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Review

Light That Reflects Your Effort

MUHAMMAD SARWAR KHAN, YUSUF ZAFAR AND KAUSER ABDULLAH MALIK†
National Institute for Biotechnology & Genetic Engineering (NIBGE), P. O. Box 577, Jhang Road Faisalabad, Pakistan; †Pakistan Agricultural Research Council, Islamabad

ABSTRACT

The use of a non-toxic visual marker is advantageous, for discerning transgenic cells and removing untransformed or non-expressing cells, tissues or organs, over genes required substrates and cofactors for their expression to report. The green fluorescent protein (*gfp*) of the jellyfish, *Aequorea victoria*, has recently been used as a reporter gene in plants and animals. In plants it has extensively been used in plant transformation experiments, localization of proteins to organelles and to study gene expression. Visual marker like FLARE-S (Khan & Maliga, 1999) made it possible to differentiate transformed from untransformed plastids resulted in chimeras in leaves of monocot as well as dicot plants. Unlike tobacco and Arabidopsis, plant regeneration from leaves in monocots (cereals) is not possible, therefore, it is difficult to make transformants homoplasmic. To make rice transformants homoplasmic fluorescent marker like FLARE-S is required which enables selection of fluorescent cells on the medium. Moreover, fluorescent antibiotic resistance marker will enable extension of plastid transformation to other cereals, where plastid transformation is not associated with a readily identifiable phenotype. Furthermore, GFP has multiple isoforms with distinct spectral properties could be useful to track numerous proteins in living cells.

Key Words: Light effects; Genetics

With the advancement in our understanding to engineer multigenic traits make it necessary to increase our repertoire of genes, in parallel with the development of a better understanding of factors which control the concerted expression of multiple transgenes. Introduction and stacking of such genes requires handy means to report their presence into the genome. Vital reporter genes undoubtedly contribute to the development of such technology by serving as tools for visual monitoring of transgene expression in transformed cells, tissues and organisms. A number of genes have been used to study gene expression, in plants as well as animals, as reporters. For example, the genes encoding β-glucuronidase (*uidA,* Jefferson *et al.,* 1986) and β-galactosidase (*lacZ*, Miller *et al.,* 1970), chloramphenicol acetyl transferase (*cat*) and neomycin phosphotransferase (*nptII*, Hererra-Estrella *et al.,* 1983; Fraley *et al.,* 1983), nopaline synthase (*nos*, Depicker *et al*., 1983; Bevan *et al.,* 1983) and octopine synthase (*ocs*, Johnson *et al*., 1974) have been used as reporter genes for transformation. Of these genes, *uidA* has successfully been expressed transiently and stable in a variety of organisms (Staub & Maliga, 1994; Seki *et al.,* 1995). However, histochemical detection of GUS in plant organelles requires prolonged incubation because the envelope membranes of the organelles act as a selective barrier to substrate penetration. Moreover, in plants chlorophyll bleaching is required to make GUS staining more effective using either ethanol or chloral hydrate (Jefferson, 1987). Furthermore, chemicals and physical procedures used in the staining disrupt cell ultrastructure Baulcombe *et al*., 1995). The use of a non-toxic marker to identify transgenic cells after transformation is an effective procedure for discerning transformed cells/organs and removing untransformed or non-expressing cells, tissues or organs. The green fluorescent protein (*gfp*) of the jellyfish, *Aequorea victoria*, has recently been used as a reporter gene in plants and animals (Baulcombe *et al*., 1995; Haseloff & Amos, 1995; Hu & Cheng, 1995; Niedz *et al*., 1995; Rizzuto *et al*., 1995; Chiu *et al*., 1996; Haseloff *et al*., 1997). *gfp* provides an easily scored cell-autonomous genetic marker in plants and has major uses in monitoring gene expression and protein localization at high resolution. It allows direct imaging of the fluorescent gene product in living cells without the need for prolonged and lethal histochemical staining procedures (Chalfie *et al*., 1994). The chromophore forms autocatalytically in the presence of oxygen and fluoresces green (508 nm) on absorption of blue light or UV light of 395 nm. The green fluorescent protein is very stable to denaturants, such as 1% SDS, a range of pH and to proteases (Prasher *et al*., 1992). Moreover, GFP fluorescence is confined to cells with no leakage unlike GUS to neighbor cells.

