Plant Cell Rep (2006) 25: 450–456
DOI 10.1007/s00299-005-0088-z

GENETIC TRANSFORMATION AND HYBRIDIZATION

Danilo D. Fernando · Javonna L. Richards · Julie R. Kikkert

In vitro germination and transient GFP expression of American chestnut (*Castanea dentata*) pollen

Received: 17 May 2005 / Revised: 6 October 2005 / Accepted: 26 October 2005 / Published online: 9 December 2005

Abstract The development of the male reproductive structures of American chestnut (*Castanea dentata*) is described to advance our understanding of its reproductive behavior. This information has been vital in the development of a strategy to collect pollen grains from male catkins suitable for *in vitro* germination and transformation experiments. Cutting male catkins into small segments and rolling them over a culture plate resulted in evenly dispersed and large amounts of pollen with minimal unwanted accessory floral parts. To optimize pollen viability, the effect of various storage conditions on *in vitro* germination was examined. Our results showed that initial storage at 4°C for 2 weeks significantly increased percent germination as compared to freshly collected pollen and those stored directly at −20°C or −80°C. This also means that for long-term storage of American chestnut pollen, the catkins should first be kept at 4°C for a couple of weeks and then at −80°C. The use of pollen grains with high viability is necessary for the transformation of American chestnut pollen. To optimize pollen transformation via particle bombardment, the effects of target distance, target pressure, and pollen developmental stage were examined. Statistical analysis showed that bombardment of ungerminated pollen at 1,100 psi resulted in the highest percent transient GFP expression (4.1%).

Keywords American chestnut · *Castanea dentata* · Pollen · Transformation · Viability

Communicated by S.A. Merkle

D. D. Fernando (✉) · J. L. Richards
Department of Environmental and Forest Biology, State University of New York College of Environmental Science and Forestry,
241 Illick Hall, 1 Forestry Drive, Syracuse, NY 13210, USA
e-mail: fernando@esf.edu
Tel.: +315-470-6746
Fax: +315-470-6934

J. R. Kikkert
Department of Horticultural Sciences, New York State Agricultural Experiment Station, Cornell University,
Geneva, NY 14456, USA

Introduction

Chestnut blight is caused by *Cryphonectria parasitica*, a fungal pathogen introduced from infected Japanese chestnut (*Castanea crenata*) trees before the turn of the previous century. Fifty years after the discovery of the disease, the American chestnut (*C. dentata*) was decimated from its entire range (Berry 1954). Research on the restoration of the American chestnut has focused on biological control and back-cross breeding. Strains of *C. parasitica* with reduced pathogenecity have been successfully used to treat the European chestnut (*C. sativa*), but not the American chestnut. It has been reported that vegetative incompatibility amongst the strains prevents the transmission of hypovirulence (Anagnostakis 1987; MacDonald and Fulbright 1991). As for resistance breeding, this strategy requires screening large numbers of progeny over six generations, each of which requires a minimum of 6 to 7 years. It is expected that progenies from intercrossed third backcrossed trees will be ready for reintroduction into the wild very soon; these seeds will represent the beginning of the fifth generation in the breeding process and constitute 93.75% American chestnut genes (Hebard 2001). However, as pointed out by Hebard (2001), besides the problem with the lengthy process, there is also the possible existence of a race structure in the pathogen that could overcome the resistance genes being bred into American chestnuts.

Resistance genes of potential use in American chestnut restoration have been developed, including those that encode magainin and cecropin-like antimicrobial peptides that confer antifungal activities (Powell et al. 1995; Liang et al. 2001). Although these genes have not yet been expressed in American chestnut, their antifungal activities have been demonstrated in other woody plants (Liang et al. 2002). Given a successful method to insert resistance genes into American chestnut through genetic engineering, the restoration of this highly valued species could be expedited with its genome remaining intact and undiluted. However, the genetic engineering approach typically depends on an *in vitro* regeneration process. Unfortunately, American

chestnuts are difficult to regenerate *in vitro* (Merkle et al. 1991; Carraway et al. 1994; Maynard et al. 1998; Xing et al. 1999; LaPierre 2003; Polin 2004).

One strategy that is independent of tissue culture steps with the added benefit of producing nonchimeric plants is pollen transformation. The use of haploid pollen grains as carriers of foreign DNA also would allow rapid production of transgenic plants and consequently, reduced chances of somaclonal variation. Pollen is a natural vector for direct gene transfer because it is involved in the normal sexual reproductive process in plants. Transformed pollen has been successfully used to generate transgenic plants such as *Nicotiana glutinosa* (van der Leede-Plegt et al. 1995), *N. tabacum* (Touraev et al. 1995, 1997; Aziz and Machray 2003), and *Pinus sylvestris* (Aronen et al. 2003).

