Journal of Fluorescence, Vol. 15, No. 5, September 2005 (© 2005)
DOI: 10.1007/s10895-005-2977-5

Laser-Induced Fluorescence Imaging and Spectroscopy of GFP Transgenic Plants

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Received March 30, 2005; accepted July 26, 2005

Green fluorescent protein (GFP) and other fluorescent protein bioreporters can be used to monitor transgenes in plants. GFP is a valuable marker for transgene presence and expression, but remote sensing instrumentation for stand-off detection has lagged behind fluorescent protein marker biotechnology. However, both biology and photonics are needed for the monitoring technology to be fully realized. In this paper, we describe laser-induced fluorescence imaging and laser-induced fluorescence spectroscopy of GFP-transgenic plants in ambient light towards the application of remote sensing of transgenic plants producing GFP.

KEY WORDS: Transgenic; fluorescence; plants; remote sensing; green fluorescent protein.

INTRODUCTION

Transgenic plants and other organisms could conceivably be monitored using the green fluorescent protein (GFP), from the jellyfish *Aequorea victoria* [1–3]. GFP has been shown to be practical for monitoring gene presence and expression of genetically linked proteins in several instances [4–6]. Indeed, there have been several recent advances in instrumentation for the detection and quantifying GFP in whole, intact plant organs at very short standoff or when plant organs (e.g., stems and leaves) can be physically manipulated [2,7]. Systems for macroscopic detection and quantification of fluorescent proteins include high-intensity UV lamps, spectrofluorometers (e.g., FluoroMax, Jobin Yvon and Glen Spectra, Edison, NJ, USA), a hand-held field portable spectrofluorometer (e.g., GFP Meter, OptiSciences, Tyngsboro, MA, USA) [7], and scanning laser systems (e.g., FluorImager, Molecular Dynamics, Sunnyvale, CA, USA). While these are practical for research, they are all suboptimal for wide-area, remote sensing applications of plant signatures such as engineered fluorescence. Furthermore, better platforms are required for detecting GFP in environmental applications. A hand-held 365 nm UV lamp, such as a UVP Model B 100 AP (UVP, Upland, CA, USA), allows for quick detection of GFP in whole plants, but it only works in the dark or in a darkened environment. Spectrofluorometers and fluorescence imaging systems are capable of detecting the presence of GFP and also allow for quantification of fluorescent tissues [4,8–10], but are lab-based instruments. The OptiSciences GFP Meter [7] is relatively inexpensive, can be used in daylight and in the field, but, like other spectrofluorometers, researchers must physically contact leaves for taking measurements.

Laser-based detection instrumentation for the imaging of GFP and other fluorescent molecules in organisms has been limited thusfar to the microscopic level. For example, confocal scanning microscopy has been

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used to detect GFP in plants [11,12] and a GFP-tagged biocontrol agent (*Clonostachys rosea*) on plants and soil [13]. Concurrent with the technical developments of laser-induced imaging, it seems reasonable to apply advanced photonics to the macroscopic and stand-off detection of GFP-transgenic organisms; it could be useful in the wide-area detection of GFP transgenic plants. Since GFP can be fused to other transgenic proteins of interest, essentially any gene/protein could be monitored in a generalized model. Remote sensing capabilities could be useful in phytosensing applications such as the detection of GFP-induction in pathogen-infected plants [14], or simply monitoring gene flow movement [1,5,8]. This paper describes the application of laser-induced fluorescence spectroscopy (LIFS) and laser-induced fluorescence imaging (LIFI) of GFP in plants in ambient light. These technologies have been used to model detection strategies toward an effective, scalable, field-deployable system [15], and the work described here applies it directly to the detection of GFP in transgenic plants.

MATERIALS AND METHODS

Laser-Induced Fluorescence Spectroscopy (LIFS) and Laser-Induced Fluorescence Imaging (LIFI)

The instrumentations described here were similar to those used in other recent studies [16–18] (Fig. 1). Leaf fluorescence measurements of plants were collected by exciting shoots of plants with either a tripled Nd:YAG laser (355 nm) or a excimer-pumped dye laser (390 nm). The fluorescence, generated in response to pulsed laser excitation, could be measured under daylight (shaded sun) conditions. However, in the research reported here, ambient room light was used.

