

## Transgene Dispersal Through Pollen

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### Summary

Techniques used for the transfer of novel genes into host plant genomes have created new possibilities for crop improvement. The implementation of transgenic crop species into agriculture has introduced the possibility of transgene escape into the environment via pollen dispersal. Although the movement of pollen is a critical step in transgene escape, there is currently no system to monitor transgenic pollen movement under field conditions. The development of an effective *in vivo* monitoring system suitable for use under field conditions is needed for research and commercial purposes so potential risks can be quantified and evaluated. This chapter describes the development of a model system using green fluorescent protein (GFP) expression in pollen as a marker to monitor pollen distribution patterns. A pollen specific promoter was used to express the GFP gene in tobacco (*Nicotiana tabacum* L.). GFP was visualized in pollen and growing pollen tubes using fluorescent microscopy. Furthermore, the goal of this research was to compare the dynamics of pollen movement with that of gene flow by using another method of whole plant expression of GFP (see Chapter 15) to estimate out-crossing frequencies by progeny analysis. Pollen movement and gene flow were quantified under field conditions. Pollen traps were collected and screened for presence of GFP-tagged pollen using fluorescence microscopy. Progeny from wild type plants were screened with a hand held ultraviolet light for detection of the GFP phenotype.

**Key Words:** Gene flow; green fluorescent protein; *Nicotiana tabacum*; out-crossing; pollen flow; transgenic.

### 1. Introduction

Over the past decade, the use of molecular techniques in plant breeding has led to the widespread use of transgenic crops in agriculture. These technological advances present new opportunities for developing plants that are resistant to pests and diseases, better able to withstand stressful environments, and have

the capacity to produce better quality food products. As with many technical advances in agriculture and biotechnology, concerns are raised about the potential consequences of these developments to the environment.

One of the principal concerns of genetically modified crops is the likelihood and possible consequence of the introduced transgenes being transferred through pollen dispersal to wild relatives or nontransgenic crops. For pollen-mediated gene flow to occur among plant populations, dispersal of pollen to a different population must occur with successful fertilization of an ovule. Therefore, a complete description of gene flow in plants must include an assessment of the relative importance of pollen as the agent of gene flow. Currently, there are few systems for the direct monitoring of pollen movement under field conditions. Previous attempts to measure gene flow have evolved around the analyses of genetic markers (1). For instance, population genetic structure gathered from isozyme surveys that can be fit to data models of population differentiation have been used (2). Other research approaches have concentrated exclusively on gene flow by using paternity exclusion analysis (3–6) or microsatellite markers (7). These systems have limitations because they are species-specific, requiring the use of expensive assays that cannot yield results in real time or in the field. More recently, visual markers such as GFP have been proposed for use, using whole plant expression to monitor gene flow under agricultural conditions (8–10). This method has been used successfully to assess out-crossing events in canola (*Brassica napus*) under field conditions (11).

A direct method could be the use of GFP-tagged pollen to monitor pollen movement under field conditions. This system would allow the quantification of pollen flow directly from a group of individuals in the field and would determine the distance and directional patterns of pollen dispersal within a plant population. GFP expression in plant pollen will not only enable the tracking of pollen movement but also can be used to differentiate between pollen from individual plants of the same species. GFP-tagged pollen could also be used to assess multiple pollination mechanisms. Because GFP can be expressed in pollen under the control of a pollen specific promoter, a system to monitor and detect pollen distribution and gene flow patterns can be developed on a large scale, thus revealing answers to many questions involving ramifications of the introgression of transgenic crop species into the environment and to evaluate the adequacy of current isolation distances for the prevention of outcrossing.

In current research, we used the pollen-specific LAT59 promoter to express GFP in pollen grains of tobacco (*N. tabacum*), an easily engineered model plant (12). The tomato LAT59 promoter (13) is a pollen-active promoter that is preferentially expressed in the anthers and pollen of tomatoes (13). The LAT59 promoter controls the expression of a gene that shows similarity to two regions

found to have conserved sequences between all *Erwinia* pectate lyases. LAT59 has a 61 and 54% similarity to regions I and II, respectively, which have been described for seven pectate lyases of *Erwinia chrysanthemi* and *E. carotovora* (14). To compare pollen movement in the field with actual out-crossing events, we describe methods to determine pollen-mediated gene flow of transgenic tobacco to nontransgenic tobacco at various distances from a source population using whole plant expression of GFP. Gene flow was determined by screening progeny from wild type recipient plants growing at various distances from the source population.

