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Green Fluorescent Protein in Transgenic Plants

Brassica *Transformation*

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1. Introduction

Until the heterologous expression of *Aequorea victoria* green fluorescent protein (GFP) was demonstrated, scientists working with transgenic organisms had no good alternative to using destructive visible genetic markers. Genes coding luciferase *(1)* and β-glucuronidase *(2)* are the most popular destructive marker genes that have been successfully used in transgenic plants. Although these markers code for sensitive enzymes that have linear dose responses, they require expensive substrates, and are limited to laboratory uses. Most of all, they cannot be used to assay living tissue directly.

GFP offers the possibility to assay vital cellular functions, to determine the transgenic status of plants, and to monitor plant transgene expression in real time, in live cells or intact plants. This chapter focuses on the use of GFP as an enabling biotechnology in the production of transgenic plants, especially *Brassicas*. GFP offers the plant biotechnologist the tool to produce plants in the absence of, or in conjunction with, antibiotic or herbicide markers for selection. It also offers a mechanism to quickly identify transgenic plants in mixed populations. GFP will prove to be an important tool for the making and monitoring of transgenic crops and trees, in the future *(3,4)*.

Several GFPs have been shown to be useful in plants. The earliest useful variant was mGFP4, a near-wild-type version that had an altered plant-recognized cryptic intron *(5)*. Unfortunately, this GFP was neither bright nor very stable. Improved versions of mGFP4 (mGFP5 and mGFP5-ER) have wild-type chromophores, but have the following mutations: V163A, S175G, and I167T *(5,6)*. These mutations confer increased folding at warm temperatures, equal

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and dual excitation peaks at 395 and 475 nm, and an emission peak at 509 nm *(6)*. The endoplasmic reticulum version has a signal sequence and HDEL retention signal for targeting GFP to the endoplasmic reticulum. Human codon-optimized S65T mutants have also been useful in plants *(7,8)*. Versions of S65T GFP have a single excitation peak at 489 nm and a red-shifted excitation optimum to (a green) 511 nm *(8)*. Another good choice for plants is the commercially available (Clontech) enhanced GFP, which has the S65T as well as the F64L and Y145F mutations, and is human codon-optimized *(9)*. Other researchers have produced mutants that have been useful in plants *(10,11)*. Recently, GFPs from other organisms have been cloned *(12)*. Plant-optimized GFP, and yellow fluorescent proteins may be expected to be better in plant applications than those currently available. In fact, a priori, *Renilla reniformis* GFP, which has recently been made commercially available by Stratagene, has spectral qualities that should make it brighter in heterologous systems *(13)*. Fluorescent proteins that emit in the yellow and orange spectra have promise in transgenic plant work.

GFP has been used in plant transformation systems as a transformation marker in soybean *(14)*, sugarcane *(15)*, orange *(16)*, tobacco *(17)*, wheat *(18)*, and apple *(19)*, to name a few species. In certain instances, GFP has been used as the sole selectable marker in transgenic plants, demonstrating that a visual marker could be used instead of antibiotic or herbicide selection. Thus far, GFP as the sole selection marker has been proven useful mainly in monocots such as sugarcane *(20)*, barley *(21)*, rice *(22)*, and oats *(23)*. The dicot exception in this case is citrus *(16)*, in which the transformation frequency was compared between GFP-only and GFP plus antibiotic selection. The researchers found that the transformation frequency was the same, but curiously, there were fewer GFP-positive shoots per experiment, using GFP selection *(16)*. One of the benefits of using GFP as the selectable marker is that high-expressing events can be selected very early in the tissue culture and regeneration process.

In this chapter, methods are described that the authors' group has used to transform members of the mustard family (*Brassicaceae*), using GFP-only and GFP in conjunction with antibiotic selection. This lab has produced transgenic *Brassicas* using antibiotic selection *(24)*, and is now using GFP to show proof-of-principle in *Brassica napus*, and also to extend the *Brassica* transformation procedure to a wild relative of the same genus: *Raphanus raphanistrum* (syn. *Brassica kaber*). Various experiments have been performed to demonstrate the efficiencies of GFP-only, or GFP-plus-antibiotic selection. Experiments described here employ a plasmid with GFP and an antibiotic selectable marker, but the goal is to use GFP as the sole selectable marker. Avoiding the use of antibiotic selection could address the criticism of biotechnology opponents who

fear that the horizontal transfer of antibiotic resistance genes could cause medical and ecological emergencies.

