Improved regeneration and *Agrobacterium*-mediated transformation of wild strawberry (*Fragaria vesca* L.)

Phillip A. Wadl

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Richard E. Veilleux, Chairman
Vladimir Shulaev
Joel L. Shuman
Jeremy Pattison

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Abstract

The Rosaceae contains many important commercially grown fruit crops. No comprehensive genomics platform is currently under development for fruit crops, giving functional genomics studies with wild strawberry (*Fragaria vesca* L.) the potential of identifying genes important in fruit crops. *Fragaria vesca* has a small genome size compared to the cultivated strawberry, *Fragaria ×ananassa* Duch. (164 vs. 600 Mbp per 1C nucleus). This feature, in addition to a short life cycle (12-16 weeks) and small plant size make *F. vesca* a good candidate for a model plant for genetic and molecular studies. The specific objective of this work was to develop an efficient high-throughput *Agrobacterium*-mediated transformation protocol to generate an insertional mutant population to support the justification of *F. vesca* as a model organism for rosaceous crops. The transformation techniques described by Alsheikh et al. (2002) and Oosumi et al. (2005) were modified and applied to a range of germplasm obtained from the USDA National Germplasm Repository. We found that the modifications made to the Alsheikh protocol were unsuccessful when applied to our germplasm. With the Oosumi et al. (2005) protocol, transformation efficiencies ranging from 11 to 100% were obtained for two accessions when explants were exposed to varying durations on TDZ containing medium during shoot regeneration. The transformation efficiency was given as the mean number of GFP^+ plants obtained per primary explant cultured. Multiplex PCR, for amplification of the *hpt*II and *GFP* genes, was

performed on a random sample of GFP^+ plants to verify insertion of the T-DNA. The statistical power of our experiment was insufficient to detect treatment effect but based on our findings the transformation efficiencies were high enough to justify PI 551572 for use in the high throughput transformations that are required to generate a population of insertional mutants large enough for gene discovery in *F. vesca.*

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Chapter 1

Literature Review

The strawberry belongs to the genus *Fragaria* closely related genera to *Duchesnea* and *Potentilla* within the Rosaceae family (Hancock, 1999; Marta et al., 2004). In 1966 there were eleven recognized species in the genus *Fragaria (Darrow, 1966)*, by 1990 there were at least 15 species recognized (Hancock, 1990) and in 1999 there were 19 species recognized (Hancock, 1999). Strawberry species are found throughout the world and the taxonomic classification of the genus is still evolving. A range of ploidy levels is observed within the genus, with naturally occurring diploid, tetraploid, hexaploid, and octoploid species, as well as interspecific hybrids with intermediate ploidy levels (Potter et al., 2000).

The cultivated strawberry, *Fragaria ×ananassa* Duch., is an important commercial fruit crop grown worldwide – 3,491,324 metric tons/yr (FAOSTAT, 2004). The United States is the leading strawberry producing nation (1,004,110 metric tons/yr) (FAOSTAT, 2004) and California is the world's leading strawberry producing region. Currently the narrow genetic base of strawberry breeding material places a limit on genetic improvement by traditional breeding methods (Galletta and Maas, 1990). Plant transformation in strawberry may provide a means of introducing new material into the existing gene pool or of revealing variation that is already present in the gene pool of strawberry species.

Fragaria ×ananassa is an octoploid (2n=8x=56) species and the high ploidy level makes genetic and molecular studies difficult. *Fragaria vesca* L. is a

diploid (2*n*=2*x*=14) species with a small genome size compared to the cultivated strawberry, *F.* ×*ananassa* (164 vs. 600 Mbp per 1C nucleus). This feature, in addition to a short life cycle and small plant size make *F. vesca* a good candidate for a model plant for genetic and molecular studies of fruit crops.

Plant transformation was defined by van den Eede et al. (2004) as the stable incorporation and expression of foreign genes. Genetic transformation of plants can be accomplished by two methods, either by direct gene transfer or *Agrobacterium*-mediated gene transfer. Direct gene transfer is commonly employed in monocot species that are not amenable to *Agrobacterium* transformation. Microinjection, electroporation, polyethylene glycol (PEG) induced DNA transfer, and microprojectile bombardment are all techniques used in direct gene transfer.

Agrobacterium tumefaciens is a naturally occurring, soil-borne bacterium that causes crown gall disease in a wide range of dicot species. The bacterium contains a Ti plasmid (tumor inducing) that interacts with the plant cells to provide a convenient mechanism for gene transfer into cells. During bacterial infection of plant tissue the T-DNA (transfer DNA), a mobile segment of Ti plasmid, is transferred to the plant cell nucleus and integrated into a plant chromosome. Several reviews are available that describe *Agrobacterium* biology in detail (Gelvin, 2003; Zupan et al., 2000; Zupan and Zambryski, 1997).

Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily

(Gelvin, 2003). As a genus, *Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species (Anderson and Moore, 1979; Decleene and Deley, 1976; Gelvin, 2003; Porter, 1991). The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984, has been extended to over 120 species in at least 35 families (Birch, 1997). Birch (1997) noted that successes include most major economic crops, vegetables, ornamental, medicinal, fruit, forestry, and pasture plants. For the purposes of plant genetic engineering, the most important aspect may be the host range of different *Agrobacterium* strains (Gelvin, 2003).

Strawberry was the first crop to be micropropagated, or regenerated *in vitro,* on a large scale and the application of molecular technologies in small fruit research has increased steadily over the past 10 years (Hokanson and Maas, 2001). *In vitro* regeneration and transformation systems for the cultivated, octoploid strawberry have been reviewed (Hancock, 1999; Hokanson and Maas, 2001; Jain and Pehu, 1992; Joshi et al., 2003). These reviews have for the most part not covered *in vitro* regeneration and transformation systems for diploid *Fragaria* species. Several groups have pursued *in vitro* studies with *F. vesca*, as a model system to optimize transformation protocols (Alsheikh et al., 2002; El Mansouri et al., 1996; Greene, 1988; Haymes and Davis, 1998; Zhao et al., 2004).

Greene (1988) observed nearly 100% shoot formation on callus of *F. vesca* 'Baron Solemacher' leaf explants when cultured on MS basal medium

supplemented with 10 µM IAA and 10 µM BA. When 'Baron Solemacher' leaf explants were infected with *Agrobacterium tumefaciens* only transformed callus was recovered. El Mansouri et al. (1996) demonstrated shoot regeneration of 98% for *F. vesca* leaf explants when cultured on MS basal medium supplemented with 1.43 µM IAA and 17.6 µM BA after 8 weeks of culture. This protocol was used to transform leaf disks with *Agrobacterium tumefaciens* and the transformation percentage (7%) was based on β-glucuronidase (GUS) expression. Haymes and Davis (1998) were the first to show the suitability of *F. vesca* 'Alpine' for transgenic research by demonstrating the transmission of the *gus* and *npt*II genes to the R_1 progeny. No transformation percentage was reported by Haymes and Davis (1998). Alsheikh et al. (2002) obtained optimal transformation efficiency (15%) when leaf disks of *F. vesca* were transformed and regenerated on MS basal medium supplemented with 1.2 µM IBA and 13.3 µM BA. Zhao et al. (2004) reported transformation efficiencies ranging from 47% to 76% for two *F. vesca* accessions when leaf and petiole explants were transformed and regenerated on MS basal salts and B5 vitamins supplemented with 1.5 µM IBA and 10 µM thidiazuron (TDZ). Oosumi et al. (2005) reported transformation efficiency of 100% when leaf explants of *F. vesca* were transformed and regenerated on MS basal medium supplemented with 13.3 µM BA and 1.2 µM IBA. These results are consistent with the reports of shoot regeneration and transformation efficiency of the cultivated strawberry. Hokanson and Maas (2001) noted that most researchers have found that *Fragaria*

genotypes exhibit variable levels of regeneration efficiencies from different plant parts, as well as genotype specific responsiveness to transformation.

Our eventual goal is to develop a comprehensive functional genomics platform for fruit crops using *F. vesca* as a model plant species in order to discover novel gene functions for economically important traits in major fruit crops. *Agrobacterium*-mediated plant transformation will be used to generate the insertional mutants needed for gene discovery. The specific objective of this work was to develop an efficient high-throughput *Agrobacterium*-mediated transformation protocol to support the long-range goal of gene discovery.

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Chapter 2

Introduction

The characteristics of a model plant species are small size (both physical and genomic), short generation time, good classical genetics, amenable to transformation, insertional mutant availability, a strong research community, expressed sequence tag (EST) libraries and microarrays available, and a sequenced genome. *Arabidopsis thaliana* has all of the above.

Most of the gene discovery tools of plant functional genomics have been developed in studies of the model plant, *A. thaliana*. *Arabidopsis*, being a cruciferous plant, has a dry fruit (silique) that is not representative of many species of horticultural importance where fleshy fruit comprise the edible product. Although genomic studies are currently under development for fruit crops, a population of insertional mutants has not been developed for strawberry or any other rosaceous crop, giving functional genomics studies with *Fragaria vesca* L. (wild strawberry) the potential of identifying genes important in fruit crops.

The family Rosaceae contains the major temperate fruit crops such as apple, peach, cherry, plum, apricot, almond, pear, brambles and strawberry. Dirlewanger et al. (2004) note that most of these species are woody perennials with a long intergeneration period due to their juvenile phase and large plant size, making them poor candidates for a model species. The diploid species *F. vesca* possesses several features that make it a potential model not only for strawberry but also for crops in the family Rosaceae (Sargent et al., 2004). *Fragaria vesca* has a small genome size compared to the cultivated strawberry, *F. ×ananassa*

(164 vs. 600 Mbp per 1C nucleus). In addition to a short life cycle (12-16 weeks), small plant size (both physical and genomic) and a fleshy fruit make *F. vesca* a good candidate for a model plant for genetic and molecular studies.

The main obstacle to genetic transformation of fruit tree species is the regeneration of transformed plants (Perez-Clemente et al., 2004). Several groups have demonstrated that *F. vesca* is amenable to transformation and regeneration (Alsheikh et al., 2002; El-Mansouri et al., 1996; Haymes and Davis, 1998; Zhao et al., 2004). There are two diploid strawberry linkage maps available (Davis and Yu, 1997; Sargent et al., 2004); these linkage maps will aid map-based cloning of economically important genes in strawberry. Also, clonal propagation of strawberry provides an added advantage of allowing clonal replicates to be used in field or greenhouse trials (Sargent et al., 2004). In *Arabidopsis* many genes have been isolated from T-DNA tagged lines demonstrating the importance of developing large populations of insertional mutants (Dekker, 2005).

El-Mansouri et al. (1996) reported the *Agrobacterium*-mediated transformation of *F. vesca* leaf explants with the *npt*II and *uid A* genes in the vector pBI121. Explants were selected on medium containing 25 $mg \cdot L^{-1}$ kanamycin and 500 $mg \cdot L^{-1}$ carbenicillin. After 4 weeks of culture the carbenicillin concentration was cut in half. The final efficiency of the transformation experiment determined by kanamycin resistant plants was 8% and 7% when the *β*-glucuronidase (GUS) expression assay was used to determine transformation efficiency. The presence of the *npt*II gene was documented by polymerase chain reaction (PCR).

Haymes and Davis (1998) reported the stable *Agrobacterium*-mediated transformation of the *gus* and *npt*II genes into *F. vesca* 'Alpine' plants with the vector pBI121. This transformation protocol was a modification of the protocol of Nehra et al. (1990b). First generation transformants were vegetatively propagated from kanamycin resistant calli. Amplified product via PCR indicated the presence of the *gus* and *npt*II genes in five clones. Southern blot analysis suggested two sites of insertion for the *npt*II gene. When the primary transformants were selfed, the progeny segregated in a 15:1 transgenic to non-transgenic ratio (0.5>P>0.25); consistent with the independent segregation of two hemizygous transgene insertions. These results demonstrate that *Agrobacterium*-mediated transformation can insert stable and heritable genes into *F. vesca*.

