Bio-molecular Engineering Verified by High Sensitivity Detection

Claudia Lee[1], Andrew Zepfel[2], Nina Timberlake[3], Larry Grill[4], Sean Gallagher[1]
[1]UVP, LLC
[2]Claremont McKenna College
[3]Scripps College
[4]Pitzer College

Abstract
An interdisciplinary, industry-academic collaboration was conducted to aid students at the Pitzer College Vaccine Development Institute and the Claremont Colleges to study the progression of Tobacco Mosaic Virus (TMV) on plants. Symptoms of the virus, including discoloration and mottling, usually takes weeks to develop. The students genetically inserted the gene for green fluorescent protein (GFP) into the virus genome. The genetic modification allows the viral infection to be detected within a few days via fluorescent imaging. The detection of the fluorescence was aided by UVP, LLC. Fluorescent images of a tobacco relative (*Nicotiana benthamiana)* agroinfected with GFP-TMV was captured daily using an macro imaging system to document the intensity and area of the viral expression. The quantitative data is used to observe the movement and expression of the virus under various conditions and when changes are made to the viral gene sequences. Students were exposed to the technical aspects of fluorescence imaging besides being able to rapidly verify their biological work.

Introduction

The Vaccine Development Institute at Pitzer College aims to produce low cost vaccines by genetically engineering Tobacco Mosaic Virus to express immunogenic epitopes on the surface of the viral coat protein. Vaccines and therapeutic drugs produced in this manner have previously been shown to be highly efficient in eliciting an immune response (Kohl et al., 2006). Moreover, this production method is significantly faster and less costly than other more traditional methods involving bacterial or mammalian cells (Grill, Palmer & Pogue, 2005).

TMV is a rod-shaped RNA virus that infects members of the *Solanaceae* family of plants, which includes tobacco, tomatoes, and potatoes. The virus causes mottling and discoloration of the leaves, but is not fatal to the infected plant. These symptoms appear in new growth and often take weeks to become clearly apparent. In order to more quickly detect and quantify the spread of TMV through a plant, the virus was tagged with GFP and visualized using an UVP BioSpectrum® Imaging System. The GFP tag allows for detection of the virus in plant tissue as early as two days post-infection, and for monitoring the spread of the virus through the plant. Early detection confirms the success of cloning procedures and the correct functioning of the virus.

Method

Engineering the TMV genome

GFP was enzymatically inserted into the PacI-AvrII-NotI multiple cloning site of the pJL TRBO vector (Figure 1). The pJL TRBO vector, as previously described by Lindbo (2007), was designed to maximize production of the inserted protein. It contains the entire TMV genome, except the gene encoding the viral coat protein, bordered by 25-bp repeat sequences, which enable the incorporation of the viral genes into the plant genome after infection. Competent *Agrobacterium tumefaciens* cells were transformed by heat-shocking with the TRBO-GFP construct. *A. tumefaciens* is a plant bacterium that utilizes a tumor-inducing (Ti) plasmid to transfer genes directly into the host's genome. After transformation, the bacteria can be cultured and injected directly into the leaves of *Nicotiana benthamiana* plants. Plants are maintained in a controlled environment at 27°C with 16 hours of light per day. GFP expression in the plant tissue is visible 2 days post-infection (dpi).

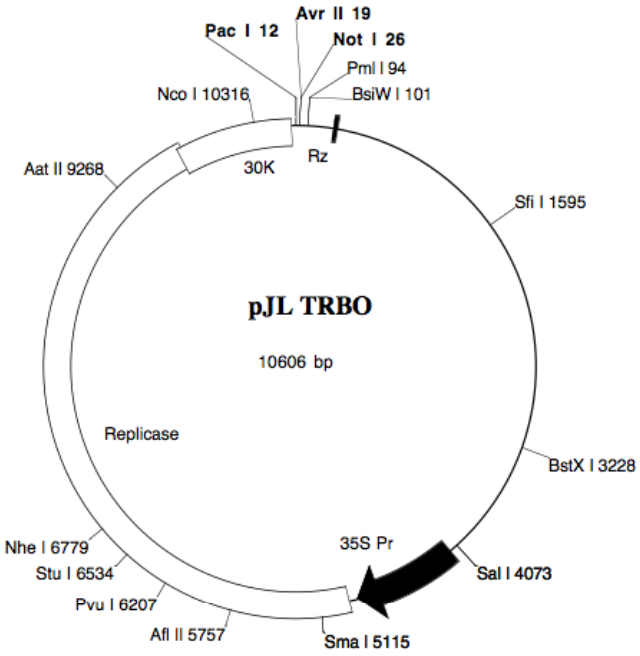

Figure 1. pJL TRBO vector with PacI-AvrII-NotI multiple cloning site. Replicase and 30K protein are part of the TMV genome. TMV coat protein is not present. 25-bp repeats are not shown.