This study was conducted to begin development of an optimized pollen transformation system that can be used as an alternative approach to produce disease resistant American chestnuts. To do this, information on the development of the catkins and pollen grains is necessary. Since successful pollen transformation depends on a reliable pollen collection strategy and optimized pollen viability, several methods to collect and store pollen from catkins were investigated. To produce transgenic American chestnut through pollen transformation, a particle bombardment protocol optimized to facilitate the insertion of foreign genes into pollen must first be established. Therefore, the effects of bombardment parameters (target distance and helium pressure) and pollen developmental stages (ungerminated and germinated pollen) on transient GFP (green fluorescent protein) expression were examined.

Materials and methods

Pollen sources, storage, and collections

The development of the male catkins was monitored from early June to early August from the three most prolific catkin producing American chestnut trees (A5, D11, and E14) growing at SUNY-ESF's Heiberg Memorial Forest in Tully, NY, USA. Mature catkins were collected around the third week of July and either used directly for experiments (e.g., germination tests or bombardments) or stored. Prior to storage, catkins were dried by spreading them out on clean white paper for 2 h at room temperature. Catkins intended for storage were placed in glass Petri dishes and sealed with parafilm. Percent viability of pollen from freshly collected catkins was compared to those stored directly under the following conditions: (1) 4°C for 2 weeks; (2) −20°C for 2 weeks; (3) −80°C for 2 weeks; (4) −20°C for 1 year; and (5) −80°C for 1 year. Percent pollen viability was also determined from those catkins initially stored at 4°C for 2 weeks and then subjected to the following conditions: (1) −20°C for 2 weeks; (2) −80°C for 2 weeks; (3) −20°C for 1 year; and (4) −80°C for 1 year.

Four different methods of collecting pollen grains from catkins were tested: (1) the catkins were laid on a clean glass plate overnight and the pollen scraped off onto a solid pollen germination medium with a razor blade; (2) the catkins were shaken for 3–5 min over a glass plate and the released pollen was scraped off onto a solid pollen germination medium with a razor blade; (3) the catkins were shaken for 3–5 min over a cardboard folder and the collected pollen was sieved through a fine nylon mesh (21 μm) onto a solid pollen germination medium; and (4) the catkins were cut into 1-in. segments, placed gently onto solid pollen germination medium using forceps, and rolled to release pollen from the anthers.

Pollen germination medium, culture and morphology

The pollen germination medium used was a modified Brewbaker and Kwack (BK) (1963) with 10 mg/L H_3BO_3, 30 mg/L $Ca(NO_3)_2$, 20 mg/L $MgSO_4$, 10 mg/L KNO_3, supplemented with 51.3 g/L sucrose, 27.4 g/L mannitol, and 4 g/L Phytagel. The pH was adjusted to 5.8 before autoclaving. Pollen was dispensed onto the solid germination medium and incubated at 25°C in the dark for 4 h. Pollen germination was examined using a Leica DMLB compound light microscope. Pollen was considered germinated if the pollen tube was at least as long as the length of the pollen grain. Percent germination was calculated by dividing the number of germinated pollen grains by the total number of pollen grains examined. Three replicate plates were examined per storage treatment and 1,000 pollen grains were counted at random locations throughout each culture plate.

Anthers of American chestnut bearing pollen grains were dehydrated overnight using tetramethylsilane (Ted Pella Inc., CA) and critical point dried with liquid nitrogen under vacuum. The specimens were mounted on metal stubs, sputter coated with a thin film of gold, and examined with an ETEC Autoscan scanning electron microscope. Representative photomicrographs of pollen grains were taken using Polaroid film.

DNA construct and preparation

The plasmid construct used was pBIN *35S-mgfp5-ER* (provided by Dr. J. Haseloff, University of Cambridge, UK). Plasmid DNA was transformed into *Escherichia coli* strain DH5α following a standard protocol (Sambrook et al. 1989). DNA isolation was performed using a Qiagen Hi-Speed Maxi Kit (Qiagen Inc., Chatsworth, CA). Purity and concentration of isolated DNA was determined using 260/280 absorbance ratios measured with a SmartSpec spectrophotometer (Bio-Rad, Hercules, CA).

Plasmid DNA was precipitated onto sterile gold particles according to the method of Vidal et al. (2003). Gold particles 0.6 μm in size (BioRad, Hercules, CA) were dried by placing 30 mg particles in a glass vial and heating them in an oven at 180°C for 12 h. The gold particles were surface sterilized with isopropanol, washed three times in sterile deionized distilled water, resuspended in 0.5 mL of 50% (v/v) glycerol, dispensed into 50 μL aliquots, and stored at 4°C until used. The gold particles were resuspended and

24 μg plasmid DNA (pBIN *35S-mgfp5-ER*) was added followed by 50 μL of 2.5 M $CaCl_2$ and 20 μL 0.1 M spermidine (Sigma Aldrich), and finally loaded onto macrocarrier disks. The final amount of DNA per shot was 3 μg.

