Notes

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Use of Green Fluorescent Protein as a Molecular Tag of Protein Movement *In vivo*

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There are a number of species of proteins in plant cells. In most cases researchers are focusing their interest on one or several proteins and observing what it is or what it is doing for the organism to live. In most cases, researchers raised antisera against the protein of interest. After raising the antisera, they would try to detect the protein(s) using the antisera by immunostaining after fixing the cells.

Recently the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has drawn much attention in biology [1]. It is now widely recognized that GFP is very feasible for use by reseachers who are investigating the functions of proteins or looking at the traffic of proteins *in vivo*. No extra special substrate/chemical substances to detect the signal are needed. The protein will not diffuse or penetrate into other places. Direct visualization of gene expression or localization of proteins by fusing with GFP is therefore possible without cell lysis or fixation. One thing we have to do is to fuse the target protein with the GFP protein by molecular recombination technique at the cDNA level. Here we will describe how we manipulated GFP genes to look at the intercellular and intracellular movement of a protein of tomato mosaic virus (ToMV). The protein is called a movement protein (MP), which functions to move ToMV from one initially-infected cell to surrounding non-infected cells. By using GFP we could observe dynamic shift of localization of the protein [2, 3].

1. *Choice of the version of GFP*

From the literature one can see that there are a growing number of "plant optimised" GFP at present. Sometimes the selection of a GFP from many variations is important. The optimization is based on different aspects. Please refer to **Table 1**. Amino acid substitutions are shown by one-letter symbols. Some of them are the optimization of codon adapted for plant (plantinized) not for jelly fish so as to be expressed efficiently in plant systems. It is claimed that aberrant RNA processing may interfere with GFP expression studies in plants. The modification of a cryptic *Arabidopsis thaliana* intron sequence gave a so-called Haseloff type GFP.

Mutations that shift light emission and improve strength of fluorescence (*e. g.* S65T; wild type serine at amino acid 65 was substituted by threonine) are now widely introduced [4].

Table 1.

Summary of GFP variants which are modified for use in plant research.

variation name	amino acid substitution	nucleic acid sequence	excitation wavelength (nm)		emisson wavelength (nm)	references
			UV	blue		
wild type	—	wt	395	475	508	Gene **111**, 229-233(1992)
S65T	S65T	wt	—	490	510	Nature **373**, 663-664(1995)
mGFP 4	—	Haseloff-type	395	475	508	Trends Genet. **11**, 328-329(1995)
G3	S65A/Y145F	Haseloff-type	—	undetermined	undetermined	Ogawa & Umesono, unpublished
cycle 3	F99S/M153T /V163A	random-PCRed	385	488	510	Nature/Biotech. **14**, 315-318(1996)
sGFP(S65T)	S65T	plantinized	—	490	510	Cur. Biol. **6**, 325-330(1996)
smRS-GFP	F99S/M153T /V163A/S65T	Haseloff-type	—	488	undetermined	Weeds World **3**, 43-48(1996)
Y66H	Y66H	wt	382	—	447	PNAS. **91**, 12501-12504(1994)
BFP	Y66H/Y145F	wt	380	—	445	Cur Biol. **6**, 178-182(1996)

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Cycle 3 mutant emits somewhat stronger fluorescence than the wt GFP when excited in the UV (395 nm) partly because this GFP has less tendency to form non-fluorescing aggregates [5, 6]. However, it is less intense than *e. g.* S65T, when excited in the blue light at 488 nm. If you want to excite with a hand-held UV light, cycle 3 mutant would be best for the purpose. If you plan to do fluorescence microscopy work with FITC-adapted filters or FACS, smRS-GFP [6] or G3-GFP (**Table 1**) would be the best choice. Spectral characteristics of both variants match common FITC filter sets.

It has been documented that S65T has an extinction coefficient of 39, 200 M-1 cm-1 at 490 nm allowing that measurement of extinction coefficients are very subject to conditions. Niswender *et al.* reported that for quantitative imaging, 1 μM of cytoplasmic wtGFP is required [7]. The detection limits should be much lower for the bright chromophore variants of GFP with S 65 T mutation.

