A Synthetic Green Fluorescent Protein Gene for Plant Biotechnology

Yasuo NIWA*

Laboratory of Plant Cell Technology, Graduate School of Nutritional and Environmental Sciences,
University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

*Corresponding author E-mail address: niwa@u-shizuoka-ken.ac.jp

Received 29 October 2002; accepted 2 December 2002

Abstract

Since Green Fluorescent Protein (GFP) from *Aequorea* jellyfish emits green fluorescence without any additional substrates or co-factors, it has emerged as a powerful new reporter for use in a variety of organisms. However, improvements such as the alteration of the excitation spectrum and the elimination of a cryptic intron site were necessary for its efficient use in plants. An engineered synthetic GFP with a S65T mutation (replacement of the serine in position 65 with a threonine) in the chromophore has provided up to 100-fold brighter fluorescent signals than the wild-type jellyfish GFP sequence without any toxic effects. The sGFP(S65T) has been widely used for studies such as localization analysis and promoter assays. This sGFP(S65T) is also an ideal candidate for the development of transformation methods for crops and trees in which it has previously proved difficult to obtain transformants via conventional methods. A non-invasive, quantitative detection technique for this GFP has been achieved in conjunction with a fluorescent imaging system.

Key words: green fluorescent protein, localization analysis, plant biotechnology, promoter assay, reporter gene, sGFP(S65T), transformation marker, visible marker.

Abbreviations

sGFP, synthetic green fluorescent protein; S65T, replacement of the serine in position 65 with a threonine.

Introduction

One of the important objectives of plant biotechnology is the establishment of novel plants with favourable characteristics. A general procedure for the achievement of this purpose is summarized in **Fig. 1**. Needless to say, proteins play essential roles in cellular activities, the genetic information for which is specific for individual species. The possibility of analysing proteins in detail has led to increased understanding of the processes of life. The 2002 Nobel Prize for chemistry was awarded to three persons, including the Japanese scientist, Koichi Tanaka, for their evolutionary methods for the analysis of proteins. It would be useful if proteins could be visualized *in vivo*. Many marker genes have been developed to visualize protein activity, however, substrates are needed in all cases.

Green Fluorescent Protein (GFP; **Fig. 2**) was first discovered by Shimomura et al from *Aequorea* jellyfish (Shimomura et al., 1962) as a companion

protein to aequorin in 1962. After thirty years, the crucial breakthroughs came with the cloning of the gene by Prasher et al (Prasher et al., 1992) and the demonstration that expression of the gene in other organisms such as *C. elegans*, *Drosophila*, and *E. coli* creates fluorescence. Therefore, the gene contains all the information necessary for the post-translational formation of the chromophore, and no

Fig. 1 Possible contribution of sGFP(S65T) to the establishment of novel plants with favorable traits.

Fig. 2 Schematic three-dimensional structure of GFP.

The chromophore (space-filling) is located in the center of a barrel-like structure formed by eleven β -sheets (ribbon).

jellyfish-specific enzymes are needed. The GFP consists of 238 amino acid residues and the chromophore is a *p*-hydroxybenzylideneimidazolinone formed from residues 65–67, which are Ser–Tyr–Gly in the wild-type protein. The wild-type GFP emits green fluorescence when excited with blue or UV light without any additional substrates or co-factors. The broader application of GFP in higher organisms requires higher expression efficiency and fluorescence intensity, especially under blue light, to minimize photobleaching and phototoxicity. Additionally, eliminating an inadequate splice site (**Fig. 3**) is essential to express functional GFP in plants (Haseloff *et al.*, 1997)