Using either excitation wavelength prompt emission of leaves and stems were collected with an imager (LIFI) and a spectrometer (LIFS). The Nd:YAG laser and LIFI imager were packaged in a single portable system used in field collections whereas the spectrometer and detector of the LIFS system were integrated in a second enclosure. The LIFI detector shared the same tripod as the laser head and the LIFS optics and the tripod was not moved between LIFI and LIFS measurements. When 390 nm laser light was desired, the output from the excimer-pumped dye laser replaced the Nd:YAG as the light source on the tripod.

Laser Sources

The Nd:YAG laser (355 nm) configuration was modified for this application (Ultra-CFR400, Big Sky Laser Technologies, Bozeman, MT, USA). Custom optics systems were supplied for the laser projector system to remove first- and second-harmonic light from the beam and to project a uniformly illuminated rectangle of light on the target area with no observable speckle. The Nd:YAG laser produced 7-ns wide pulses of UV light at an output of 10 to 18 mJ/pulse at a rate of 6 Hz. The repetition rate was limited by the available image processing card and not the laser system. The LIFS/LIFI measurements were made at a distance of 1.0 m for these studies. The imaged and illuminated field was about 60 cm × 45 cm at that distance, which was effective for good spatial resolution for imaging small scene features. The LIFS-viewed area was a 10 cm circle located at the center of the imaged area. The standard projection size was set by the choice of lens on the laser output module and imaging receiver. By increasing the lens focal lengths of both the imager and projector simultaneously the LIFI system can be used at longer distances. A similar LIFI system has been used to image from helicopters at approximately 90 m above the targets in which both projection and receiver lenses had been adjusted to create a 3.3-m diameter field (Di Benedetto *et al*. unpublished data).

Based on our preliminary emission studies, 390 nm excitation gave a better signal-to-noise ratio than 355 nm excitation when imaging GFP in transgenic plants (data not shown). To produce 390 nm light, a Lambda Physik Model LPX210i 308 nm XeCl excimer laser was used to pump a Lambda Physik 2002 dye laser. The dye laser produced approximately 8 mJ of 390 nm light. This was passed through a liquid light guide and projected as a uniform source onto the plants. The 390-nm projector was boresighted to the other system components to avoid parallax and shadowing.

Detection Systems

The LIFI and LIFS detectors were intensified CCD cameras that could be gated to less than 100 ns. Each had an intensifier coupled to a charge coupled device (CCD) camera. Although the LIFS spectrometer system used a cooled, slow scan CCD camera, the LIFI ICCD detector produced 30-frame/s video in a standard RS-170 format.

Overall system timing was synchronized to the RS-170 vertical reset timing since the latter is controlled by a highly accurate clock. Using this accurate time marker, the laser trigger and the LIFS acquisition system were timed to the LIFI imager, preventing any long-term timing drift. Although the different systems were initiated and started by the video clock, the nanosecond timing of the cameras to the laser was achieved by timing the two intensifiers of the ICCD cameras directly to the

