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TECHNICAL NOTE

GFP-tagged pollen to monitor pollen flow of transgenic plants

L. C. HUDSON, D. CHAMBERLAIN* and C. N. STEWART JR
Department of Biology, University of North Carolina at Greensboro, Greensboro, NC, 27402–6174 USA

Abstract

In this study, the pollen-active LAT59 promoter from tomato was used to express a green fluorescent protein (GFP) encoding gene in *Nicotiana tabacum* (tobacco) pollen. This promoter is preferentially expressed in anthers and pollen. Pollen in transgenic plants segregated in a 1 : 1 Mendelian ratio, and the plants were polymerase chain reaction (PCR)-positive. GFP-tagged pollen was developed as a tool for tracking the movement of transgenic plant pollen in the environment. Specifically, it should be a useful tool for characterizing the spatial distribution patterns of transgenic pollen, to determine pollination mechanisms, to monitor the effects on nontarget organisms, and to monitor gene flow in field conditions.

Keywords: gene flow, GMO, green fluorescent protein, pollen flow, pollinators, transgene monitoring

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As the result of the widespread use of transgenic crops, the potential escape of transgenes into the natural environment has become a growing concern. Because some engineered crop species have wild relatives growing in close proximity, the risks of transgene escape causing fitness-enhancement of weedy relatives warrants the need for an *in vivo* gene monitoring system suitable for use in the field. Another concern is nontarget risks, such as side-effects of Bt-transgenic pollen on beneficial or ecologically important insects. An important tool for monitoring possible introgression of genes such as herbicide, disease, insect, and drought resistance into weedy relatives is green fluorescent protein (GFP) (Stewart 1996). The gene encoding GFP was isolated and cloned from the jellyfish *Aequorea victoria*, and several spectral variants have been developed. GFP is a 27-kDa monomer that fluoresces green under long wave ultraviolet or blue light. (Chalfie *et al*. 1994). GFP does not require a cofactor for fluorescence, which makes it an effective *in vivo* marker of gene expression (Leffel *et al*. 1997). GFP is a valuable tool used to assess frequency of stable transformation during tissue culture and in monitoring the gene flow of transgenic plants in the environment (Stewart 1996). This paper demonstrates that pollen tagging with GFP is a viable and useful approach to visualize transgenic pollen for tracking.

In this study, the tomato LAT59 promoter (Twell *et al*. 1989) was used to express the variant *mGFP5-ER* in *Nicotiana tabacum* (tobacco) pollen and visualized on honeybees. This pollen-active promoter controls the expression of a gene that is a member of a family of pectate lyase-like proteins, which is preferentially expressed in the anthers and pollen of tomatoes (Twell *et al*. 1989). GFP-expressing pollen under the control of pollen promoters were not found to impair reproductive function of tobacco (Ottenschlager *et al*. 1999). The expression of GFP in the pollen of plants will enable scientists to track the movement of pollen, to differentiate between pollen from individual plants of the same species, to determine pollination mechanisms, and to study spatial patterns of pollen with respect to a plants location in the field. We describe proof-of-concept experiments showing the potential of such a system.

The CaMV35S promoter cassette from the *Agrobacterium tumefaciens* expression vector pBIN mGFP5-ER (Haseloff *et al*. 1997), was excised by a *Hin*dIII and *Bam*HI restriction digest. The LAT59 promoter was ligated into the vector to replace CaMV35S promoter. The plasmid was renamed

Correspondence: Neal Stewart. Fax: 1 336 334 5839; E-mail: nstewart@uncg.edu
*Present address: Department of Medicine, Division of Cardiology, Duke University Medical Center, DUMC 3104, Durham, NC 27710 USA.

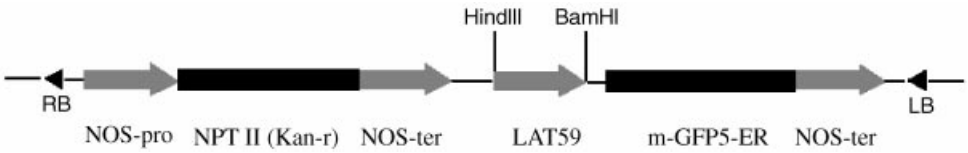

Fig. 1 The LAT59 pollen-active promoter was subcloned into the *Agrobacterium tumefaciens* expression vector pBinGFP5ER in the place of the CaMV35S promoter to create pBinDC1 as shown.

pBINDC1 (Fig. 1). This vector contained an NPTII cassette (kanamycin resistance) that was under the control of the nopaline synthase promoter and terminator.