2. *Construction of virus mutants containing of movement protein-GFP fusion proteins*

Four primers were prepared to construct ToMV MP : GFP fusions (**Fig. 1**). Primer pglyGFP includes a *Sac*I site (lowercase; the site is not found in the ToMV full-length cDNA clone), sequences directing the synthesis of polyglycine (underlined) and followed by N-terminal sequences from GFP; AGTCAAgagct-cATTTCTGGTGGTGGTGGTATGAGTAAAG-GAGAAGAACT. Primer G 2+3 includes a *Bst*EII site (lowercase; unique site in the ToMV full-length cDNA clone) and antisense GFP C-teminal sequences; AAGggttaccTTATTTGTATAGTTCATC CATGCCATG. High-fidelity PCR [8] to amplifiy G 3 GFP sequences were performed with the two above primers and G3 GFP plasmid DNA as a template. The products were restricted with *Sac*I and *Bst*EII overnight.

Primer L392EcoRV has a sequence of TACGGAT-CATTGACATATgatatcGGA, which is colinear with ToMV genomic sequences [9] and covers an *Eco*RV site (lowercase) located at n.t. 392. Primer L2-SacI has the sequence TACATgagctcATCCGCGACCGAC-GTCTCG, which has an antisense sequence of ToMV and is designed to introduce a *Sac*I site (lowercase) just after amino acid 260 th of the MP and to fuse MP and GFP in the same reading frame. A fragment encompassing MP and upstream genomic sequence was made by high-fidelity PCR [8] using two primers, L392EcoRV and L2-SacI, and wild type ToMV cDNA (pTLW 3)[8] as a template. The PCR products were restricted with *Sac*I and *Acc*III (n. t. 3759; another unique site in the ToMV full-length cDNA clone).

The *Acc*III (n. t. 3759)-*Bst*EII (n. t. 5799) fragment in pTLW3 was replaced with *Acc*III/*Sac*I restricted PCR fragments containing the MP gene and a *Sac*I/*Bst*EII restricted GFP fragment. The resultant virus LQwt : Gfus virus would produce a MP trucated at the C-terminus by 4 amino acids and fused with G3 GFP, but lacked an intact CP (**Fig. 1**).

3. *Observation of GFP localization*

(1) Observation of whole plants

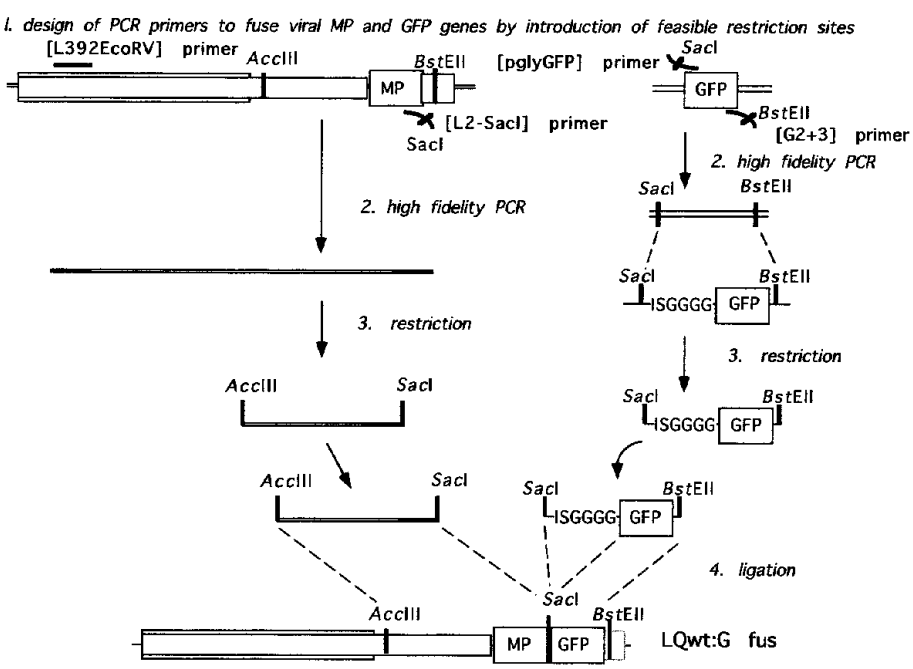

Fig. 1 Scheme of construction of virus expressing MP : GFP fusion proteins.