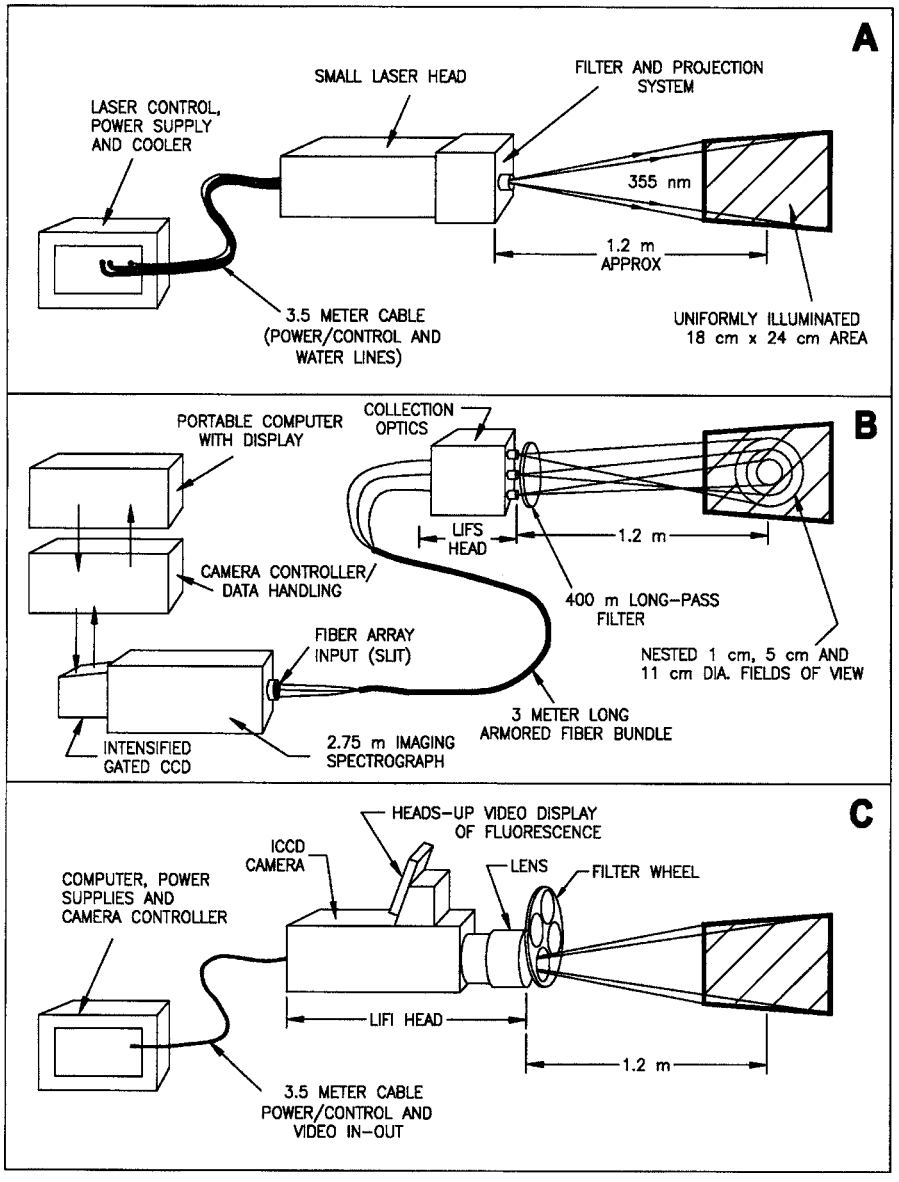

Fig. 1. LIFS/LIFI system schematic. Panel A shows the laser illumination system which is based on a 355-nm tripled Nd-YAG laser. Panel B shows the LIFS system. Note that the LIFS system captures three views of plants simultaneously. By comparing the different fields of view, the researcher can investigate some of the spatial irregularities and determine if pixel size is a factor. Panel C is a photo of the LIFI system used in these studies.

laser output. Laser/detector timing was verified using an avalanche photodiode and an oscilloscope.

LIFI Detection System

The LIFI detection system used a gated CCD camera system (NVSI Camera Systems, Fayetteville, North Carolina, USA) to collect fluorescence images of UV-illuminated plants. The spectral response of the intensifier extended from 400 to 900 nm.The CCD camera was directly coupled to the intensifier through a fiber optic taper (i.e., a minifier). The RS-170 format CCD camera had a 3:4 image aspect ratio which was matched the rectangular UV-illuminated footprint from the Nd:YAG laser. Fluorescence signals were imaged into the CCD camera through a C-mount camera lens (50 mm, *f*-stop 0.85 lens). A manually operated filter wheel in front of the CCD camera lens was used to hold the narrow-bandpass filters

required for fluorescent imaging of plant canopies. In previous work, the LIFI unit collected images of the fluorescing vegetation in four separate spectral bands, centered at 450, 525, 680, and 740 nm, by using a filter wheel in front of the intensified CCD camera. For this project, however, three images were collected at 480, 510, and 550 nm. A 420 nm bandpass filter was also placed directly in front of the imaging lens to more completely block excitation laser light pollution.

The LIFI data collection process was manually reset for each output bandwidth that then used an electronic switch that initiated a series of automatic events. When the acquisition was initiated, images were collected by an 8-bit frame grabber and stored in digital memory as separate frames. The gated intensifier (electronic shutter) of the CCD camera opened for 100 ns in which the open window was timed to coincide with the return of fluorescence light from the target. For measurements made at greater distances, the intensifier opening would be delayed to account for the transit time to and from the target. Immediately following the illumination frame, a "laser-off" image was electronically captured. This background image had close to identical solar photon contamination so that a limited degree of subtraction was needed to image fluorescence. Typically, a series of 10 fluorescent images plus 10 background images were collected. After the frames were collected, the sequential background images were subtracted from fluorescent images, summed into a 16-bit buffer and stored to disk in binary format. This process used custom software and hardware written and built by the DOE Special Technologies Laboratory (Santa Barbara, CA, USA) by the authors. This custom software was built around the commercially available ENVI/IDL software (Research Systems, Boulder, CO, USA), and was written for processing LIFI images. In addition, ENVI header files were stored during acquisition to decrease image processing key strokes. Image post-processing consisted of the following: (a) images were adjusted to a common *f*-stop rating of 1.4; (b) regions of interest were manually selected for each set of images selected; and (c) band intensities and ratios were estimated from each of the imaged fluorescence bands. The three-color images shown in this paper were composites of three filtered images in the automated acquisition routine.

