SHORT COMMUNICATION

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Effects of pollen-synthesized green fluorescent protein on pollen grain fitness

Abstract Genetically engineered pollen with a visible marker gene could be useful to monitor the movement of transgenic pollen provided there are no negative physiological or fitness effects of expressing such a gene. In this study, we measured the fitness of *Nicotiana tabacum* cv. Xanthi pollen expressing the marker gene green fluorescent protein (GFP). Average pollen tube germination frequencies and pollen tube growth rates in vitro were measured in three different types of plants: (1) plants producing GFP in pollen cells only (LAT59-GFP), (2) plants synthesizing GFP under the control of a constitutive promoter (CaMV 35S) in which no GFP was produced in pollen, and (3) non-transgenic plants. Pollen synthesizing the GFP protein did not differ significantly in average pollen germination frequencies from pollen without GFP ($P=0.65$). Average pollen tube growth rates over a 5-h period did not differ significantly between transgenic and non-transgenic types ($R^2=0.89$, 0.98, and 0.95, respectively, for GFP-tagged, 35S-GFP, and wild type). Overall, GFP expression in pollen grains of tobacco was not found to have an effect on pollen fitness under the controlled experimental conditions of this study.

Keywords *Nicotiana tabacum* · GFP · LAT59 promoter · Pollen tube · Pollen fitness

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Introduction

The male gametophyte of a flowering plant is a highly specialized tissue that is essential for reproductive success. A mature pollen grain is responsible for the recognition of a compatible stigma and delivery of sperm nuclei to the plant ovule (Cheung 1996). Tobacco pollen is essentially a bicellular structure, independent of the sporophyte, originating from two meiotic and one mitotic division. A tobacco pollen grain consists of only two cells—a vegetative nucleus and a generative cell—surrounded by a relatively thick exine cell wall (McCormick 1993). The rise of the haploid microspore after the first two meiotic divisions is considered to be the start of plant gametophyte gene expression and development, which makes the male gametophyte an attractive experimental system for various issues in plant molecular biology.

Being bicellular, tobacco pollen is an ideal model in the study of pollen tubes. Techniques for in vitro and semi vivo germination of pollen are well established in tobacco, and elongated tubes can be easily obtained (Mulcahy and Mulcahy 1985). *Arabidopsis thaliana* and some *Brassica* species are members of the 30% of angiosperm plants species that have tricellular pollen and do not germinate well in vitro (Preuss et al. 1993). In vitro growth of most tricellular pollen types subsides in less than 1 h; however, bicellular pollen tubes routinely elongate for over 5 h (Hoekstra 1979).

Although in vitro germination provides a controlled experimental environment, tube growth in vitro does not completely mimic growth in vivo. Under optimum culture conditions, in vitro pollen tubes reach approximately 30–40% of in vivo lengths (Read et al. 1993). Although pollen-pistil interactions are not duplicated in vitro, in many species, including tobacco, pollen germination and tube growth are robust under experimentally defined conditions, rendering in vitro-based studies relevant to the in vivo situation (Taylor and Hepler 1997).

As a molecular marker, green fluorescent protein (GFP) has many applications that could not have been imagined 10 years ago. GFP has been used as a molecular

marker in many organisms (Chiu et al. 1996; Muldoon et al. 1997) and has been optimized for use in plant systems (Chui et al. 1996; Pang et al. 1996; Reichel et al. 1996; Haseloff et al. 1997; Davis and Vierstra 1998; Haseloff 1999; Stewart 2001, and references therein). The expression of GFP in pollen has applications that include use as a marker for gene expression during pollen development (Benedikt et al. 1998), reproductive biology (Ottenschlager et al. 1999) and pollen dispersal patterns under field conditions to assess risk of gene flow and non-target effects (Hudson et al. 2001).

In order to use genetically engineered pollen for risk assessment studies, there is a need to assess the effects of recombinant foreign proteins in reproductive cells. If GFP is cytotoxic to pollen, its use as a pollen marker would be compromised. GFP cytotoxicity has been documented in mammalian cells (Liu et al. 1999), and could potentially have adverse affects on regulation or disruption of pollen cell development, leading ultimately to cell death.