A synthetic GFP with S65T mutation

I have previously shown that wild-type GFP signals are detectable in a transient expression

system in *Arabidopsis* (Sheen *et al.*, 1995). However, it was concluded that an increase in expression efficiency and quantum yield achieved by blue light excitation would make GFP substantially more useful as a vital marker in plants. First, a synthetic GFP gene with preferred codon usage for higher organisms was made as an sGFP (**Fig. 3**). Then a S65T mutation (replacement of the serine in position 65 with a threonine) was introduced by site-directed mutagenesis to create sGFP(S65T) (**Fig. 3**) according to the observation by Heim *et al.* (Heim *et al.*, 1995). An engineered sGFP(S65T) sequence with codons optimal for high expression of eukaryotic proteins has provided up to 100-fold brighter fluorescent signals than the wild-type jellyfish GFP sequence in plants (Chiu *et al.*, 1996). This S65T mutation additionally causes characteristics such as faster chromophore formation, slower photobleaching, and a single excitation peak ideal for fluorescein isothiocyanate (FITC) filter sets. Moreover, sGFP(S65T) has no toxic effects to plants without the use of any targeting signals (Niwa *et al.*, 1999). At least in rice, sGFP(S65T) is brighter than mgfp5, a thermostable folding mutant (Siemering *et al.*, 1996), under blue light excitation (personal communication from Dr. Ugaki at Tokyo University).

There are three types of sGFP(S65T)-containing vectors (**Fig. 4A**). Two of them originally constructed by Dr. Sheen (pblue-sGFP(S65T)-nos3'KS = sKS and pblue-sGFP(S65T)-nos3'SK = sSK) are promoter-less vectors suited for promoter analysis. The nucleotide sequence between the multiple cloning site of the Bluescript vector and the sGFP(S65T) gene are shown in **Fig. 4B** (Niwa unpublished result and personal communication from Dr. Nonomura at National Institute of Genetics). An unexpected *Eco*RI site was found in the downstream region of the nopaline synthase gene

Fig. 3 Schematic structure of various GFP genes.

Chromophore of Ser–Tyr–Gly and cryptic intron are represented as boxes shown in the figure. *Nco*I and *Bsr*GI restriction sites and the S65T mutation site are indicated at their respective positions. Signal peptide and ER retention sequences are shown as SP and HDEL, respectively.

Fig. 4 Physical maps of sGFP(S65T)-containing plasmids (A) and of its sequences (B–D).

The synthetic GFP(S65T) gene and polyadenylation signal sequence from the nopaline synthase gene (*nos3'*) were inserted into a modified Bluescript vector in opposite orientations to each other (pblue-sGFP(S65T)-*nos3'*KS = sKS and pblue-sGFP(S65T)-*nos3'*SK = sSK). An unexpected *EcoRI* site found in the downstream region of *nos3'* of the sSK vector is represented as **EcoRI*. The sGFP(S65T) gene was driven by the cauliflower mosaic virus 35S promoter (PCaMV35S) with the omega sequence from TMV (PCaMV35Somega-sGFP(S65T)-*nos3'* = pTH2). The pTH2 is a pUC-derived vector and the original *SalI* site at the multiple cloning site was removed. Nucleotide sequence and corresponding amino acids and restriction sites of the upstream region of the sGFP(S65T) gene in the sSK vector (B). Nucleotide sequences and corresponding amino acid residues are numbered and restriction sites of N-terminal (C) and C-terminal (D) regions of sGFP(S65T) in pTH2 vector are shown.

(*nos*) terminator of the sSK vector (personal communication from Dr. Kitamoto at Tokyo University). The CaMV35Somega-sGFP(S65T)-*nos3'* plasmid named pTH2 is a pUC-based vector containing the CaMV35S promoter, the omega sequence from tobacco mosaic virus (TMV), sGFP(S65T), and the polyadenylation signal sequence from *nos*. The pTH2 vector is widely used

for localization analysis (Table 1) and as a marker for gene transfer (Table 2).

Localization analysis

Since the sGFP(S65T) gene product shows no-toxic side effects without any targeting signals, it is ideal for cellular and sub-cellular localization analysis. Examples of the use of sGFP(S65T) in this

Table 1 Intracellular compartments targeted and visualized by sGFP(S65T)