LIFS Detection System

The LIFS detector collected leaf fluorescence from a 10-cm diameter circle centered in the laser-illuminated area. The collected light from the LIFS optical system was transferred via a 3-m fiber optic bundle to the input slit of a 275 cm focal length spectrograph (Model SP-275, Acton Research, Acton, MA, USA). Dispersed light at the output side of the spectrograph was detected by a gated intensified CCD camera (Princeton Instruments, Trenton, NJ, USA) with 256 × 1024 pixels of resolution. Though the detector and spectrometer are capable of recording light from 400 to 800 nm with an effective resolution of 3 nm, the LIFS detector optic used a laser-blocking filter with a cut wavelength of 450 nm (Schott KV450). This served to block both 355 and 390 nm excitation without generating fluorescence from the filters themselves. Since the intensifier of the ICCD camera was electronically gated (shuttered) and synchronized with the laser, the LIFS detector exposure interval could also be sufficiently short to reject ambient background light reflected from the surface. Shutter-open times were 100 ns windows in which the start of the detection window coincided with the arrival of the laser pulse on the target; the LIFS shutter (intensifier) was open for 600 ns for every second (100 ns for each of 6 laser pulses/s). Since the ICCD detector of the LIFS is a cooled, slow scan device, the CCD can record over many seconds with minimal read noise, allowing for hundreds of laser pulses to be recorded per each read of the camera. Since many laser pulses can be collected during a single acquisition, signal-to-noise ratio greatly improves with increased dwell time on a target, enabling GFP measurements in ambient light.

Transgenic Plants

Brassica napus (canola) cv. "Westar" plants transgenic for GFP (*mgfp5-er*) under the control of constitutive *CaMV* 35S promoter were used in this study [4,8]. The mGFP5-ER variant has equal excitation peaks in both blue and UV wavelengths (465 and 395 nm optima, respectfully). The transgenic plant event that was used in the descriptions in this paper was the canGFP2 event [8] a moderate to high expressing line for *gfp* and one in which there exists intrinsic variation in expression among plants. The line was segregating, and thus the pool of plants contained hemizygous plants (with one copy of the transgene) that are known to fluoresce at an increment of half the amount of homozygous plants [5] (with two copies) and homozygous plants. Therefore, the plant population had individuals with 0, 1, and 2 copies of the transgene. Plants were grown on an enclosed patio that received full sunlight in Santa Barbara, CA, USA. They were grown in 4-L pots in a soil-less bark-based potting mix. The plants analyzed in this study were mature with flower bolts removed.

In other experiments presented here, potted *Nicotiana tabacum* cv. 'Xanthi' (tobacco) plants were grown under an enclosed canopy in Edgefield, SC, USA. The

transgenic event tobGFP3 was used [4], and these plants have the identical construct as described for the transgenic canola plants. In addition to analyzing the fluorescence of the tobacco plants with LIFS and LIFI, a FluoroMax, (Jobin Yvon and Glen Spectra, Edison, NJ, USA) spectrophotometer was used [7] as another fluorescence measurement tool.

For both the canola and tobacco studies, enzyme-linked immunosorbant assays (ELISA) were performed to quantify GFP produced in each plant sample. Prior research has shown that ELISA and Fluoromax data are positively correlated, thus yielding external standards for LIFS and LIFI data [6]. In all these studies, NIH and USDA regulations were followed when working with recombinant DNA and transgenic plants.

RESULTS AND DISCUSSION

The purpose of this study was to assess whether LIFS and LIFI could be used in the remote sensing of GFP transgenic plants when GFP was produced at appropriate physiological levels. At face value, these optical technologies are well suited for the stand-off detection of organisms synthesizing GFP or other fluorescent proteins because of the thoroughly characterized spectral properties of these molecules. Previous spectrophotometric measurements on plants have shown that GFP fluorescence at 505–510 nm can be readily discriminated from plant autofluorescence with appropriate excitation wavelengths [2,5,7,19]. Although leaf-level spectrophotometric measurements provide the optimal geometry for molecular-level characterization, LIFI and LIFS has the potential to provide a canopy-level perspective relatively quickly and on a large scale in applicable environments.