Bombardment, apparatus and parameters

Pollen grains from three randomly chosen catkins were dispensed per pollen germination plate. To eliminate genotypic effect, the three catkins represented three different genotypes. Three plates were bombarded per treatment condition. Three trials were done for each experiment. Pollen grains were either bombarded immediately after dispensing on culture medium (ungerminated) or after 2–4 h of incubation at 25°C (germinated).

The biolistic device used was the prototype of the commercially available PDS-1000/He (BioRad) developed and located at the New York State Agricultural Experiment Station, Cornell University, Geneva, NY, USA. Bombardment was done immediately after DNA-coated gold particles were loaded onto macrocarriers to minimize DNA degradation and adhesion of gold particles to the membrane (Kikkert et al. 2005). In all experiments, the particle gun was set to the following conditions according to Kikkert et al. (2005): 1 cm distance between rupture disk and macrocarrier, 1 cm macrocarrier flight distance, and 28 in. Hg vacuum pressure. The effects of three target distances (5, 8, and 11 cm) and two helium pressures (1,000 and 1,100 psi) on transient GFP expression were examined.

Post-bombardment pollen handling and analysis of GFP expression

After bombardment, pollen was incubated at 25°C for 4 h and then overnight at 4°C to retard the growth of bacteria and fungi that impede observation. Examination for GFP expression was done using a Leica DMLB fluorescence microscope. GFP expression was detected under oil immersion objective and performed in the dark. To eliminate false positives, several complementary control measures were implemented including: (1) use of a Piston GFP filter set designed to minimize autofluorescence (Chroma Technologies Corp., Rockingham, VT). This filter set has excitation and emission spectra of 470±20 nm (blue light) and 515±15 nm (green light), respectively; (2) scoring of GFP expression only in pollen tubes since the pollen wall is highly autofluorescent; and (3) use of negative controls such as examining unbombarded pollen and pollen bombarded only with gold particles (no plasmid DNA included). Fresh onion leaves bombarded with pBIN *35S-mgfp5-ER* served as a positive control; this gave an indication of the activity of the plasmid DNA and success of bombardment conditions.

The percentage transient GFP expression was calculated based on the number of intensely green fluorescing pollen tubes out of the total number of germinated pollen grains counted. Per experiment, 150 germinated pollen grains were counted. Three replicate plates were done per experiment.

Pollen germination rates after various storage conditions were analyzed using multivariate ANOVA procedures and differences detected with Tukey's honestly significant difference test using SPSS software (SPSS Inc. 2002). Interactions between helium pressure, target distance, and pollen developmental stage were determined using multivariate analysis based on a three-way 2×2×3 factorial design.