Subcellular compartment	References for examples
Cell – plate	Nishihama <i>et al.</i> , 2002; Yokoyama and Nishitani, 2001
Endoplasmic reticulum	Hayashi <i>et al.</i> , 2001; Hong <i>et al.</i> , 1999; Koizumi <i>et al.</i> , 2001; Okushima <i>et al.</i> , 2002; Saito <i>et al.</i> , 1999; Tamura <i>et al.</i> , 2001; Takeuchi <i>et al.</i> , 2000
Golgi apparatus	Nebenfuhr <i>et al.</i> , 1999; Takeuchi <i>et al.</i> , 2002
Microfilament	Chen <i>et al.</i> , 2002
Microtubule	Kumagai <i>et al.</i> , 2001; Marc <i>et al.</i> , 1998
Mitochondrion	Beardslee <i>et al.</i> , 2002; Chang <i>et al.</i> , 1999; Christensen <i>et al.</i> , 2002; Nakazono <i>et al.</i> , 2000; Niwa <i>et al.</i> , 1999; Okada <i>et al.</i> , 2000; Versaw and Harrison, 2002
Nucleus	Chui <i>et al.</i> , 1996; Fukaki <i>et al.</i> , 2002; Igarashi <i>et al.</i> , 2001; Ito <i>et al.</i> , 2002; Iwakawa <i>et al.</i> , 2002; Kitakura <i>et al.</i> , 2002; Kosugi and Ohashi, 2002; Ohta <i>et al.</i> , 2000; Okada and Toriyama, 2001; Tamura <i>et al.</i> , 2002; Yanagisawa, 2001
Plasma membrane	Chaumont <i>et al.</i> , 2000; Ivanchenko <i>et al.</i> , 2000
Plastid	Araki <i>et al.</i> , 2000; Chang <i>et al.</i> , 1999; Chui <i>et al.</i> , 1996; Ho <i>et al.</i> , 1998; Ishiguro <i>et al.</i> , 2001; Isono <i>et al.</i> , 1997; Iuchi <i>et al.</i> , 2000; Kohchi <i>et al.</i> , 2001; Muramoto <i>et al.</i> , 1999; Niwa <i>et al.</i> , 1999; Obara <i>et al.</i> , 2002; Okada <i>et al.</i> , 2000; Sitthithaworn <i>et al.</i> , 2001; Takechi <i>et al.</i> , 2000
Peroxisome	Mano <i>et al.</i> , 1999; Mano <i>et al.</i> , 2002
Protein body	Personal communication from Dr. Tanaka at Kyoto Prefectural Univ.
Vacuole	Mitsuhashi <i>et al.</i> , 2000; Morita <i>et al.</i> , 2002; Saito <i>et al.</i> , 2002; Ueoka – Nakanishi <i>et al.</i> , 2000

manner include nuclear, plastidic (Chui *et al.*, 1996) and mitochondrial (Niwa *et al.*, 1999) localization studies. **Table 1** summarizes the intracellular localization analysis experiments with sGFP(S65T).

For the purpose of nuclear localization analysis, it should be noted that the monomer size of GFP is small enough to diffuse into the nucleus through nuclear pores. Therefore, the GFP itself localizes not only in the cytoplasm, but also in the nucleus. Of course, if a nuclear localizing signal is fused to sGFP(S65T), most of the green fluorescent signals are detected in nucleus (Chui *et al.*, 1996), however, it is important to eliminate its passive penetration into nucleus. One of the solutions to this problem was achieved by the creation of a GFP–GUS fusion construct to increase the molecular weight of the GFP (Grebenok *et al.*, 1997; Kosugi and Ohashi, 2002). A similar chimeric gene has also been made by Dr. Ugaki at Tokyo University (personal communication).

Macromolecular trafficking within the sieve ele-

ment–companion cell complex, phloem unloading, and post–phloem transport were studied using the sGFP(S65T) under the control of the *AtSUC2* promoter (Imlau *et al.*, 1999). Plasmodesmata of the sieve element–companion cell complex, as well as plasmodesmata into and within sinks, allow trafficking of the 27–kD non–phloem GFP protein.

Transformation marker

Transformation has been performed with selection markers that can distinguish transformed from non–transformed cells via the observation of different growth rates on selection medium (**Fig. 5**). Although this method is powerful and efficient in certain well technically–developed model plants, it is sometimes very difficult to apply to various plants such as crops. The main reason for this is the decrease in regeneration efficiency due to the effects of chemicals used for selection. Analysis of the expression of the marker genes within the candidate cells and plants is also necessary. Since I

