BioFeature

Applications of Green Fluorescent Protein in Plants

Staci M. Leffel, Stephen A. Mabon and C. Neal Stewart, Jr.

University of North Carolina Greensboro, NC, USA

ABSTRACT

Green fluorescent protein (GFP) is increasingly being used in plant biology from the cellular level to whole plant level. At the cellular level, GFP is being used as an in vivo reporter to assess frequency of transient and stable transformation. GFP has also proven to be an invaluable tool in monitoring trafficking and subcellular localization of protein. At the organ level and up, many exciting applications are rapidly emerging. The development of brighter GFP mutants with more robust folding properties has enabled better macroscopic visualization of GFP in whole leaves and plants. One interesting example has been the use of GFP to monitor virus movement in and among whole plants. GFP is also emerging as a powerful tool to monitor transgene movement and transgenic plants in the field. In a proof-of-concept study, tobacco was transformed with a modified version of the GFP gene controlled by a constitutive (35S) promoter. GFP expression in progeny plants ranged from 0% to 0.5%, and approximately 0.1% GFP was the minimal amount needed for unambiguous macroscopic detection. GFP is the first truly in vivo reporter system useful in whole plants, and we project its usefulness will increase even further as better forms of GFP genes become available.

INTRODUCTION

Green fluorescent protein (GFP) has emerged as a powerful new tool in plant biotechnology research and applications. The purpose of this review is to briefly chronicle GFP research in plants and to summarize some of the bold directions this relatively new tool is taking plant biotechnology.

GFP was first cloned and sequenced from the cnidarian, *Aequorea victoria*, in the early nineties (32). Since then, it has been expressed in a variety of organisms, ranging from bacteria (19), fungi (41), invertebrates (30), vertebrates (49) and plants (12,34,37,44). Wild-type GFP is a 27-kDa monomer consisting of 238 amino acids that has the unique characteristic of emitting green light when excited with UV (360–400 nm) or blue (440–480 nm) light. GFP is the only well-characterized example of a protein that displays strong, visible fluorescence without any additional substrates or co-factors (14). It maintains this fluorescence even after prolonged incubation in strong denaturing agents such as 6 M guanidine HCl, 8 M urea or 1% sodium dodecyl sulfate (SDS) (4). GFP has a broad range of pH stability, retaining conformation from pH 5.5–12.0, and is extremely thermostable, surviving temperatures up to 65°C (4).

In the jellyfish *A. victoria*, its role is to transduce the blue chemiluminescence of another protein (aequorin) into green light (22,23). The light absorbing agent of the protein, the chromophore, is covalently attached to the protein and is formed by a post-translational modification in which a tripeptide within the protein is cyclized and oxidized (7,13,27,38). This chromophore is protected inside the protein, whose molecular structure has been recently elucidated (27,46). The overall shape of the protein consists of eleven strands of β-sheet forming the shape of a cylinder, capped with α-helices on the top and bottom (46). These α-helices also form a scaffold for the chromophore, which is located near the geometric center of the protein (46). This structure represents a new protein fold nicknamed the β-can (46). Oxygen has a paradoxical relationship with the protein, for it is believed to be necessary for correct protein folding, but its contact with the chromophore will quench fluorescence (46).

GFP IN CELLS

GFP Optimization

Green fluorescent protein retains fluorescence when fused to another protein both on the N- and C-terminal ends, which makes it an attractive fluorescent tag to monitor subcellular activities such as gene expression, protein-protein interactions and protein trafficking and localization (5,10,15, 37). These applications have been improved by the development of chromophore-mutated versions of GFP, in which single amino-acid substitutions have produced forms of the protein with shifted excitation peaks. Wild-type GFP has two excitation points at

about 395 and 470 nm, and it emits the most amount of green light at 508–509 nm (5,45). A blue-shifted variant has been produced by substituting histidine (Y66H) or tryptophan (Y66W) for tyrosine at position 66 in the chromophore (14). These mutants, called blue fluorescent proteins (BFPs) (14) were created to enhance GFP as a molecular tool by providing a second visibly distinct color for in vivo visualization of subcellular activities (13). Other mutations made by exchanging amino acid serine 65 to cysteine (S65C) and threonine (S65T) have increased the magnitude of green fluorescence, which has significantly improved the detection levels of the GFP (34). In addition, other modifications, such as F64L and