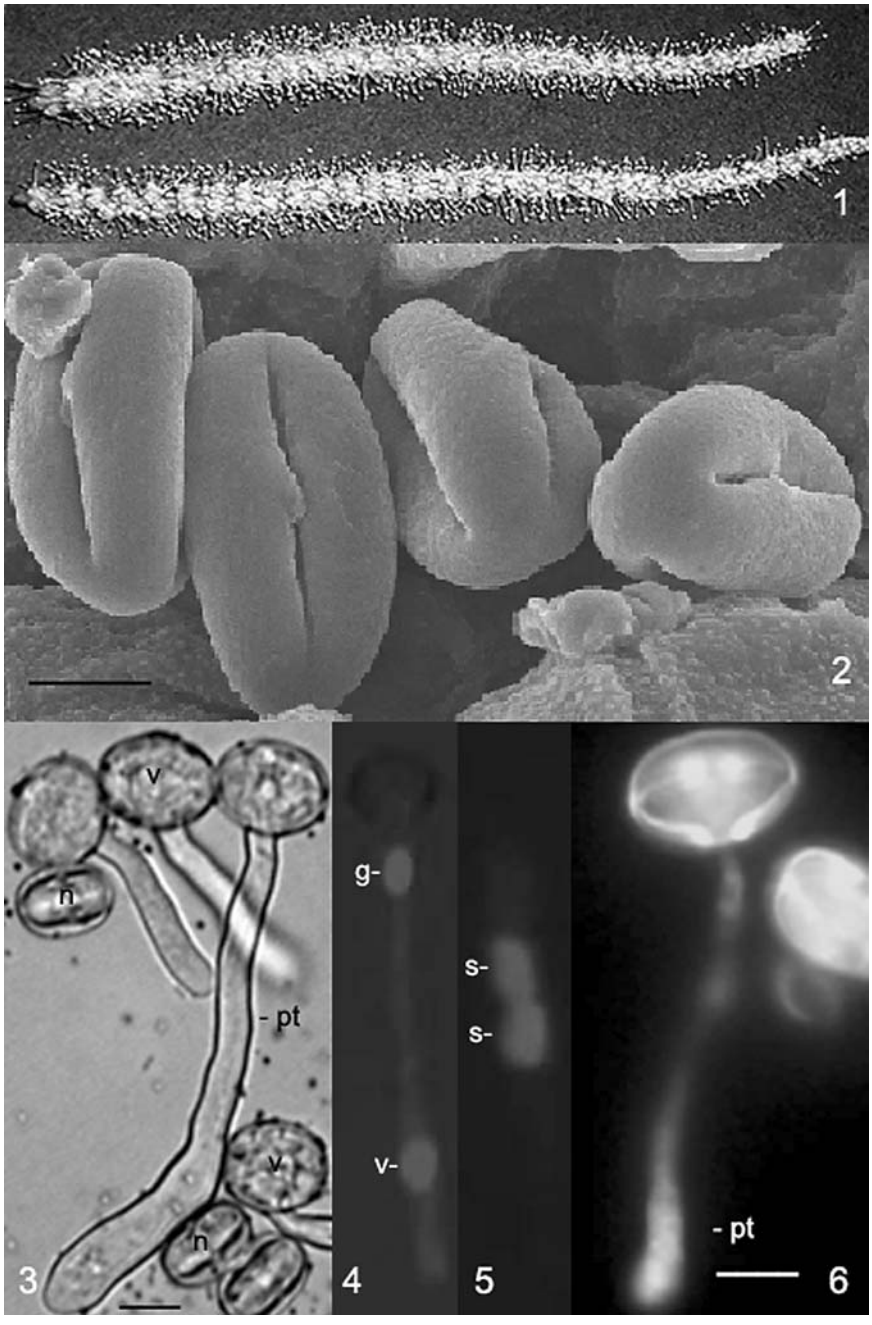
Results

The male catkin primordia were evident starting in early June, after the leaves were fully developed. Staminate flowers generally mature acropetally along a catkin, i.e., the first flowers to open are usually those at the proximal end, while the last ones are usually those at the distal end (Fig. 1). Male flowers are creamy-white to pale yellow and hairy in appearance due to the anthers and very long filaments, respectively. The flowers are strongly scented when fully developed. Maturation of the male catkins at Heiberg Memorial Forest began in early to mid-July and was completed by late July. By early August, all anthers of American chestnut had dehisced.

Dry pollen grains of American chestnut have a deflated appearance and are 15 μm in length (Fig. 2). Once hydrated, the pollen expands and assumes an oval to more or less round shape (Fig. 3). Non-germinating pollen grains do not expand as much as the germinated pollen (Fig. 3). At anther dehiscence, American chestnut pollen grains are binucleate, containing generative and vegetative nuclei; following germination in culture, the generative nucleus divides to form two sperm cells (Figs. 4 and 5).

Of the four different methods for collecting pollen grains from catkins that were tested, the use of catkins cut into small segments and rolled on culture medium resulted in large amounts of evenly dispersed pollen with minimal accessory floral parts. This technique also involved the least amount of steps. With the glass plate method, the pollen grains adhered to the glass plate and razor blade; therefore collection of large numbers of pollen for *in vitro* experiments was difficult. Also, leaving the catkins overnight rendered all pollen nonviable. When catkins were shaken over a cardboard folder, reasonably large quantities of pollen were collected, but substantial accessory floral materials were also included making pollen examination and counting difficult. Sieving the accessory floral parts resulted in the loss of many pollen grains.

The developmental stage of the catkins directly impacted pollen collection. Attempts to collect pollen from immature, greenish catkins were futile, as minimal pollen was collected. Efforts to collect pollen from expired, bright yellow to brown male catkins resulted in small numbers of pollen that were not viable. In addition, pollen was mixed with large amounts of accessory materials such as anthers and filaments. Therefore, catkins that were creamy-white to pale yellow as described earlier were optimal for pollen collection with reduced accessory floral parts.



Figs. 1–6 Development and transient GFP expression of the male reproductive structures of American chestnut. **Fig. 1** – Male catkins showing anthesis in acropetal sequence. **Fig. 2** – Scanning electron micrograph of pollen grains (*Bar*=4 μm). **Fig. 3** – Germinated (v) and ungerminated (n) pollen showing pollen tube (pt), subglobose shape of viable pollen (v), and unexpanded nonviable *pollen* (n) (*Bar*=10 μm). **Fig. 4** – DAPI stained pollen tube showing a generative (g) and a vegetative nucleus (v). **Fig. 5** – DAPI stained pollen tube showing two sperm cells (s). **Fig. 6** – Pollen tube showing transient GFP expression (*Bar*=7 μm)

American chestnut pollen grains were successfully germinated on a modified Brewbaker and Kwack (1963) medium. Using freshly collected pollen, germination was observed as early as 30 min after dispensing on solid pollen germination medium and incubating at 25°C in the dark. With pollen stored at 4°C, germination occurred within 1 h of dispensing on germination medium while pollen stored at −20°C or −80°C took 3 h or more to germinate. To compensate for these differences and maximize germination, scoring of pollen for germination rates or GFP expression was done after overnight incubation.