Recombinant *E. coli* cells that fluoresce bright:

The green fluorescent protein has successfully been expressed to screen *E. coli* cells, to compare promoters strength and document gene expression levels under different promoters; promoters of bacterial and bacteria-like origin (Hiberred *et al*., 1998). Moreover, plant transformation vector constructs containing gene (s)

linked with gfp, particularly designed to transform organelles like plastids, to confirm that the *gfp* constructs able to produce functional green fluorescent protein, expression can be examined in *E. coli* (Khan, 2000). This protein gives handy screening of recombinant *E. coli* colonies due to fluorescence under blue light or UV light of 395 nm. Thus selection and screening of colonies containing gene linked with fluorescent marker will enable to differentiate cloned and wild-type colonies avoiding the need to use substrate to document gene expression like *lac-Z* expression requires substrate for blue white selection/screening.

Several chloroplast promoters have been shown to direct the transcription initiation of reporter genes in prokaryotic cells (Erion *et al*., 1983; Boyer & Mullet, 1986; Thompson & Mosig, 1988) on the basis of sequence similarities of putative chloroplast promoter regions with *E. coli* promoter regions, and on the ability of prokaryotic RNA polymerase to recognize certain chloroplast promoters (Erion *et al*., 1983; Sugita & Sugiura, 1996). The *gfp* has been used as visual marker to compare expression levels of the reporter and selection genes under species-specific i.e. bacterial promoters and using heterologous promoters i.e. bacteria-like plastid promoters (Khan, 2000; Khan & Maliga, 1999). To confirm that the *gfp* constructs able to produce functional green fluorescent protein, expression is examined in *E. coli*. Electro-competent cells of *E. coli* strain DH5α were electroporated with plasmids containing *gfp* and without *gfp*, and plated on LB-agar plates containing antibiotics. The *E. coli* colonies which grew overnight at 37˚C were selected and analysed for inserts by fluorescence and by digestion of DNA with appropriate enzymes, obtained by miniprep method. Cells from positive colonies were grown on LB-agar plates containing drug for 20 h at 37˚C and the *E. coli* colonies were examined under a hand-held long-wave UV lamp. Colonies containing the gfp under bacterial promoter and bacteria-like promoter fluoresced green on excitation with long-wave UV light but those without gfp did not fluoresce (Fig. 1). Liquid cultures (5 ml) of all three strains were grown in the presence of antibiotic at 37˚C for 2-3 h to an OD600 of 0.5 measured using a Perkin-Elmer Lambda 9 spectro-photometer. The relative fluorescence intensities at 508 nm after excitation at 395 nm were measured using a Perkin-Elmer LS spectro-fluorimeter. GFP promoter, *rrn* (pMSK24) and plasmid without gfp. fluorescence was 6-fold higher in cells transformed with plasmid where expression of *gfp* was controlled by the bacterial *trc* promoter compared with cells transformed with plasmid where *gfp* expression was controlled by the chloroplast ribosomal RNA promoter (Fig. 2). This confirms that the chimeric *gfp* constructs were functional and suggests that the *trc* promoter is stronger than the chloroplast *rrn* promoter in *E. coli*. A number of other genes like *aadA*, *nptII* and *kan* have been expressed in *E. coli* under bacteria-like promoters, moreover, some complementation experiments have been conducted in *E. coli*.

Fig. 1. GFP expression in *E. coli* cells to compare promoters strength. GFP gene under control of bacterial promoter, *trc* (pMSK18), bacteria-like

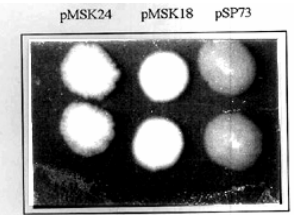
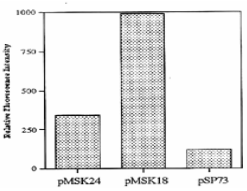


Fig. 2. Fluorescence intensities show that the *trc* promoter appeared to be more stronger than *rrn*.



Transformed animal cells that fluoresce bright:

Down the road ultimate objectives of the biotechnology and genetic engineering is to decipher what and how events occur inside the living cells or organisms. Biological research in living cells has been exceedingly difficult in the absence of readily available, user-friendly, noninvasive techniques. A major step forward in the effort to understand the biology of the living cell, an extremely useful tool for a number of biotechnological applications has recently been made with use of visual detection of cellular events in their natural environment using green fluorescent protein. Several examples of GFP expression in nematodes, flies and animal cells (MacGregor & Caskey, 1989; Lin *et al*., 1994; Wang *et al*., 1994; Moss *et al*., 1996; Valdivia & Falkow, 1997) are available in literature, as author has more concern with plants therefore use of GFP in plants is being discussed in details in this review.

Multiple isoforms of GFP with distinct spectral properties are available to track numerous proteins in living cells. These isoforms can be used in energy transfer experiments to study physical proximity of two proteins. Can be used to study protein degradation in living cells, protein localization to membranes, to study muscle development in animals. And there are so many

aspects concerning molecular biology where GFP can be used to uncover them.