Alsheikh et al. (2002) reported *Agrobacterium*-mediated transformation of *F. vesca* and *F. v. semperflorens* leaf disks and petiole sections with the *gus* and *npt*II genes in the binary vector pCIRCE-GUS. Both *F. vesca* forms showed sensitivity to kanamycin and due to this a low kanamycin concentration (10 $mg \cdot L^{-1}$) was used initially and later raised from to 25 $mg \cdot L^{-1}$ over a period of 8 weeks. In addition, the carbenicillin concentration was 500 $mg \cdot L^{-1}$ for the first 4 weeks of culture and was cut in half for the next 4 weeks of culture. A transformation efficiency of 15% was obtained by selecting the correct explant type and age, explant orientation, *Agrobacterium* density and inoculation time, and phenolic compounds for bacterial virulence induction. The integration of the *npt*II gene

was verified by PCR and integration of the *gus* gene was confirmed by Southern analysis.

Zhao et al. (2004) demonstrated phloem-specific expression of the binary vector pBISPG using leaf explants of *F.×ananassa* 'Hecker' and 'La Sans Rivale' and leaf and petiole explants of *F. vesca* 'Alpine' accessions FRA197 and FRA198. Explants were subjected to a 3-day pre-selection treatment and after the pre-selection treatment the explants were cultured on regeneration medium containing 50 $mg \cdot L^{-1}$ kanamycin. The explants were subcultured to fresh medium every 2 weeks for a total of 8 weeks and regenerated shoots were transferred to multiplication medium with antibiotics for continued selection. Transformation rate was calculated by the percent of the explants that regenerated shoots on medium containing kanamycin. Transformation rates ranged from 7.4% to 76.3% depending on the genotype and explant type used. All putative transgenic plants tested positive for the presence of the *gus* gene when PCR was performed. Histochemical GUS staining demonstrated phloem-specific expression. The results demonstrate that the binary vector pBISPG may be useful in developing strawberry resistant to phytoplasmas.

Oosumi et al. (2005) reported *Agrobacterium*-mediated transformation of *F. vesca* leaf explants with the *gfp* and *hpt*II genes in the binary vector pCAMBIA-1304. Explants were selected on medium containing 4 $mg \cdot L^{-1}$ hygromycin. The final transformation efficiency was based on the frequency of explants that yielded GFP^{+} shoots. A transformation efficiency of 100% was achieved for 6 of 14 accessions tested.

The development of an efficient and reliable transformation procedure is the first step in creating a population of *F. vesca* insertional mutants. Ricardo et al. (2003) note that transformation success is highly dependent on the particular strawberry genotype used and a protocol developed for one genotype is not necessarily applicable to other genotypes. The reports on *Agrobacterium*-mediated transformation of *F. vesca* all used different genotypes and different transformation protocols, so it was critical for the success of this project to first select the genotype that is most responsive to transformation and regeneration.

A transformation system needs to approach the ease and efficiency of the *in planta* system used for *Arabidopsis* prior to developing a population of insertional mutants of *F. vesca* as a genomics tool. Feldmann and Marks (1987) developed a protocol for transforming *Arabidopsis* with *Agrobacterium* that avoided the tissue culture step that is common for generating primary transformants of most plant species. Using the *in planta* technique, Alonso et al. (2003) generated approximately 150,000 transformed *Arabidopsis* plants that were estimated to contain 225,000 independent T-DNA insertions. This population of insertional mutants was created in 4 months from 400 individual plants and the probability of mutating any single gene in the population was 96.6% based on a genome size of 125 Mbp, average gene length of 2 kb, and a random T-DNA insertion events. We estimate that a population of 255,000 independent T-DNA transformed lines is needed to mutate any single gene with 95% probability in *F. vesca*.

One of the requirements of a model species is a short generation time (seed to seed). *Fragaria vesca* has a generation time of 12-16 weeks, which is only 4-8 weeks longer than *Arabidopsis.* The time of emergence of strawberry seedlings may vary in the same lots from 10 to 140 days from date of planting (Scott and Ink, 1948). Scott and Ink (1948) use sulfuric acid scarification in the germination of seed from two crosses and after 16 days obtain an average of 60% germination from the two crosses. Scott and Ink (1955) demonstrated that soaking seeds in a chlorine solution for less than 8 hours could increase seed germination of strawberry. Miller et al. (1992) developed a protocol that involved the cutting of surface sterilized achenes across the embryo axis and then placing the shoot apex/radicle-containing sections on semisolid MS medium lacking growth regulators. Achenes began germination in as little as 5 days after culture and achieved maxium germination (97% to 100%) in less than 2 weeks.

To begin this project, seed germination experiments were conducted on a range of germplasm obtained from the USDA National Germplasm Repository, Corvallis, Ore., to determine if stratification is required. The next experiments were to apply the techniques described by Alsheikh et al. (2002) to a range of germplasm obtained from the USDA National Germplasm Repository. Shoot regeneration experiments were conducted to compare the regeneration potential of *F. vesca* 'Alpine' using a medium described by Passey et al. (2003) and the medium that gave Alsheikh et al. (2002) the greatest regeneration frequency after the initial experiments were conducted. Preliminary results from the shoot regeneration experiments of 'Alpine' were then applied to a modified

transformation protocol developed by Oosumi et al. (2005). The specific objective of this work was to develop an efficient high-throughput *Agrobacterium*-mediated transformation protocol to generate an insertional mutant population of *F. vesca* to support the justification of *F. vesca* as a model organism for rosaceous crops.

Materials and Methods

Plant material and seed germination

Seeds of *Fragaria vesca* L. were obtained from the USDA National Clonal Germplasm Repository, Corvallis, Ore. and a commercial seed company (Table 1), and sterilized by a wash in water (1 h), immersion in 70% ethanol (20 s), stirring in 1% sodium hypochlorite containing 1 $g \cdot L^{-1}$ Tween-20 (15 min) and rinsing in sterile water (four times). The seeds were then planted directly on MS basal medium (Murashige and Skoog, 1962), ten seeds per 175 mL glass jar or 10 seeds per petri plate for germination containing 20 mL of MS basal medium. Seedlings germinated in jars were transferred individually to 175 mL glass jars containing 40 mL of MS basal medium and grown in an incubator under a 16 h photoperiod at 22ºC. Seedlings germinated in petri plates were then transferred to sterile Fafard 3B medium and grown in a growth chamber under an 11 h photoperiod at 22ºC (day)/16ºC (night). These seedlings were used as the explant source for transformation experiments.

Seed was harvested from greenhouse grown plants of five accessions obtained from the USDA National Germplasm Repository and soaked in 1% sodium hypochlorite solution (Scott and Ink, 1955) or 1% calcium hypochlorite solution for 4 h. Four replicates of 25 seed were placed onto moist germination

paper in sterile glass petri plates (PYREX® USA No. 3250), sealed with Parafilm and kept at room temperature. Seed germination was counted 7, 11, 14, 17, 21, 27, 30, and 34 days after planting.

Transformation procedures as outlined by Alsheikh et al. (2002)

The binary vector pCAMBIA-1304 (GenBank accession: AF234300, Center for the Application of Molecular Biology to International Agriculture, Black Mountain, Australia), containing *mgfp5-gus*A-His6 fusion, was introduced into *A. tumefaciens* strain LBA4404 carrying the ternary plasmid pBBR1MCS-5.virGN54D containing constitutive *virG* mutant gene (van der Fits et al., 2000) by Dr. Teruko Oosumi. Cryopreserved scrapings of *Agrobacterium tumefaciens* strain LBA4404 were inoculated into 10 mL of liquid LB medium supplemented with 50 $mg \cdot L^{-1}$ kanamycin and grown overnight at 28°C, with shaking at 200 rpm. Cultures were centrifuged for 20 min at 4,000×*g*. The bacterial pellets were then resuspended and diluted to OD_{595}=0.1 in liquid MS20 medium [MS basal medium containing 2% (w/v) sucrose, pH 5.2].

Leaf discs (4 mm diameter) and petiole sections (5 mm long) were excised from 8-week-old *in vitro* cultures. Forty explants of each type were cultured in 9 cm petri dishes containing 30 mL of shoot regeneration medium (MS basal medium containing 2.5 $g \cdot L^{-1}$ Gellan Gum, 2.22 µM BA and 1.22 µM IBA). The plates were sealed with 3M Micropore tape and incubated for 2 days under standard growth room conditions. Explants were transfected in 50 mL beakers containing 15 mL *Agrobacterium* suspension and shaken at 30 rpm for 20 min at 25°C. The explants were then blotted dry on sterile filter paper and placed into

175 mL glass jars containing 40 mL shoot regeneration medium at a density of 10 leaf discs or 20 petiole sections per jar and incubated under low light at 24°C for 3 days. The explants were then washed twice (1 h per wash) in a carbenicillin solution (500 $mg \cdot L^{-1}$, pH 5.2), and returned to 175 mL jars containing shoot regeneration medium supplemented with 500 $mg \cdot L^{-1}$ carbenicillin and incubated under low light at 24°C. Five days later, the explants were transferred to 175 mL jars containing shoot regeneration medium supplemented with 10 $mg \cdot L^{-1}$ kanamycin and 500 $mg \cdot L^{-1}$ carbenicillin and cultured under the standard growth room conditions. Kanamycin selection was increased by 5 $mg \cdot L^{-1}$ at 2-week intervals (up to 25 $mg \cdot L^{-1}$), while carbenicillin was lowered to 250 $mg \cdot L^{-1}$ after 4 weeks. Shoots that regenerated after 8 weeks were rooted in MS basal medium supplemented with 15 $mg \cdot L^{-1}$ kanamycin. The modifications of this protocol are outlined in Figure 1.

Shoot regeneration to improve regeneration frequency

It was discovered in an experiment to examine the transient expression of *Agrobacterium* construct LBA 4404 pHB2892 containing the green fluorescent protein (GFP) that the time needed for shoot regeneration could be decreased from an average of 8-10 weeks in MS basal media supplemented with 1.22 µM IBA and 13.3 µM BA (Alsheikh et al., 2002) to an average of 4-6 weeks with media supplemented with 4.54 µM thidiazuron (TDZ) and 0.9 µM 2,4-D as outlined by Passey et al. (2003).

Leaf (4 mm diameter) and petiole (5 mm long) sections were excised from 8+ week-old in vitro seedlings. Leaf discs were placed abaxial side up

and petiole sections were placed horizontally at a density of ten leaf discs and 20 petiole sections per 175 mL glass jar containing 20 mL MS basal medium supplemented with the plant growth regulators IBA/BA or TDZ/2,4-D, and solidified with 2.5 $g \cdot L^{-1}$ Gellan Gum (Caisson Laboratories, Inc.). Each treatment included 100 leaf discs and 100 petiole sections that were subcultured to fresh medium at 2-week intervals. The number of explants regenerating shoots was counted after 4, 6, and 8 weeks. Single regenerated shoots were excised from callus tissue and cultured on MS basal medium solidified with 2.5 $g \cdot L^{-1}$ Gellan Gum for rooting.

Transformation procedures as outlined by Oosumi et al. (2005)

The binary vector pCAMBIA-1304 (GenBank accession: AF234300, Center for the Application of Molecular Biology to International Agriculture, Black Mountain, Australia), containing *mgfp5-gus*A-His6 fusion, was introduced into *A. tumefaciens* strain GV3101 (pMP90) (Koncz and Schell, 1986) carrying the ternary plasmid pBBR1MCS-5.virGN54D containing constitutive *virG* mutant gene (van der Fits et al., 2000) and this vector was kindly provided by Dr. Teruko Oosumi. Cryopreserved scrapings of *Agrobacterium tumefaciens* strain GV 3101 (pMP90) pCAMBIA-1304 were inoculated into 2 mL of liquid LB medium supplemented with 20 $mg \cdot L^{-1}$ rifampicin, 25 $mg \cdot L^{-1}$ gentamycin, and 50 $mg \cdot L^{-1}$ kanamycin and grown for 22 h at 30ºC, with shaking at 240 rpm. Cultures were centrifuged for 3 min at 13,000 rpm. The bacterial pellets were then resuspended in 15 mL liquid MS basal medium containing B5 vitamins and 2% sucrose, pH 5.5.