Control for quantitative fluorescence imaging

Two days after the transfection, glowing patterns can be observed when the plants were illuminated with a UV handheld lamp. To verify that the visually detected pattern was TMV-GFP, a leaf was removed and placed in a spectro-fluorometer to acquire its emission spectrum. As shown in figure 2, the visually glowing area (area 1) significantly increased in emission intensity at about 520nm relative to the non-glowing area (area 2). 520nm is the signature wavelength of GFP.

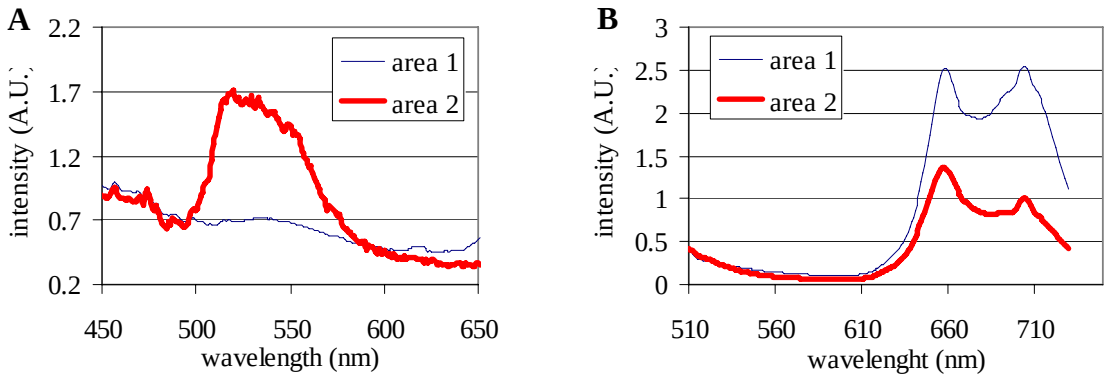

Figure 2, Emission spectra of an infected leaf under A) 365nm UV and B) 480nm excitation illumination. Area 1 expressed visually detectable fluorescence. Area 2 was far from the point of infection and is used as a reference.

GFP could be excited by blue (480nm) light as well as UV. To compare the difference, an emission spectral scan was also taken using 480nm excitation wavelength. When excited with 480nm, the inelastic scattering of the excitation light spread over the wavelength of GFP emission. The signal to background ratio is reduced. It was determined from the emission scan that 365nm UV should be used for excitation and an emission filter passing light between 500 to 600nm should help discerning the GFP signal. A shorter wavelength (<500nm) contains mostly the scattered excitation light. The longer wavelength region (>600nm) has signal irrelevant of the infection. Its spectral signature matches the autofluorescence of chlorophyllchlorophyll (Halfhill et al., 2003).

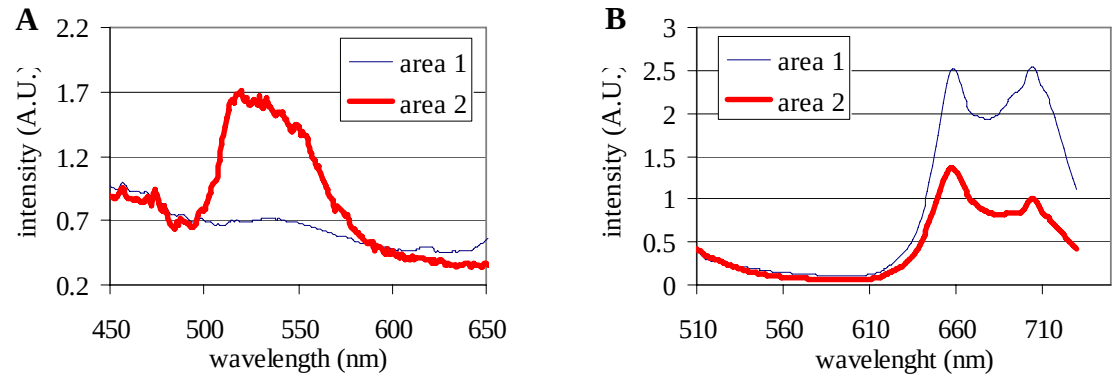

Figure 2, Emission spectra of an infected leaf under A) 365nm UV and B) 480nm excitation illumination. Area 1 expressed visually detectable fluorescence. Area 2 was far from the point of infection and is used as a reference.

