Development of Fibre Optic Spectroscopy For Detection of Genetically Modified Plants

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ABSTRACT

In this paper, fibre optic spectroscopy (FOSpectr) was developed for detection and quantification of recombinant green fluorescent protein (EGFP) in transgenic tobacco plants. *In vitro* detection was first carried out to optimize the sensitivity of the optical system. The bacterial expression vectors, pEGFP and pDsRED, were transformed into *Escherichia coli* host cells and fluorescent proteins were produced following induction with IPTG. Soluble EGFP and DsRED proteins were isolated from lysed bacterial cells and successfully purified by size separation under non-denaturing electrophoretic conditions and quantified. The purified proteins were serially diluted for quantitative analysis by fibre optic spectroscopy using different light sources, namely, blue LED (475 nm), tungsten halogen (350 – 1000 nm) and double frequency Nd:YAG green laser (532 nm). Tungsten halogen was found to be unsuitable for excitation of both EGFP and DsRED. Blue LED and green laser were the most suitable for excitation of EGFP and DsRED, respectively. The minimum concentration of EGFP detectable with blue LED excitation was 7.5 μg/ml whereas that for DsRED under excitation by green laser was 3.75 μg/ml.

To determine the capability of spectroscopy detection *in planta*, transgenic tobacco plants expressing EGFP were first imaged under a fluorescence microscope. This was to select a panel of transformed plants expressing varying levels of the fluorescent protein. These plants were then screened via FOSpectr. The results showed that the amplitude of the fluorescence emission signal obtained from FOSpectr correlated well with the level of EGFP expressed as indicated by fluorescence microscopy. Thus, proof-of-concept for the use of FOSpectr as a potentially powerful tool for screening transgenic plants were provided in this project.

Key words: Fluorescent proteins; fiber optics spectroscopy; genetically modified plants; selection marker;

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1. INTRODUCTION

Global advances in biosensing and biomarker technologies are geared towards development of innovative techniques that provide cost-effective, high-throughput, instantaneous, sensitive and non-invasive measuring and detection devices for biological applications. In keeping with current trends in technology development, this work focuses on developing an optical-cum-fluorescent protein marker system as an alternative to antibiotic resistance selection for identifying genetically engineered plants. Currently, genetically engineered plants are usually identified with the aid of a selectable marker gene that confers distinct properties on the modified plants. Antibiotic resistance genes are commonly used for such a purpose. Thus, when such a gene is successfully introduced into a plant, that modification event can easily be identified by virtue of the plant's ability to survive in the presence of antibiotics in the growing medium. However, there are public concerns and objections associated with the use of antibiotic resistance markers. These include the fear that such genes may escape from the modified plants by horizontal gene transfer and be incorporated into disease-causing pathogens, thus rendering antibiotic treatment ineffective (Grumet and Gifford, 1998). Other concerns relate to food safety issues (Dhanda, 2002). Although, there is as yet no solid scientific basis for such fears, public sentiment should still be treated with due deference and potential risks addressed. Thus, in the scientific arena, an array of alternative approaches has evolved to address these concerns. These include the development of antibiotic resistance marker excision strategies (Corneille *et al.* 2001; Iamtham and Day, 2000; Puchta, 2000; Gleave *et al.* 1999) or the use of alternative scorable markers for identifying genetically modified plants (Kunkel *et al*. 1999; Ebinuma *et al*. 1997).

In this work, we describe a novel optical approach used in conjunction with fluorescent protein markers that not only directly addresses public and biosafety concerns but also provides researchers with a powerful tool for instantaneous, non-invasive screening for successful transformation events. Since its molecular characterization in the early 1990s (Prasher *et al*. 1992) and its heterologous expression in bacteria and nematodes shortly thereafter (Chalfie *et al*. 1994), the green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, has been widely adopted as a biological marker in protein localization studies and reporter of gene activity (Tsien, 1998; Chiocchetti, *et al*. 1997; Welsh and Kay, 1997; Gerdes and Kaether, 1996). Since toxicity of highly expressed GFP in transgenic animals have not been observed (Amsterdam and Hopkins, 1998), it would be reasonable to expect that the cognate gene could be incorporated into plants for selection purposes without posing undue additional food safety issues associated with consumption of GFP-modified plants. A red-shifted GFP variant, EGFP, was used in our work so that excitation of the fluorophore could be achieved with blue light at 488nm instead of ultraviolet light, thus obviating potential UV-mediated damage of living cells. In addition, EGFP fluoresces up to 35 fold more strongly than wild-type GFP (Cormack *et al*. 1996). This brighter variant would thus greatly enhance the prospects of its detection *in planta*.