N. tabacum cv 'Xanthi' was transformed with pBINDC1 using the *Agrobacterium*-mediated leaf-disc transformation method (Horsch *et al*. 1985). Transgenic plants were selected on media containing kanamycin (200 mg/L) and timentin (400 mg/L). Shoots arising from leaf discs were rooted on agar solidified MSO medium (McCormick *et al*. 1986). After the plantlets formed roots, they were transferred to soil and grown to maturity under growth chamber conditions. Upon maturation, the plants were examined with a hand held long wave ultraviolet light (UVP model B-100AP 100 W: 365 nm) to test for the constitutive expression of GFP. Pollen was collected and observed under an epifluorescent (FITC filtered) microscope with blue light to determine if pollen was expressing GFP.

To verify the transgenic status of the plants, polymerase chain reaction (PCR) analysis was performed using GFP-specific primers (Fig. 2). DNA was isolated from transgenic plants (Stewart 1997) and the GFP fragment was amplified using PCR under conditions of 95 °C for 5 min, 54 °C for 1 min, and 72 °C for 2 min with the forward primer 5′-CCTTAAGGATCCAAGGAGATATAACAATGA-3′ and reverse primer 5′-CCGGTTGAGCTCTTAAAGCTCAT-CATGTTT-3′.

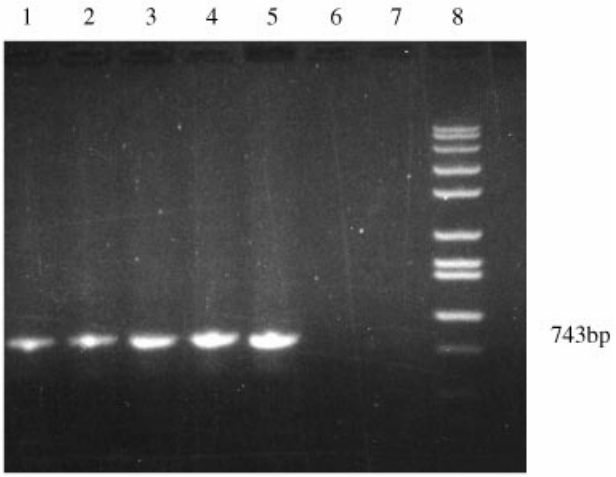

Fig. 2 Polymerase chain reaction analysis of transgenic tobacco. Lane 1 contained a positive control of purified mGFP5ER plasmid. Lanes 2–5 contained genomic DNA from the putative transgenic tobacco plants. Lanes 6 and 7 contained the negative controls of purified water and control tobacco, respectively. Lane 8 contained a high-low marker. The 743 bp band shared by lanes 2–5 illustrates that the transgene was present in the genome.

Transgenic flowering plants were placed in a cage with 24 bees for 7 days. Bees were captured in Petri dishes and placed in a –20 °C freezer. Bees were placed on glass slide and examined under an epifluorescence microscope with a FITC filter set to determine the presence of GFP pollen on wing, head, and leg areas.

Out of approximately 100 leaf disks, coincubated with *Agrobacteria*, 12 transgenic plants were recovered. The 12 plants were morphologically identical to untransformed control plants except for green fluorescent pollen. DNA isolated from each transgenic plant was PCR positive for the GFP sequence (Fig. 2). One line of trangenic plants were screened under ultraviolet light to determine if leaf, stem, root, seeds, or pollen areas were expressing GFP. The transformants exhibited no green fluorescence other than in pollen, which would suggest that the GFP protein is not being expressed (data not shown). Transgenic and non-transgenic pollen grains are identical under white light. In contrast, when GFP is expressed in plants under the control of 35S promoter, green fluorescence is evident under ultraviolet light in shoots and flowers but not pollen (Harper *et al*. 1999; Harper & Stewart 2000).

Examination of 180 pollen grains from a LAT59-GFP transformed plant showed that 96 pollen grains contained the GFP gene. Under the goodness-of-fit Chi-squared test a *P*-value of 0.31 indicated that a 1 : 1 Mendelian ratio of the pollen grains expressed GFP (Fig. 3A). This resulted in the insertion of the GFP gene on one chromosome. It is possible for the GFP gene to insert into many different chromosomes; in this case the result would not be a 1 : 1 ratio. Honeybees (*Apis mellifera*) carrying transgenic pollen on leg and head areas were easily identified (Fig. 3B).