The goal of this investigation was to test for any fitness costs caused by the presence of GFP in pollen cells of *Nicotiana tabacum* cv. Xanthi. In order to answer this question, pollen tube germination frequencies and growth rates were compared among the following: (1) tobacco pollen synthesizing GFP under the control of the tomato LAT59 pollen-specific promoter (GFP-tagged); (2) pollen from tobacco synthesizing GFP under the control of the constitutive CaMV 35S promoter (35S-GFP), in which no GFP is produced in pollen; and (3) a nontransgenic control.

Materials and methods

Plant material

Seeds collected from the GFP pollen T_0 generation were sterilized with a 70% ethanol solution for 1 min, 20% bleach with 0.001% Tween 20 for 8 min, then washed three times with sterile distilled water. Seeds were grown on MS medium (Murashige and Skoog 1962) containing kanamycin at 200 mg/l to select transgenic T_1 plants (Hudson et al. 2001). This process was repeated until seedlings from the T_3 generation were produced. These T_3 tobacco plants were homozygous for the GFP locus and five homozygous plants from a single transgenic event were used in this study. The plants were observed to be morphologically and reproductively normal as compared with nontransgenic plants.

Five 35S-GFP plants were also analyzed for fitness and subjected to the same selection regime. These plants express the GFP gene constitutively (Leffel et al. 1997; Harper et al. 1999); however, GFP was not synthesized in the pollen of the transgenic tobacco (Fig. 1). Five nontransgenic tobacco cv. Xanthi plants were also analyzed as a negative control.

Pollen germination frequency

Pollen from the three types of plants was extracted during anthesis at 9:00 am for 5 consecutive days by gently tapping flowers directly above Petri dishes. Pollen grains were then transferred from Petri dishes to Falcon plate wells, and 100 μl BK medium (Brewbaker and Kwack 1963) was added to three replicates per plant (one replicate per well). The Falcon plate was placed in an incubator at 25°C for 3 h. After 3 h, 15 μl BK medium with pollen grains was placed on a microscope slide for observation. Pollen tubes were counted at 100× magnification under a microscope. A digital camera (Olympus Q color 3) and imaging system was used to project the image onto a computer monitor for observation and easier tallying. Germination frequencies were obtained by taking the total number of pollen grains on the computer screen divided by the number of germinated pollen grains. The on-screen field of vision was chosen randomly for observation.

Pollen tube growth rate

The same plants were used to determine pollen tube growth rates. Pollen was extracted daily at 9:00 am during anthesis as before. Each replicate consisted of five Falcon plate wells, each representing a specific time point (1–5 h). At hourly intervals, 30 μl pollen/BK mixture was taken from the Falcon well and placed on a microscope slide. After photomicroscopy as above, pollen samples were discarded. This procedure was repeated for a total of 5 h. At each time point, fresh pollen tubes were measured on the computer monitor with a ruler; measurements were converted into actual lengths by using a Hausser Scientific Brightline Hemacytometer of known grid size. Pollen tube growth rate was calculated over a 5-h period for the three tobacco types.

Results

As expected, GFP fluorescence was observed in all pollen grains produced by the homozygous GFP-tagged tobacco plants when viewed using an epifluorescence microscope under blue light excitation observed using an FITC filter set. GFP fluorescence was also observed in the pollen tubes of GFP-tagged pollen, whereas its presence was not observed in 35S-GFP or nontransgenic tobacco pollen (Fig. 1).

Average pollen tube germination frequencies were calculated by taking the number of germinated pollen grains present after 3 h and dividing it by the total number of pollen assayed (Table 1). GFP-tagged, 35S-GFP and nontransgenic tobacco plants had average pollen tube percent germination frequencies of 52.7%±11.1, 57.1%±14.7, and 53.5%±11.2, respectively (Fig. 2). Analysis of variance test showed no significant differences among average pollen tube germination frequencies for each tobacco type (GFP-tagged, 35S-GFP, and nontransgenic) by day and by plant ($P=0.65$).