LIFS data patterns taken on GFP transgenic canola and tobacco match the spectral data taken with other spectrofluorometers in which the plants must be physically handled to take measurements (Figs. 2(C), 3–5; see also refs [5] and [7]). The data here revealed two prominent signals: the green GFP emission band (507 maximum) and the four bands expected for UV excitation of plants. In addition to the red chlorophyll emissions at 685 and 740 nm, emission from the 450 and 525 nm bands of plants are always observed and can be problematic for detection of the 510 nm emission from the GFP fluorophore. By capturing images with two filters adjacent to the central 510 nm peak (480 and 525 nm), LIFI images could readily detect the GFP signature as a peak overlapping the 450 and 525 nm plant emissions. Since the LIFS spectrum captured the full spectrum of both the GFP and endogenous plant signatures, LIFS spectra (collected as an averaged area of the plant leaf) verified that the LIFI images were specifically differentiating GFP signals from endogenous plant spectra. On a plant that produced relatively low amounts of GFP fluorescence, "green" images was observed in stems and apical meristems more so than leaf blades (Fig. 2(A)), which is to be expected given that the 35S promoter is especially active in these tissues compared with leaves. Furthermore, Halfhill *et al*. [19] thoroughly characterized GFP fluorescence in 35S-GFP transgenic canola and found that stems and apical meristems were the optimal targets for detection. Here, we found a range of GFP expression patterns were detectable in GFP-transgenic canola, with LIFS and LIFI data showing congruent results. Plants with highest GFP emission peaks shown by LIFS were also imaged "greener" by LIFI (Fig. 3). The expression of GFP was shown to be moderate as measured by ELISA (0.377 ng GFP/μg total extractable soluble protein; SD = 0.220, $n = 15$), which was similar to previous results (e.g., [5,6,19]). For the canGFP2 transgenic canola event, GFP concentrations ranged fivefold, which was consistent with the variation detected with LIFS and LIFI data shown here.

Similar results were found in the GFP tobacco study. LIFS and LIFI could detect GFP in transgenic plants in leaves (Figs. 4 and 6). In this experiment the LIFS data matched those taken with the Fluoromax spectrophotometer (compare Figs. 4 and 5). All of the GFP tobacco plants showed green fluorescence using LIFI, whereas there was an absence of imaged green fluorescence in non-transgenic plants (Fig. 6). ELISA results showed that the tobacco plants had higher GFP concentration in leaves with less relative variation (1.29 ng GFP/μg total extractable soluble protein; SD = 0.65, $n = 12$) compared with the canola experiment. Green fluorescence could be detected in all GFP transgenic plants using LIFI indicating promise for using this technology for sensing GFP in transgenic plants.

These results demonstrate a new utility for laser-induced fluorescence techniques that might be used in standoff detection mode with transgenic plants. LIFS and LIFI have been successfully used in several non-transgenic biological applications, such as the analysis of plant stress [18,20–24], monitoring of aquatic systems [25], and the detection of pathogenic bacteria in environmental samples [16].

Multi-band LIFI data are collected as a series of spectral or temporal bands that are processed using techniques similar to multispectral analysis. In the ground-based systems such as the one used here, laser-induced fluorescence spectroscopy (LIFS) is often collected simultaneously with imagery to record the fluorescence spectra (400–750 nm) of a single feature in the outdoor

