$ calli. With lower concentrations of hygromycin, only small GFP$^+$ spots were found on large masses of callus. Neither GFP$^+$ callus nor GFP$^+$ spots were observed when explants were cultured on a non-selective medium. GFP$^+$ callus formed on 67% of explants selected with 4 mg l^{-1} hygromycin the most efficient concentration tested. Similar experiments were carried out using LBA4404.pBBR1MCS-5.virGN54D containing the same binary vector and the same trend was observed (data not shown).

To test the effect of hygromycin concentration on shoot regeneration of selected GFP$^+$ calli, we continued hygromycin selection until 10 weeks after infection. Most GFP$^+$ calli had developed meristematic nodules 9 weeks after infection and 4–8% of explants formed GFP$^+$ shoots (Fig. 3e, f). Almost all GFP$^+$ calli selected with 0.5, 1, or 2 mg l^{-1} hygromycin had formed shoots by 16 weeks after infection. However, only 63% (GV3101) and 67% (LBA4404) of the GFP$^+$ calli selected with 4 mg l^{-1} hygromycin had formed shoots by that time.

Analysis of transgenic 'Alpine' plants

Once rooted, transformed plants grew vigorously. GFP expression was detected in the whole shoot before rooting. After rooting, however, GFP expression was only clearly detected in organs with low chlorophyll accumulation: folded leaves, leaf tips, roots, petals, fruit, and the base of the plant (Fig. 4). Anthocyanin also made GFP detection more difficult. For GFP detection we used the FITC/TRITC dual filter, narrow band-pass GFP filter, and a long-pass GFP filter. With the first two filters, the detectable GFP signal was low in tissues containing chlorophyll or anthocyanin.

T-DNA insertions were confirmed by PCR amplification analysis. PCR amplification was carried out using a primer set specific to the *hpt* gene. The expected 402 bp band was found in a random sample of ten GFP$^+$ plants and the plasmid pCAMBIA-1304 but it was absent in the untransformed control plant (Fig. 5).

Transformation of 14 *F. vesca* accessions

Our main objective for optimizing the strawberry transformation system was to develop a collection of insertional mutant lines in *F. vesca* for gene tagging and other genomic studies. In addition to high transformation efficiency, the strawberry accessions used for these studies should possess additional properties, including rapid shoot growth, day neutrality, short life cycle, inducible runnering, abundant seed production, and high germination efficiency. To identify *F. vesca* accessions best suited for genomic studies and generation of the T-DNA-tagged mutants, we utilized the newly developed transformation protocol to screen 14 diverse *F. vesca* accessions (Table 1) obtained from the NCGR. For these and all subsequent transformation experiments, we used plants grown in a growth chamber instead of in vitro plants due to the relative ease of growing plants, their more rapid development from seed, and the more durable explants that were obtained from them.

All accessions except PI 551833 initiated callus earlier than 'Alpine'. In many cases, callus initiated from the edge of the vascular tissue and expanded to the leaf lamina. For PIs 602924, 602931, 551834, and 551572, callus initiated on primary explants 4 days after infection and covered the explants by the time they were transferred to selective medium 6 days after infection. GFP expression was detected as small spots on the explants of these accessions about 2 weeks after infection. In all other accessions except PI 551833, GFP expression was detected 3 weeks after infection. On average, GFP