Instrumentation for time lapsed, non-invasive, in-vivo whole plant imaging

To monitor the expression and propagation of the virus over time in one live *A. tumefaciensN benthamiana* plant, an UVP BioSpectrum Imaging System was used to perform fluorescence imaging on the infected plant. Built-in 365nm overhead UV was used for excitation. To ensure the maximal dynamic range, the system was set to automatically acquire fluorescent images using a series of 10 different exposure time settings (25msec to 12.8sec). The cooled CCD camera has no noticeable noise at the long exposure time. The plant was set on the mechanical lift inside the imaging darkroom such that the height of the leaves of interest can be controlled to keep a constant distance with the UV light source and the camera. When the intensity of the excitation light and the distance of the observation are held at constant, the fluorescence intensity is mostly varied by the concentration of virus.

Results

Leaves of the plants were infected with bacteria which contains the GFP-TMV RNA. The bacteria can either be injected orwasere infiltrated using the agro -infection method, as shown in figure 3A. The plants were grown under normal control condition and daily moved to the imaging system for measurements. As early as 48 hours after infection, fluorescence can be observed around the point of infection (figure 3B). Unintentionally, one leaf was showing signs of direct viral infections without the punch mark from agro infection. This can be created from an accidental drop of the infectious fluid on the leaf. This leaf showed fluorescence two days later. The speed of propagation and the intensity of fluorescence are both higher in the stems than in the leaves (figure 3C).

The fluorescence images allows quantitative studies of the viral expression. On different leaves of the same plant, the fluorescence around the infiltration area was observed two days earlier than the infection (figure 4A). The averaged intensity in all detected areas shows that the amount of detected GPF-TMV reaches its maximum 4-dpi. If the bacteria were initially infiltrated, the fluorescence doesn’t reach maximum until seven days passed infection. Figure 4B shows the average intensity of five infected areas Ffrom two different plants, five infection points showed maximal expression at the same dpi.