Surface sterilized leaf sections or primary explants (1 mm wide) were excised from 6-7-week-old growth chamber grown plants and infected in the *Agrobacterium* suspension for 20 min at 25ºC, shaking every 5 min. The primary explants were then blotted dry on sterile filter paper and placed in 100x25 mm petri plate on 25 mL of co-cultivation medium (Table 2) at a density of 40-50 primary explants per plate and incubated in the dark for 2 days at 25ºC. The primary explants were washed in carbenicillin solution (500 $mg \cdot L^{-1}$, pH 5.5) for approx 30 min, shaking every 5 min and then the explants were returned to 100x25 mm petri plates containing 40 mL shoot induction medium (Table 2) and incubated under low light at 23ºC for 7 days. After 7 days of culture on SIM-I the primary explants were cut into secondary explants by cutting between the veins of the primary explants with a #10 surgical blade. Secondary explants were then placed in 100x25 mm petri plates onto 40 mL of SIM-II (Table 2) at a density of 50 secondary explants per plate and incubated under low light for 2 weeks at 23ºC. At 23 days secondary explants were cut into tertiary explants by dividing individual pieces of callus tissue and then placing tertiary explants on fresh SIM-II and incubating under low light for 2 weeks at 23ºC. Callus tissue was screened for GFP activity after 5 weeks of culture and GFP+ tertiary explants were selected and transferred to SIM-III (Table 2) and incubated under low light for 2 weeks at 23ºC. The GFP^{+} tertiary explants were transferred to fresh SIM-III every 2 weeks and incubated in the normal light of the incubator until GFP+ shoots grew large enough (2-5 mm) for excision. Once shoots were large enough, single shoots were cut from tertiary explants and placed onto 40 mL root induction

medium (Table 2) in 100x25 mm petri plates and incubated at 23ºC in the normal light of the incubator. Well-rooted independently transformed plants (~ 5 cm) were selected and transferred to 175 mL glass jars containing 40 mL of MS basal medium and incubated for about 2 weeks. After 2 weeks plants were transferred to sterile Fafard 3B medium and grown under shade in the greenhouse for 7-10 days and then moved into the full light of the greenhouse.

Six transformation experiments were conducted to shorten the time of shoot regeneration using two different shoot regeneration media in eight treatments. The growth regulators BA and IBA were used in one medium and TDZ and 2,4-D were used in the other medium (Table 3). Each experiment consisted of eight plates of 50 secondary leaf explants, two of which were cultured for the entire 8 weeks on either BA/IBA medium or TDZ/2,4-D medium (Table 3). The remaining six plates received different durations of exposure to TDZ over the 8 week incubation (Table 3).

GFP screening during regeneration

Callus tissue of all explants was visually screened for GFP expression after 5 weeks of culture. An Olympus fluorescent microscope SZX-RFL3 fitted with 100 W mercury burner with FITC/TRITC dual filter set was used to visualize GFP fluorescence.

Molecular analysis of putative transgenic shoots

Genomic DNA was isolated from leaves of GFP^+ and untransformed plants using a modified CTAB method (Doyle and Doyle, 1987) and a modified rapid

CTAB method (Doyle and Doyle, 1987; Lodhi et al., 1994; Porebski et al., 1997). Genomic DNA (100 ng) and primers pairs (20 pmol/primer) for the *npt*II gene (forward 5´-TATGACTGGGCACAACAG´; reverse 5´-GTATCCATCATGCGTGATGC´) were added to puRETaq Ready-To-Go PCR Beads (GE Healthsciences) and a RoboCycler® Gradient 40 Temperature Cycler with the Hot Top Assembly (Stratagene) was used for PCR amplifications using the following cycle: 94ºC, 30 s; 54ºC, 30 s and 72ºC for 30 cycles. A 12.5 µL aliquot of each PCR reaction was analyzed by 1% agarose gel electrophoresis using 1X TBE buffer. The expected product size of amplification for the *npt*II gene was 293 bp.

Genomic DNA (100 ng) and primers pairs (20 pmol/primer) for the *hpt*II gene (forward 5´-GAATCGGTCAATACACTACA´; reverse 5´-TCTGATAGATTGGTCAAGA´) and the *gfp* gene (forward 5´-GAATACAACTACAACTCCCA´; reverse 5´-GTAATGGTTGTCTGGTAAAA´) were added to puRETaq Ready-To-Go PCR Beads (GE Healthsciences) and a RoboCycler® Gradient 40 Temperature Cycler with the Hot Top Assembly was used for PCR amplifications using the following cycle: 94ºC, 1 m; 54ºC, 1 m and 72ºC, 1 m for 35 cycles. As a positive control, 20 ng of pCAMBIA-1304 plasmid DNA was used. A 20 µL aliquot of each PCR reaction was analyzed by 2% agarose gel electrophoresis using 1x TAE buffer. The expected product size of amplification was 411 bp for the *hpt*II gene and 177 bp for the *gfp* gene.

GUS staining

Histochemical GUS staining of leaves taken from greenhouse-grown putative transgenic strawberry plants was performed as described by Jefferson et al. (1987) using X-Gluc and plant material was observed under a microscope.

Flow cytometry

The ploidy level of regenerated shoots was determined by flow cytometry. Analysis of samples was carried out on a Coulter Epics XL Flow Cytometer. Three flow cytometry protocols were modified in an attempt to achieve consistent results. The first protocol to be tested and modified was described by Owen et al. (1988) for potato. This protocol was modified by the addition of 5% PVP-40 to the chopping buffer. Next, the protocol developed by Meng and Finn (2002) for *Rubus* was tested and modified by using leaf material grown in the dark for 1 week. The third protocol to be tested and modified was developed Brandizzi et al. (2001) for strawberry and the only change to this protocol was using leaf material that was grown in the dark for 1 week.

Data analysis

All data were analyzed using SAS GLM Version 9. Means were separated using Ryan-Einot-Gabriel-Welsch Multiple Range Test. Growth regulator effects on shoot regeneration and effects of seed pre-treatment on seed germination were tested for significance at the $P<0.05$ level.

Results

Seed germination

Ten accessions of *F. vesca* were obtained from the USDA National Germplasm Repository, Corvallis, Ore. and used in seed germination studies, planted directly on MS basal medium, ten seeds per petri plate or stratified on moist germination paper for 10 weeks at 4ºC prior to plating on MS basal medium (Table 4). Germination ranged from 0% to 80% when seeds were directly planted on MS basal medium and from 0% to 100% when seeds were stratified for 10 wks at 4ºC. Four accessions (PIs 551573, 551783, 551833 and 602923) responded favorably to stratification with a mean of 40% germination without and 92% with stratification. Open-pollinated 'Alpine' (PI 616581) did not germinate at all and only a single seedling of PI 616862 was obtained. Stratification had little or no effect on three other accessions (PIs 551572, 548865 and 616674). Plants obtained from these experiments were used as explants for *Agrobacterium*-mediated transformation following the procedures outlined by Alsheikh et al. (2002).

A 1% sodium hypochlorite soaking method developed by Scott and Ink (1955) or a 1% calcium hypochlorite solution were applied to five different accessions of *F. vesca* germplasm. Seed from each accession was subjected to a 4-h soak in each treatment. ANOVA was conducted on seed germination data separately for each of the 8 days (Table 5). Statistical analysis of seed germination data revealed significant variation for accession, treatment and the interaction of accession x treatment. The treatment effect was only significant at day 7. The interaction of accession x treatment was significant through day 14 but not afterwards. Accession variation was significant for all days but by day 27

there were no significant differences among four of five accessions, with PI 551573 exhibiting significantly lower germination than the others.

Germination ranged from 20% to 95% at 14 days across all accessions for both treatments applied (Figure 2). PI 551573 reached only 30% germination in either treatment just as it had done in the preliminary trial without stratification. The other four accessions all germinated at greater than 75% after 34 days with either sodium or calcium hypochlorite treatment. However PI 551572 was the only accession to germinate at greater than 90% within 14 days. The choice of sodium or calcium hypochlorite made little difference for most accessions except PI 602923, which germinated more quickly with the sodium hypochlorite treatment and for PI 602578 the final germination percentage was higher with the sodium hypochlorite treatment.

Transformation procedures as outlined by Alsheikh et al. (2002)

Leaf discs (n=491) and petiole sections (n=273) were inoculated in ten separate transformation experiments following the transformation protocol developed by Alsheikh et al. (2002). For all experiments there was GFP^+ callus present after 5 weeks on leaf and petiole explants. Shoot regeneration on selection medium was slow (12+ weeks) and when genomic DNA was isolated from 15 shoots and PCR was performed there was no amplification of the *npt*II gene.

Shoot regeneration

We found that the time needed for shoot regeneration could be decreased from an average of 8 to 10 weeks in media supplemented with 1.22 µM IBA and 13.3 µM BA to an average of 4 to 6 weeks with media supplemented with 4.54 µM TDZ and 0.9 µM 2,4-D as outlined by Passey et al. (2003). For *F. vesca* 'Alpine,' regeneration frequency at 6 weeks for leaf or petiole explants on medium supplemented with IBA/BA was 8% (n=180) compared to 27% (n=210) on medium supplemented with TDZ/2,4-D (Figures 3, 4 and 5). Regardless of the medium composition or explant source, all cultures had regenerated by 8 weeks after initiation.

Modifications of Oosumi et al. (2005) protocol

A promising strawberry transformation protocol developed by Oosumi et al. (2005) became available during the course of this research project. The following experiment was initiated to try to superimpose the benefits of earlier regeneration on efficient transformation of two accessions of *F.* vesca. Treatments consisted of varying durations on TDZ containing medium during the 8-week culture (Table 3). Both accessions initiated callus prior to subculture onto SIM-I. For PI 551572 callus initiation was 2 days faster than PI 551792. The callus first formed on the cut and wounded edges of the explants and gradually covered the rest of the explant. Callus present on PI 551792 was green and compact compared to PI 551572 where the callus was whitish green and friable. The differences in callus made subculturing more difficult for PI 551572.

Callus of PI 551792 (n=356) and PI 551572 (n=1432) were screened for GFP expression. ANOVA was conducted on the number of GFP^+ shoots and GFP^+ plants obtained per primary explant for PIs 551792 and 551572. For PI 551792 statistical analysis revealed no significant variation between the two experiments conducted for both observations. Significant variation was detected at 95% probability among treatments for number of GFP^+ shoots obtained per primary explant but not for number of GFP^+ plants obtained per primary explant (Table 6). Statistical analysis of PI 551572 using a nested design revealed significant variation among the four experiments for the number of GFP^+ shoots obtained per primary explant but no significance was detected for cocultivation effect nested within the four experiments, treatment and the interaction of experiment and treatment (Table 7). The small sampling size of PI 551792 did not allow statistical analysis using a nested design.

The treatment effects on GFP expression are shown in Tables 8 and 9. For PI 551792 two treatments (2B6Z and 8B) resulted in the highest percentage of explants expressing GFP^+ callus, while four other treatments (8Z, 5Z3B, 3Z5B and 2B1Z5B) GFP^+ callus expression was less than 55%. Expression of GFP^+ callus for PI 551572 ranged from 40-77%, three treatments were less than 50% (3Z5B, 2B6Z and 2B3Z3B), while the other five treatments (8Z, 5Z3B, 2Z6B, 2B1Z5B and 8B) GFP expression was greater than 50% and GFP expression was 77% with treatment 5Z3B. Expression of GFP^+ callus was of equal intensity for both accessions. After GFP screening, secondary explants that were GFP^+

callus were cut into tertiary explants and the GFP^- explants were discarded (Figure 6).