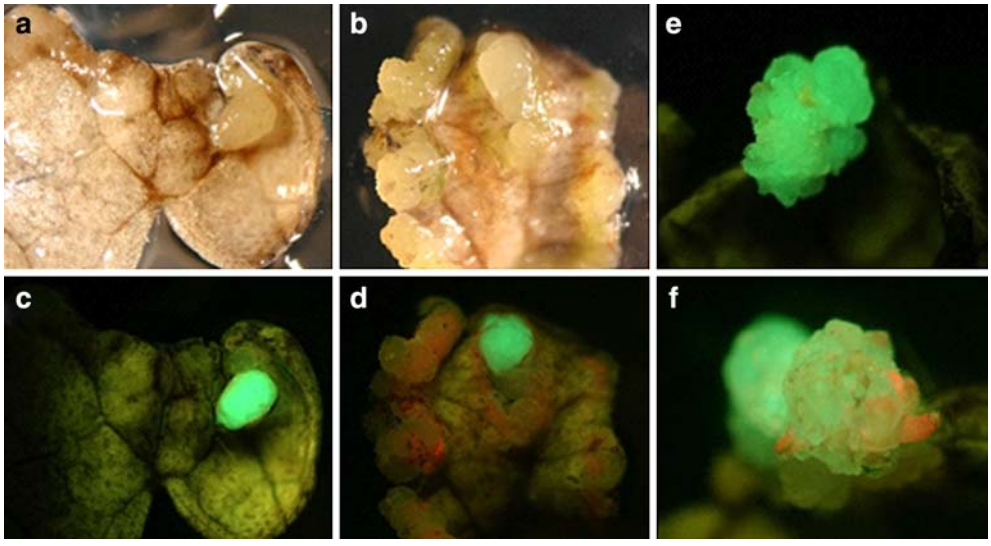

Fig. 3 Green fluorescent protein and hygromycin selection of transgenic calli. **a**–**d** Globular calli developing on *F. vesca* leaf explants cultured on shoot induction medium containing different concentrations of hygromycin 5 weeks after infection. Callus on medium containing 4 mg l^{-1} hygromycin (**a**, **c**), calli on medium containing 2 mg l^{-1} hygromycin (**b**, **d**), and GFP expression in them (**c**, **d**). **e**, **f** GFP expression in different phases of transgenic calli development on shoot induction medium containing 4 mg l^{-1} hygromycin. Embryonic callus 7 weeks after infection (**e**) and regenerating callus 9 weeks after infection (**f**)