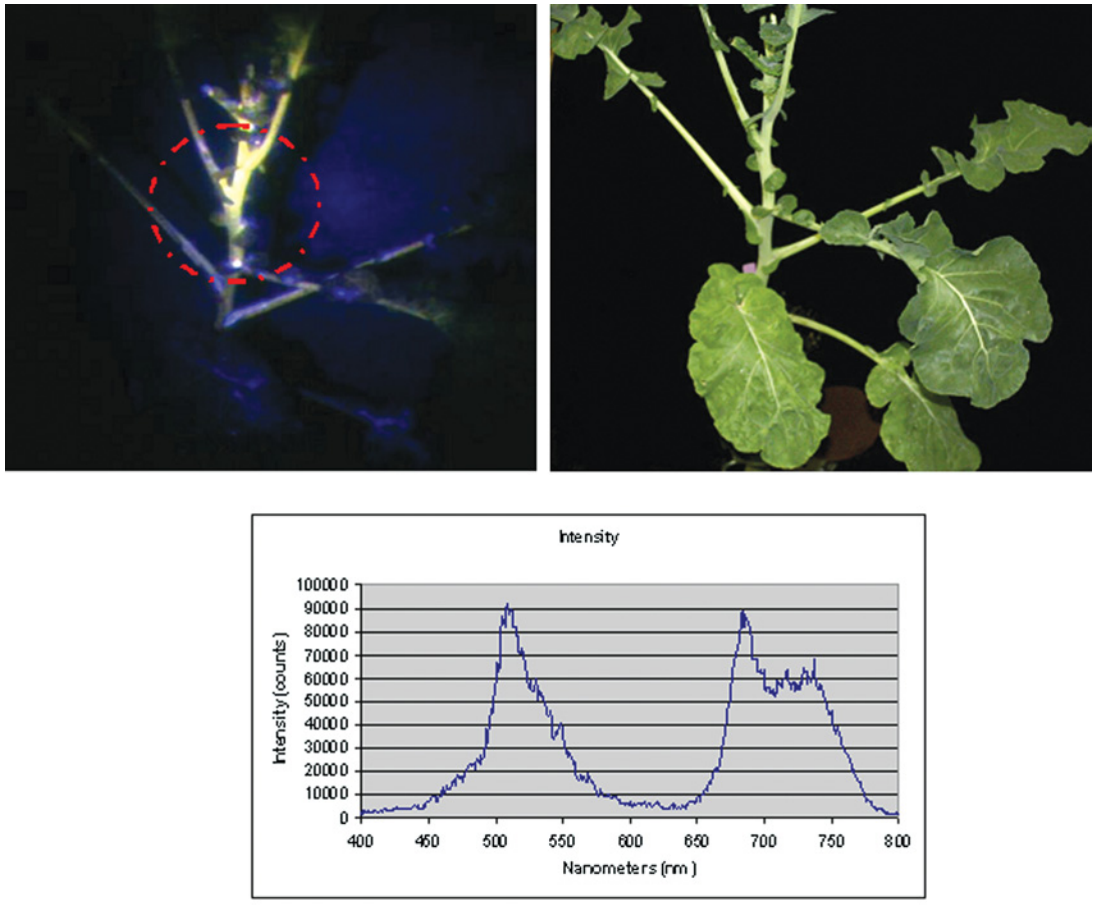

Fig. 2. A sample canola plant analyzed for GFP fluorescence. Laser-induced fluorescence imaging at 1 m under ambient room light (A) and without laser-induction (B). Laser-induced fluorescence spectra are in panel C.

scene. LIFI/LIFS has proven to be a complimentary diagnostic for vegetation state when combined with more traditional reflectance measurements and laboratory fluorescence spectra (see Schueger [18] for a discussion). While reflectance values typically vary from a few percent to 60–70%, fluorescence yields can vary over many orders of magnitude, thus indicating why fluorescence system development is often focused on a single need (or phenomenon) such as measuring vegetation stress. However, GFP or other fluorescent proteins cannot be measured using reflectance methodologies, but they can provide significant predictable spectral signatures as targets for detection over a somewhat predictable background, which is one reason why GFP is a widely popular reporter gene [2]. But see Halfhill *et al*. [19] for a discussion on prediction of GFP fluorescence in a known plant background.

In this particular biological/electro-optical system, the excitation wavelength of 355 nm from the tripled Nd:YAG laser was suboptimal for the detection of mGFP5-ER, since its excitation optimum is 395 nm. When a 390 nm dye laser was substituted for the Nd:YAG as an excitation source, there was a ca. 10-fold increase in contrast signal-to-noise ratio observed. The data indicate the techniques tested were effective at a standoff distance of 1 m (Figs. 2–4, 6). But because there is a linear decay relationship between signal-to-noise ratio between distance as well as for laser power (data not shown), before the technology could be deployed in the field, the system would need to be optimized specifically for the fluorescent molecule of choice (e.g., GFP) to be detected.

In addition to optimizing instrumentation to detect a specific fluorescent protein signature, the fluorescent protein itself should be optimized to match available instrumentation. Whereas many scientists have worked to decrease the Stokes shift for GFP by mutagenesis for red-shifted excitation [26–28], it is conceivable that the excitation optimum of GFP might be UV shifted to utilize the attractive properties of the Nd:YAG laser. Alternatively, a red fluorescent protein might be the ideal molecule for