Shoot regeneration for both accessions did not occur in any of the treatments prior to 5 weeks. Shoot regeneration for PI 551792 occurred on treatments 8Z and 8B by 7 weeks and by 9 weeks for the remaining treatments. Once shoots regenerated, elongation was slow (4 to 6 weeks) and the shoots were small and implanted within the callus making the excision of single shoots difficult. The mean numbers of GFP^+ shoots and GFP^+ plants regenerated for each treatment of PI 551792 are shown in Table 8. Only treatments 8Z and 8B produced a mean of more than one GFP^+ shoot per primary explant. Treatments 5Z3B and 2B1Z5B resulted in the lowest mean number of GFP^+ shoots obtained, while treatments 3Z5B and 2B6Z produced the same mean number of GFP^+ shoots. The mean number of GFP^+ plants obtained per primary explant was less than the mean number of GFP^+ shoots obtained per primary explant for all treatments. Although ANOVA indicated significant variation among treatments for the number of GFP^+ shoots obtained per primary explant, REGWQ failed to separate the means. Duncan's multiple range test revealed that treatments 8B and 8Z were best, followed by 3Z5B and 2Z6B and treatments 2B1Z5B and 5Z3B were the worst when the number of GFP^+ shoots obtained per primary explant were observed. Only treatment 8B resulted in a mean of greater than one GFP^+ plant per primary explant, while for three treatments (8Z, 3Z5B and 2B6Z) the mean number of GFP^+ plants obtained per primary explant ranged from 0.54 to 0.69. The two treatments (5Z3B and 2B1Z5B) that exhibited the

lowest mean number of GFP^+ shoots obtained per primary explant also produced the lowest mean number of GFP^+ plants obtained per primary explant. Although no significance was detected among treatments, the mean number of GFP^+ plants obtained per primary explant for treatment 8B was nine times greater than the poorest treatment (1.00 and 0.11 GFP^+ plants per primary explant for treatments 8B and 2B1Z5B, respectively).

Shoot regeneration for PI 551572 occurred by 6 weeks for treatments 5Z3B and 8B and by 8 weeks for the remaining treatments. Shoots of PI 551572 required only 2 to 4 weeks to grow large enough to divide into single shoots for root induction. The mean number of GFP^+ shoots and GFP^+ plants regenerated for each treatment of PI 551572 are shown in Table 9. The mean number of GFP^+ shoots obtained per primary explant was greater than one for six of eight treatments. Only treatment 5Z3B resulted in a mean number of GFP^+ plants obtained per primary explant greater than one. For the remaining treatments the mean number of GFP^+ plants obtained per primary explant ranged from 0.18 to 0.69. Although no significance was detected among treatments, the mean number of GFP^+ plants obtained per primary explant for treatment 5Z3B was six times greater than that for the poorest treatment (1.19 and 0.18 GFP^+ plants per primary explant for treatments 5Z3B and 2B6Z, respectively).

Single GFP^+ excised shoots formed roots within 2 to 4 weeks and were ready for the greenhouse about 1 month after root induction. All rooted plants were screened for GFP expression in roots prior to being moved to the greenhouse. Initially plants that screened negative for GFP expression were

discarded and considered to be escapes, but later in the process GFP^- plants were kept to verify if these plants were true escapes. Plants that screened GFP^+ were moved to the greenhouse and were self-pollinated and the T_1 seed was collected. No plants of PI 551792 obtained from any treatment produced fruit within 20 weeks and so were discarded (Table 8). For PI 551572, the treatment effect on the percent of GFP^+ plants producing fruit is shown in Table 9. Treatment 2B6Z resulted in the lowest percent (43%) of GFP^+ plants producing fruit, treatments 2B1Z5B and 3Z5B resulted in the greatest percent (89 and 86, respectively) of GFP^+ plants producing fruit and the remaining five treatments ranged from 53-69%. All T_0 plants of PI 551572, regardless of the treatment, produced fruit within 12 wks after acclimatization to the greenhouse.

Flow cytometry

A total of 121 strawberry plants was analyzed to determine ploidy level using flow cytometry (Table 10). Flow cytometry was performed on eight different occasions over a span of 23 months using three modified chopping buffers (Brandizzi et al., 2001; Meng and Finn, 2002; Owen et al., 1988). In late winter 2004, eight samples of *F. vesca* L. 'Alpine' were analyzed by flow cytometry to determine if the protocol developed by Owen et al. (1988) was applicable to *F. vesca* L. 'Alpine'. The monoploid potato control gave readable peaks in both treatments (Fig 7-i and 7-iii). Without 5% PVP-40, the *F. vesca* sample did not produce clear peaks (Fig 7-ii). For strawberry this protocol was successful only when 5% PVP-40 was added to the chopping buffer (Fig 7-iv). During the summer of 2004, 64 of 66 *F. vesca* 'Alpine' plants were analyzed unsuccessfully

using this modified chopping buffer. Then in early winter of 2004 this modified chopping buffer was tested on strawberries known to vary for ploidy level (Figure 8). For control *F. vesca*, clear 2x, 4x and 8x peaks were obtained (Fig 8-i). One regenerant was found to be diploid (Fig 8-ii) and another regenerant was found to be tetraploid (Fig 8-iii). As expected *F. ×ananassa* 'Chandler' was found to be octoploid (Fig 8-iv).

In the spring of 2005, 18 samples of PI 551572 were analyzed using the three modified flow cytometry protocols listed in the materials and methods section. Flow cytometry was unsuccessful for the three modified flow cytometry protocols tested. Then in the fall of 2005, 17 samples of PI 551572 were analyzed using the modified flow cytometry protocol developed by Owen et al. (1988). These results were consistent with the results of the previous eight experiments conducted on flow cytometry. To summarize flow cytometry, the results of all the experiments proved unreliable at best. There were some when the ploidy on up to half of the samples was determinable but others when the ploidy of only a few samples analyzed could be determined.

GUS staining

GFP^+ (n=11) and GFP^- (n=25) T_0 plants of PI 551572 were stained for GUS expression. None of the 25 GFP^- plants showed GUS detection and nine of 11 GFP^+ plants tested positive for GUS expression. All of these plants were further analyzed by multiplex PCR using primers designed to amplify the *hpt*II and *gfp* genes.

Molecular analysis of PI 551572 T_0 plants

GFP$^+$ (n=15) and 25 GFP$^-$ (n=25) T_0 plants were analyzed using multiplex PCR for the detection of the *hpt*II and *gfp* genes. It was expected that the GFP$^+$ plants integrated the T-DNA from GV3101 pCAMBIA-1304 containing the *hpt*II, *gfp*, and *gus* genes and that the GFP$^-$ plants do not carry the T-DNA and, hence *hpt*II, *gfp*, and *gus* genes were not present in genomic DNA. Multiplex PCR of the *hpt*II and *gfp* genes revealed that for 11 of 15 GFP$^+$ plants the *hpt*II gene was present. Ten of the GFP$^+$ plants contained both the *hptII* gene the *gfp* gene (Figures 9 and 10). When multiplex PCR was performed on GFP$^-$ plants 3 of 25 plants contained the *hpt*II gene and 2 of 3 plants that contained the *hpt*II gene also contained the *gfp* gene (Figures 9 and 10).

Discussion

Various strawberry genotypes have been extensively studied with respect to transformation and regeneration (Alsheikh et al., 2002; Barcelo et al., 1998; El-Mansouri et al., 1996; Greene, 1988; Haymes and Davis, 1998; James et al., 1990; Mathews et al., 1995; Nehra et al., 1990a; Nehra et al., 1990b; Passey et al., 2003; Ricardo et al., 2003; Sorvari et al., 1993; Zhao et al., 2004). Reported transformation efficiencies for *F. vesca* range from as low as 7% (El-Mansouri et al., 1996) to as high as 100% (Oosumi et al. 2005). Transformation efficiency for strawberry in all cases investigated, varied depending on what explant type and genotype were used. The reported transformation efficiency of PIs 551572 and 551792 stress the importance of selecting the appropriate genotype prior to beginning large scale transformation experiments. Oosumi et al. (2005), by screening 14 accessions of *F. vesca* provided the most comprehensive

supporting information regarding the genotypic response to transformation of strawberry. The two genotypes tested in this study were also tested by Oosumi et al. (2005) and our results of greater than 100% transformation efficiency of these accessions are consistent with their findings.

The introduction of the synthetic cytokinin thidiazuron has had dramatic effects on woody plant micropropagation (Huetteman and Preece (1993). The search query “thidiazuron and shoot regeneration” generated 216 matches from the ISI Web of Science Database and these matches dated from 1991 to 2005. Thirty-four matches were published research within the family Rosaceae, with the *Rosa*, *Malus*, *Rubus*, *Prunus*, and *Fragaria* genera generating more than ¾ of the matches. Debnath (2005) demonstrated the regeneration of *in vivo* stipules of *F. x ananassa* Duch. ‘Bounty’ when cultured on medium supplemented with TDZ. Our modifications to the Oosumi et al. (2005) protocol were developed in response to the reduction in shoot regeneration time of *F. vesca* 'Alpine' leaf disc and petiole sections when cultured on MS basal medium supplemented with 4.54 µM TDZ and 0.9 µM 2,4-D (Figure 5) without obtaining the numerous dense small shoots regenerated with prolonged TDZ exposure (Figures 3 and 4). The eight treatments were designed to include different durations of TDZ exposure from *Agrobacterium* inoculation to shoot induction. Although many observations were conducted per treatment, the mean of GFP^+ plants obtained per primary explant was not significant among treatments. Obtaining a high number of GFP^+ plants per primary explant is critical for developing the ~255,000 T-DNA tagged lines needed for gene discovery in *F. vesca*. For the accessions investigated the

means of GFP^+ plants obtained per primary explant varied fro six to nine-fold, indicating there was little evidence of any general effect of TDZ on strawberry regeneration after transformation. Although PI 551792 responded favorably to transformation with treatment 8B, the insertional mutants derived from this accession did not produce fruit eliminating PI 551792 as a candidate for use in strawberry genomics studies. For PI 551572 treatment 5Z3B resulted in the greatest yield of GFP^+ fruitful plants (Table 9); hence this is our recommended protocol for future transformation of PI 551572. In addition to having high transformation efficiency, a model species requires a short generation time (seed to seed). Our results prove that in addition to the high transformation efficiency, PI 551572 demonstrates the short generation time required of a model species.

Multiplex PCR of transgenic plant material allows the simultaneous detection of various parts of the inserted T-DNA (Mannerlof and Tenning, 1997; Tao et al., 2001; Xu et al., 2005). Successful multiplex PCR requires the design of primers such that the formation of primer dimers is minimal and the expected size of amplification products varies sufficiently to allow for separation when electrophoresis is performed. In addition, the thermocycler conditions must be optimized prior to beginning large-scale analysis of transgenic plant material. Multiplex PCR has the advantage of reducing the time required to screen transformed plant lines. This method of screening transformed plants also reduces the possibility of escapes and plants exhibiting poor marker gene expression from infiltrating T_1 seed lots. Our results revealed 11 anomalous plants among 40 plants screened. The GFP^+ plants that did not show the

presence of the *hpt*II or *gfp* genes can be explained in two ways. Either the genomic DNA was of poor quality and no amplification of the *hpt*II or *gfp* genes occurred or the GFP screening was wrong and these anomalous GFP^+ plants escaped both hygromycin and GFP selection. The anomalous GFP^- plants could have resulted from a mistake during GFP screening, incomplete T-DNA integration and gene silencing.

Transgene expression has been positively correlated to the number of copies of T-DNA present in transgenic plants (Afolabi et al., 2004; Atkinson et al., 1998; Elmayan and Vaucheret, 1996; Fagard and Vaucheret, 2000; Finnegan and McElroy, 1994; Gittins et al., 2003; Hobbs et al., 1990; Hobbs et al., 1993; Lechtenberg et al., 2003; Matzke and Matzke, 1995; Nagaya et al., 2005; Peach and Velten, 1991; Schubert et al., 2004). The effects of ploidy have also been implicated in transgene expression (Beaujean et al., 1998; Mittelsten Scheid et al., 1996). Other authors have proposed that plants are activating defense mechanisms in response to infection by *Agrobacterium* (Finnegan and McElroy, 1994; Matzke and Matzke, 1995). We have identified 11 plants that do not fit the expected transgene expression patterns assuming that these plants contain a single copy of T-DNA. Performing Southern blot analysis on the questionable plants would answer the question of T-DNA copy number effect on transgene expression. Grevelding et al. (1993) demonstrated in *Arabidopsis* that explant type had an effect on the number of T-DNA copies inserted. Root derived transgenic plants contained fewer multiple insertions of the T-DNA (36%) compared to leaf disc derived plants (89%). More experimentation is needed for

strawberry to determine if leaf discs are the best explant type for use in transformations. Unfortunately, ploidy determination using three different flow cytometry protocols has proved unreliable. By developing a reliable flow cytometry protocol the ploidy of transformed plants might be correlated to T-DNA copy number.