2. OBJECTIVES

The overall objective of this project is to develop an optical system based on fibre optic spectroscopy that allows for instantaneous and non-invasive detection of fluorescence emission signals from EGFP-modified tobacco plants. In a previous paper, we described the purification of bacterially-expressed green fluorescent protein and its subsequent use for optimizing the optical parameters of the fibre optic system for *in vitro* fluorescence detection (Liew *et al*. 2002). In this paper, we further describe how the optimized FOSpectr system evaluate the performance of the optical system by cormparing *in planta* emission signals obtained from FOSpectr with the fluorescence images obtained from fluorescence microscopy.

3. METHODOLOGY

3.1 Fibre Optic Spectrometer Set-up

The optical system for direct detection of fluorescence emission either from whole plant tissues or liquid samples was developed using off-the-shelf components. Essentially, it comprised 200 micron bifurcated optical fibres cable arranged in a "Y" configuration, with 6 optical fibres for illumination and one for reading. One end of the fibre optic cable is coupled to the excitation light source (blue LED, principal spectral line at 475 nm). The other end is coupled to a miniature double channel spectrometer: one channel monitors the excitation light source while the other monitors the fluorescence signal from the sample. The absolute values from these 2 channels was then calculated and quantified. The spectrometer was also fitted with 100 micron grating and CCD detectors providing an accuracy of ±0.40nm over a visible spectrum range of approximately 400nm to 800nm. A data acquisition card is used to establish communication between the computer and the spectrum. Band pass filters were used to enhance and isolate signals obtained from the emission spectrum, by making use of the fact that most biological labelers have a larger fluorescence Stokes shift.

3.2 Construction of chimaeric EGFP gene for plant expression

The structure of the EGFP gene construct for high level constitutive expression in plants is shown in Figure 1. The protein-coding region of EGFP contained in the plasmid, pEGFP (Clontech), was amplified by PCR and directionally inserted downstream of the synthetic 5' non-coding leader sequence of TMV RNA in the plant binary vector, pTMV35S (Liew, 1995). The resultant recombinant vector, pGLOWGreen, thus contained the chimaeric CaMV 35S promoter-TMV leader-EGFP-trasnscript 7 terminator fusion between the border sequences of the binary vector. An *nptII* gene, sited downstream of the right border sequence, encodes the enzyme neomycin phosphotransferase which confers genetically modified plant tissues with high endogenous resistance to the antibiotic kanamycin.

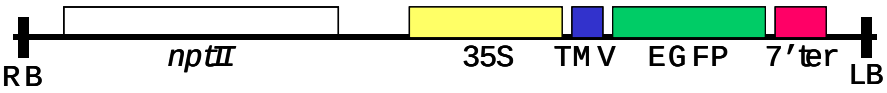

Figure 1: Assembly of chimaeric genes between the right and left border sequences of the recombinant plant binary vector, pGLOWGreen.

3.3 Regeneration of tobacco EGFP transformants

The chimaeric EGFP gene was introduced into the tobacco nuclear genome via *Agrobacterium*-mediated gene transfer. Briefly, tobacco leaf disks were cocultivated with the transformed *Agrobacterium tumefaciens* harbouring pGLOWGreen. Whole transformed plants were regenerated following callus induction and organogenesis under kanamycin selection. Stable integration and expression of the EGFP gene was confirmed by fluorescence microscopy.

3.4 Plant Expression of Fluorescent Proteins

Independently transformed tobacco plants expressing different levels of EGFP were assessed visually under the fluorescence microscope and segregated into weak, moderate or strong expressing lines. Classification of these lines on the basis of EGFP expression at the protein level was further fine tuned via Western analysis of leaf protein extracts with GFP antiserum. This preliminary grouping of transformed lines was to evaluate the capabilities of the fibre optic system for *in planta* detection and quantification of fluorescence levels. Untransformed wild-type tobacco plants were used to determine the levels of background fluorescence or

noise signals. Various parts of the plant were randomly selected and illuminated with blue light emitted from the fibre optic probe. Fluorescence emission signals were detected by the reading fibre within the same probe and spectral data were captured by a customized software installed in a laptop computer.