This chapter describes experimental methods on the construction of a transformation vector using a pollen specific promoter to express GFP in pollen grains. It also discusses the instrumentation and methods used to visualize GFP in pollen and pollen tubes. We describe an experimental field design, which can be used to track pollen movement under field conditions with pollen-tagged tobacco or similar plants. Finally, we describe the use of whole plant expression of GFP to detect gene flow under field conditions.

## 2. Materials

### 2.1. Plasmid Construction

1. Plasmid construct pBINmGFP5-ER (15) containing the CaMV35s promoter and an *nptII* kanamycin resistance cassette.
2. Plasmid construct containing the LAT59 promoter (13).
3. Restriction enzymes *Hind*III and *Bam*HI (Promega, Madison, WI).
4. T4 DNA ligase (Promega).

### 2.2. Plant Transformation

1. Surface sterilized seeds (20% bleach and 0.001% Tween-20 solution for 8 min) from *Nicotiana tabacum* cv "Xanthi."
2. Murashige and Skoog (MS) basal media (16) is used for seed germination.
3. All plant medium use 0.2% Gelrite gellan gum as a solidifying agent, and all agents are autoclaved prior to media being poured into plates with the exception of antibiotics.
4. *Agrobacterium tumefaciens* strain GV 3850 containing the pBINDC1 expression vector (12) with the LAT59 pollen specific promoter controlling the mGFP5-ER gene along with an *nptII* cassette for kanamycin selection.
5. DBI medium containing 1 mg/L of indoleacetic acid for tobacco shoot organogenesis (leaf as explant source).
6. MSO medium for rooting.
7. Antibiotics kanamycin (Sigma, St. Louis, MO) and timentin (GlaxoSmithKline, Philadelphia, PA).
8. 100-mm Petri dishes and GA 7 Magenta boxes for tissue culture.
9. Laminar flow hood.

### 2.3. Fluorescence Microscopy

1. An epifluorescence microscope (Olympus Reflected Fluorescence system BX51) under blue light using a fluorescein isothiocyanate (FITC) filter set was used to visualize GFP expression in pollen.
2. Pollen was photographed on microscope slides using a digital camera (Olympus Q color 3 with Q Capture software).
3. BK medium: 10% sucrose, 100 mg/L of boric acid, 300 mg/L of calcium nitrate, 200 mg/L of magnesium sulfate, 100 mg/L of potassium nitrate (17) for pollen tube germination.

### 2.4. Plant Material

1. GFP pollen specific tobacco plants containing the pBINDC1 plasmid (PGFP).
2. Tobacco plants containing the pBINmGFP5-ER plasmid and expressing GFP throughout the entire plant (WPGFP).

### 2.5. Field Experiment

1. The experimental field design was based on Saegritz et al. (18) and consisted of a central donor plot split into four quadrants.
2. Two quadrants of the center donor plot contained PGFP tobacco plants.
3. The two remaining two quadrants contained WPGFP tobacco plants.
4. Each of the four quadrants contained six rows with eight tobacco plants per row, giving a total of 192 transgenic tobacco plants located within the center donor plot.
5. A pollen trap was placed in eight different directions (N, S, E, W, NW, SW, NE, and SE) at distances of 5, 10, 15, 20, and 25 m from the center donor plot as well as inside each quadrant within the center plot to measure wind dispersed pollen flow.
6. Replicate pollen traps were constructed from double-sided sticky adhesive tape on glass microscope slides.
7. Slides were covered with petroleum jelly and attached to vertical wooden stakes with collection heights of 50 and 100 cm from the soil surface.
8. Pollen dispersion was measured from the onset of anthesis.
9. Two wild-type recipient tobacco plants cv "Xanthi" were placed in a spatial grid around the center donor plot in eight directions at distances of 10–100 m from the center plot.
10. 10 × 15 seeds germination paper (Anchor Paper Co., St. Paul, MN).
11. 0.2 g/L of calcium sulfate ( $\text{CaSO}_4$ ).
12. A hand-held long wave ultraviolet light, (model B-100AP 100 W: 365 nm, UVP, Upland, CA).

## 3. Methods

### 3.1. Plasmid Construction

The CaMV 35S promoter cassette from the *Agrobacterium tumefaciens* expression vector pBINmGFP5-ER (courtesy of J. Haseloff) was excised by a

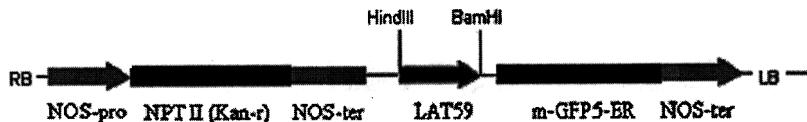


Fig. 1. Construction of pBINDC1 plasmid. The LAT59 pollen-active promoter was subcloned into the *Agrobacterium tumefaciens* expression vector pBINmGFP5-ER in the place of the CaMV35s promoter to create pBINDC1 as shown above.