Zhao et al. (2004) have reported the transformation rate of *F. vesca* FRA 197 at 62% when *in vitro* leaf explants were regenerated on medium containing TDZ and IBA. FRA 197 is the local inventory ID of accession PI 551572 that Oosumi et al. (2005) reported transformation efficiency of 100% when *in vivo* leaf explants were regenerated on medium containing BA and IBA. Using the transformation protocol outlined by Oosumi et al. (2005), we demonstrate that there are not only genotypic factors present that influence transformation efficiency, but that the composition of the regeneration medium also influences the transformation efficiency of PIs 551792 and 551572. The statistical power of our experiment was insufficient to detect treatment effect but based on our findings the transformation efficiencies are high enough to justify PI 551572 for use in the high throughput transformations that are required to generating a population of insertional mutants large enough for gene discovery in *F. vesca*.

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Table 1: Inventory of *F. vesca* accessions.

Accession number*	Seed source	Runnering habit	Fruit color
PI 551573	Hawaii, USA	yes	white
PI 551783	Oregon, USA	NT**	NT**
PI 551792	Finland	yes	red
PI 551833	New Mexico, USA	yes	red
PI 548865	Ecuador	yes	red
PI 616581	Open pollinated 'Alpine'	no	white
PI 602923	Europe-seed clone of 'Alpine'	no	red
PI 616862	Bolivia	yes	red
PI 551572	Hawaii, USA	yes	white
PI 551834	Germany	no	red
PI 602578	Europe	no	white
'Alpine'	Tradewinds Seed Co., USA	no	white

* All germplasm obtained from USDA National Clonal Germplasm Repository, Corvallis, Ore., except for 'Alpine'.
** Not tested.

Table 2: Media used for experiments following the Oosumi et al. (2005) *Agrobacterium*-mediated transformation protocol.

Media code[a]	Growth regulator[b]				Antibiotic[b]	
	BA	TDZ	IBA	2,4-D	Cb	Hg
Co-cultivation[c]						
CCM-Z		4.54		0.9		
CCM-B	13.3		1.2			
Shoot induction						
SIM-I-Z		4.54		0.9	500	4
SIM-I-B	13.3		1.2		500	4
SIM-II-Z		4.54		0.9	250	4
SIM-II-B	13.3		1.2		250	4
SIM-III-Z		4.54		0.9	100	
SIM-III-B	13.3		1.2		100	
Root induction						
RIM			0.98		100	

[a] All media contain MS salts and B5 vitamins and are solidified with 0.2% Gellan Gum (Caisson Labs Inc.) unless otherwise stated.
[b] Growth regulator values are given in µM and antibiotic values are given in milligrams per liter
[c] Solidified with 0.7% agar

Table 3: Schematic of media transfers and treatments applied to Oosumi et al. (2005) protocol.

Day 1	40-50 primary explants infected and cultured cultured							
	CCM-Z[a]				CCM-B[a]			
Day 3	40-50 primary explants transferred to							
	SIM I-Z[a]				SIM I-B[a]			
Trt[b]	8Z	5Z3B	3Z5B	2Z6B	2B6Z	2B3Z3B	2B1Z5B	8B
Day 10	Z	Z	Z	B	Z	Z	Z	B
Day 24	Z	Z	B	B	Z	Z	B	B
Day 38	Z	Z	B	B	Z	Z	B	B
Day 52	Z	B	B	B	Z	B	B	B
Day 66	Z	B	B	B	Z	B	B	B
Day 80[c]	Z	B	B	B	Z	B	B	B

[a] CCM-Z = co-cultivation medium containing 4.54 µM TDZ and 0.9 µM 2,4-D; CCM-B = co-cultivation medium containing 13.3 µM BA and 1.2 µM IBA; SIM I-Z = shoot induction medium containing 4.54 µM TDZ and 0.9 µM 2,4-D; SIM I-B = shoot induction medium containing 13.3 µM BA and 1.2 µM IBA

[b] At day 10 the primary explants are cut into secondary explants and cultured at a density of 50 secondary explants/ plate/ treatment

[c] At day 80 shoots that are large enough (~ 3-5 mm) are transferred to root induction medium and regenerating shoots smaller than 3 mm are transferred to fresh SIM every 2 weeks until shoots are large enough for root induction

Table 4: Comparison of seed germination rate of ten *F. vesca* accessions when stratified or not stratified at 4ºC for 12 weeks and then planted on MS basal medium.

Accession number	Seed source	Germination percentage without stratification	Germination percentage with stratification
PI 551573	Hawaii, USA	30	80
PI 551783	Oregon, USA	10	90
PI 551792	Finland	70	80
PI 551833	New Mexico, USA	60	100
PI 548865	Ecuador	80	100
PI 616581	Open pollinated 'Alpine'	0	0
PI 602923	Europe-seed clone of 'Alpine'	60	100
PI 616674	Bolivia	60	30
PI 616862	Plovdiv, Bulgaria	10	0
'Alpine'	Tradewinds Seed Co., USA	54	Not tested
Mean*		43	64

* n=10 seeds per treatment

Table 5: ANOVA of seed germination of five accessions of *F. vesca* after 4h treatment with either 1% sodium hypochlorite or 1% calcium hypochlorite. Seeds (n=25) were planted in four replicates for each treatment.

Effect		Day 7	Day 11	Day 14	Day 17	Day 21	Day 27	Day 30	Day 34
Source	df	MS	MS	MS	MS	MS	MS	MS	MS
Accession	4	67.4*	313.8*	333.3*	359.5*	360.4*	387.2*	397.3*	378.7*
Treatment	1	65.0*	0.2 ns	0.1 ns	0.4 ns	1.2 ns	6.4 ns	3.6 ns	2.5 ns
Acc x Trt	4	68.3*	50.5*	30.2*	7.9 ns	1.8 ns	4.1 ns	3.8 ns	4.9 ns
Error	30	4.0	5.6	5.7	6.7	8.1	8.7	8.6	8.4

An asterisk (*) following a value denotes a significance at P<0.001 and ns following a value denotes no significance.

Table 6: ANOVA of transformation efficiency of PI 551792 with the binary vector pCAMBIA-1304.

Effect		GFP^+ shoots per primary explant	GFP^+ plants per primary explant
Source	df	MS[1]	MS[1]
Expt	1	0.01 ns	0.04 ns
Trt	5	0.38*	0.16 ns
Error	1	0.32	0.03

[1]An asterisk (*) following a value denotes a significance at $P<0.05$ and ns following a value denotes no significance at $P<0.05$.

Table 7: ANOVA of transformation efficiency of PI 551572 with the binary vector pCAMBIA-1304.

Effect		GFP^+ shoots per primary explant	GFP^+ plants per primary explant
Source	df	MS[1]	MS[1]
Expt	3	2.55*	0.29 ns
Cocult(Expt)	4	0.07 ns	0.19 ns
Grtrt	3	0.27 ns	0.25 ns
Expt x grtrt	9	0.17 ns	0.11 ns
Error	31	0.45	0.29

[1]An asterisk (*) following a value denotes a significance at P<0.05 and ns following a value denotes no significance at P<0.05.

Table 8: Transformation efficiency of PI 551792 with the binary vector pCAMBIA-1304.

Treatment	% explants with GFP+ callus[1]	Mean number of GFP+ shoots obtained per primary explant[2]	Mean number of GFP+ plants obtained per primary explant [2]	% GFP+ fruitful plants[3]
8Z	55 (36/65)	1.43 a	0.64 a	0 (0/9)
5Z3B	42 (41/97)	0.26 a	0.15 a	0 (0/4)
3Z5B	52 (16/31)	0.85 a	0.69 a	0 (0/9)
2Z6B	C*	-	-	-
2B6Z	89 (32/36)	0.85 a	0.54 a	0 (0/7)
2B3Z3B	C	-	-	-
2B1Z5B	49 (45/91)	0.30 a	0.11 a	0 (0/3)
8B	94 (34/36)	1.46 a	1.00 a	0 (0/13)

[1] Numbers in parentheses are GFP+ explants / total explants
[2] Means with the same letter are not significant at P<0.05 level
[3] Numbers in parentheses are GFP+ fruitful plants / total GFP+ plants
* Contaminated cultures

Table 9: Transformation efficiency of PI 551572 with the binary vector pCAMBIA-1304.

Treatment	% explants with GFP+ callus[1]	Mean number of GFP+ shoots obtained per primary explant[2]	Mean number of GFP+ plants obtained per primary explant[2]	% GFP+ fruitful plants[3]
8Z	68 (124/182)	1.08 a	0.69 a	61 (11/18)
5Z3B	77 (139/180)	1.54 a	1.19 a	64 (20/31)
3Z5B	48 (84/175)	1.04 a	0.40 a	86 (18/21)
2Z6B	69 (123/177)	0.92 a	0.56 a	64 (14/22)
2B6Z	44 (80/180)	0.59 a	0.18 a	43 (3/7)
2B3Z3B	40 (73/182)	1.33 a	0.58 a	53 (16/30)
2B1Z5B	54 (94/175)	1.10 a	0.49 a	89 (17/19)
8B	60 (108/181)	1.15 a	0.67 a	69 (18/26)

[1] Numbers in parentheses are GFP+ explants / total explants
[2] Means with the same letter are not significantly at $P<0.05$ level
[3] Numbers in parentheses are GFP+ fruitful plants / total GFP+ plants

Table 10: Results of flow cytometry analysis of 121 strawberry samples using three different protocols.

Protocol[1]	Accession	Date	No. of samples analyzed	Readable samples	Ploidy estimate of readable samples	
					2x	4x
Owen et al. (1988)	'Alpine'	2/18/04	8	4	4	-
Owen et al. (1988)	'Alpine'	6/17/04	40	0	-	-
Owen et al. (1988)	'Alpine'	6/25/04	26	2	-	2
Owen et al. (1988)[2]	'Alpine'	12/03/04	3	1	1	-
	PI 602924		5	4	2	2
	PI 551792		3	2	1	1
Owen et al. (1988)	PI 551572	5/10/05	6	0	-	-
Meng and Finn (2002)	PI 551572	5/10/05	6	0	-	-
Brandizzi et al. (2001)	PI 551572	5/10/05	6	0	-	-
Owen et al. (1988)	PI 551572	11/16/05	17	3	2	1
Total			121	16	10	6

[1] A monoploid potato was used in every experiment as a control.
[2] One sample of *F.* ×*ananassa* 'Chandler' was analyzed and the ploidy estimate was 8x.