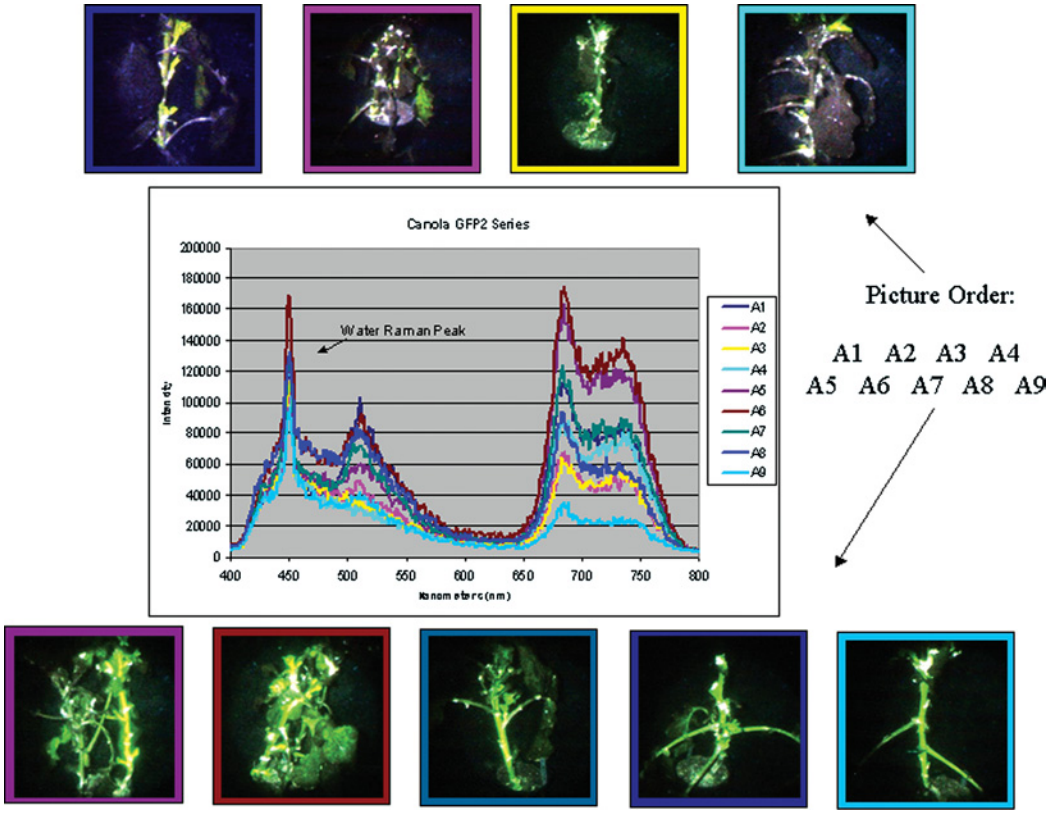

Fig. 3. Laser induced fluorescence imaging and spectrometry (LIFI and LIFS) of multiple canGFP2 canola plants at 3.3 m distance. Each image is an "RGB" false color composite and stretched 2% stretch to increase contrast. When the images are collectively stretched to the same brightness, several are practically dark due to the lack of GFP in the green band (525 nm).

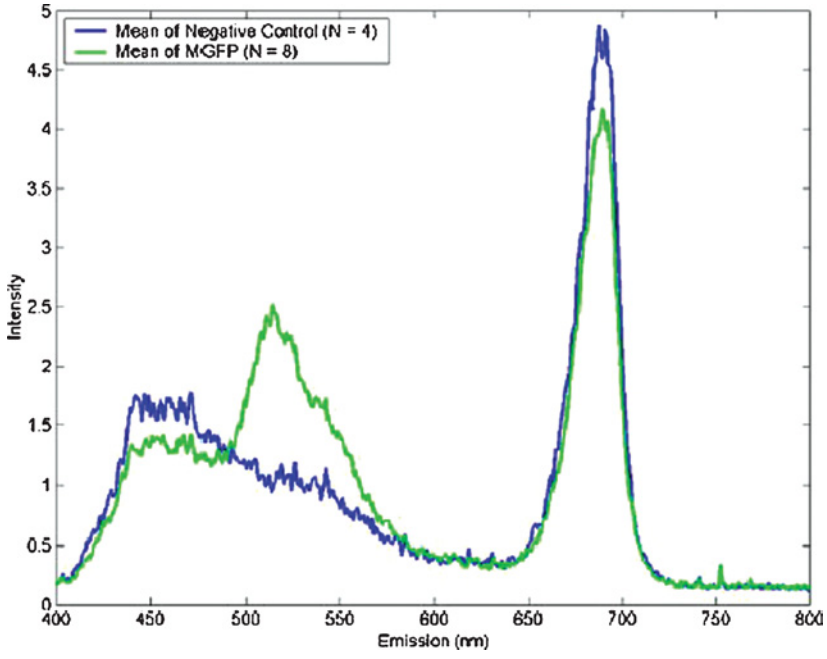

Fig. 4. LIFS data of tobGFP3 tobacco plants.