Figure 1. Photographs of green GFP transgenic and red non-transgenic tobacco plants under 365-nm UV illumination. Plants were characterized by visual inspection for green fluorescence using a hand-held, long-wave UV spotlight (Model B-100AP 100 W: 365-nm wavelength; UVP, San Gabriel, CA, USA). Each plant was classified as high, medium, low or not fluorescent according to "greenness" of the leaves under UV light compared to control plants. (A) Highly fluorescent (right) and nonfluorescent (left) influorescences and (B) leaves are shown. Plants were illuminated by two UVP spotlights at 1 m distance. Photos were taken using a 35-mm single reflex camera with Fuji 100 ASA color film, at *f* stop 11 and 60-s exposures with a yellow filter on a macro lens.

S65T, have improved the folding in EGFP, an enhanced version of the protein (8,47).

Though the wild-type gene has been expressed in both monocot and dicot cells (34), expression has generally been low or unstable in plants (16). Therefore, modified versions of the gene, such as *mgfp4*, were produced when an 84-bp sequence necessary for proper protein folding and expression was found to be mis-spliced in *Arabidopsis* (11). The sequence similarity to known plant introns caused it to be spliced-out during transcription, so Haseloff and colleagues reduced its AU content and reduced the chance of the sequence being excised, thereby restoring proper expression in the plant (12). To help improve visible fluorescence from GFP, Haseloff et al. has also recently transformed *Arabidopsis* with a modified GFP construct (*mgfp5-er*) that targets the protein to the endoplasmic reticulum, resulting in high, in vivo, expression in plants (12). In addition, this construct also has V163A and S175T mutations that enhance folding, stability and hence in vivo fluorescence (39).

Transformation Tool

The fact that GFP requires no additional substrates or co-factors to fluoresce and its ability to be expressed in a variety of organisms has made it a valuable universal reporter gene for scientific research (2,5,31). One obvious use for GFP is as a β-glucuronidase (GUS) substitute in transient and stable expression assays. For example, in the first studies using GFP in plant cells, it was shown that wild-type GFP was fluorescent in orange (25) and *Arabidopsis* (16) protoplasts. Modified versions have been utilized to a greater extent in maize (6,29,34) and alfalfa protoplasts (34), and the cells of *Arabidopsis* (11, 12,28,37), corn (28), rice (24,48), soybean (30), spruce and pine (44). It is conceivable that GFP can be used in place of selectable markers, such as those conferring antibiotic or herbicide resistance to recover transformed cells, tissues, organs and ultimately plants. For example, in transformation systems in which in vivo technology is used, such as bombardment of meristems (20,21), it would be much more cost-ef-

ficient to use an in vivo marker such as GFP rather than GUS. Chimeric transformants can be identified easily and nondestructively and en masse. Thus, GFP may reduce the cost of transformation and make transformation systems of economically less important crops, model and wild plants more feasible.
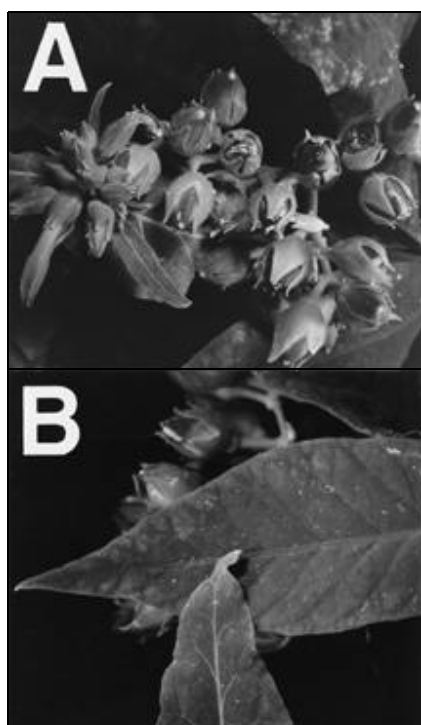
Cell Biology

Beyond transformation studies as noted above, GFP has been used in plants mainly as a subcellular targeting tool and as a marker gene (11). GFP has been targeted to the endoplasmic reticulum (3,12) and fused to proteins containing nuclear localization sequences (9) and to mitochondrial targeting signals (17) to study intracellular protein targeting. In a cell cycle study, GFP has been fused to phragmoplastin, a protein targeted to the cell plate (10). In this case, the chimeric gene proved valuable to monitor early events in cell plate formation. These studies, especially the last, demonstrate the power of GFP in cell biology inasmuch that cells do not have to be destroyed during assay, and that GFP may potentially be fused to any peptide.