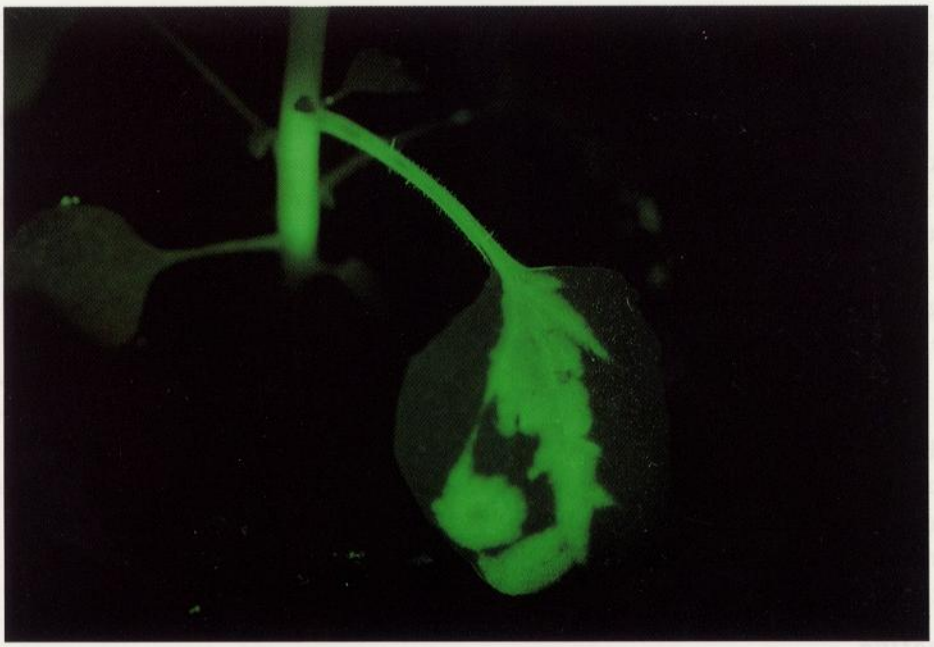

Fig. 2 Detection of spread of ToMV expressing GFP during multiplication by UV-illumination to plant tissue.

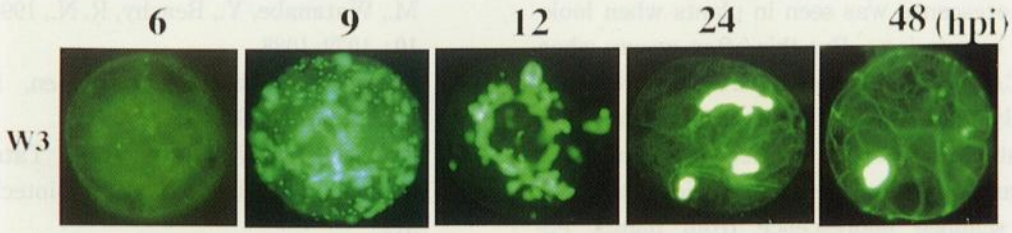

Fig. 3 Observation of time-dependent change of localization of ToMV MP : GFP in BY-2 tobacco protoplasts.

A hand-held UV illuminator is adequate for visualization of wild-type GFP expression. Figure 2 represents an example which shows spread of ToMV expressing GFP in infected *Nicotiana benthamiana* tissue. A good light source for exciting GFP by visible light is a tungsten halogen lamp used for fiber optic illuminators or even slide projectors. A xenon arc lamp would be another possibility if higher luminous flux is needed. A Kodak slide projector with an optical filter like a 488DF22 narrow bandpass filter in the slide slot, matched to the excitation wavelength of the S65T GFP (488 nm). To visualize and cut off the illuminating blue light, two Kodak No. 12 or 58 gelatin filters taped to a UV face shield as a convenient way to hold the filters.