*HindIII* and *BamHI* restriction digest. The LAT59 promoter (courtesy of S. McCormick) was ligated into the vector to replace CaMV35S promoter. The plasmid was renamed pBINDC1 (Fig. 1) (12). This vector contained an *nptII* cassette (kanamycin resistance) that was under the control of the nopaline synthase promoter and terminator.

### 3.2. Plant Transformation

*Nicotiana tabacum* cv "Xanthi" was transformed with pBINDC1 (12) using the *Agrobacterium*-mediated leaf-disk transformation method (19). Transgenic plants were selected on MS media (15) containing kanamycin (200 mg/L) and timentin (400 mg/L). Shoots arising from leaf discs were rooted on agar solidified MSO medium (20). After the plantlets formed roots, they were transferred to soil and grown to maturity under growth chamber conditions.

### 3.3. Fluorescence Microscopy

For observing GFP in pollen, freshly dehisced pollen grains were removed from anthers and placed on microscope slides. No staining or cover slide was necessary (see Note 1). GFP-tagged pollen was viewed under blue light conditions using an epifluorescent microscope (see Note 2). To observe GFP expression in growing pollen tubes, pollen grains were removed from anthers and placed in BK media (see Note 3). After 2 h, 30 µL of the BK pollen mixture was placed on microscope slide (see Note 4). Pollen tubes were observed at 100× magnification under a microscope (see Note 5). A 16 ms exposure time was used when photographing pollen under white light conditions and 2.75-s exposure under blue light conditions (see Note 6).

### 3.4. Plant Material

The field design of this experiment incorporated two types of transgenic tobacco. Tobacco plants expressing GFP throughout the entire plant (WPGFP) contained the *mgfp5-er* transgene, driven by the CaMV 35S constitutive promoter. WPGFP tobacco plants were used to measure gene flow in the field.

Homozygous WPGFP seeds were germinated on MS medium containing kanamycin 200 mg/L as a selection agent. After germination, seedlings were transferred to soil and the phenotype was confirmed by GFP visualization with a handheld, long-wave UV light (*see Notes 7–14*). Plantlets were placed in the greenhouse until transferred to the field sites.

GFP pollen-specific tobacco plants (PGFP) expressed the *mgfp5-er* transgene, driven by the LAT59 pollen specific promoter. PGFP tobacco plants expressed the GFP protein exclusively within pollen grains and were used to measure pollen movement in the field. Homozygous PGFP seeds ( $T_2$ ) were germinated on MS media with 200 mg/L of kanamycin. After germination, plantlets were placed in soil and grown in greenhouse conditions until planted at the field sites.

### 3.5. Field Design

Pollen flow was measured with pollen traps to sample pollen distribution at specified distances (*see Notes 15–17*). Pollen slides were collected at 24-, 48-, and 72-h periods after pollen shed from the donor tobacco population within the center plot. The presence of GFP-tagged pollen was assessed by screening the slides collected from the field site. Slides were screened using an epifluorescent microscope with blue light at 100 $\times$ –400 $\times$  magnification without staining. Gene flow was measured by analyzing progeny from the wild-type recipient plants for the GFP phenotype. Seed capsules were harvested from the receptor plants, which surround the donor plot at various distances and directions. The progeny from these seeds were screened using either the germination paper method, or the soil germination method. Using the germination paper method seeds were germinated in a dark incubator at 27°C on filter paper soaked in a 0.2 g/L calcium chloride solution. In the soil germination method, seeds were germinated in soil and grown under greenhouse conditions. After 3 wk gene flow was quantified by progeny analysis of seedlings from recipient plants and plants expressing GFP in the pollen (PGFP) within the donor plot. Seedlings were screened for the GFP phenotype with a handheld UV light. Outcrossing frequencies were calculated from the summed progeny at each coordinate and represent the average outcrossing frequency per plant.

## 4. Notes

1. When viewing GFP-tagged pollen under the microscope, it is not necessary to use a stain, such as aniline blue. Spread pollen evenly over the slide and do use a not cover slip. When the pollen grains become crowded on a slide, a cover slip tends to mash the pollen and makes it difficult to see each grain clearly. We found that a magnification of 400 $\times$  was most effective for viewing GFP in pollen; however, it is visible at lower magnifications (i.e., 40 $\times$ –100 $\times$ ).