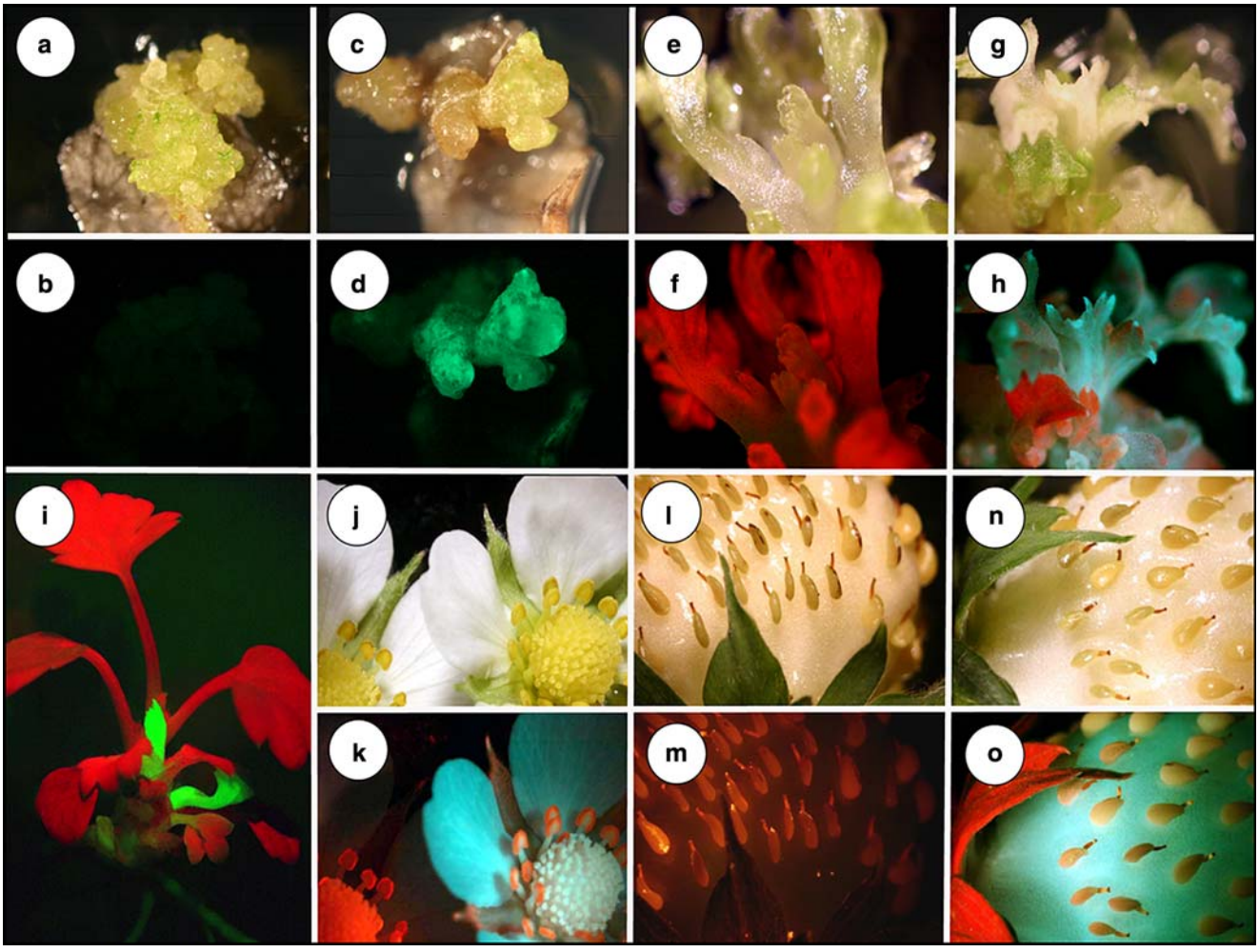

Fig. 4 Green fluorescent protein expression in *F. vesca* transformants. **a**, **b** regenerating untransformed callus; **c**, **d** regenerating transformed callus; **e**, **f** untransformed shoot developing from a leaf explant; **g**, **h** transgenic shoot developing from a leaf explant; **i** young transgenic plant; **j**, **k** flowers of untransformed (*left*) and transgenic (*right*) plants; **l**, **m** fruit of untransformed plant; **n**, **o** fruit of a transgenic plant. GFP expression was visualized using GFP long-pass filter (**b**, **d**, **f**, **h**, **i**, **m**, **o**) or FITC/TRITC filter (**k**)

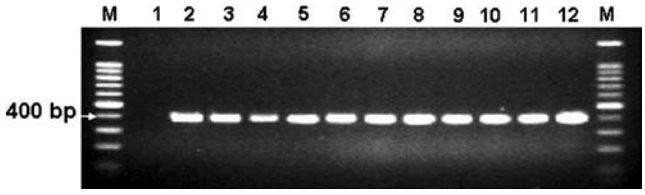

Fig. 5 PCR amplification of the *hpt* gene in transgenic 'Alpine' strawberry. *Lanes M* 100 bp DNA ladder (Promega). *Lane 1* untransformed control 'Alpine' plant. *Lanes 2–11* GFP^+ 'Alpine'

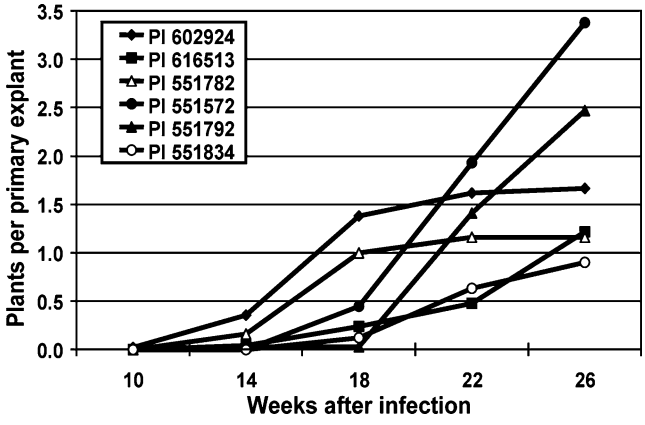

Fig. 6 Variation in the regeneration frequency of rooted transgenic plants per primary explant among six accessions of *F. vesca* 10–26 weeks after infection with *A. tumefaciens* GV3101 containing pCAMBIA-1304