WHOLE PLANT FLUORESCENCE

Disease Movement

While most of the research to date with GFP in plants has been performed at the cellular level, perhaps the most exciting applications will be at the organ level and higher. For all these studies, only modified versions of GFP have been used because of the requisite high expression. Especially exciting is the prospect of using GFP in monitoring and studying pathogen infection of plants. For example, viral infections in plants have been studied using GFP by replacing coat-protein genes from potato virus X (PVX) with the fluorescent protein and monitoring virus-infected tissue with microscopy to study virus movements (2) and by fusing the protein-to-movement proteins to determine how a virus spreads through and among host cells (15,17). This approach can also be used macroscopically. For

BioFeature

Table 1. Summary of GFP Transgenic Plant Progeny Data: Means and Standard Deviations

Primary Transgenic Fluorescence Class[a]	Progeny Transgenic Fluorescence Class[a]	Progeny Fluorescence Spectrometry Data[b]	Progeny GFP Expression[c]	Seed Yield[d]
High (4)	3.14 ± 1.16	1.57 ± 0.50	0.19 ± 0.19	1.03 ± 1.38
Low (1)	1.92 ± 0.86	1.43 ± 0.30	0.12 ± 0.18	2.65 ± 2.07
Control (0)	0 ± 0	1.35 ± 0.28	0 ± 0	1.80 ± 0.56

[a]Plants were grouped according to relative visual observation and a scale from 0–4 (in parentheses), with 0 being red and 4 being green under UV illumination; e.g., highly fluorescing plants appeared green under UV light (365 nm) as in the green plant in Figure 1, low fluorescence was intermediate between red and green, etc. Control plants were non-transgenic and appeared red.

[b]Fluorescence spectrophotometry was performed as another assay for visual fluorescence of GFP in plant extract samples as prepared below. Extraction for fluoresence spectrometry were performed from leaves that were harvested on dry ice and stored at -80°C. Samples and GFP standards were excited at 380 nm with the emission scan measured from 400–575 nm. Fluorescence in arbitrary units ($\times 10^5$) with excitation at 380 nm and detection at 502 nm.

[c]Gene expression is represented as % GFP of total extractable protein as determined by immunostained protein blot analysis. A 0.1-g (1.5 cm^2) portion of the fourth leaf from the apex leaf was homogenized with 600 µL extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 10 mM 2-mercaptoethanol, 18% glycerol) on ice. Samples were clarified by centrifugation at 16 400× g for 5 min. Protein blot analysis was performed using 2 µg of total protein in each sample lane and was analyzed according to published methods (33). Recombinant GFP protein (CLONTECH) was used for standards. Immunostaining was performed by applying each of the following antibodies sequentially using methods published elsewhere (33): rabbit poly-clonal anti-GFP serum (1:3000 dilution; CLONTECH), goat-anti rabbit (Sigma Chemical, St. Louis, MO, USA) and rabbit anti-goat/alkaline phosphatase (Sigma Chemical). GFP protein was detected by exposing the nitro-cellulose membrane to nitro blue tetrazolium/bromochloroindolyl phosphate substrate for approximately 4 min. Total protein was quantified using the Bradford assay. Densitometric analysis was used to quantify immunostained GFP.

[d]Seed weight per plant at the end of the growing season of plants grown in a greenhouse and common garden in 1-gallon plastic pots.

example, researchers at the Scottish Crop Research Institute have fused coat protein with GFP and a protein of interest to use in a production system of foreign proteins in plants, a GFP overcoat (26,42). Under UV illumination, viral movement can be easily seen in whole plants; hence, bioproduction of the protein of interest can be monitored in real time.

Ecological Monitoring

One use for GFP in whole plants is to use it as an in vivo marker to monitor transgene spread in the environment. Since some engineered crop species have weedy relatives growing nearby, researchers and environmentalists are concerned that fitness-enhancing transgenes will be introgressed into wild populations (35,40), which could foreseeably alter natural and agricultural ecosystems, a likely situation (1,18,36). One ramification of large-scale releases is the possibility of increased invasive-

ness and competition of transgenic weeds containing a transgene conferring an increment of fitness. No tracking system is in place to monitor transgene introgression into unintended hosts; however, GFP is the best candidate for this application (43).