2. When using an epifluorescence microscope, it is necessary to turn on the light source approx 30 min prior to viewing the specimen to allow proper warm-up of the burner. In general, when using epifluorescence microscopes, leaving the light source on for at least 30 min will prolong bulb life.
3. Pollen tube germination requires the use of a pollen tube germination media. Several variations of BK media exist so it is important to review the current literature to choose a variant of BK media that is optimal for the plant species being used. These variations have been modified to be more effective for pollen tube germination in specific plant species. Freshly dehisced pollen and fresh germination media must be used when germinating pollen tubes for best results. Depending on the species, binucleate pollen grains will germinate and tubes will grow in excess of 5 h. Many plant species with trinucleate pollen will germinate and grow but will have less longevity.
4. Pollen grains viewed under dry conditions will have a different shape (oblong) from that of hydrated pollen (round) in an aqueous solution. This is important when screening for pollen on pollen traps, as petroleum jelly will hydrate the pollen grains.
5. GFP can be visualized in the pollen tubes during any time of growth. No cover slip or stain is required. We found that  $\times 100$  magnification was best for visualizing GFP in pollen tubes.
6. When photographing GFP in pollen grains, exposure time is crucial. Wild-type tobacco pollen grains have slight autofluorescence under blue light, which might be confused with the GFP phenotype in photographs when using different exposure times. We found 16 ms to be the optimal exposure time for photographing pollen under white light conditions. The optimal exposure time for photographing GFP-tagged pollen under blue light was 2.75 s.
7. We have used two methods to screen large numbers of seedlings for the GFP phenotype: sowing seeds on germination paper and soil.
8. One strength of the germination paper method includes the ability to rapidly screen thousands of seedlings in a relatively small space by a single researcher. This method has been efficient for seeds produced under ideal conditions, that is, clean and healthy seeds. This method is also especially effective for large seeded plant species, such as many from the genus *Brassica*.
9. The germination paper method also has shown some weaknesses when the seeds are dirty (as is often the case with field collected material) or produced from plants grown in suboptimal conditions. In these cases, the germination paper can grow a large amount of contamination from dirty seed, which interferes with seedling health and the ability to accurately score the GFP phenotype. Also, when the seeds are from a sick parental plant, the seedlings are often of poor health, and grow poorly on the germination paper. Plant health is important when screening for GFP, and suboptimal seeds and seedlings will reduce the ability to accurately score the presence or absence of GFP.
10. The soil germination method is good for small seeded plants that require a period of growth before the GFP status can be determined. In the case of tobacco and

*Arabidopsis thaliana*, seedlings from these plants require several weeks of growth before they are large enough to accurately screen for GFP. In these cases, sowing the seeds on soil under greenhouse conditions is an efficient method to produce material suitable for GFP screening.

11. The soil germination method also has some difficulties, including sowing seeds at proper densities and the space required for large numbers of plants. With regard to sowing density, a balance must be reached between the numbers of seedlings in each container compared with the ability to accurately screen each plant for GFP. If the seedlings are at extreme densities, they will crowd each other and it will be difficult to see each plant to score the GFP phenotype. If the density is too low, the greenhouse space will become a limiting factor.
12. We have found that screening a large number of plants on soil requires several researchers. In our case, we found that it was most efficient to have people dedicated to UV screening in a dark environment and others dedicated to bringing and removing plant containers to be screened.
13. Multiple UV lights may also be employed to increase the accuracy of scoring low expressing GFP individuals. From our experience, the power and number of UV lamps can be increased to help discern between plants that exhibit slight differences in fluorescence.
14. Overall, one of the most important factors in the ability to screen GFP is overall plant health. Plants grown in suboptimal conditions are very difficult to screen for GFP.
15. Wild-type plants placed at coordinates around the center plot of transgenic plants must be germinated and planted in the field at the same time as those in the center plot to ensure coinciding flowering times. Planting large numbers of wild-type plants at the coordinate locations increases the amount of seeds that can be collected and screened. This increases the chance of detecting a rare out-crossing event. However, increasing the number at each coordinate could also limit the ability to detect an outcrossing event because cross-pollination will be occurring between wild-type plants at each coordinate. It is important to balance the number of plants at each location to maximize the amount of seeds that can be collected without decreasing chances of outcrossing between the transgenic and wild-type plants in field plot.
16. To use GFP-tagged pollen to effectively monitor pollen movement, it is our suggestion to use a plant species that is known to outcross under field conditions. Homozygous plants must be used in the field experiments. In homozygous plants, 100% of the pollen will express GFP maximizing the ability to see pollen movement. We used tobacco as a model plant, with designs toward employing the system for monitoring canola pollen.
17. Many types of pollen traps exist that could be used to track pollen movement. To maximize the chance of seeing pollen movement in the field, pollen traps need to be appropriate heights depending on the plant species being used. Placing traps around the center plot at a high density will ensure maximum efficiency.

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