Table 2 Plant and other species successfully used

Species	References for examples
<i>Arabidopsis thaliana</i>	Chui <i>et al.</i> , 1996; Kinoshita <i>et al.</i> , 2001; Niwa <i>et al.</i> , 1999
Barley (<i>Hordeum vulgare</i>)	Cho <i>et al.</i> , 2002; Gubler <i>et al.</i> , 2002
Bean (<i>Vicia faba</i>)	Marc <i>et al.</i> , 1998
(<i>Glycine max</i>)	Personal communication from Dr. Furutani at Kyoto Prefectural Institute of Agricultural Biotechnology
Fern (<i>Adiantum capillus</i>) ¹⁾	Personal communication from Dr. Kanegae at Tokyo Metropolitan Univ.
Lily (<i>Lilium longiflorum</i>)	Personal communication from Dr. Tanaka at Yokohama City Univ.
Maize (<i>Zea mays</i>)	Chang <i>et al.</i> , 1999; Chui <i>et al.</i> , 1996; Yanagisawa, 2001
Moss (<i>Physcomitrella patens</i>)	Hara <i>et al.</i> , 2001; Kabeya <i>et al.</i> , 2002
Onion	Chui <i>et al.</i> , 1996
Petunia (<i>Petunia hybrida</i>)	Personal communication from Dr. Tanaka at Institute for Advanced Technology, Suntory Ltd.
Poplar	Personal communication from Dr. Sakai at Kyoto Univ.
Rice (<i>Oryza sativa</i>)	Hagenbeek <i>et al.</i> , 2000; Hasizume <i>et al.</i> , 1999; Jang <i>et al.</i> , 2002; Okushima <i>et al.</i> , 2002; Personal communication from Dr. Morita at Kyoto Prefectural Univ.
Spiderwort (<i>Tradescantia reflexa</i>)	Personal communication from Dr. Takumi at Kobe Univ.
Sweet potato (<i>Ipomoea batatas</i>)	Personal communication from Dr. Mistukawa at Toyota Central R&D Labs., Inc.
Tamarix (<i>Tamaricaceae Tamarix</i>)	Personal communication from Dr. Yoshida at Taisei Co.
Tea (<i>Camellia sinensis</i>)	Niwa unpublished results
Tobacco (<i>Nicotiana tobacum</i>)	Chen <i>et al.</i> , 2002; Chui <i>et al.</i> , 1996; Kohchi <i>et al.</i> , 2001; Okada and Toriyama, 2001; Yang <i>et al.</i> , 2001
(<i>Nicotiana glauca</i>)	Kobayashi <i>et al.</i> , 2001
Torenia (<i>Torenia fournieri</i>)	Personal communication from Dr. Kobayashi at Osaka Univ.; Personal communication from Dr. Tanaka at Suntory Ltd.
Verbena (<i>Verbena hybrida</i>)	Tamura <i>et al.</i> , 2002
Wheat (<i>Triticum aestivum</i>)	Madin <i>et al.</i> , 2000, Personal communication from Dr. Yoshida at Hokkaido National Agricultural Experiment Station
Yeast (<i>Saccharomyces cerevisiae</i>)	Nakamura <i>et al.</i> , 1997; Versaw and Harrison, 2002
Fungus (<i>Aspergillus nidulans</i>),	Valdez-Taubas <i>et al.</i> , 2000
(<i>Aspergillus niger</i>)	Gordon <i>et al.</i> , 2000
(<i>Aspergillus oryzae</i>)	Personal communication from Dr. Gomi at Tohoku Univ.
(<i>Magnaporthe grisea</i>)	Balhadere <i>et al.</i> , 2001; DeZwaan <i>et al.</i> , 1999
Human	Kuruto-Niwa <i>et al.</i> , 1998

¹⁾ Growth was severely affected in stable transformants

Fig. 5 Comparison of conventional transformation procedures using a selectable marker (A) and the novel method using the directly visible sGFP(S65T) (B).

already mentioned that the sGFP(S65T) produces a very bright signal without any toxicity, it therefore has the potential to resolve these difficulties. In the case of cultured tobacco cells, GFP-positive cells could be distinguished from non-transformed cells under non-selective conditions (Niwa *et al.*, 1999). The sGFP(S65T) gene is ideal as a non-invasive visualization marker for both transient systems and stable integration into chromosomes. It has also been used to develop novel gene delivery methods. The sGFP(S65T) gene is also used in a MAT vector system for generating marker-free transgenic plants (Sugita *et al.*, 2000; Endo *et al.*, 2002).