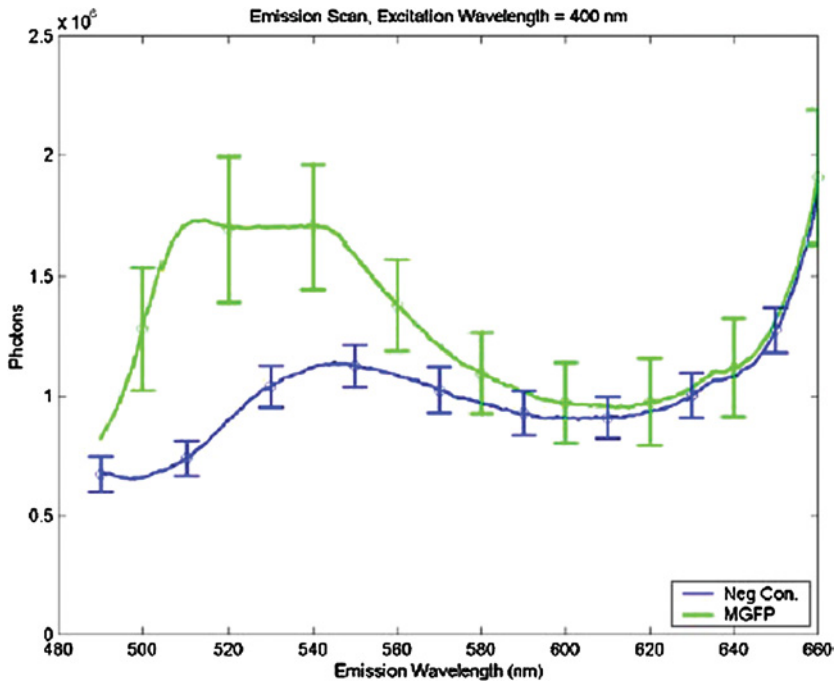

Fig. 5. Fluoromax data of tobGFP3 tobacco plants.

laser-induced detection in transgenic plants, since a doubled Nd:YAG laser (532 nm) could be used for excitation in the green spectra, yielding twice the power as in tripled mode (355 nm). Indeed, in addition to the mutagenesis of GFP to shift its spectral properties, there have been significant efforts to discover and clone genes encoding a rainbow of new fluorescent proteins (e.g., [29–31]) which might ultimately be better candidates for LIFS/LIFI monitoring than GFP. It is quite evident that biology and photonic research must be performed in tandem to design optimum systems for bioreporting.

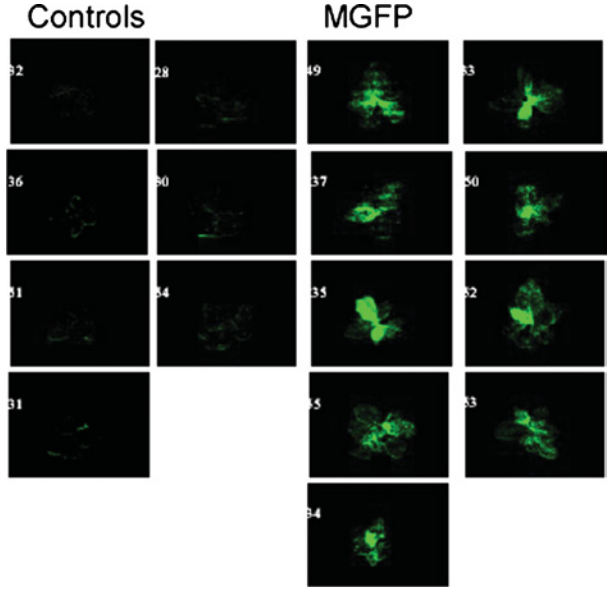

Fig. 6. LIFI data of several non-transgenic tobacco plants (*left*) and tobGFP3 tobacco plants (*right*).

ACKNOWLEDGMENTS

We thank Jim Haseloff for the gift of the *mgfp5-er* construct. We also would like to thank Steve Jones and Keith Williams for assistance in data acquisition and general aid in this project. This work was supported by a grant from the Defense Advanced Research Projects Agency (USA) and the US Army.

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