Substantial variations were recorded in the germination rates of American chestnut pollen from trial to trial from each of the storage conditions examined (Table 1). Genotypic differences were not tested as a source of variation because mixed pollen sources (from three genotypes) were used in all germination tests and bombardment experiments. Storage at 4°C for 2 weeks resulted in a significantly higher germination rate (48%) compared to all the other storage treatments examined (Table 1). The lowest mean germination percentage (8%) was found in pollen that was directly stored for 1 year at −20°C; this was significantly lower than all the other storage treatments examined (Table 1). Storage of catkins at 4°C for 1 month was tested but resulted in poor germination rates since the pollen dried up too much or microbes developed

Table 1 The effects of catkin storage conditions on percentage pollen germination in American chestnut

Storage conditons	Directly stored	With initial storage at 4°C for 2 weeks
Freshly collected	33±9 a	33±9[a] a
4°C for 2 weeks	48±8 b	48±8 b
−20°C for 2 weeks	20±6 c	25±7 b
−80°C for 2 weeks	32±5 a	43±4 c
−20°C for 1 year	8±7 d	10±8 d
−80°C for 1 year	19±5e	27±5e

[a]Mean percent germination ± standard error. Values within each column that are followed by the same letter do not differ significantly at $p<0.05$ ($n=3000$)

excessively on the catkins (data not shown). Higher percentage viability was achieved from those pollen, which were initially stored at 4°C for 2 weeks (Table 1).

GFP expression, based on the implementation of several control measures, was observed as intensely green glowing pollen tubes (Fig. 6). Mean percentage GFP expression in each of the three target distances bombarded at 1,000 and 1,100 psi using germinated and ungerminated pollen is presented in Fig. 7. In the pollen tubes, which were unbombarded or bombarded with gold particles only, GFP expression was not observed.

Transient GFP expression was higher at 1,100 psi in both ungerminated and germinated pollen as compared to 1,000 psi ($p<0.0001$). Transient GFP expression was observed in all target distances in both germinated and ungerminated pollen. In spite of the apparent differences in the mean percent transient GFP expression between the three target distances examined (Fig. 7), statistical analysis indicated that there were no significant differences among these settings ($p<0.342$).

To examine the relationship between bombardment parameters and pollen developmental stage, transient GFP expression was compared for bombarded ungerminated and germinated pollen. Mean percent transient GFP expression was higher when ungerminated pollen was bombarded as compared to bombarding germinated pollen in most treatments ($p<0.023$). At 1,100 psi, bombarding ungerminated pollen yielded a higher mean percent transient GFP expression in all the three target distances compared to using germinated pollen.

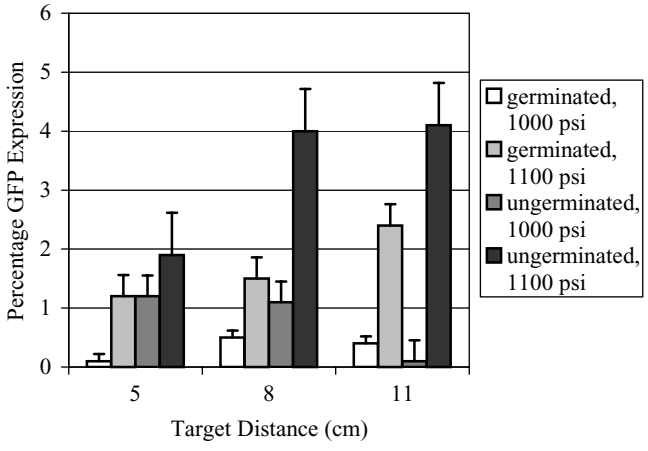


Fig. 7 Effects of target distance and helium pressure on GFP expression using germinated and ungerminated American chestnut pollen

Discussion

Collection of large quantities of American chestnut pollen was very difficult because of their small size, limited amount, nonuniform developmental stage along a catkin and between catkins, and short-term viability. It was also complicated by the presence of large quantities of accessory floral parts even after following published protocols (Jaynes 1974; Rutter 1990; Maynard 1991). Similar results were achieved from three of the four techniques that were tested in this study. However, putting catkin segments directly on the culture plate and rolling them over the medium resulted in large amounts of pollen grains that were evenly dispersed and virtually free of accessory floral parts. In other woody species such as poplars (Stanton and Villar 1996), willows (Kopp et al. 2002), and conifers (Bramlett and Matthews 1991; Fernando et al. 1998, 2000) collection of large quantities of pollen is routine and high germination rates are typically achieved.