Pollen tube growth was observed over a 5-h period. Measurements were taken hourly from a total of 6,242 grains of GFP-tagged pollen, 6,980 grains of 35S-GFP pollen, and 6,228 grains of nontransgenic pollen. Regression analyses were performed on the three tobacco types in order to calculate an average pollen tube growth rate. Pollen from GFP-tagged pollen plants had an average tube length of 0.40±0.09 mm at 5 h and an average growth rate of 0.09 mm/h ($R^2=0.90$). Pollen from 35S-GFP plants had an average pollen tube length of 0.41±0.09 mm at 5 h and an average growth rate of 0.10 mm/h ($R^2=0.98$). Nontransgenic plant pollen had an average tube length of 0.40±0.05 mm and an average pollen tube growth rate of 0.09 mm/h; i.e., no significant differences ($R^2=0.95$) (Fig. 3).

Fig. 1A–F Green fluorescent protein (GFP) is detectable in pollen tubes of pollen-tagged GFP transgenic plants. **A**, **B** Germinated pollen from LAT59-GFP tobacco under white (**A**) and blue (**B**) light. **C**, **D** Germinated pollen from 35S-GFP tobacco under white (**C**) and blue (**D**) light. **E**, **F** Germinated pollen from nontransgenic tobacco plants under white (**E**) and blue (**F**) light. Pollen tubes were germinated in BK medium and pictures were taken under 100× magnification with a 16 ms exposure time under white light conditions (**A**, **C**, **E**), and 2.75 s under blue light (**B**, **D**, **F**)
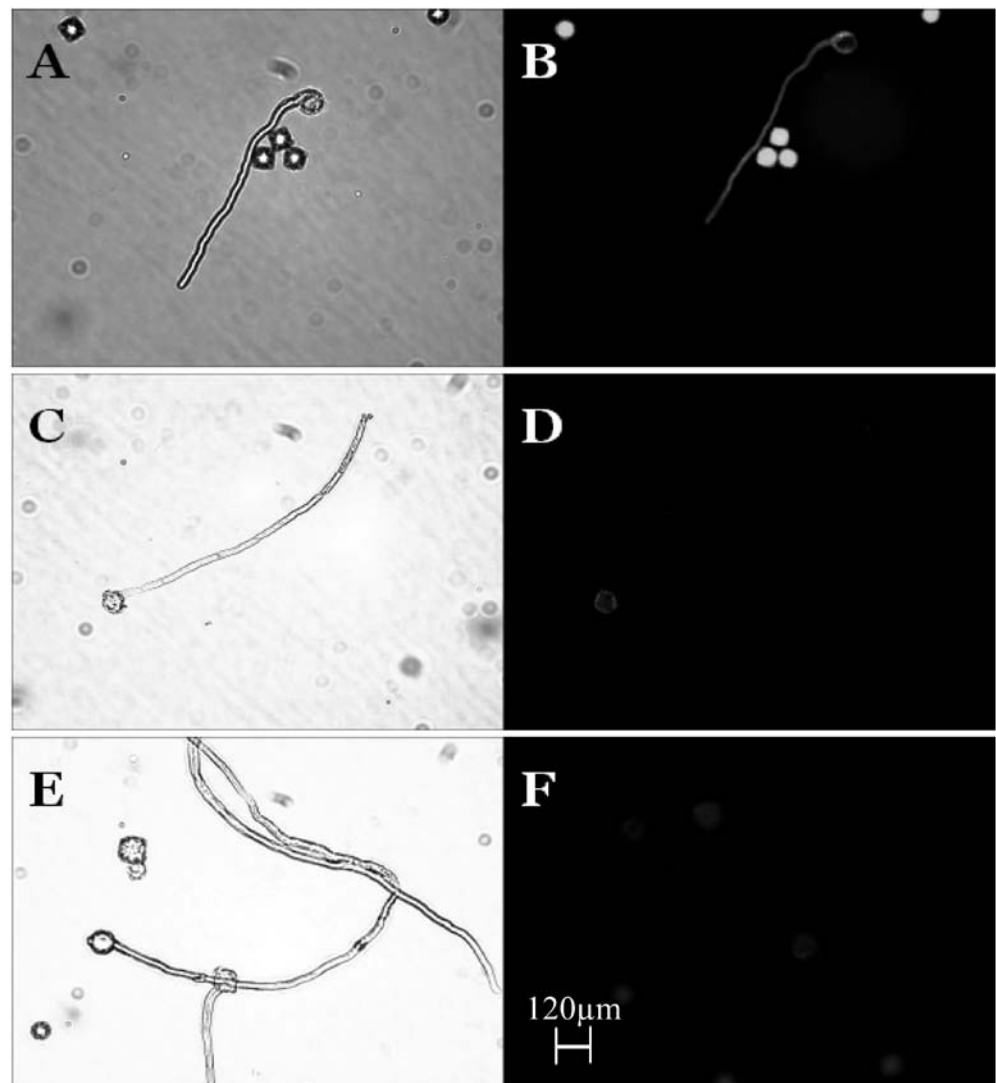