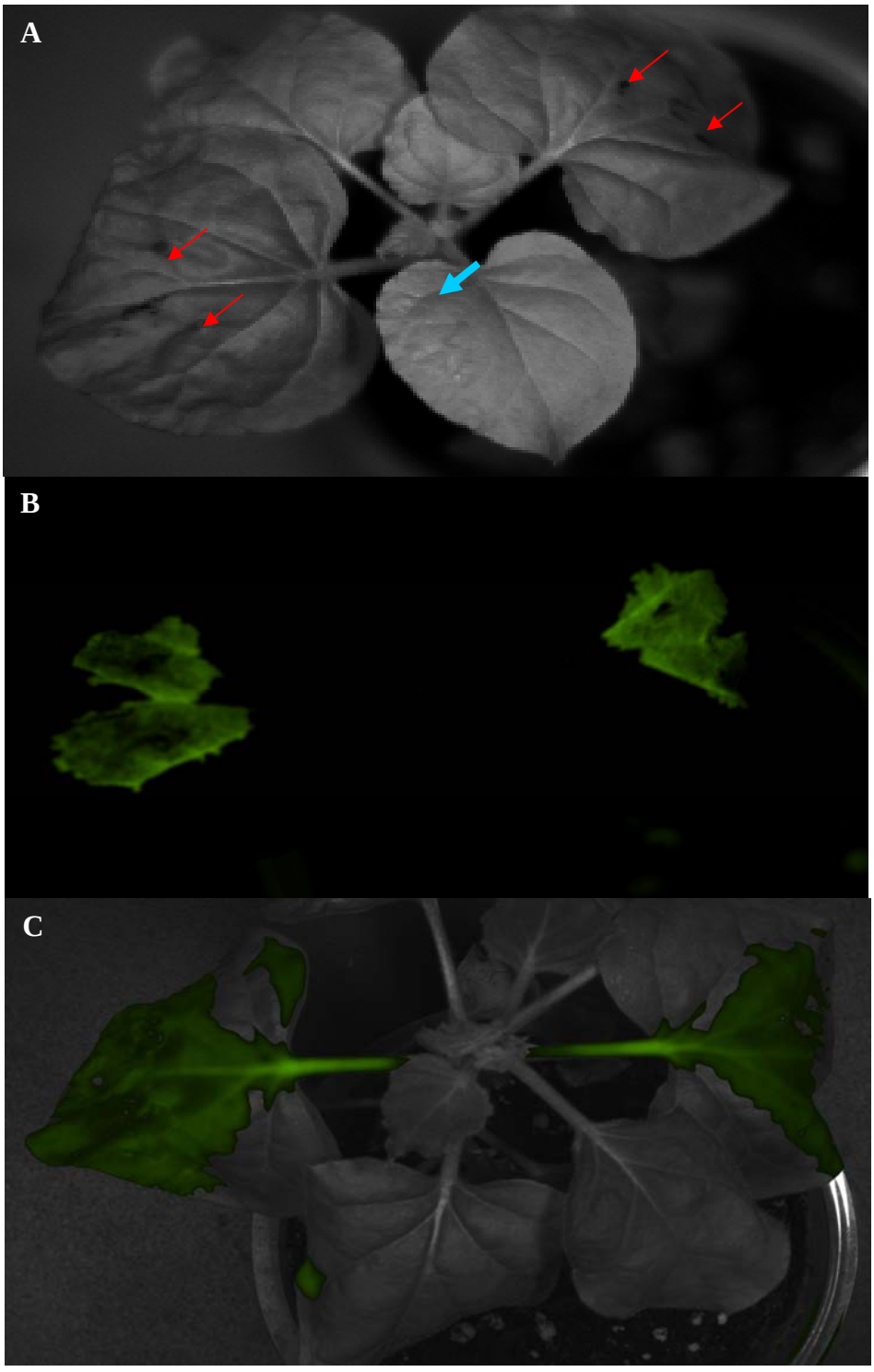

Figure 3A), white White light image of one plant 24 hours after infection. The thin red arrows indicate the point of injectioninfiltration, and the blue arrow points to an infiltrationarea of initial viral replicationviral infection point without the agro infection punch mark. **B)** the fluorescent image of the same plant at 2-dpi in green pseudo color. **C)** the overlay of the fluorescent image and the white light image at 10-dpi.