To test the practicality of using GFP as a transgene monitoring tool, we have engineered tobacco (*Nicotiana tabacum* cv. Xanthi) for whole-plant fluorescence using *mgfp4*, a mutagenized version of the gene that retains UV excitation under the control of the 35S promoter. The goal is to provide an in vivo, real-time, scorable marker gene to monitor transgenic plants in the field. When a plant transgenic for the GFP gene is excited with a hand-held, long-wave UV light (365 nm), the plant appears green compared to non-transgenic plants, which appear red due to the red autofluorescence of chlorophyll (Figure 1). To visibly detect GFP expression in the plant, protein expression must be high enough to mask this red

autofluorescence (43) (Figure 1). Several practical problems present themselves in visualizing stably transformed plants for GFP and engineering a transgene tracking system. Two of these are addressed below.

Is fluorescence stable across generations? We have produced over 200
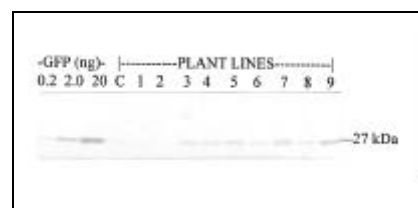
Figure 2. Immunostained protein blot analysis of plants synthesizing GFP. Protein standards are wild-type GFP from CLONTECH Laboratories (Palo Alto, CA, USA). Plant samples identities follow: lane C, non-transgenic control; lanes 1 and 2, nonfluorescing transgenics; lanes 3 and 4, low-fluorescing transgenics; lanes 5 and 6 medium-fluorescing transgenics; lanes 7–9, high-fluorescing transgenics. Sample 8 represents expression of 0.1% *mgfp4*, and 10 represents expression of approximately 0.5%.

independent lines of transgenic tobacco. In analyzing approximately 20 of these lines representing a range of fluorescence and growing out progeny, we have determined that the transgene is stably expressed in whole-plants-based visual assessment, fluorescence spectrophotometry and Western blot analysis (Table 1). We currently have fourth-generation plants that are fluorescent. There do seem to be environmental interactions associated with fluorescence phenotypes, but these are independent of transgenic line effects.

How much GFP must be produced to be unambiguously visualized in whole plants? Protein analysis was conducted on transgenic plants to quantify GFP protein expression and to compare these data to visual fluorescence data (Table 1).

Approximately 0.1% mGFP4 expression is sufficient to yield highly fluorescent plants, assuming all species are in the active (fluorescent) form on the basis of visual and immunostain data comparisons (Table 1; Figure 2). Fluorescence was positively correlated with protein expression ($P > 0.05$; Figure 2). Fluorescence spectrophotometry

confirmed the visible fluorescence data (Figure 3). The fluorescence spectrophotometry was valuable for comparative purposes, but was not useful to quantify the number of green fluorescent molecules per unit leaf area because the extinction coefficient of the wild-type standard was presumed different than the recombinant GFP in plants. Fluorescence spectrophotometric scans also reveal endogenous fluorescent compounds in leaves. Also, since leaves were frozen before extraction, there may have been protein degradation before measurement.

In conclusion, green fluorescent protein is stably inherited in progeny and is useful as a tag to mark transgenic plants in vivo. We (43) and others (12, 28) have shown that whole plant fluorescence with GFP is possible. This, to our knowledge, is the first report to show how much GFP must be synthesized in plants to be reliably visualized.