Table 1 Pollen grain germination frequency of three tobacco lines. Data were collected 3 h after treatment with germination medium, and represent five discrete samples from five different plants per line

Tobacco line	Total number pollen grains	Number of pollen grains germinated
LAT59-GFP	13,306	7,013
35S-GFP	13,807	8,080
Nontransgenic	13,678	7,606

Discussion

The synthesis of GFP under the control of the LAT59 pollen-specific promoter in pollen grains of *N. tabacum* cv. Xanthi does not decrease pollen fitness. Levels of GFP under the control of the LAT59 promoter are not toxic and do not hamper the development or function of tobacco pollen. The data suggest that GFP can be used as a marker of gene expression in plant reproductive biology for studies addressing growth of pollen tubes through the style, fertilization events, and development of zygotic embryos. GFP can also be used as a marker in pollen, thereby enabling monitoring of pollen movement under

field conditions to assess possible gene flow and non-target risks of transgenic crops. In our results, green fluorescence was evenly distributed in the cytoplasm of all pollen grains from plants expressing *mgfp5-er*, which is targeted to the endoplasmic reticulum, under the control of the LAT59 pollen-specific promoter in transformed homozygous tobacco plants. Fluorescence of GFP was expressed in the mature tube of GFP-tagged pollen. This was expected, and is consistent with data indicating that the LAT59 gene is expressed after microspore mitosis and reaches a maximum expression level in mature microspores (Mascarenhas 1990). The LAT59 gene also encodes a pectin-degrading enzyme (Wing et al. 1989)
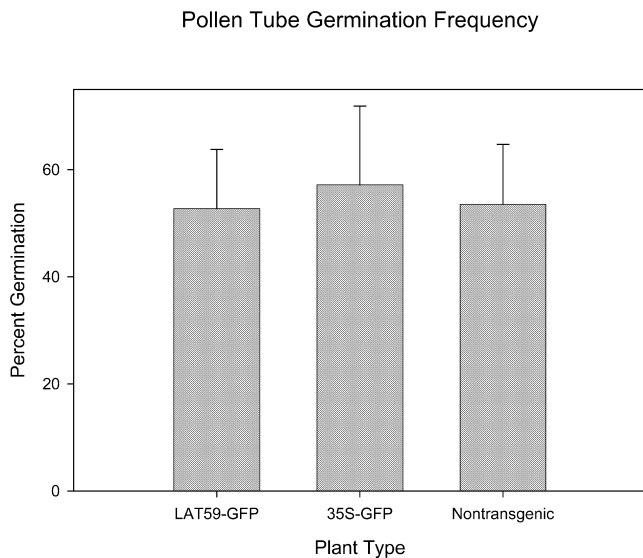
Pollen Tube Germination Frequency