Transformed plant cells that fluoresce bright:

Obtaining an early, clear and strong signal, to screen transformed cells and to document gene expression in plants, is clearly advantageous over genes required substrates and cofactors for their expression to report. This is particularly preferred to establish new transformation protocols and to identify transformed cells, and to do genes fusion studies. A number of plant species have been genetically modified for different reasons but the use of antibiotic resistance genes is not acceptable by people due to the possibility of gene (s) escape from genetically modified plants to the near relatives or to the feeding animals including human being. A number of NGO's are against the release of GM crops into the open environment. Therefore, it would be advantageous to use either plant gene conferring natural resistance or gene that is nontoxic to animals/human being as a replacement of selectable marker. Such a gene, as mentioned above, is GFP that has also been used successfully to monitor gene dissemination from GM crop to near relatives like oilseed rape into wild type *Brassica rapa* (Scott & Wilkinson, 1999). Virus movements in plants have been detected using GFP as probe in live tissues of tobacco *Nicotiana benthamiana* (Baulcombe *et al*., 1995; Casper & Holt, 1996). Rapid screening of transformed plant cells has been reported using GFP as a visual marker (Vain *et al*., 1998).

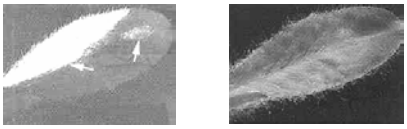
Present use of fluorescent marker GFP in plants:

With the development of biotechnology and compelling demand to express multiple genes controlling multiple traits, into the same recipient plant favors the engineering of organelle genome of the plants. The ability for genetic transformation of the plastid genome in higher plants is extremely attractive and important to the development of transgenic traits that may be difficult or impossible to achieve by nuclear transformation. The plastid genome of higher plants is an attractive target for crop engineering, since proteins in chloroplasts may accumulate to high levels, multiple genes may be expressed as polycistronic units, and lack of pollen transmission in most cultivated crops results in natural gene containment. Plastid transformation is accomplished through a multi-step process. Plastid transformation vectors containing a selectable marker gene and passenger gene (s) flanked by homologous plastid targeting sequences (Khan & Maliga, 1999) are introduced into the plastids by biolistic DNA delivery (Svab & Maliga, 1993; Khan & Maliga, 1999) or PEG treatment (Golds *et al.*, 1993; Koop *et al.*, 1996) methods.

The green fluorescent protein has successfully been expressed in *E. coli* and chloroplasts of tobacco, rice (Khan & Maliga, 1999), different plastid types (Hibberd *et al*., 1998) and potato (Siderov *et al*., 1999) using chloroplast as well as bacterial-specific expression signals. It was therefore expected that genes would be expressed from bacterial promoters in chloroplasts. This protein has been expressed in plastids transiently (Hibberd *et al*., 1998) as well as stable expression of *gfp* in chloroplasts under the control of such bacterial promoters have been obtained successfully.

The selectable marker genes confer resistance to drugs in plastids. These drugs inhibit chlorophyll accumulation and shoot formation on plant regeneration media. Unlike tobacco and Arabidopsis, in rice a readily identifiable tissue culture phenotype such as shoot regeneration is not available. Marker gene recipient cell go through phases of embryogenesis and organogenesis before regenerate to green shoots. During the time of embryogenesis and organogenesis, wild-type and transformed plastids and plastid genome copies gradually sort out. The extended period of genome and organeller sorting yields chimeric plants consisting of sectors of wild type and transgenic cells. In the chimeric tissue antibiotic resistance conferred by marker gene (s) is not cell autonomous: transplastomic and wild type sectors are both green due to phenotypic masking by the transgenic tissue. Chimarism necessitates a second cycle of plant regeneration on a selective medium. In the absence of a visual marker this is an inefficient process and end up in heteroplastomic tissues or plants (Khan & Maliga, 1999) that were only identified by FLARE-S (Fluorescent antibiotic resistance enzyme conferring resistance to spectinomycin and streptomycin) expression (Fig. 3).

Fig. 3. Tobacco leaves showing chimeric nature of cells. Bright color indicates gfp expression while dark (red) chlorophyll fluorescence.



Unlike tobacco and Arabidopsis, plant regeneration from leaves in cereals is not possible, therefore, it is difficult to make transformants homoplasmic. To make rice transformants homoplasmic FLARE-S containing fluorescent cells will be screened using fluorescent detection system. Single cell will divide to form a colony on selection medium and that can regenerate into possible homoplastomic shoot. Furthermore, fluorescent antibiotic resistance marker will enable extension of plastid transformation to other cereals, where plastid

transformation is not associated with a readily identifiable phenotype.

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