2. Materials

1. Surface-sterilized seeds (20% bleach solution for 5 min) from *B. napus* cv Westar.
2. Marashige and Skoog (MS) basal medium ***(25)*** for seed (hypocotyl explant source) germination. All plant tissue culture plates are produced using 0.2% Gelrite gellan gum (Sigma, St. Louis, MO) as a gelling agent. All agents are autoclaved, except kanamycin, before media is poured into plates.
3. MS basal medium with 1 mg/L, 2,4-D (MSD1) for 24 h preconditioning hypocotyls, and postco-cultivation.
4. *Agrobacterium tumefaciens* strain GV 3850 containing pBin *mgfp5-er* (35S promoter controlling *mGFPer* gene with linked NOS promoter-controlled *nptII* for kanamycin selection [**Fig. 1**]).
5. *Agrobacterium* solution (10^8 cells/mL in liquid MS basal medium with acetosyringone 0.05 m*M*) for co-cultivation with hypocotyls.
6. MSD1 media containing 400 mg/L Timintin to select against *Agrobacterium,* and with or without 20 mg/L kanamycin to select for transformed cells. No kanamycin is used for GFP-only selection.
7. CSRA: MS basal media containing 4 mg/L 6-benzylaminopurine, 2 mg/L zeatin, 5 mg/L silver nitrate, and with or without the above antibiotics to promote organogenesis.
8. CSRB: MS basal media containing 4 mg/L 6-benzylaminopurine, 2 mg/L zeatin, with or without antibiotics.
9. CSE: MS basal medium containing 0.05 mg/L 6-benzylaminopurine plus antibiotics for shoot elongation.
10. MSR: MS basal media containing 0.1% indole burtyric acid plus antibiotics to promote root development.
11. 100-mm Petri dishes and GA7 Magenta boxes for tissue culture.
12. Standard dissecting microscope and Spectroline BIB-150 UV lamp.
13. Laminar flow-hood.

3. Methods

3.1. GFP Transformation and Selection in Brassica (24)

1. Seeds are germinated on MS basal media. Zygotic hypocotyls were dissected and chopped into 1-cm-long segments. The hypocotyls segments were placed in a Petri dish containing the *Agrobacterium* inoculum in liquid MS basal medium for 30 min. Periodically shake the segments gently during the 30 min inoculation time Transfer the explants to MSD1 for 1 d, then to MSD1 plus one or no antibiotics (no kanamycin was in the media when using GFP selection only).
2. After 3 d, transfer the tissue to CSRA to initiate shooting. There is a considerable time delay (a few weeks) between shoot initiation and shoot formation using this procedure.

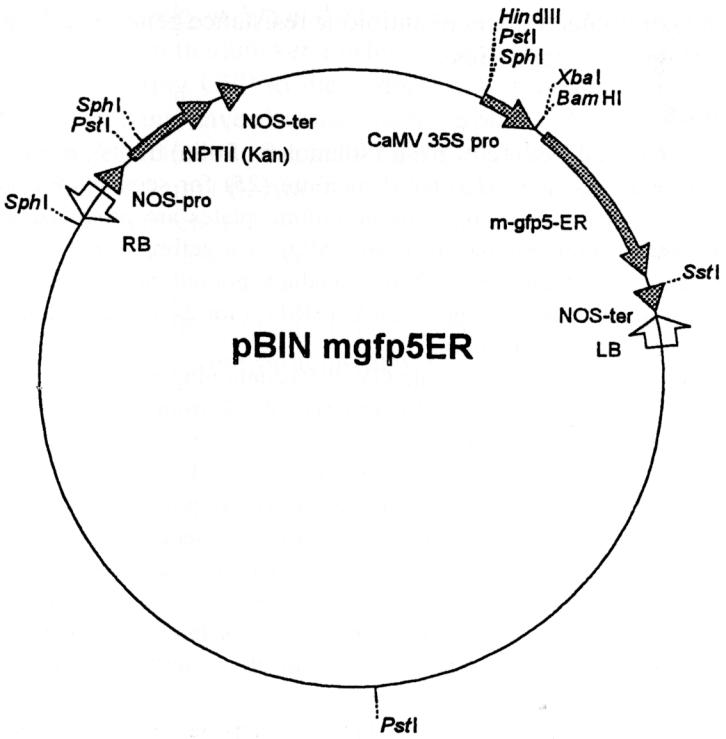

Fig. 1. The binary plasmid, pBin mgfp5ER, which was used for the plant transformation experiments (courtesy of Jim Haseloff). Kanamycin selectable (*nptII*) gene is under the control of the NOS promoter, and the endoplasmic reticulum targeted *GFP* gene is under the control of the 35S promoter from the cauliflower mosaic virus.