Fig. 2 Average percent germination frequency (±SD) of pollen from five plants per type after 3 h in BK pollen tube germination medium
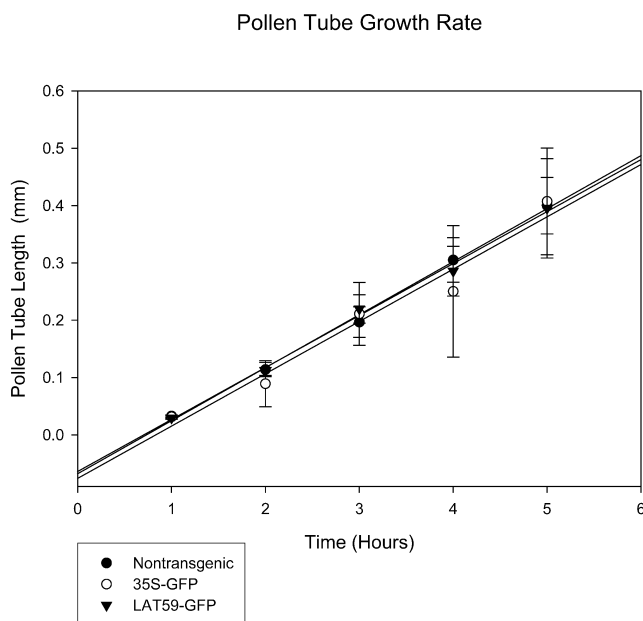
Pollen Tube Growth Rate

Fig. 3 Regression analysis of average pollen tube growth rates in BK germination medium for LAT59-GFP 35S-GFP, and nontransgenic plants were 0.092 mm/h (R^2=0.90), 0.103 mm/h (R^2=0.98), and 0.094 mm/h (R^2=0.95), respectively. Samples were taken from five plants per type

whose presence would be required for pollen tube synthesis and solubilization of cell wall material during the emergence and rapid growth of the pollen tube through the pistil.

High levels of GFP expression have been reported to have a detrimental effect on living cells (Liu et al. 1999) including pollen. In one study, pollen having very bright GFP fluorescence contained thick actin cables, had slow cytoplasmic streaming, and tended to cease growth prematurely (Benedikt et al. 1998). However, the negative effect may be attributed to the talin protein that was fused with GFP (used mark actin filaments) and not to GFP per se. Harper et al. (1999) showed that whole plant expression of GFP had no fitness costs to field-grown tobacco plants. Consistent with this latter study, in which the same GFP variant was used, the presence of GFP in pollen did not impair pollen development, pollen germination, or tube growth. Our findings are supported by previous work by Ottenschlager et al. (1999) who found no toxic effects of GFP expression controlled by other pollen-specific promoters in the pollen of several different plant species including tobacco. In this study, average pollen tube germination frequencies of GFP-tagged pollen were not significantly different from those of non-fluorescent pollen, and there was little variation between plants or between days on which pollen extraction took place. Pollen tube growth rates between the three types were statistically consistent and, at 5 h, pollen tubes from each type had grown to approximately the same lengths. GFP expression was not detected in 35S-GFP pollen, as also observed in previous studies using the same promoter (Twell et al. 1989; Mascarenhas and Hamilton 1992; Stoger et al. 1992; Wilkinson et al. 1997).

The promoters used to regulate transgenes in pollen could be important in agricultural applications of transgenic crops. These promoters could be used as tools to assess the dispersion of pollen of transgenic crops within the environment and patterns of successful germination. The LAT59 promoter isolates expression of GFP solely to pollen grains and could be used as a model system to track pollen movement in the field, an important component of introgression from transgenic crops, which is of significant concern (Stewart et al. 2003). According to this study, GFP has no effect on tobacco pollen, and GFP-expressing pollen can easily be distinguished from wild type pollen. GFP-tagged pollen is therefore a good candidate for use in model systems to determine outcrossing distances by tracking pollen flow in the field, or to determine any effects that the presence of a non-endogenous protein in pollen could have on pollinating insects.