There is great controversy about the introduction of genetically modified plants into the environment. One important issue is the movement of transgenes from crops to wild relatives. We have proposed using whole-plant expressing GFP to monitor gene flow in the field (Stewart 1996; Leffel *et al*. 1997; Harper *et al*. 1999). However, GFP-tagged pollen may be used to monitor pollen flow directly and in mixed populations and communities; there is no current system for direct monitoring of pollen movement.

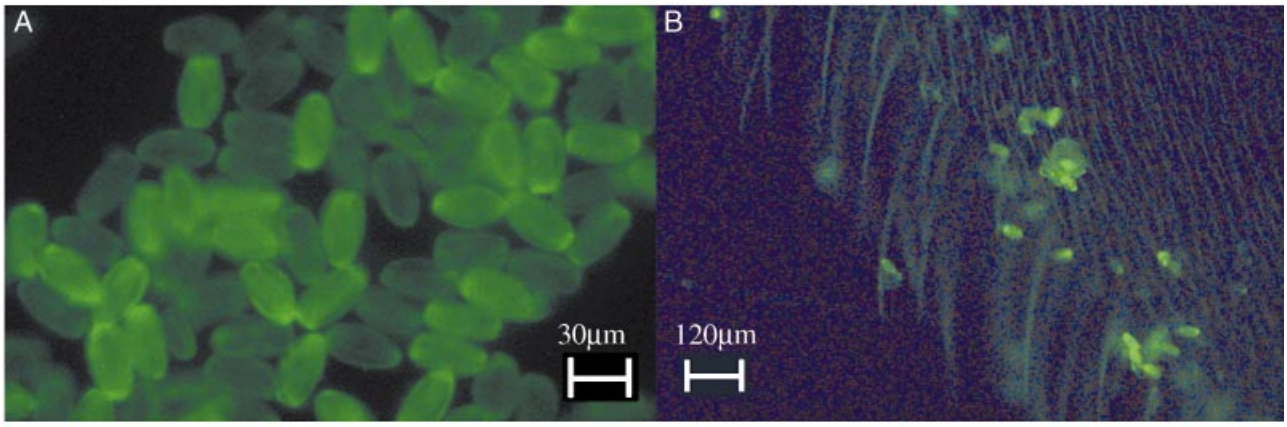

Fig. 3 *Nicotiana tabacum* (tobacco) cv 'Xanthi' transgenic pollen shown under epifluorescence microscopy using blue light excitation (FITC filter set). (A) 400× magnification demonstrating 1 : 1 Mendelian segregation. (B) 100× magnification showing transgenic and nontransgenic segregating pollen on a bee leg.

This study demonstrates that the visualization of GFP pollen is possible in nature.

There are several basic and applied applications of GFP-tagged pollen because GFP can be seen in real time and can be used in large-field level situations nondestructively. Systems that are currently being used to determine pollination mechanisms are ineffective in the field because of the small size and mobility of pollinators and pollen. Highly variable markers such as microsatellites are available for some plant species but PCR is laborious and does not provide *in vivo* and real time results. Systems such as pollen traps to monitor wind pollination cannot differentiate between plants of the same species. Although, the use of cytoplasmic male sterile (CMS) plants can provide an indirect method to differentiate between transgenic and nontransgenic pollen from the same species, GFP-tagged pollen provides a much more efficient method. It is feasible that noninvasive monitoring of the presence of GFP-tagged pollen on insects could be done automatically using photonic systems. Therefore, we could use this system to learn more about the pollinator/pollination system. When a pollinator, such as a bee, is introduced to the flowers of a plant expressing GFP pollen, the movement of the pollen can be tracked throughout the environment. Pollinators could be used to estimate the amount of pollen flow from a pollen-donor population of transgenic plants to nontransgenic plants grown at specified distances from the transgenics.

While GFP-tagged pollen will benefit risk assessment research in the near-term, such a system might be an enabling tool in the commercial monitoring of transgenic crop pollen flow. One example would be to track pollen flow from transgenic to organically grown fields containing the same crop. Likewise, pollen containment could be assessed using traps and pollen movement via wind and insects could be documented.

Further studies will involve an analysis of the progeny from the first line of plants obtained and a comparison of the strength of this pollen promoter to other pollen specific promoters. We will also begin pollen distribution and gene flow monitoring experiments using canola (*Brassica napus*) as a model crop in the field under a variety of conditions.

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