Catkin storage conditions affected pollen viability. The mean percentage pollen germination in freshly collected catkins was 33%. This represents the typical amount of viable pollen available per catkin at the stage of collection. In American chestnut, we have observed non-uniform developmental stages of pollen grains along a catkin, i.e., some pollen grains have already dried-up and others have just dehisced, while the rest are still enclosed within the anthers. The proportion between these stages varied from day to day resulting in a wide range of germination rates (6–68%). Mean percentage pollen germination (48%) was higher in catkins that were stored at 4°C for 2 weeks. Storage at 4°C seemed to prolong the viability of pollen grains available at the time of collection. It also probably allowed more pollen grains, which were still enclosed within the anthers during collection to be released during storage, therefore increasing the total number of viable pollen grains. Short-term storage at 4°C also improved the viability of pistacio (*Pistacia vera*) pollen (Vaknin and Eisikowitch 2000).

Storage of American chestnut pollen directly at −20°C for as short as 2 weeks significantly reduced pollen viability. Reduced pollen viability also directly correlated with the duration of storage. The sensitivity of American chestnut pollen to storage and handling has been previously reported and believed to be related to the moisture levels in the catkins (Rutter 1990; Maynard 1991). Viability of American chestnut pollen is reduced both by freezing samples that are too wet and by allowing the pollen to become too dry. Maynard (1991) reported that allowing catkins to dry overnight resulted in the complete loss of viability, and this was confirmed by our study. In flowering dogwood (*Cornus florida*), storage of pollen at −20°C also resulted in drastic reduction in germination (Craddock et al. 2000).

If pollen viability is maximized prior to storage at subfreezing temperatures, the recovery of viable pollen after storage is also maximized. Therefore, to store American chestnut pollen, it is recommended that the catkins be placed initially at 4°C for 2 weeks followed by storage at −80°C. Extended storage at 4°C will dry up the catkins excessively causing cessation of pollen viability or the catkins become overgrown with microbes. The use of low humidity environment conferred by frost-free freezers or desiccants may also help extend pollen viability (Hanna 1994).

Optimization of a protocol for transforming American chestnut pollen grains required the selection of a suitable reporter gene. GFP was chosen since its detection does not require a cofactor or substrate (Prasher 1995) and can be easily done under a fluorescence microscope (Cubitt et al. 1995). However, the principal advantage of using GFP in this study was that transformed pollen could be analyzed in a nondestructive assay. Maintaining pollen viability while screening for transient transformation events is essential if the pollen grains are to be used in controlled pollinations. As previously reported, GFP does not seem to affect the viability or normal development of pollen (Ottenschlager et al. 1999; Hudson et al. 2001 Hudson and Stewart 2004). The normal progression of germination and tube development with incorporated GFP strongly suggests that transformed American chestnut pollen would be viable for use in artificial pollinations and subsequent fertilization of receptive female flowers.

American chestnut pollen grains were examined 4 h after bombardment but no GFP expression was observed; expression was observed only after an additional overnight incubation. Therefore, examination for GFP in our study was done 16–24 h post-bombardment. In the pollen of *Tradescantia paludosa* and *N. tabacum*, initial GFP expression was detected as early as 3 h postbombardment, but highest GFP accumulation, and therefore strongest GFP expression occurred between 15 and 60 h postbombardment (Keller and Hamilton 1998; Wang et al. 1998).

The promoter used to drive GFP expression in American chestnut pollen was the constitutive promoter *CaMV 35S*. This promoter has also been successfully used to drive reporter gene expression in pollen grains of a wide range of species (Nishihara et al. 1993; Hay et al. 1994; Li et al. 1994; Fernando et al. 2000; Aronen et al. 2003). However, higher gene expression has been reported when pollen or anther-specific promoters have been used in place of constitutive promoters (Twell et al. 1989; Barinova et al. 2002). It may be of interest for future work on microprojectile bombardment of American chestnut pollen to investigate whether the use of pollen-specific promoters such as *ZM13* or *LAT52* would enhance reporter gene expression. In addition, it may be beneficial for the restoration of American chestnut to limit reporter gene expression in the pollen grains.

Helium pressure and developmental stage of American chestnut pollen significantly affected transient GFP expression, whereas target distance did not. GFP expression was significantly higher when 1,100 psi was used compared to 1,000 psi. Russell et al. (1992) reported that higher bombardment pressures resulted in increased transient gene expression in *N. tabacum*. However, higher pressures cause more cell damage and death in American chestnut. In *Picea glauca* pollen, optimal reporter gene expression was observed between 400 and 900 psi (Li et al. 1994). Helium pressures of 900 and 1,300 psi were recommended for pollen of *Pinus sylvestris* and *Picea abies*, respectively (Haggman et al. 1997). Highest efficiency transient expression in pollen of *Antirrhinum majus* occurred at 1,000 psi (Barinova et al. 2002). Optimization of bombardment parameters such as helium pressure and target distance is clearly important because no single setting is best for pollen of all species. The bombardment parameters that worked for American chestnut pollen are within the range of what was reported in other species.