References

Benedikt K, Spienlhofer P, Chua N (1998) A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes. Plant J 16:393–401

Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and tube growth. Am J Bot 50:589–865

Cheung AY (1996) Pollen-pistil interactions during pollen tube growth. Trends Plant Sci 1:45–51

Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6:325–330

Davis SJ, Vierstra RD (1998) Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis thaliana*. Plant Mol Biol 36:521–528

Harper BK, Mabon SA, Leffel SM, Halfhill MD, Richards HA, Moyer KA, Stewart CN Jr (1999) Green fluorescent protein as a marker for expression of a second gene in transgenic plants. Nat Biotechnol 17:1125–1129

Haseloff J (1999) GFP variants for multispectral imaging of living cells. In: Kay S, Sullivan K (eds) Methods in cell biology, vol 58. Academic Press, New York, pp 139–151

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci USA 94:2122–2127

Hoekstra FA (1979) Mitochondrial development and activity of binucleate and trinucleate pollen during germination in vitro. Planta 145:25–36

Hudson LC, Chamberlain D, Stewart CN Jr (2001) GFP-tagged pollen to monitor pollen flow of transgenic plants. Mol Ecol Notes 1:321–324

Leffel SM, Mabon SA, Stewart CN Jr (1997) Applications of green fluorescent protein in plants. Biotechniques 23:912–918

Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ (1999) Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 260:712–717

Mascarenhas JP (1990) Gene activity during pollen development. Annu Rev Plant Physiol Plant Mol Biol 43:317–338

Mascarenhas JP, Hamilton DA (1992) Artifacts in the localization of GUS activity in anthers of Petunia transformed with a CaMV 35S-GUS construct. Plant J 2:405–408

McCormick S (1993) Male gametophyte development. Plant Cell 5:1265–1275

Mulcahy GB, Mulcahy DL (1985) Ovarian influences on pollen tube growth, as indicated by the semivivo technique. Am J Bot 72:1078–1080

Muldoon RR, Levy JP, Kain SR, Kitts PA, Links CJ Jr (1997) Tracking and quantification of retroviral-mediated transfer using a completely humanized, red-shifted green fluorescent protein gene. Biotechniques 22:162–167

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Ottenschlager I, Barinova I, Voronon V, Dahl M, Heberle-Bors E, Touraev A (1999) Green fluorescent protein (GFP) as a marker during pollen development. Transgenic Res 4:279–294

Pang S-Z, DeBoer DL, Wan Y, Ye G, Layton JG, Neher MK, Armstrong CL, Fry JE, Hinshee MA, Fromm ME (1996) An improved green fluorescent protein gene as a vital marker in plants. Plant Physiol 112:893–900

Preuss D, Lemieux B, Yen G, Davis RW (1993) A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signaling during fertilization. Genes Dev 7:974–985

Read SM, Clark AE, Bacic A (1993) Stimulation of growth of cultured *Nicotiana tabacum* W38 pollen tubes by polyethylene glycol and Cu salts. Protoplasma 177:1–14

Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, Schell J, Reiss B, Maas C (1996) Enhanced green fluorescence protein mutant in mono- and dicotyledonous plant cells. Proc Natl Acad Sci USA 93:5888–5893

Stoger E, Benito-Moreno RM, Ylstra B, Vicente O, Bors EH (1992) Comparison of different techniques for gene transfer into mature and immature tobacco pollen. Transgenic Res 1:71–78

Stewart CN Jr (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep 20:376–382

Stewart CN Jr, Halfhill MD, Warwick SI (2003) Transgene introgression from genetically modified crops to their wild relatives. Nat Rev Genet 4:806–817

Taylor LP, Hepler PK (1997) Pollen germination and tube growth. Annu Rev Plant Physiol Plant Mol Biol 48:461–491

Twell D, Wing RA, Yamaguchi J, McCormick S (1989) Isolation and expression of an anther-specific gene from tomato. Mol Gen Genet 217:240–245

Wilkinson JE, Twell D, Lindsey K (1997) Activities of CaMV35S and nos promoters in pollen: implications for field release of transgenic plants. J Exp Bot 48:265–275

Wing RA, Yamaguchi J, Larabell SK, Ursin VM, McCormick S (1989) Molecular and genetic characterization of two pollen expressed genes that have sequence similarity to pectate lyases of the plant pathogen *Erwinia*. Plant Mol Biol 14:17–28