calli had developed nodules 6 weeks after infection and initiated shoots 8 weeks after infection. Since many leaf segments had multiple GFP^+ calli with apparent independent cellular origin on the secondary explants after 8 weeks of culture, we cut the secondary explants into tertiary explants to separate well-isolated GFP^+ calli, thus increasing the chance of regenerating several independent transformed plants from a single primary explant. After 10 weeks of culture, regeneration of most of the accessions had occurred on the tertiary explants. The ratio of the number of shoots obtained on tertiary explants at week 12 to the original number of primary explants is shown in Table 2.

More than 4,000 independent calli expressing GFP occurred on the explants of PIs 551572 and 551792. Hundred percent of PI 551572 secondary explants had GFP^+ calli and many of the secondary explants had GFP^+ calli that were too numerous to count, while the frequency of secondary explants with GFP^+ calli of PI 551792 was 91%. While untransformed calli of PI 551792 died after the explants were transferred to selective medium, most calli of PI 551572 were friable 6 weeks after infection and untransformed cells were still surviving, causing us to extend the selection period for PI 551572 an additional 2 weeks. Hygromycin concentration was reduced to 2 mg l^{-1} during the extended selection period. Shoot regeneration of PI 551572 began 8 weeks after infection. Shoots of PI 551572 were large enough for root induction 3–4 weeks after they appeared. On the other hand, shoots of PI 551792 were small and embedded in the calli, which kept growing even after shoots developed from them. The shoots required about 6 weeks to grow large enough for root induction.

The developmental rate varied among accessions. Most GFP^+ calli of PIs 602924, 602931, and 616513 developed meristematic nodules 5 weeks after infection and shoots appeared 6–7 weeks after infection. The explants of PI 602924 produced callus only from the edges of veins and formed large chimaeric calli with respect to GFP expression. Consequently, a single large callus could regenerate both GFP^+ and GFP^- shoots. Shoots of PI 602924 were large enough for root induction 3–4 weeks after they first appeared. On the contrary, shoots of PIs 551834, 602578, 602931, and 616513 were small and needed about 6 weeks to grow enough for transfer to RIM. In the case of PIs 551890 and 551791, GFP^+ calli did not form any shoots until 14 weeks after infection. In addition, the shoots of these two accessions needed 8–10 weeks to grow sufficiently for transfer to RIM.

The final transformation efficiency of each accession based on GFP expression after 6 months of selection is shown in Table 2. Since the primary explants were cut twice during the 8-week incubation between *Agrobacterium* infection and shoot regeneration, we can express transformation efficiency based on the frequency of primary, secondary or tertiary explants that yielded GFP^+ shoots. In a typical experiment, a single primary explant (a leaf strip 1–1.5 mm wide by 5–15 mm long) would yield 4–6 secondary explants. Due to independent development of multiple GFP^+ calli on one secondary explant, each secondary explant could yield 0–4 tertiary explants. The cuts were executed to separate obviously independent calli expected to have derived from independent transformation events. The final transformation efficiency based on shoots obtained per primary explant was greater than 100% for six accessions. The frequency of tertiary explants that yielded GFP^+ shoots ranged from 3 to 88%. PI 551572 was the most easily transformed accession with a transformation rate of four rooted plants per primary explant (Fig. 6). Although the number of GFP^+ calli positively correlated with final transformation efficiency 5 weeks after infection, some accessions that appeared to have high transformation ability (measured as the frequency of GFP^+ calli) did not have high yield of transformed plants. For example PI 602931 produced more than ten GFP^+ calli per primary explant by week 5, but its final transformation efficiency was lower than that of PI 551782, which produced only two GFP^+ calli per primary explant. Accessions with high transformation efficiency tended to regenerate GFP^+ shoots early. These results indicated that high regenerability is required for high transfor-