(2) Observation at a microscopic level

Much weaker fluorescence can be observed at the cell level using a fluorescence microscope or FACS. Leica fluorescence stereomicroscope based on the MZ12 with the GFP fluorescence module is suitable for the observation of S65T GFP. This GFP module is well designed for observation.

Whole mounts of live plant tissue can be sectioned optically using a laser-scanning confocal microscope to allow the analysis of cellular and subcellular detail, despite autofluorescence, refractile nature and light scattering properties of plant cell walls. It potentially allows the precise monitoring of dynamic events in living plants.

If details of fluorescence localization is necessary, fixation with 4% paraformaldehyde is adequate for the initial try. There is perhaps a slight fading of fluorescence after fixation, but this is not a big problem. Other fixatives like methanol are likely to fade the GFP fluorescence. One interpretation for this is that GFP alone cannot be fixed by cold methanol as expected for a non-interactive protein. But GFP fusion constructs like with some cytoskeletal or other methanol stable structure are clearly visible.

If available, Nomarski device option would give a detailed image. Figure 2 shows how ToMV MP accumulates and behaves in tobacco BY-2 protoplasts after LQwt : Gfus virus infection. We could see that MP is first synthesized and scattered in the whole cytoplasm, then forms bright larger structures and finally associates with filamentous cytoskeletons. Association with microtubules was confirmed by im-

munostaining with anti-tubulin antibody [2].

For long observation we should beware of desiccation of samples. To avoid this, sealing the coverslips on wet mounts of GFP expressing cells is necessary. Nail polish is known as the easiest way to seal, but some brands are claimed to quench the fluorescence. A 1 : 1 : 1 mixture (weight) of vaseline, lanolin and parafin wax works fine for this purpose. The mixture is heated gently on a hot plate until melted and used to seal the coverslips. It sets immediately upon cooling.

(3) Photorecording

Any of the color slide or color print films will have a very high green efficiency that coincides with the GFP emission maximum. The film peak sensitivity occurs at about 530 nm.

4. *Troubleshooting*

(1) Autofluorescence of plants

Some people claimed that green or yellow endogenous fluorescence was seen in plants when looking for GFP expression. But this often occurs when using FITC/FACS filter sets. The fluorescence comes mainly from the cell wall and is sometimes absent or patchy. When we applied monochromatic light of a range of 380-540 nm to various kinds of plants and scanned fluorescence from plants, endogenous fluorescence which might disturb GFP observation comes in red only from chlorophyll in chloroplasts.

(2) No fluorescence from fusion constructs

It is accepted that N-terminal fusion proteins are more likely to fail in fluorescing than C-terminal fusion constructs. Besides, the use of a 6-10 amino acids stretch of glycine and/or alanine linker between the target protein and GFP might help GFP tag to fluoresce. GFP takes a rigid high-ordered structure to fluoresce. It is quite possible that existence of glycine/alanine stretch allows the original protein portion in the fusion protein to form respective structures and functions.

When PCR technique is applied to fuse GFP to the protein of interest, 3′ untranslated sequence originated from *A. victoria* should not be included to express GFP from a heterologous promoter. It suppresses the expression of GFP in plants (C. Holt, unpublished data).

(3) Reduced viability of GFP expressing plants

It is reported that it is difficult to regenerate fertile plants efficiently from the bright transformants. It is possible that high levels of GFP expression are mildly toxic or interfere with regeneration.

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Erratum

S. Nakamura, T. Iwai, R. Honkura, M. Ugaki, M. Ohshima and Y. Ohashi, **Four Chitinase cDNAs from *Chenopodium amaranticolor***, *Plant Biotechnology*, **14**, 85–86 (1997).

The sentence, "A polyclonal antibody against CAM 18 was used as the.", in legend of Fig. 2 in page 86 should become "A polyclonal antibody against CAM18 was used as the primary antibody.".