There are several other questions that must be answered before GFP can be used reliably in whole-plant applications. *What is the relationship between light quality and light quantity that plants used for growth and fluores-*
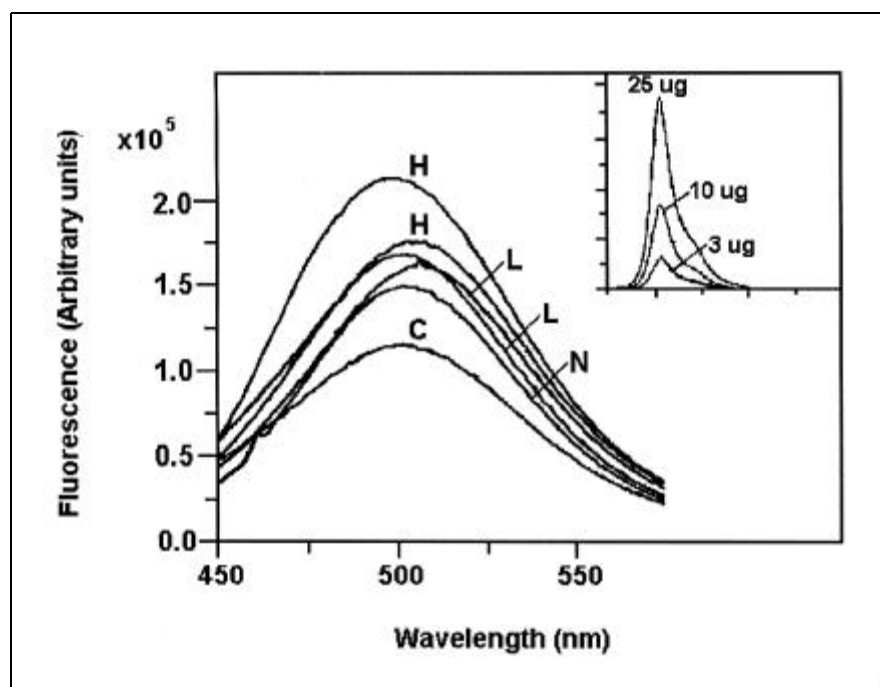
Figure 3. Fluorescence spectrophotometric analysis of GFP transgenic plants. Wild-type GFP standards (inset) and plants were excited at 380 nm with the emission scan measured from 400–575 nm, with excitation peak at 502 nm. C = control non-transgenic plants; N = GFP-transgenic plants that were indistinguishable visually from C; L = low fluorescence-group plant by visual observation; H = high fluorescence-group plant by visual observation.

BioFeature

cence? We have noticed that plant growth conditions, namely irradiance, can cause apparent switch-on/switch-offs of fluorescence. Under prolonged periods of cloudiness, we observe that previously highly fluorescent plants are scored low-to-no fluorescence. While it seems that light quantity is the major factor, we cannot rule out light quality factors at this point. *Is there any yield drag associated with GFP fluorescence and expression?* Field experiments need to be performed to test whether GFP decreases plant growth, seed yield and photosynthesis. Likewise, GFP dynamics in the field need to be documented. While much more needs to be done in biotechnology, much of the needed technology for commercial applications has to be developed in remote sensing, optics, computer technology and spectral analysis. For example, at least one company is in the process of developing a portable fluorometer designed to measure GFP in intact leaves in the field.

It is foreseeable that in 21st century agriculture, GFP will be a valuable tool in several arenas. It is envisaged that GFP could be used to monitor pathogen spread across whole fields. Pathogens, insects and other stresses might trip inducible promoter-fused GFPs in transgenic plants that would serve as an early-warning system for crop failure, enabling the grower to take timely action. Different colored fluorescence proteins might be used to code for various pests and stresses. GFP biotechnology should be useful to monitor other agricultural biotechnologies and guard against transgene flow to weedy, wild relatives that might disrupt ecosystems. These systems would certainly increase crop yields, decrease chemical inputs and protect natural ecosystems, thereby increasing sustainability of agriculture. We tip our hats to the jellyfish.

REFERENCES

1. **Arias, D.M. and L.H. Reiseberg.** 1994. Gene flow between cultivated and wild sunflowers. Theor. Appl. Genet. *89*:655-660.
2. **Baulcombe, D.C., S. Chapman and S. Santa Cruz.** 1995. Jellyfish green fluorescent protein as a reporter for virus infections. Plant J. *7*:1045-1053.
3. **Boevink, P., S. Santa Cruz, C. Hawes, N. Harris and K.J. Oparka.** 1996. Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells.

BioFeature

Plant J. *10*:935-941.

4. **Bokman, S.H. and W.W. Ward.** 1981. Renaturation of *Aequorea* green fluorescent protein. Biochem. Biophys. Res. Commun. *101*:1372-1380.

5. **Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science *263*:725-888.