4 RESULTS AND DISCUSSION

4.1 Expression of EGFP in transgenic tobacco

More than 20 transgenic tobacco plants lines harbouring the EGFP gene cassette were regenerated. Preliminary screening of leaf segments under the fluorescence microscope showed variations in the level of fluorescent proteins expressed amongst the plants. These could be categorized into weak, moderate and strong expressing lines according to the intensity of fluorescence emission (Figure 2). Leaves from wild-type non-transgenic tobacco plants appear red due to chlorophyll autofluorescence. Dark red autfluorescence from the chloroplasts of wild-type *Arabidopsis* leaves were also observed by Niwa et al. (1999) by a fluorescent imaging system under 488 nm Ar blue laser excitation. Niwa et al. 1999 showed that red autofluorescence intensity is consistent across various transgenic *Arabidopsis* lines and can be useful for normalizing green fluorescence intensity to increase reliability of quantitative values. Thus, in our classification of expressors, strong expressors are those where green fluorescence was expressed at significantly high enough levels to mask the red autofluorescence whereas in weak autofluorescence, appreciable red breakthrough fluorescence could be observed. Variable EGFP transgene expression can be due to a combination of events arising from copy number and plant chromosomal position of gene insertion, transcriptional activity of DNA sequences surrounding the site of insertion, locality of silencers and enhancers, and DNA methylation. Interestingly, we also observed variations in the spatial distribution of expressed EGFP at the tissue level in one of our tobacco transformants (Figure 2b). This phenomenon points to the advantage of developing the system for whole tissue or even whole plant imaging rather than point data collection.

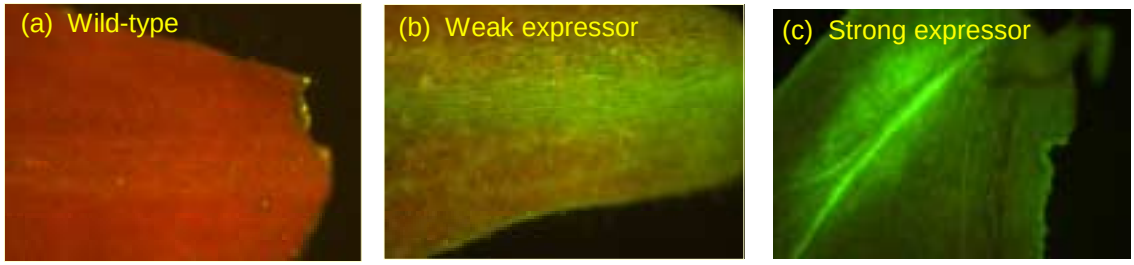

Figure 2: Fluorescence microscope images of tobacco leaves of (a) non-transgenic wild-type plant (b) weakly expressing transgenic plant (c) moderately expressing transgenic plant (d) high expressing transgenic plant (magnification 4X).

When leaf cells were viewed under high power magnification, EGFP was found to be localized primarily within the nucleus and peripheral cytoplasm. This is typical of the compartmentalisation of green fluorescent proteins in eukaryotic plant cells when subcellular targeting signals are not used (Kohler et al. 1997; Haseloff et al. 1997; Rouwendal et al. 1997; Chiu et al. 1996). Previous studies showed toxicity of wild type GFP in Arabidposis linked to GFP accumulation in the nucleus and cytoplasm. (Hasselof et al. 1997). However, Niwa et al. (1999) did not observe any GFP-mediated toxicity when a variant GFP(S65T) sequence with optimized eukaryotic codon usage was expressed at high levels in *Arabidopsis*. In our work, we observed higher incidences of morphological aberrations amongst potato transformants compared with tobacco transformants, the former displaying features like reduced plant

vigour, unusual leaf shape and development of a chlorotic appearance. Whether these phenotypic aberrations are the result of greater susceptibility of potato plants to GFP toxicity remains to be determined. However, we note that our observations were similar with those reported by Rouwendahl et al. (1997) who also found that GFP potato transformants tended to manifest poor growth compared with tobacco transformants.

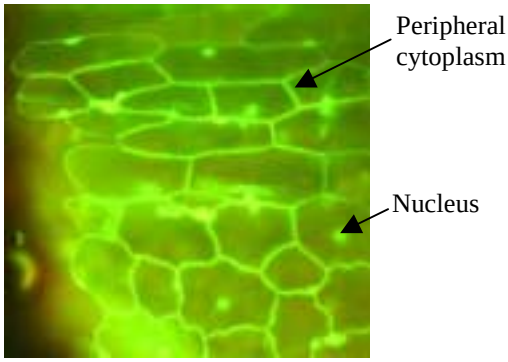

Figure 3: Fluorescence microscope image showing cellular localization of EGFP within cells of transgenic tobacco leaves (magnification 40X)