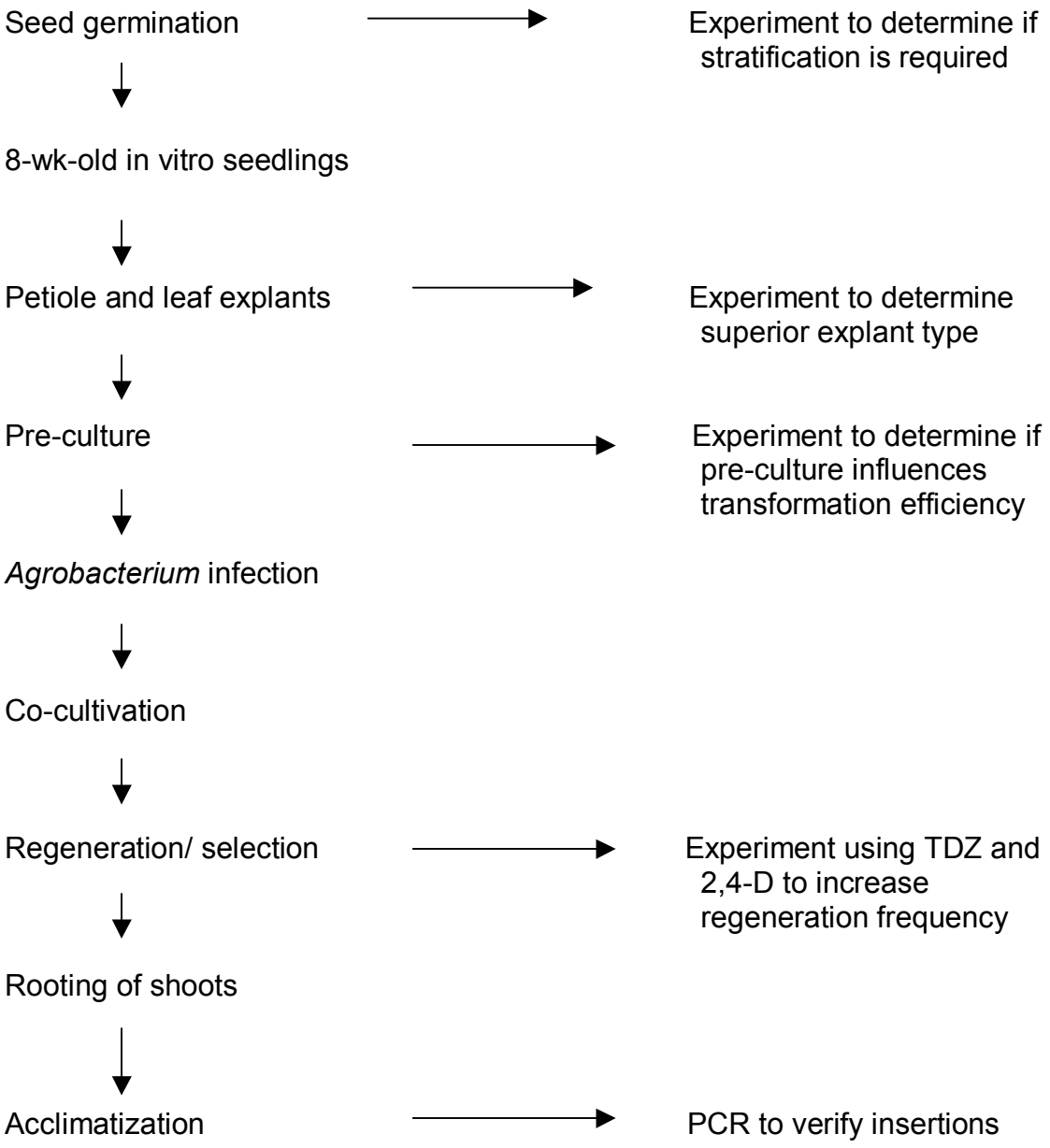
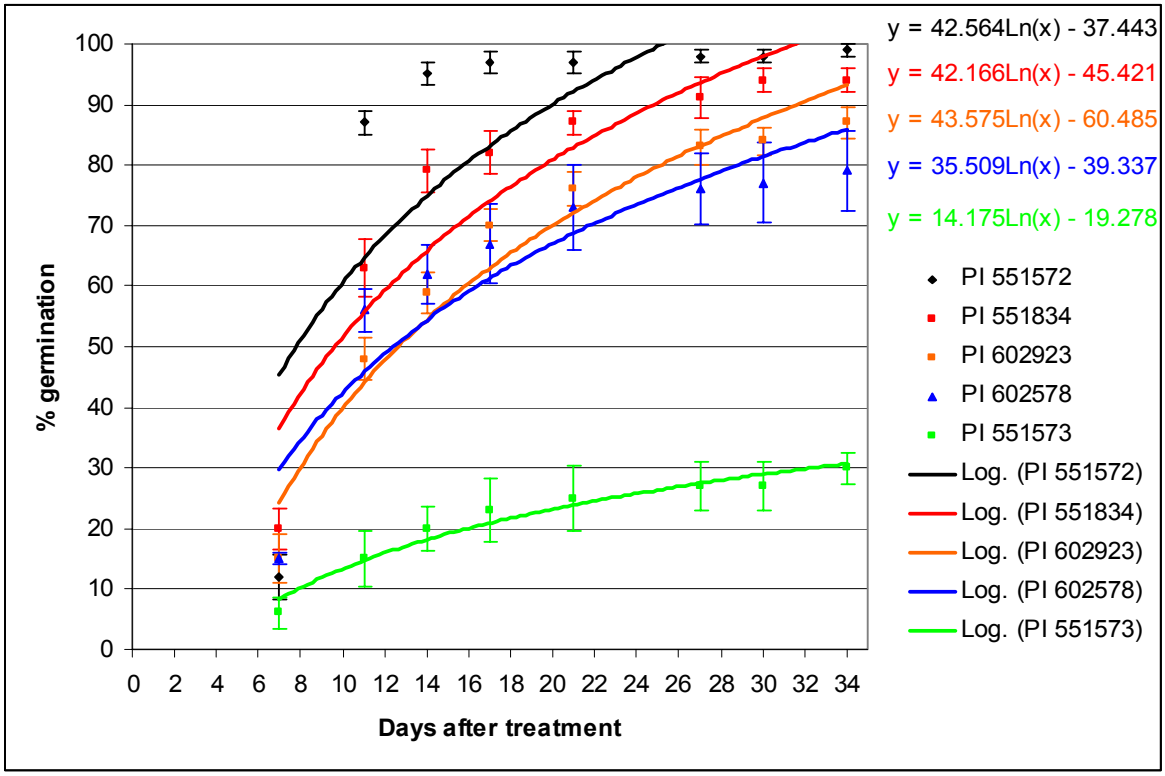


Figure 1. Flow chart showing experimental modifications of the procedures outlined by Alsheikh et al. (2002).



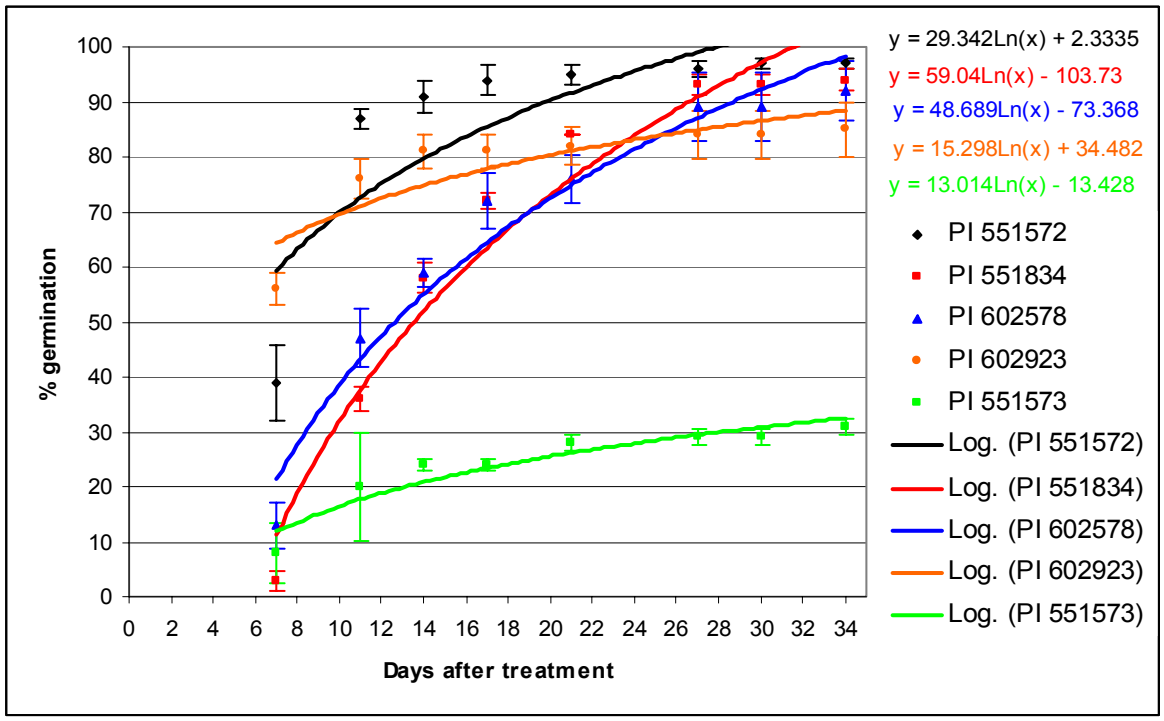


Figure 2. Percent germination of five *F. vesca* accessions over 34 days after a 4h treatment with either 1% sodium hypochlorite (top) or 1% calcium hypochlorite (bottom). Standard error bars and logarithmic trend lines are shown for each accession.

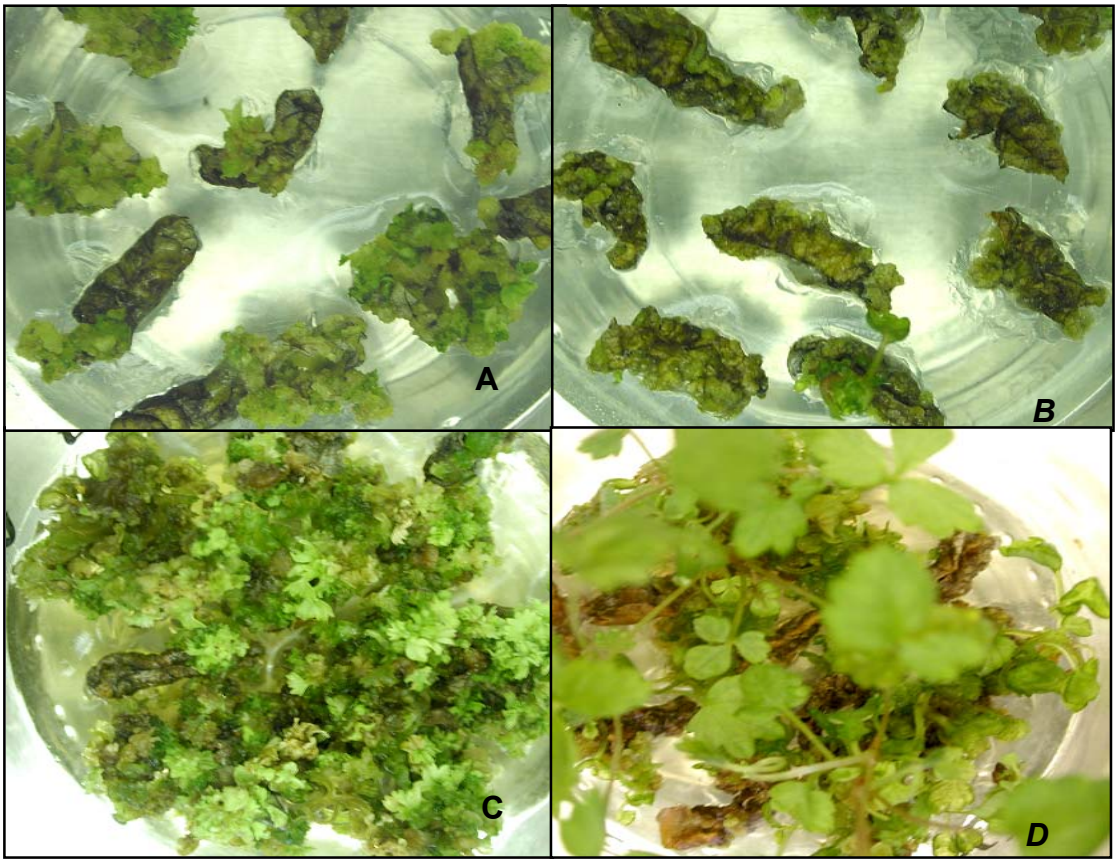


Figure 3. Shoot regeneration of *F. vesca* 'Alpine' leaf discs cultured on MS basal medium supplemented with 4.54 µM TDZ and 0.9 µM 2,4-D at 6 weeks (A) and 10 weeks (C) or 13.3 µM BA and 1.22 µM IBA at 6 weeks (B) and 10 weeks (D).