As the 35S promoter in the pBI vector (Jefferson, 1987; Jefferson *et al.*, 1987) directs expression of the GUS gene in both *Agrobacterium* and plants, it is therefore impossible to distinguish the GUS signals originating from the transformed plants from those originating from *Agrobacterium*. Therefore it is important to introduce an intron into the GUS gene in this vector (Ohta *et al.*, 1990). GFP will localize to both the nucleus and the cytoplasm regardless of the promoter used. In the case of sGFP(S65T) driven by the 35S promoter of pTH2 however, there seems to be no need to introduce an intron as the gene does not seem to be functional in *Agrobacterium*, or at least only weakly functional (Niwa unpublished result; personal communication from Dr. Sugaya at Saitama University). The solely nuclear localization of this GFP additionally provides evidence that the GFP expression can be attributed to the transformed plants as opposed to that of *Agrobacterium*. In the case of sGFP(S65T) driven by the 35S promoter of pBI, GFP expression does occur in *Agrobacterium*, however again it is possible to distinguish GFP expression attributable

to the transformed plant cells from that attributable to the *Agrobacterium* from the nuclear localization of the GFP. However, the introduction of an intron into the GFP gene would eliminate the possibility of expression of GFP in *Agrobacterium*, and thus ensure that all GFP expression could be attributed to the transformed plant cells. The sGFP(S65T) gene containing such an intron has been constructed by Dr. Mitsukawa at Toyota Central R&D Labs., Inc. (personal communication). Reasons as to why the 35S promoter of pTH2 is dysfunctional in *Agrobacterium* is not known, although there are two differences between the 35S promoter of pTH2 and that of pBI vectors. The 35S promoter of pTH2 is shorter (Fig. 4) than that of pBI vectors and contains the omega sequence from TMV.

Detections of sGFP(S65T)

Because the spectrum of sGFP(S65T) is almost identical that of FITC, fluorescence microscopy and confocal laser scanning microscopy is adequate for the detection of GFP fluorescence for analysis at the cellular level. When the sGFP(S65T) gene is used for plant biotechnology such as transformation marker and promoter analysis, the non-invasive detection system at the tissue and whole plant level is essential. The FluorImager (Fig. 6), a fluorescent

Fig. 6 The FluorImager, used for non-invasive quantitative detection of sGFP(S65T).

imaging system, is suited to this purpose. The measurement of organ-specific expression of sGFP(S65T) has been demonstrated (Niwa *et al.*, 1999). Homozygous plants could be distinguished from heterozygous plants and fully fertile progenies could be obtained from the analyzed plants. In the case of *Arabidopsis*, about 3000 seedlings can be quantitatively detected at any one time without any lesions.

Once the post-translational maturation of chromophore takes place, sGFP(S65T) can be directly detected after SDS-PAGE using systems such as the FluorImager (Niwa unpublished results) and UV trans-illuminator. Immunological detection of sGFP(S65T) has been achieved by both polyclonal and monoclonal antibodies from MBL (Medical and Biological Laboratories Co., Ltd. Nagoya, Japan; unpublished results).

Performance of sGFP(S65T)

The use of sGFP(S65T) has been attempted in a wide variety of plants. Plant species in which its use has been successfully applied are summarized in **Table 2**. They include model plants, crops, and trees. Moreover, the sGFP(S65T) is also used in fungi, yeast, and human cultured cells. In the case of *Adiantum capillus*, a transient expression system has proved successful, however, the growth of the ferns was severely affected for stable transformation (personal communication from Dr. Kanegae at Tokyo Metropolitan University). It is not known whether this observation is specific to sGFP(S65T) or general phenomenon of GFP. The nature of GFP, which absorbs blue light as an emission light may influence the growth of ferns.