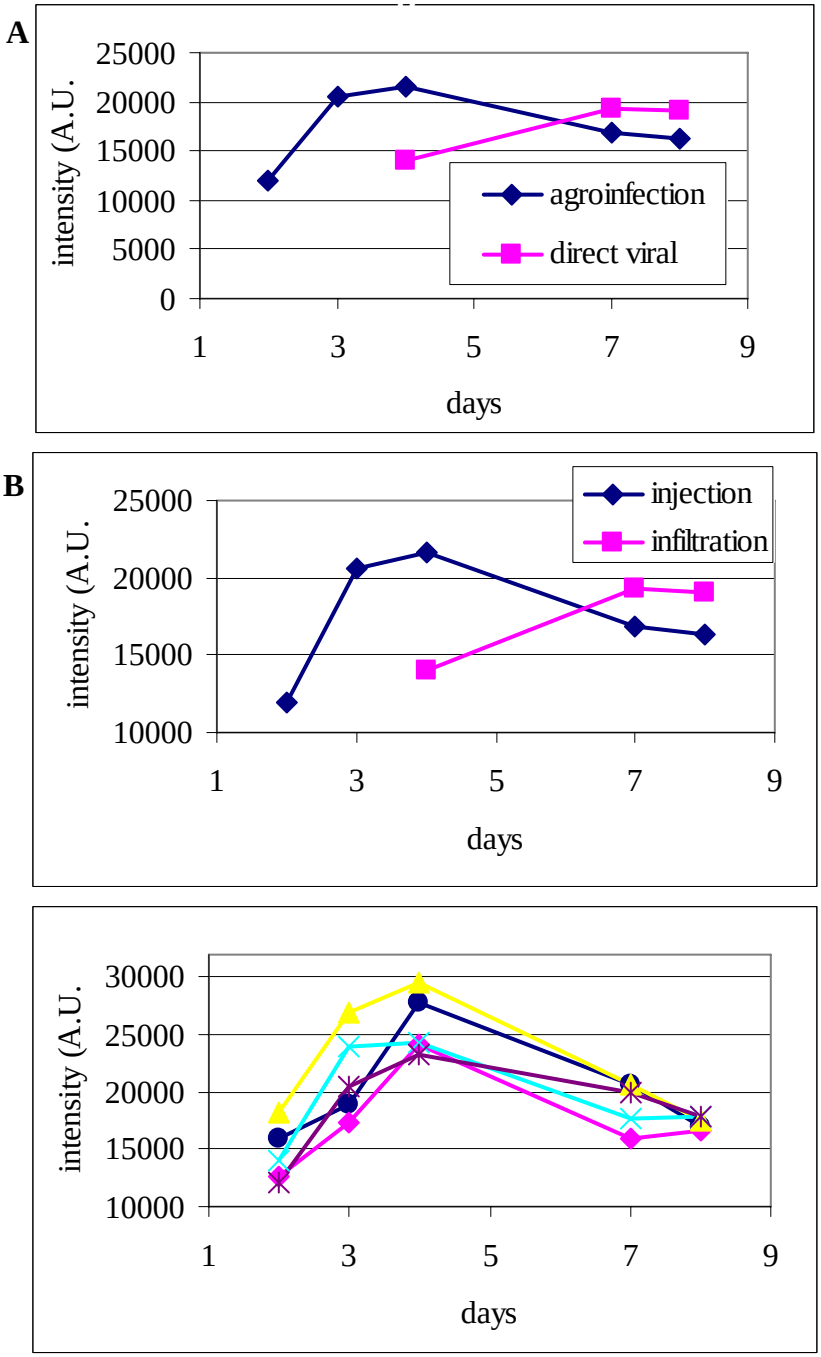

Figure 4, quantitative comparison of viral expression from **A)** different infection methods on the same plant and **B)** injected infection on five leaves of two different plants.

The result of the fluorescence macro imaging suggests that using the designed TMV plasmid would give the highest yield if the plant tissue was harvested at 4-dpi.

Discussion

GFP as a quantitative reporter in live plants

Lindbo (2007) had previously published on using the pJL TRBO vector to report the viral expression in live plants. The amounts of GFP destructively extracted from plants were used to compare the effectiveness of pJL TRBO and other vectors. Although the intensity of fluorescent images shown by Lindbo do seem to correlate with the amount of GFP extracted, it was impossible to compare the day-to-day increase of the viral expression on the same leaf using the destructive method.

To report the viral expression quantitatively with the non-invasive fluorescent method, several variables must be well controlled. Because the produced fluorescence is the function of excitation power and concentration of GFP, the intensity of the excitation illumination per unit area on the sample should be held at constant. In our setup, the UV light source was fixed at the top of the imaging cabinet, and the plant was placed on a computer control lift which can be lowered to maintain a constant distance between the light source and the plant despite its growth over time. This allows the possibility of maintaining the constant excitation power level.

In figure 4A, the infected areas reached their maximums at different dpi. As it is expected that the process should be accelerated when the virus was introduced with agroinfection, the temporal difference of the two curves serves as a control which promises that the increase in fluorescence is from increase of viral expression and not the fluctuation of the imaging setup. Furthermore, as shown in figure 4B, several infected areas on different plants all demonstrated the same temporal behavior: the averaged intensity in all detected areas shows that the amount of detected GPF-TMV reaches its maximum 4-dpi. This is consistent with Lindbo's finding.

Conclusion

Fluorescence intensity acquired from leaves infected with GFP-TMV can be used to monitor protein production repeatedly and non-invasively. The result of the fluorescence macro imaging suggests that using the designed TMV plasmid and agroinfection method would give the highest yield per infected area, when plant tissue is harvested at 4-dpi.

Future work

The pJL TRBO vector is expected to have greatly reduced long range mobility (Donson et al., 1991). Quantification of GFP expression in infected plants may be used to compare the mobility of viruses with altered gene sequences.

Reference

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Biographical Information

Claudia Lee: Software program manager at UVP, LLC.
Andrew Zepfel: Student at Claremont McKenna College.
Nina Timberlake: Student at Scripps College.
Larry Grill: Director of Vaccine Development Institute at Pitzer College
Sean Gallagher: Chief technology officer at UVP, LLC.