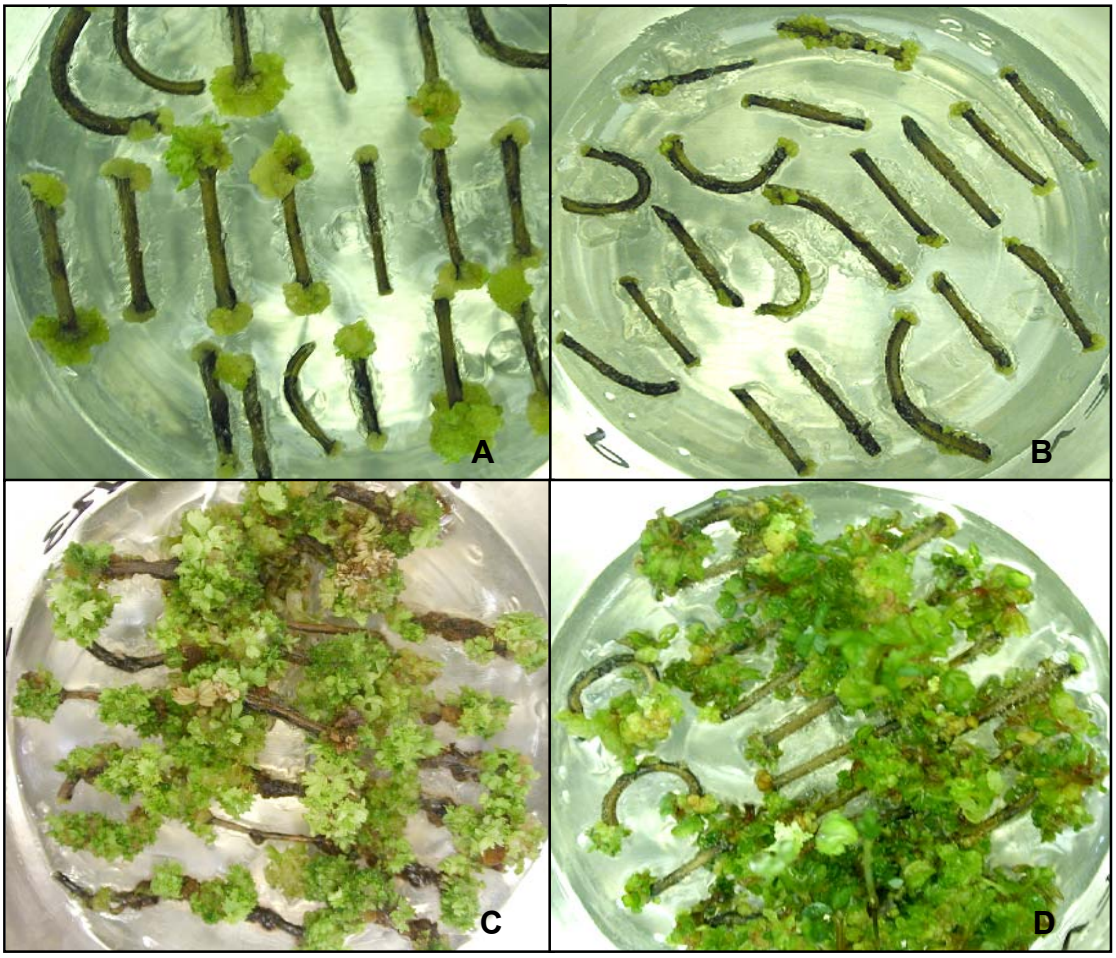


Figure 4. Shoot regeneration of *F. vesca* 'Alpine' petiole sections cultured on MS basal medium supplemented with 4.54 µM TDZ and 0.9 µM 2,4-D at 6 weeks (A) and 10 weeks (C) or 13.3 µM BA and 1.22 µM IBA at 6 weeks (B) and 10 weeks (D).

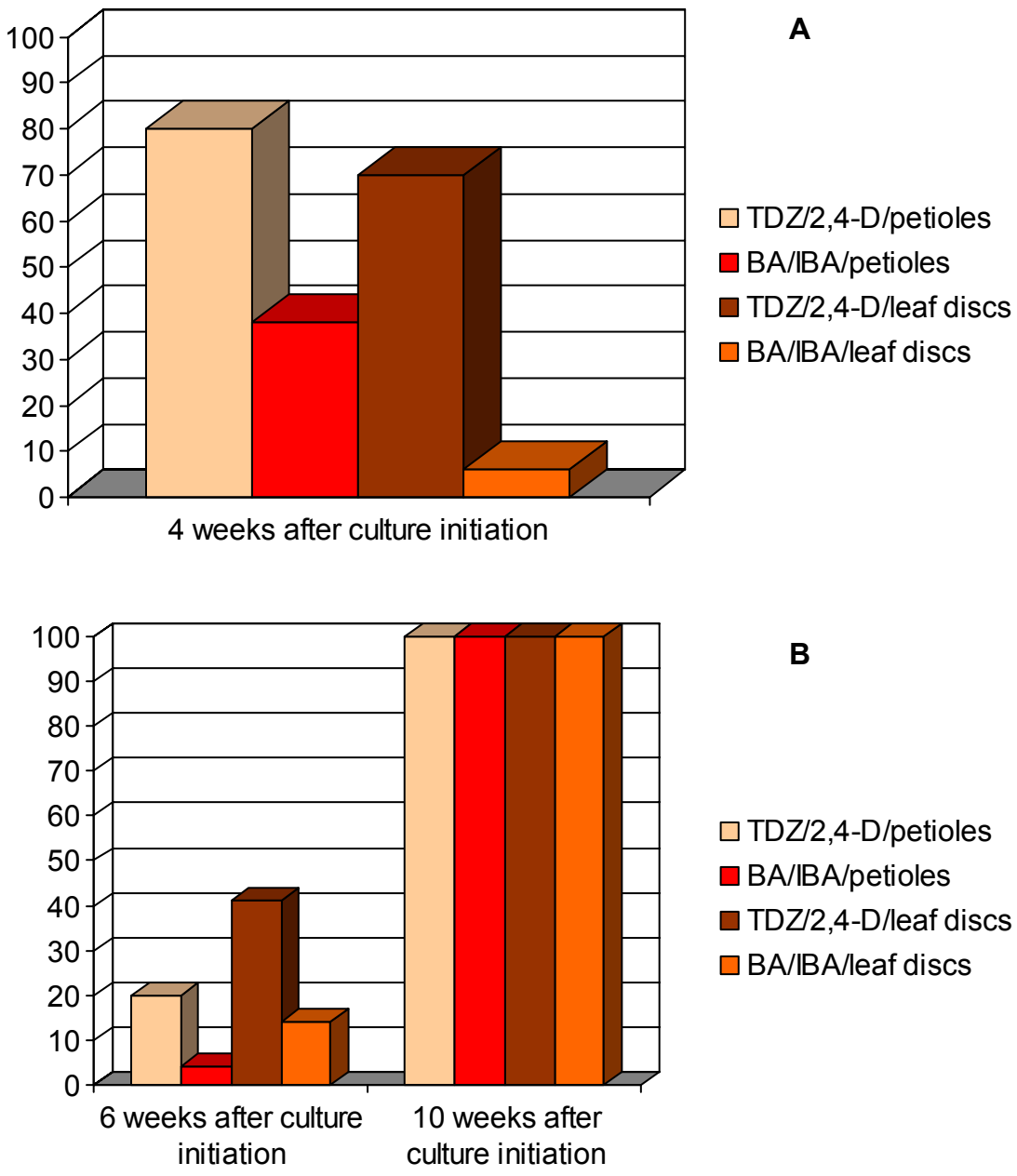


Figure 5. (A) Callus regeneration of *F. vesca* 'Alpine' leaf and petiole explants at 4 weeks after culture initiation when cultured on MS basal medium supplemented with 13.3 µM BA and 1.22 µM IBA or 4.54 µM TDZ and 0.9 µM 2,4-D. The y-axis is the percent of explants with callus. (B) Shoot regeneration of *F. vesca* 'Alpine' leaf and petiole explants at 6 and 10 weeks after culture initiation when cultured on MS basal medium supplemented with 13.3 µM BA and 1.22 µM IBA or 4.54 µM TDZ and 0.9 µM 2,4-D. The y-axis is the percent of explants with shoots.

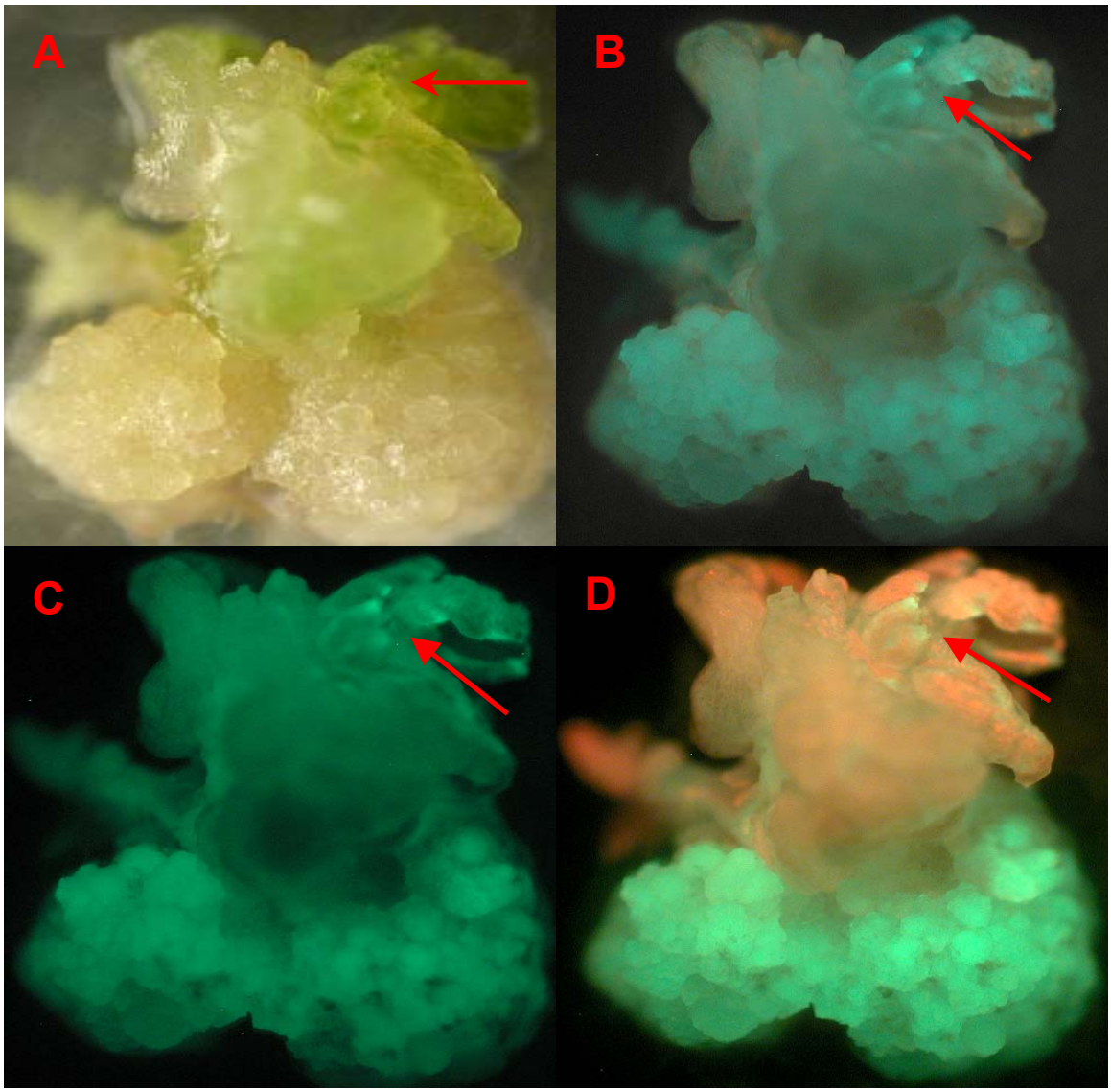


Figure 6. Shoot regeneration on GFP^{+} callus of PI 551572 transformed with *Agrobacterium* strain GV 3101 pCAMBIA-1304. A. No GFP filter. B. Dual GFP filter. C. Narrow pass GFP filter. D. Long pass filter. Pictures were taken 7 weeks after transformation.