In many woody species, ungerminated pollen grains are the preferred targets for particle bombardment (Hay et al. 1994; Haggman et al. 1997; Fernando et al. 2000; Aronen et al. 2003), and it is also the case with American chestnut. In germinated pollen, the pollen tube is fragile and has a slow growth rate since considerable pollen tube length has already been attained at the time of bombardment. On the other hand, ungerminated pollen grains are still protected by their tough pollen wall and have to go through a rapid growth rate. Therefore, they would be expected to produce greater amounts of GFP.

Results of this study may be useful in the production of large quantities of transformed American chestnut pollen. These pollen grains can be individually picked amongst untransformed pollen through the use of a micromanipulation facility. The collected transformed pollen can be applied to the stigma to attempt to generate transgenic American chestnuts seeds.

Acknowledgements The authors thank: Drs. William Powell, Charles Maynard, and Haiying Liang for their help in cloning of plasmid DNA, allowing access to the trees at Heiberg, and much other support throughout the project; Drs. Jim Haseloff and John Runions for providing the *GFP* construct; Dr. Jose Vidal for providing guidance on bombardment; and Dr. Bruce I. Reisch for supporting the project and allowing access to his laboratory. The collaboration between laboratories of BIR and DDF was made possible through the Edna Bailey Sussman Fund awarded to JLR. Funding for this project was provided by a grant from the New York Chapter of the American Chestnut Foundation to DDF.

References

Anagnostakis SL (1987) Chestnut blight: the classical problem of an introduced pathogen. Mycologia 79:23–37

Aronen TS, Nikkanen TO, Haggman HM (2003) The production of transgenic Scots pine (*Pinus sylvestris* L.) via the application of transformed pollen in controlled crossings. Transgenic Res 12:375–378

Aziz N, Machray GC (2003) Efficient male germ line transformation for transgenic tobacco production without selection. Plant Mol Biol 51:203–211

Barinova I, Zhexembekova M, Barsova E, Lukyanov S, Heberle-Bors E, Touraev A (2002) *Antirrhinum majus* microspore maturation and transient transformation *in vitro*. J Exp Bot 53:1119–1129