6. **Chiu, W.L., Y. Niwa, W. Zeng, T. Hirano, H. Kobayashi and J. Sheen.** 1996. Engineered GFP as a vital reporter in plants. Curr. Biol. *6*:325-330.

7. **Cody, C.W., D.C. Prasher, W.M. Westler, F.G. Prendergast and W.W. Ward.** 1993. Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. Biochemistry *32*:1212-1218.

8. **Cormack, B.P., R.H. Valdivia and S. Falkow.** 1996. FACS optimized mutants of the green fluorescent protein (GFP). Gene *173*:33-38.

9. **Grebenok, R.J., E. Pierson, G.M. Lambert, F. Gong, C.L. Afonso, R. Haldeman-Cahill, J.C. Carrington and D.W. Galbraith.** 1997. Green-fluorescent protein fusions for efficient characterization of nuclear targeting. Plant J. *11*:573-586.

10. **Gu, X. and D.P.S. Verma.** 1997. Dynamics of phragmoplastin in living cells during cell plate formation and uncoupling of cell elongation from the plane of cell division. Plant Cell *9*:157-169.

11. **Haseloff, J. and B. Amos.** 1995. GFP in plants. Trends Genet. *11*:328-329.

12. **Haseloff, J., K.R. Siemering, D.C. Prasher and S. Hodge.** 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. USA *94*:2122-2127.

13. **Heim, R., D.C. Prasher and R.Y. Tsien.** 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA *91*:12501-12504.

14. **Heim, R. and R.Y. Tsien.** 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol. *6*:178-182.

15. **Heinlein, M., B.L. Epel, H.S. Padgett and R.N. Beachy.** 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science *270*:1983-1985.

16. **Hu, W. and C.-L. Cheng.** 1995. Expression of *Aequorea* green fluorescent protein in plant cells. FEBS Lett. *369*:331-334.

17. **Kohler, R.H., W.R. Zipfel, W.W. Webb and M.R. Hanson.** 1997. The green fluorescent protein as a marker to visualize plant mitochondria *in vivo*. Plant J. *11*:613-621.

18. **Langevin, S.A., S.A. Clay and J.B. Grace.** 1990. The incidence and effects of hybridization between cultivated rice and its related weed, red rice, *Oryza sativa* L. Evolution *44*:1000-1008.

19. **Leff, L.G. and A.A. Leff.** 1996. Use of green fluorescent protein to monitor survival of genetically engineered bacteria in aquatic environments. Appl. Environ. Microbiol. *62*:3486-3488.

20. **McCabe, D.E. and B.J. Martinell.** 1993. Transformation of elite cotton cultivars via particle bombardment of meristems. Bio/Technology *11*:596-598.

21. **McCabe, D.E., W.F. Swain, B.J. Martinell and P. Christou.** 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/Technology *6*:923-926.

22. **Morin, J.G. and J.W. Hastings.** 1971. Energy transfer in a bioluminescent system. J. Cellular Physiol. *77*:313-318.

23. **Morise, H., O. Shimomura, F.H. Johnson and J. Winant.** 1974. Intermolecular energy transfer in the bioluminescent system of *Aequorea*. Biochemistry *13*:2656-2662.

24. **Nagatani, N., S. Takumi, M. Tomiyama, T. Shimada and E. Tamiya.** 1997. Semi-real time imaging of the expression of a maize polyubiquitin promoter-GFP gene in transgenic rice. Plant Sci. *124*:49-56.

25. **Niedz, R.P., M.R. Sussman and J.S. Satterlee.** 1995. Green fluorescent protein: an *in vivo* reporter of plant gene expression. Plant Cell Rep. *14*:403-406.

26. **Oparka, K.J., P. Boevink and S. Santa Cruz.** 1996. Studying the movement of plant viruses using green fluorescent protein. Trends Plant Sci. *1*:412-418.

27. **Ormo, M., A.B. Cubitt, K. Kallio, L.A. Gross, R.Y. Tsien and J. Remington.** 1996. Crystal structure of the *Aequorea victoria* green fluorescent protein. Science *273*:1392-1395.

28. **Pang, S.-Z., D.L. DeBoer, Y. Wan, G. Ye, J.G. Layton, M.K. Neher, C.L. Armstrong, J.E. Fry, M.A. Hinchee and M.E. Fromm.** 1996. An improved green fluorescent protein gene as a vital marker in plants. Plant Physiol. *112*:893-900.