Table 2 Transformation efficiencies of 14 *F. vesca* accessions

Accession ID	No. of primary explants	GFP^+ calli (%[a]) week 5	GFP^+ shoots (%[a]) week 12	Rooted GFP^+ shoots (%[a]) week 27
PI 551552	40	53	8	0
PI 551572	38	> 5,000	552	393
PI 551782	37	200	145	116
PI 551791	50	656	0	24
PI 551792	34	4,000	412	279
PI 551833	48	0	0	0
PI 551834	41	805	133	95
PI 551890	46	> 1,000	0	59
PI 551892	43	279	2	0
PI 602578	41	563	81	102
PI 602923	40	445	28	0
PI 602924	42	> 1,000	202	166
PI 602931	41	> 1,000	52	15
PI 616513	46	> 1,000	148	130

[a]The ratio of the number of GFP^+ calli or shoots to the initial number of primary explants

mation efficiency in *F. vesca*. For highly regenerable accessions, the duration from infection of explants to generating rooted plants was 10 weeks (PI 602924), 13 weeks (PI 616513), 14 weeks (PIs 551782 and 551792), and 16 weeks (PI 551572).

We did not obtain any GFP^+ shoots from PIs 551552, 551833, 551892, and 602923. For PIs 551552, 551892, and 602923, only a few GFP^+ shoots were obtained. However, the shoots did not grow vigorously enough to form roots. In the case of PI 551833, some small GFP spots were observed on explants 2 weeks after infection, but the explants died within 2 weeks of hygromycin selection without callus development.

We obtained many transformed cells in PI 551572 observed as GFP^+ callus. However, extensive callus formation led to the survival of many untransformed cells through the initial hygromycin selection. In order to kill untransformed and select transformed cells, we increased the hygromycin selection time. Moreover, shoot regeneration in this accession was relatively slow. This delay in shoot regeneration could have been caused by two extra weeks of hygromycin exposure. Another possibility was that the overgrowth of untransformed calli prevented the development of transformed calli. The results from our experiments with 'Alpine' were similar to those of Mathews et al. (1995) who reported that a high frequency of transformed shoots was observed when explants were initially exposed to 25 mg l^{-1} Kn compared to an initial exposure of 10 mg l^{-1} Kn in transformation of strawberry cultivars. We assumed that suppressing proliferation of untransformed cells could hasten shoot regeneration of PI 551572. Our preliminary results suggested that leaf explants transferred to selective medium containing 4 mg l^{-1} hygromycin immediately after washing-off started shoot regeneration soon after infection with the first shoots appearing within 5 weeks of screening. As a result, the minimal time from explant infection to rooted plants was shortened by 4 weeks. However, the transformation efficiency was decreased about 50% compared to screening with the pre-selection step (data not shown).

Molecular analysis of transgenic PI 551572 plants

T-DNA insertions were confirmed by PCR amplification and by Southern blot hybridization using randomly selected putative transgenic plants of PI 551572. PCR amplification of genomic DNA with *hpt* primers yielded the expected 402 bp product in all 16 GFP^+ plants but not in untransformed PI 551572 (data not shown). Southern blot hybridization analysis was performed on 6 of the 16 transformed and 1 untransformed PI 551572 plant. DNA was digested with *Hin*dIII, which has a single recognition site in the T-DNA, and hybridized to the mGFP5-specific probe. The distance between the *Hin*dIII site and the right border of the T-DNA was about 3.7 kb. In all GFP^+ plants tested, a single unique band ranging in size from 3.8 to 4.9 kb was detected (Fig. 7). This indicated that GFP^+ plants of PI 551572 were likely to be independent transformants carrying a single or low copy T-DNA insertions.