3. After another 7 d, (10 d after *Agrobacterium* transformation) transfer the tissue to CSRB. Between 2 and 4 wk GFP fluorescence will appeared in calli, then in shoots (*see* **Notes 1–3**).
4. At this point, weekly monitoring with a UV light is required to track transgenic events.
5. When the event callus (fluorescing uniformly green) is approx 0.5 cm in diameter, it is safe to isolate it from the greater tissue and transfer it onto fresh CSRB (*see* **Note 4**). Alternatively, shoots can be transferred to fresh CSRB.
6. Transgenic shoots are transferred to CSE as needed for elongation, then to MSR for rooting.
7. Visually assay for relative transgene expression by comparing GFP emission under UV illumination, thereby allowing selection of the highest-expressing events very early in the transformation process. **Figure 2** shows the product of this method for the transformation of the *Brassica* relative, wild radish (*Raphanus raphanistrum*) on CSRB.

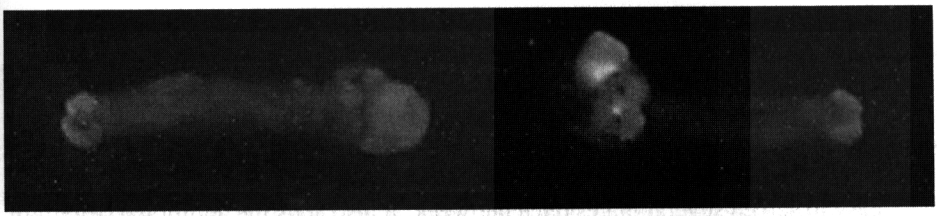

Fig. 2. *Raphanus raphanistrum* hypocotyls segments producing callus stably transformed with mGFP5er under the control of a constitutive promoter. Notice the variation of fluorescence between cut ends. GFP is visualized under UV (365 nm) illumination with no emission filter. (For optimal, color representation please see accompanying CD-ROM.)

4. Notes

1. Much of the success of GFP as an enabling technology in transgenic plants hinges on the success of seeing its production in plants. For lab work, most researchers use epifluorescence microscopes fitted with mercury lamps (~100 W) with blue filters (e.g., 470/40 nm) with 515 nm long-pass emission filters. Of course, without emission filters, one only sees blue reflectance (*see* **refs. *26–28***) for details. In using such arrangements, several researchers have reported background fluorescence that interferes with observing GFP ***(14,22,27)***. Altering filter choices, such as choosing emission filters of narrower bandwidth, or alternative emission filters should help ***(15,21)***. Empirical optimization by plant species and tissue types may need to be performed when using blue-light-excited GFPs. The choice of UV-excited GFPs, such as mGFP5, is often ignored as a viable choice by plant scientists. For example, there may be background fluorescence when excited by blue light, but not when excited by UV wavelengths.
2. If one desires to visualize whole plants or organs, then a microscope is not the best tool. For blue-excited GFPs, one can use the photonics of a microscope system, and indeed, Opti-Sciences (Tyngsboro, MA) produces a blue light source with the proper cutoff or bandpass filters for measuring GFP-transgenic plants (GF probe). For UV-excited GFPs, the authors' group and others typically use a portable UV lamp (UVP 100 AP, Upland, CA) with no emission filter, or the lighter Spectroline BIB-150 produced by Spectronics (Westbury, NY). These lamps have a 100 W mercury bulb and a 365-nm filter. The authors group and others have attempted to use less powerful UV lamps with little success. On the other side, we have combined 2–3 of the Spectroline UV lamps, to boost photon excitation irradiation, for more spectacular photographs. To effectively visualize GFP in transgenic plants, the lamp should be very bright and at the proper wavelength. Although the Spectroline or UVP lamps work well for UV excitation of GFP, they would be even more effective if they used a 395 nm filter instead of the 365-nm filter, since the former better matches GFP excitation.
3. UV protective eyewear should be used.

4. There are few tricks to keep in mind when using GFP as a selection for transformation of plants. Tracking transgenic events as early as possible, and keeping the events segregated is desirable. Isolating high-expressing events is important. However, if one excises green fluorescent tissue from the mother explant source, it may die. The authors have been unsuccessful if fluorescent *Brassica* callus is isolated, if the tissue piece is much smaller than 0.5 cm. The UV lamp makes it easy to screen several plates once per week. It also adds the additional benefit of "lighting-up" contaminants that are otherwise hard to see on Petri dishes.

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