29. **Paterson, A.H., K.F. Schertz, Y.F. Lin, S.-C. Liu and Y.-L. Chang.** 1995. The weediness of wild plants: molecular analysis of genes influencing dispersal and persistence of johnsongrass, *Sorghum halepense* (L.) Pers. Proc. Natl. Acad. Sci. USA *92*:6127-6131.

30. **Plautz, J.D., R.N. Day, G.M. Dailey, S.B. Welsh, J.C. Hall, S. Halpain and S.A. Kay.** 1996. Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells. Gene *173*:83-87.

31. **Prasher, D.C.** 1995. Using GFP to see the light. Trends Genet. *11*:320-323.

32. **Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergrast and M.J. Cormier.** 1992. Primary structure of the *Aequorea victoria* green fluorescent protein. Gene *111*:229-233.

33. **Pratt, L.H., D.W. McCurdy, Y. Shimazaki and M.-M. Cordonnier.** 1986. Immunodetection of phytochrome: immunocytochemistry, immunoblotting, and immunoquantitation, p. 50-74. *In* H.F. Linskens and J.F. Jackson (Eds.), Modern Methods of Plant Analysis. Springer, New York.

34. **Reichel, C., J. Mathur, P. Eckes, K. Langenkemper, C. Koncz, J. Schell, B. Reiss and C. Maas.** 1996. Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc. Natl. Acad. Sci. USA *93*:5888-5893.

35. **Rissler, J. and M. Mellon.** 1993. Perils Amongst the Promise. Union of Concerned Scientists, MIT Press, Cambridge, MA.

36. **Sawahel, W.A.** 1994. Transgenic plants: performance, release and containment. W.J. Microbiol. Biotechnol. *10*:139-144.

37. **Sheen, J., S.B. Hwang, Y. Niwa, H. Kobayashi and D.W. Galbraith.** 1995. Green fluorescent protein as a new vital marker in plant cells. Plant J. *8*:777-784.

38. **Shimomura, O.** 1979. Structure of the chromophore of *Aequorea* green fluoroscent protein. FEBS Lett. *104*:220-222.

39. **Siemering, K.R., R. Golbik, R. Sever and J. Haseloff.** 1996. Mutations that suppress the thermosensitivity of green fluorescent protein. Curr. Biol. *6*:1653-1663.

40. **Snow, A.A. and P.M. Palma.** 1997. Commercialization of transgenic plants: potential ecological risks. Bioscience *47*:86-96.

41. **Spellig, T., A. Bottin and R. Kahmann.** 1996. Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. Mol. Gen. Genet. *252*:503-509.

42. **Sterling, J.** 1997. Novel production systems. Genet. Eng. News *17*:8.

43. **Stewart, C.N., Jr.** 1996. Monitoring transgenic plants using in vivo markers. Nature Biotechnology *14*:682.

44. **Tian, L., A. Sequin and P.J. Charest.** 1997. Expression of the green fluorescent protein gene in conifer tissues. Plant Cell Rep. *16*:267-271.

45. **Ward, W.W., H.J. Prentice, A.F. Roth, C.W. Cody and S.C. Reeves.** 1982. Spectral perturbations of the *Aequorea* green fluorescent protein. Photochem. Photobiol. *35*:803-808.

46. **Yang, F., L.G. Moss and G.N. Phillips, Jr.** 1996. The molecular structure of green fluorescent protein. Nature Biotechnology *14*:1246-1251.

47. **Yang, T.T., L.Z. Cheng and S.R. Kain.** 1996. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acid Res. *24*:4592-4593.

48. **Yue, H., X. Xin-Ping and L. Bao-Jian.** 1997. Improved green fluorescent protein as a fast reporter of gene expression in plant cells. Biotechnol. Tech. *11*:133-136.

49. **Zolotukhin, S., M. Potter, W.W. Hauswirth, J. Guy and N. Muzyczka.** 1996. A "humanized" green fluorescent protein cDNA adapted for high level expression in mammalian cells. J. Virol. *70*:4646-4654.

Address correspondence to:

C. Neal Stewart, Jr.
University of North Carolina
Greensboro, NC 27412, USA
Internet: nstewart@goodall.uncg.edu