Screening T_1 plants for morphological mutants

Using the optimized transformation protocol, we produced over 500 transgenic *F. vesca* lines. To test whether we can find obvious morphological phenotypes associated with T-DNA insertions, we performed an initial morphological screen on T_1 progenies of 80 T-DNA insertional mutant lines. Four lines with altered leaf shapes were found about 2 months after germination (Fig. 8). At least one putative leaf mutant segregated phenotypically in a 3 normal:1 mutant ratio expected for a single insertion. We are currently performing further genetic and molecular characterizations of these putative mutants.

Discussion

Agrobacterium-mediated transformation has been used to generate transgenic strawberry plants with several

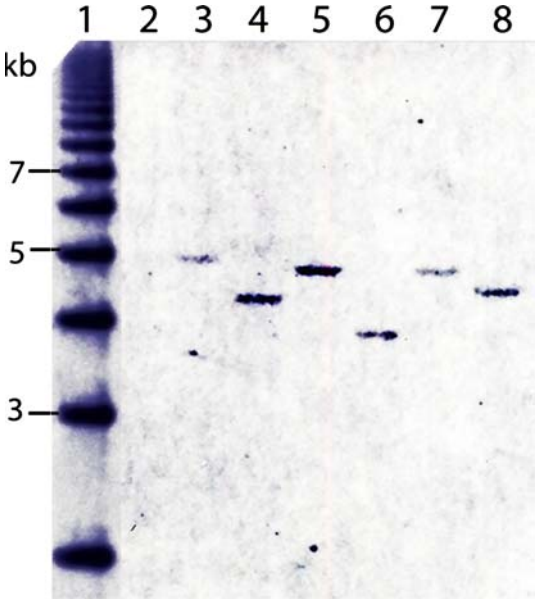

Fig. 7 Southern blot hybridization of *F. vesca* PI 551572 plants. 15 μg of genomic DNA was digested with *Hin*dIII and hybridized with an mGFP5-specific probe. *Lane 1* 1 kb ladder (1–15 kb, Takara, lowermost (1 kb) band has been cropped); *lane 2* control without DNA; *lanes 3–8* GFP^+ T_0 plants

different constructs and various germplasm (Jelenkovic et al. 1986; James et al. 1990; Nehra et al. 1990a, b; Mathews et al. 1995; El Mansouri et al. 1996; Barceló et al. 1998; Haymes and Davis 1998; Alsheikh et al. 2002; Ricardo et al. 2003; Zhao et al. 2004). However, the efficiency of transformation, i.e., the ratio of transformed plants obtained per explant cultured, though sufficient for generating transformants bearing a desirable gene for cultivar improvement, has been inadequate to generate a collection of insertional mutants, ranging from 5 to 7% (El Mansouri et al. 1996; Haymes and Davis 1998) to 68% (Zhao et al. 2004). To increase the efficiency of transformation, we imposed more stringent selection, used a more aggressive strain of *Agrobacterium*, and identified strawberry germplasm that could be easily transformed.

We conducted preliminary studies with thidiazuron (TDZ) instead of BA in the shoot regeneration medium in an attempt to hasten and increase regeneration and also used petiole explants as well as leaf explants. However, TDZ gave no better or earlier regeneration than BA and petiole explants tended to be less regenerative than leaf explants (data not shown). Therefore, in order to streamline the methods for high throughput, we opted for the less expensive cytokinin (BA) and the more regenerative explant. With time as a limiting factor rather than plant material, we needed to select the procedures that yield the highest regeneration rate. Using in vivo rather than in vitro mother plants reduced the requirement to economize on plant material since it was much simpler to propagate.