Berry FH (1954) Chestnut blight and resistant chestnuts. U S Dep Agric Bull 2068:1–21
Bramlett DL, Matthews FR (1991) Storing loblolly pollen. South J Appl For 15:153–157
Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50:859–865
Carraway DT, Wilde HD, Merkle SA (1994) Somatic embryogenesis and gene transfer in American chestnut. J Am Chestnut Found 8:29–33
Craddock JH, Reed SM, Schlarbaum SE, Sauve RJ (2000) Storage of flowering dogwood (*Cornus florida L.*) pollen. Hortscience 35:108–109
Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995) Understanding, improving, and using green fluorescent proteins. Trends Biochem Sci 20:448–455
Fernando DD, Owens JN, von Aderkas P (1998) *In vitro* fertilization from co-cultured pollen tubes and female gametophytes of Douglas fir (*Pseudotsuga menziesii*). Theor Appl Genet 96:1057–1063
Fernando DD, Owens JJ, Misra S (2000) Transient gene expression in pine pollen tubes following particle bombardment. Plant Cell Rep 19:224–228
Haggman HM, Aronen TS, Nikkanen TO (1997) Gene transfer by particle bombardment to Norway spruce and Scots pine pollen. Can J For Res 27:928–935
Hanna WW (1994) Pollen storage in frostless and conventional frost-forming freezers. Crop Sci 34:1681–1682
Hay I, Lachance D, von Aderkas P, Charest PJ (1994) Transient chimeric expression in pollen of five conifer species following microparticle bombardment. Can J For Res 24:2417–2423
Hebard FV (2001) Backcross breeding program produces blight-resistant American chestnuts (Virginia). Ecol Restor 19:252–254
Hudson LC, Stewart CN (2004) Effects of pollen-synthesized green fluorescent protein on pollen grain fitness. Sex Plant Reprod 17:49–53
Hudson LC, Chamberlain D, Stewart CN Jr (2001) GFP-tagged pollen to monitor gene flow of transgenic plants. Mol Ecol Notes 1:321–324
Jaynes RA (1974) Genetics of chestnut. Forest Service Paper WO-17. U.S. Department of Agriculture, Washington, D.C
Keller NL, Hamilton DA (1998) Transient expression of the green fluorescent protein. Sex Plant Reprod 11:163–165
Kikkert JR, Vidal JR, Reisch BI (2005) Stable transformation of plant cells by particle bombardment/biolistics. In: Peña L (ed) Methods in molecular biology, vol 286: transgenic plants: methods and protocols. Humana, Totowa, NJ, pp 61–78
Kopp RF, Maynard CA, de Niella PR, Smart LB, Abrahamson LP (2002) Collection and storage of pollen from *Salix* (Salicaceae). Am J Bot 89:248–252
LaPierre SL (2003) Studies in American chestnut (*Castanea dentata* Marsh. (Borkh.)) micropropagation and acclimatization. Masters thesis, SUNY College of Environmental Science and Forestry, Syracuse, NY
Li YH, Tremblay FM, Seguin A (1994) Transient transformation of pollen and embryogenic tissues of white spruce (*Picea glauca* (Moench.) Voss) resulting from microprojectile bombardment. Plant Cell Rep 13:661–665
Liang H, Maynard CA, Allen RD, Powell WA (2001) Increased *Septoria musiva* resistance in transgenic poplar leaves expressing a wheat oxalate oxidase gene. Plant Mol Biol 45:619–629
Liang H, Catranis CM, Maynard CA, Powell WA (2002) Enhanced resistance to the poplar fungal pathogen, *Septoria musiva*, in hybrid poplar clones transformed with genes encoding antimicrobial peptides. Biotechnol Lett 24:383–389
MacDonald WL, Fulbright DW (1991) Biological control of chestnut blight: Use and limitations of transmissible hypovirulence. Plant Dis 75:656–661
Maynard CA (1991) Chestnut pollen collection and handling. J Am Chestnut Found 5:101–106
Maynard CA, Xing Z, Bickel SL, Powell WA (1998) Using genetic engineering to help save the American chestnut: a progress report. J Am Chestnut Found 12:40–56
Merkle SA, Wiecko AT, Watson-Pauley BA (1991) Somatic embryogenesis in American chestnut. Can J For Res 21:1698–1700
Nishihara M, Ito M, Tanaka I, Kyo M, Ono K, Irifune K, Morikawa H (1993) Expression of the β-glucuronidase gene in pollen of lily (*Lilium longiflorum*), tobacco (*Nicotiana tabacum*), *Nicotiana rustica*, and peony (*Paeonia lactiflora*) by particle bombardment. Plant Physiol 102:357–361
Ottenschlager I, Barinova I, Voronin V, Dahl M, Heberle-Bors E, Touraev A (1999) Green fluorescent protein as a marker during pollen development. Transgenic Res 8:279–294
Polin LD (2004) Biolistic transformation of American chestnut (*Castanea dentata* (Marsh.) Borkh.) somatic embryos using green fluorescent protein (GFP) as a scorable marker. Masters thesis, SUNY College of Environmental Science and Forestry, Syracuse, NY
Powell WA, Catranis CM, Maynard CA (1995) Synthetic antimicrobial peptide design. Mol Plant Microbe Interact 8:792–794
Prasher DC (1995) Using GFP to see the light. Trends Genet 11:320–323
Russell JA, Roy MK, Sanford JC (1992) Major improvements in biolistic transformation of suspension-cultured tobacco cells. *In Vitro* Cell Dev Biol 28:97–105
Rutter PA (1990) A chestnut pollinator's handbook. Badgersett Research Farm, Canton, MN
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, NY
SPSS, Inc. (2002) SPSS statistical algorithms, Version 11.5 for Windows. Chicago, IL
Stanton BJ, Villar M (1996) Controlled reproduction in *Populus*. In: Settler RF, Bradshaw HD Jr, Heilman PE, Hinckley TM (eds) Biology of *Populus* and its implications for management and conservation, Part I . NRC Research Press, National Research Council of Canada, Ottawa, Ontario, Canada, pp. 113–138
Touraev A, Fink CS, Stoger E, Heberle-Bors E (1995) Pollen selection: a transgenic reconstruction approach. Proc Natl Acad Sci USA 92:12165–12169
Touraev A, Stoger E, Voronin V, Heberle-Bors E (1997) Plant male germ line transformation. Plant J 12:949–956
Twell D, Klein TM, Fromm ME, McCormick S (1989) Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. Plant Physiol 91:1270–1274
Vaknin Y, Eisikowitch D (2000) Effects of short-term storage on germinability of pistachio pollen. Plant Breed 119:347–350
van der Leede-Plegt LM, van den Ven BCE, Schilder M, Franken J, van Tunen AJ (1995) Development of a pollen-mediated transformation method for *Nicotiana glutinosa*. Transgenic Res 4:77–86
Vidal JR, Kikkert JR, Wallace PG, Reisch BI (2003) High-efficiency biolistic co-transformation and regeneration of 'Chardonnay' (*Vitis vinifera* L.) containing *npt*II and anti-microbial peptides. Plant Cell Rep 22:252–260
Wang J, Shi HZ, Zhou C, Yang HY, Zhang XL, Zhang RD (1998) β-glucuronidase gene and green fluorescent protein gene expression in de-exined pollen of *Nicotiana tabacum* by particle bombardment. Sex Plant Reprod 11:159–162
Xing Z, Powell WA, Maynard CA (1999) Development and germination of American chestnut somatic embryos. Plant Cell Tissue Organ Cult 57:47–55