4.2 In planta quantification of fluorescence using FOSpectr

Fluorescence signals at an emission wavelength of 509 nm could be detected by FOSpectr from weak and strong expressors of EGFP but not from non-transgenic wild-type plants (Figure 4a). It was observed that the amplitude of the emission signal correlated well with the strength of EGFP expression as assessed by fluorescence microscopy. Fluorescence intensities obtained from the leaf blade, main veins and stems of the same plant appeared to be fairly consistent within the various plant parts but signal amplitude from the stems tends to be higher than the other tissues (Figure 4b). The combined observations of differences in the levels of expressed EGFP between organs of the same plant described earlier as well as variations in the spatial distribution of EGFP expression within the same tissue point to the advantage of developing the system for whole tissue or even whole plant imaging rather than point data collection. Finally, a peak emission signal around 700 nm was also detected for leaf samples. This signal corresponded to autofluorescence from chlorophyll in the leaves. Future work will fine-tune quantitation of EGFP expression by correlating the amplitude of the fluorescence emission signal with actual amount of EGFP protein expressed in transformed tissues.

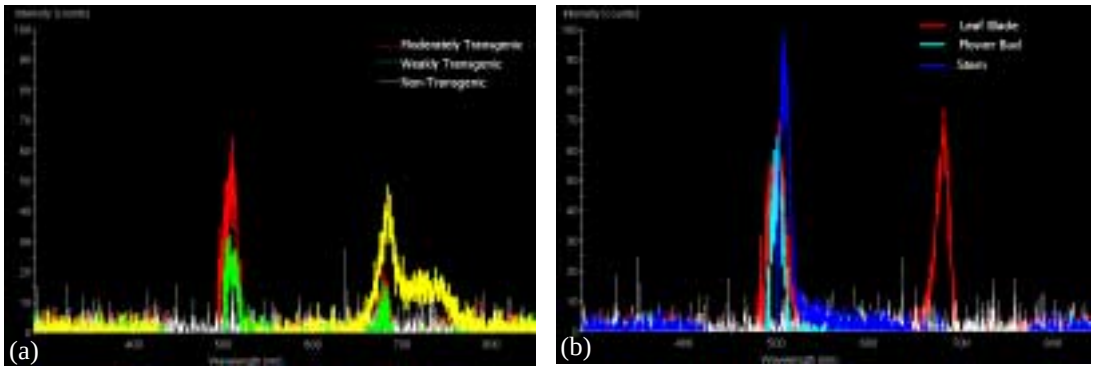

Figure 4: FOSpectr detection of EGFP fluorescence (a) from non-transgenic, weak and stronge expressors of EGFP (b) from the leaf blade, main vein and stem of a transgenic tobacco plant.

The FOSpectr approach provides significant advantage over the current method for screening GM plants based on antibiotic resistance selection as the amplitude of the emission signal provides added capability of quantifying the activity of the introduced gene. In addition, future development of a portable version of the optical system would allow instantaneous and non-destructive detection of transgenic plants even out in the field. The fluorescent scorable marker system will potentially reduce the amount of labour and time required to screen and select genetically stable and highly expressed plant lines. This approach will be a significant step towards improving public risk perception of GM crops over the current method of screening plants based on antibiotic resistance. It is also expected that this strategy could be incorporated in risk assessment programs for field-released GM plants to track transgene movement in the environment. When fluorescent protein genes are linked with stress-inducible promoters, this technology also provides the springboard to develop "Indicator Plants" for optical tracking of abiotic stress in field cultivated crops.

5. CONCLUSIONS

Green and red fluorescent proteins, EGFP and DsRED, was produced in large quantities by *E.coli* bacteria. These proteins were also successfully purified on the basis of molecular size separation under non-denaturing electrophoretic conditions without loss of fluorescence ability. EGFP was found to be most effectively excited by blue LED (488 nm) resulting in fluorescence emission at 509 nm. At an integration time of 200 msecs, the lowest detectable concentration of EGFP was found to be 7.5 μg/ml. In the case of DsRED, the most suitable excitation light source was green laser (532nm), allowing collection of distinct emission signal at a concentration as low as 3.75 μg/ml when an integration time of 600 msecs was used. A panel of weak and strongly expressing fluorescent transgenic tobacco plants were assembled on the basis fluorescence microscope imaging. The present results showed that FOSpectr could detect EGFP fluorescence signals from weak and strong expressors. Further fine-tuning of spectroscopic quantification should improve the sensitivity for *in planta* detection.

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