Risks associated with tissue culture-based transformation include escapes, chimaeric shoots, and somaclonal variation (Poethig 1989; Irish 1991; Mathews et al. 1998; Olhoft and Phillips 1999; Kaeppler et al. 2000b). The formation of chimeras during transformation has been reported in strawberry (Mathews et al. 1998) and other systems (Christou and Ford 1995; Firoozabady et al. 1995) and is considered to be one of the major problems for strawberry transformation. To minimize these risks, rapid shoot regeneration and stringent selection are required. Most of the existing *Agrobacterium*-mediated protocols for strawberry transformation are based on Kn selection (Jelenkovic et al. 1986; James et al. 1990; Nehra et al. 1990a, b; Mathews et al. 1995; El Mansouri et al. 1996; Barceló et al. 1998; Haymes and Davis 1998; Alsheikh et al. 2002; Ricardo et al. 2003;

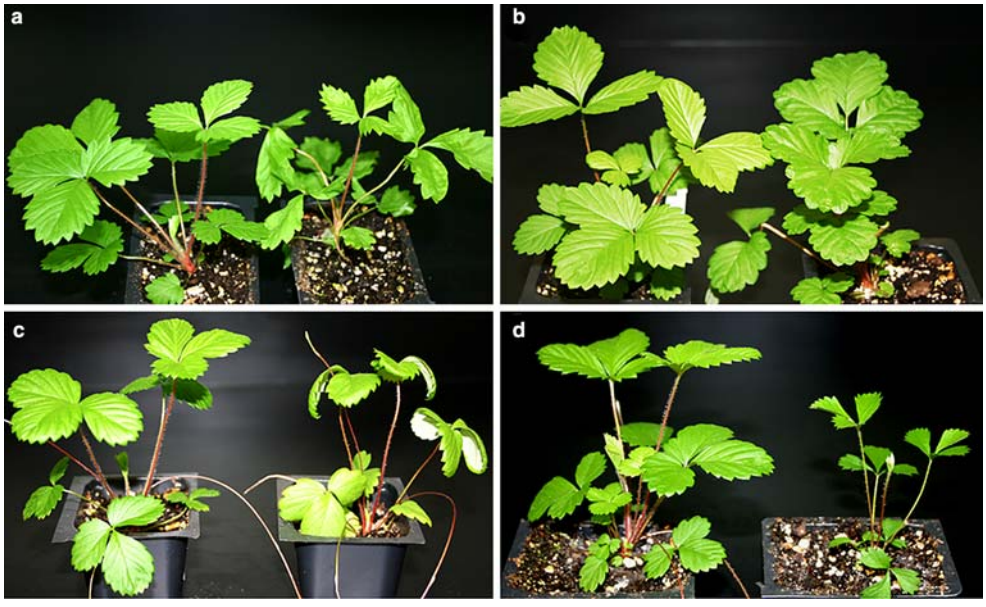

Fig. 8 Four examples of transgenic *F. vesca* plants (T_1 progeny) showing altered leaf morphology. *Each panel* shows a wild-type plant on the left and a plant of the same age and grown under the same conditions but with altered leaf phenotype on the right: **a** Narrow and crumpled leaf phenotype; **b** rounded lobe leaf phenotype; **c** curled leaf phenotype; and **d** fan-leaf phenotype

Zhao et al. 2004). Hygromycin was not previously used in *F. vesca* transformation and its use in *Fragaria* × *ananassa* has been reported by only one group (Mathews et al. 1995). We have identified the optimal concentration of hygromycin to suppress the growth of untransformed cells, yet allow efficient transgenic shoot formation. Due to the initial stringent selection with hygromycin, we were able to minimize chimaeras and escapes.

Shoot regeneration of transformed callus was inhibited by stringent selection with hygromycin. Leaf explants of 'Alpine' produced GFP^+ calli at a high frequency (67%) and proliferation of non-transformed cells was clearly suppressed by selection with 4 mg l^{-1} hygromycin. The final transformation efficiency of the explants infected with GV3101 and selected with 4 mg l^{-1} hygromycin was 42% based on rooted GFP^+ shoots (63% of GFP^+ calli). We assumed that exposure to 4 mg l^{-1} hygromycin for 9 weeks decreased overall transformation efficiency. Since only GFP^+ calli survived 5 weeks after infection, we transferred selected GFP^+ calli to non-selective medium to increase the frequency of shoot formation.