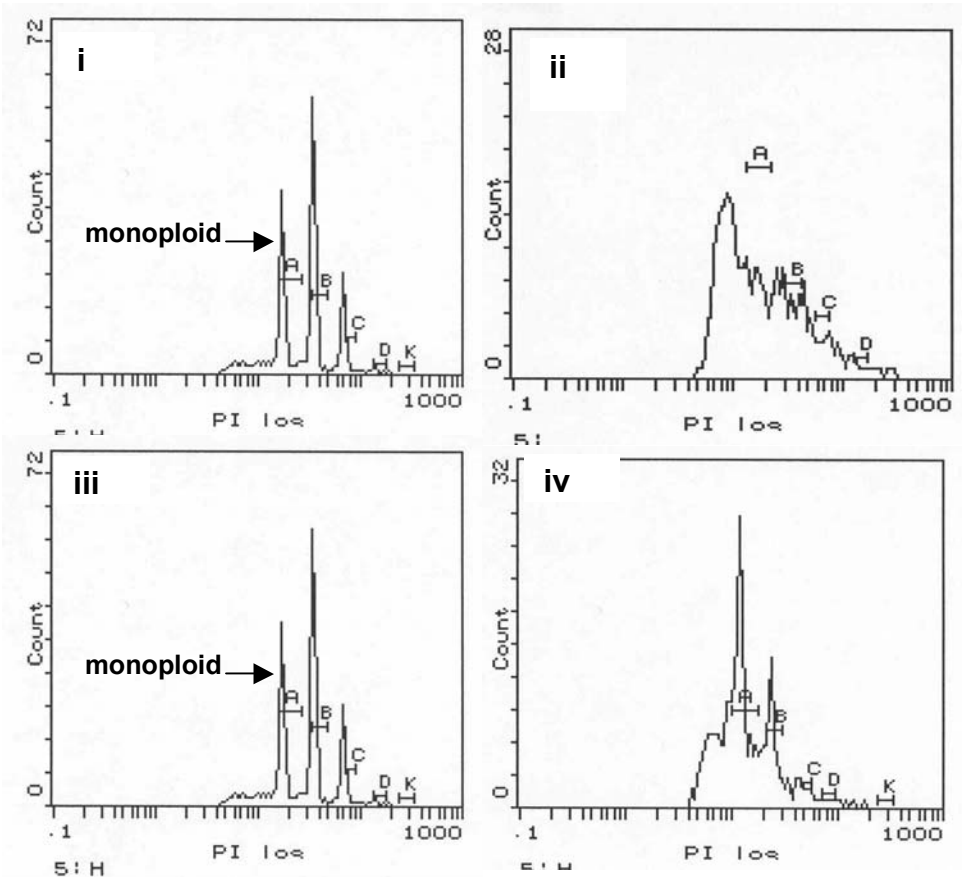


Figure 7. Flow cytometric histograms of a potato monoploid (2n=1x=12, i and iii) and a *F. vesca* 'Alpine' (2n=2x=14, ii and iv) using modified Owen et al. (1988) flow cytometry protocol. The A, B, C, and D gates represent the monoploid, diploid, tetraploid, and octoploid DNA contents for potato. The gates were set by running a monoploid (2n=1x-12) control. The count on the y-axis is the number of propidium iodine stained cell nuclei that fall into particular channels (PI log) corresponding to DNA content. The first peak (on the left) in each histogram indicates the ploidy of the plant. Subsequent peaks result from endomitosis.

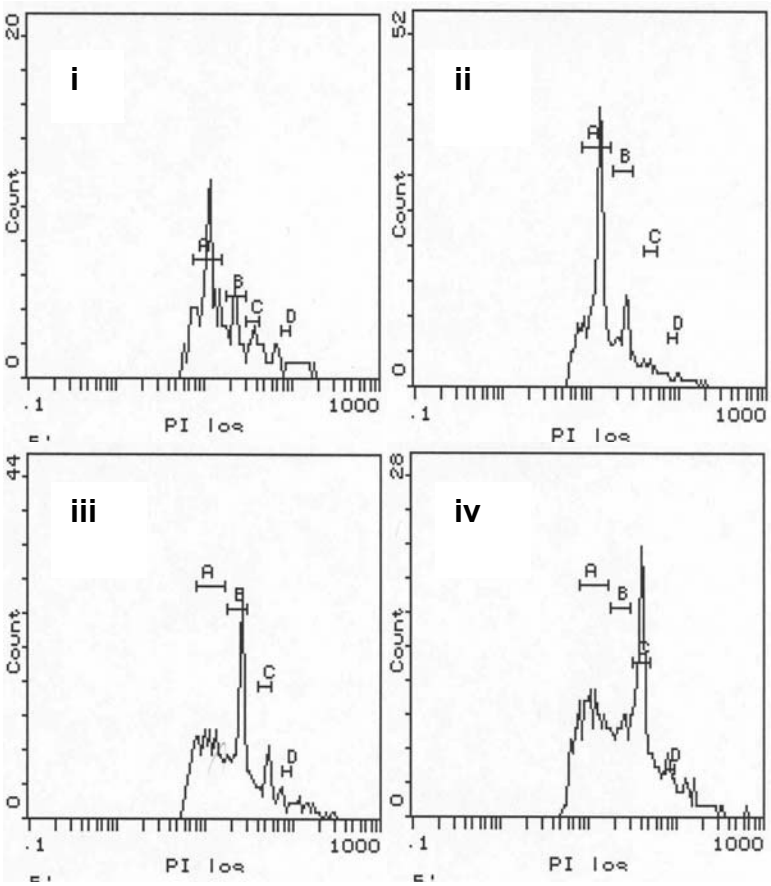


Figure 8. Flow cytometric histograms of a wild-type *F. vesca* (2n=2x=14, i), a transformed *F. vesca* (ii), a transformed *F. vesca* (iii), and a wild-type *F. x ananassa* 'Chandler' (2n=8x=56, iv) using a modified flow cytometry protocol by Owen et al. (1988). The A, B, and C gates represent the diploid, tetraploid, and octoploid DNA contents for potato. The count on the y-axis is the number of propidium iodine stained cell nuclei that fall into particular channels (PI log) corresponding to DNA content. The first peak (on the left) in each histogram indicates the ploidy of the plant. Subsequent peaks result from endomitosis.

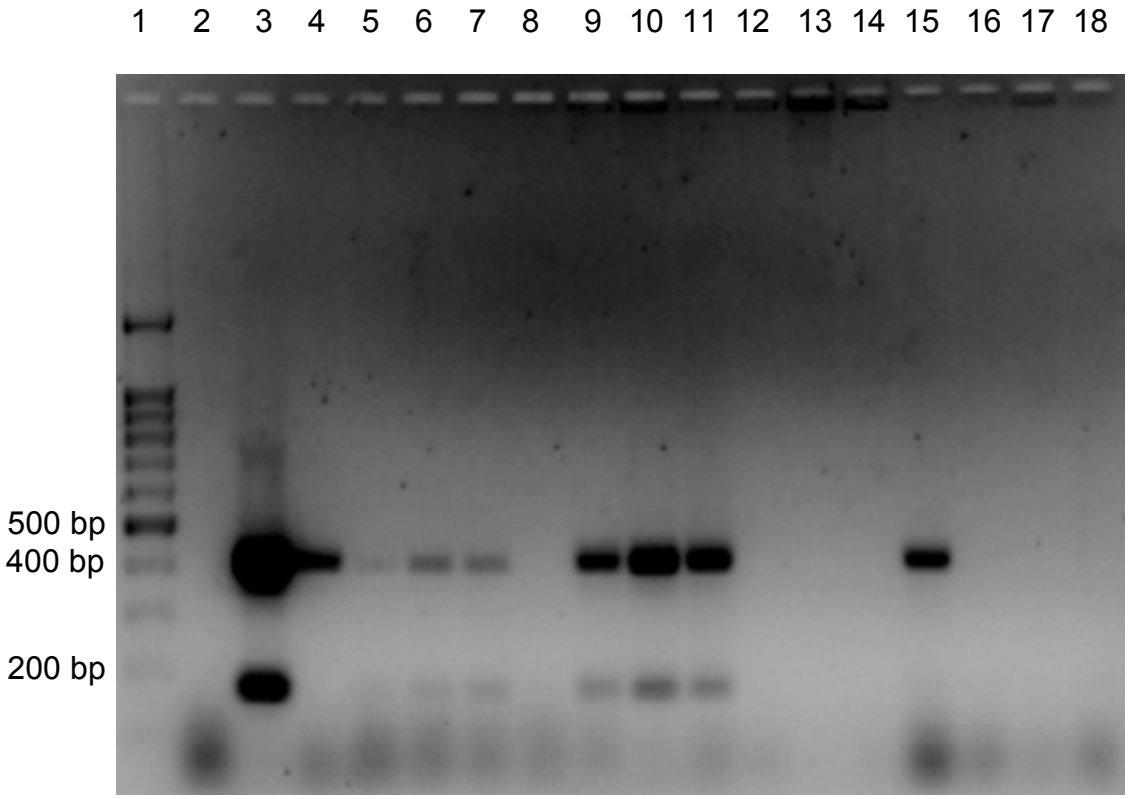


Figure 9. Multiplex PCR of the *hpt*II and *gfp* genes of PI 551572 plants that screened GFP^+ and GFP^-. 1, 100 bp ladder; 2, wild type PI 551572; 3, pCAMBIA-1304; 4-11, GFP^+ plants; 12-18, GFP^- plants. Expected size of the *hpt*II gene is 411 bp and the *gfp* gene is 177 bp.

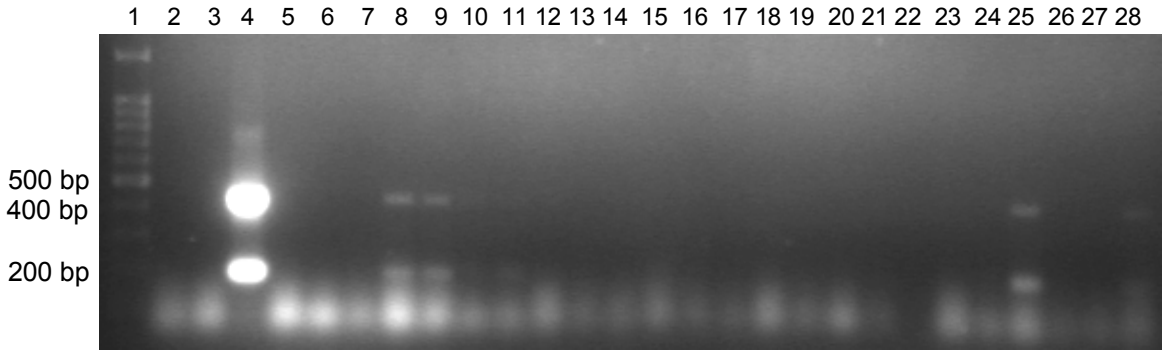


Figure 10. Multiplex PCR of the *hpt*II and *gfp* genes of PI 551572 plants that screened GFP^+ and GFP^-. 1, 100 bp ladder; 2, blank; 3, wild type PI 551572; 4, pCAMBIA-1304; 5-11, GFP^+ plants; 12-28, GFP^- plants. Expected size of the *hpt*II gene is 411 bp and the *gfp* gene is 177 bp.

Vita

Phillip A. Wadl was born in Cincinnati, Ohio on November 23, 1976. He attended public schools and graduated from North College Hill High School in 1995. Phillip graduated from Virginia Polytechnic Institute and State University in 2003 with a B.S. degree in Horticulture. In 2003 Phillip began an M.S. in Horticulture under Dr. Richard Veilleux. He received his M.S. in 2005 and is currently pursuing a Ph.D. in Plant Pathology at The University of Tennessee. Phillip and his wife, Erica, reside in Knoxville, Tennessee.