Antibiotic selection can be combined with visual selection using GFP to increase screening efficiency and improve the experimental throughput. GFP is an in vivo reporter that allows continuous monitoring of the transformation events throughout selection. GFP selection has been used as a partial or complete replacement of antibiotic selection in various plant species (Elliott et al. 1998, 1999; Ghorbel et al. 1999; Tian et al. 1999; Jordan 2000; Kaeppler et al. 2000a; Molinier et al. 2000; Halfhill et al. 2001; Stewart 2001; Zhang et al. 2001; Zhu et al. 2004). However, the use of GFP as a selectable marker has not been previously reported for strawberry. Our data indicate that GFP can be used as a visual selectable marker in *F. vesca*.

The highest rates of shoot regeneration for strawberry reported by Alsheikh et al. (2002) and Zhao et al. (2004) were achieved only by using in vitro plantlets as a source of explants. Alsheikh et al. (2002) stressed the importance of conducting transformation and regeneration of strawberry in 175 ml glass jars. The use of these culture vessels for high-throughput production of transgenic plants would require more growth chamber space than Petri dishes and generate a tedious glassware washing workload. In our experiments deep plastic Petri dishes did not inhibit the transformation efficiency and did not result in explant necrosis. Additionally, deep Petri dishes were more suitable for GFP screening of small secondary and tertiary explants plated at 50 per 10 cm Petri dish, facilitating the generation of a mutant library.

Genotypic differences in shoot regeneration ability have been reported among cultivars of *Fragaria* × *ananassa* (Nehra et al. 1989; Rugini and Orlando 1992; Passey et al. 2003; Zhao et al. 2004), subspecies of *F. vesca* (Sorvari et al. 1993; Alsheikh et al. 2002), and two accessions of *F. vesca* (Zhao et al. 2004). Highly variable differences in transformation efficiency were observed among accessions, although our protocol was effective on a range of germplasm. Of 14 accessions screened, only 4 did not produce rooted transformed plants, 7 exceeded the previously reported highest transformation efficiency for strawberries and 6 had 100% primary transformation efficiency. Of the four accessions that did not produce rooted transformants, only one did not regenerate on selective medium and did not produce GFP^+ calli or shoots. The other three regenerated in culture and produced transgenic calli and shoots, but the transformed shoots failed to root.

Our screening of diverse *F. vesca* germplasm coincidentally included accession PI 551572 that was previously used for *Agrobacterium*-mediated transformation by Haymes and Davis (1998) and Zhao et al. (2004). Of all accessions screened, PI 551572 (FRA 197) exhibited the best potential for callus formation, transformation, shoot regeneration, and shoot growth. Therefore, its final transformation efficiency was the greatest among all the accessions we tested. It has several characteristics that make it suitable for generating a collection of insertional mutant lines. The seeds of PI 551572 germinated shortly after harvest without special treatment and seedlings grew faster than other accessions, whereas many *F. vesca* accessions require extensive vernalization prior to germination. Additionally, PI 551572 is day neutral, has a short generation time, produces thousands of seeds per plant, and runners freely allowing for vegetative propagation.

Development of a highly efficient transformation protocol and identification of accession PI 551572 as the most amenable accession for transformation pave the way for generating a T-DNA mutant collection of strawberry that will in turn significantly impact functional genomic research and gene discovery in Rosaceae and other fruit crops.

Acknowledgements This project was funded by a Virginia Tech ASPIRES grant. We thank Dr. Kim Hummer, Dr. Nalha Bassil, and Bruce Bartlett at the NCGR for providing seed and in vitro cultured material of *F. vesca*. We thank Scott Rapier for help with plant maintenance in the greenhouse. We thank Dr. Jerzy Nowak, Dr. Ron Mittler, and Jim Walke for critically reading the manuscript.

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