The sGFP(S65T) has been used to develop a highly efficient cell-free and robust protein synthesis system using wheat embryos (Madin *et al.*, 2000). Heat-shock response was quantitatively detected under the control of HSP18.2 promoter (Matsuhara *et al.*, 2000). Moreover, activities of promoters such as sulphate transporters from *Arabidopsis* (Takahashi *et al.*, 2000; Yoshimoto *et al.*, 2002), *FIE* (Kinoshita *et al.*, 2001) and *MEA* (Yadegari *et al.*, 2000), rice cytochrome c (Jang *et al.*, 2002), rice actin and barley endosperm-specific hordein (Cho *et al.*, 2002), *Bra r 1* from *Brassica* (Okada and Toriyama, 2001) and NCR derived from soybean chlorotic mottle virus (Fukuoka *et al.*, 2000) have been visualized using sGFP(S65T).

Future aspects

To date, when plants contain metabolites for auto-fluorescence, whose spectrum is close to or overlapping with GFP fluorescence, for example the

roots of tobacco, mature pollens, and stigma of pistil, it is impossible to distinguish the GFP signal from the background fluorescence. A Multifluorescence-Imaging system with a META detector and emission fingerprinting from Zeiss will hopefully resolve this difficulty.

Acknowledgement

The author thanks Dr. Jen Sheen (Massachusetts General Hospital, USA) for her support during the initial stages of this study, the members of Plant Cell Technology Laboratory especially Dr. Takanori Hirano for his excellent assistance and Dr. Alison Hills (Royal Holloway University of London, UK) for her critical reading of this review.

References

- Araki, N., Kumumi, K., Masamoto, K., Niwa, Y., Iba, K., 2000. Temperature-sensitive *Arabidopsis* mutant defective in 1-deoxy-D-xylulose 5-phosphate synthase within the plastid non-mevalonate pathway. *Physiol. Plant.*, **108**: 19–24.
- Balhadere, P. V., Talbot, N. J., 2001. *PDE1* encodes a P-type ATPase involved in Appressorium-mediated plant infection by the rice blast fungus *Magnaporthe grisea*. *Plant Cell*, **13**: 1987–2004.
- Beardslee, T. A., Roy-Chowdhury, S., Jaiswal, P., Buhot, L., Lerbs-Mache, S., Stern, D. B., Allison, L. A., 2002. A nucleus-encoded maize protein with sigma factor activity accumulates in mitochondria and chloroplasts. *Plant J.*, **31**: 199–209.
- Chang, C.-C., Sheen, J., Bligny, M., Niwa, Y., Lerbs-Mache, S., David B. Stern, D. B., 1999. Functional analysis of two maize cDNAs encoding T7-like RNA polymerases. *Plant Cell*, **11**: 911–926.
- Chaumont, F., Barrieu, F., Jung, R., Chrispeels, M. J., 2000. Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. *Plant Physiol.*, **122**: 1025–1034.
- Chen, C. Y., Wong, E. I., Vidali, L., Estavillo, A., Hepler, P. K., Wu, H.-m., Cheung, A. Y., 2002. The regulation of actin organization by actin-depolymerizing factor in elongating pollen tubes. *Plant Cell*, **14**: 2175–2190.
- Cheung, A. Y., Chen, C. Y., Glaven, R. H., de Graaf, B. H. J., Vidali, L., Hepler, P. K., Wu, H.-m., 2002. Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. *Plant Cell*, **14**: 945–962.
- Chiu, W.-I., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., Sheen, J., 1996. Engineered GFP as a vital reporter in plants. *Curr. Biol.*, **6**: 325–330.
- Cho, M. J., Choi, H. W., Jiang, W., Ha, C. D., Lemaux, P. G., 2002. Endosperm-specific expression of green fluorescent protein driven by the hordein promoter is stably inherited in transgenic barley (*Hordeum vulgare*) plants. *Physiol. Plant.*, **115**: 144–154.
- Christensen, C. A., Gorsich, S. W., Brown, R. H., Jones, L. G., Brown, J., Shaw, J. M., Drews, G. N., 2002.

- Mitochondrial GFA2 is required for synergid cell death in *Arabidopsis*. *Plant Cell*, **14**: 2215–2232.
- DeZwaan, T. M., Carroll, A. M., Valent, B., James A. Sweigard, J. M., 1999. *Magnaporthe grisea* Pth11p is a novel plasma membrane protein that mediates Appressorium differentiation in response to inductive substrate cues. *Plant Cell*, **11**: 2013–2030.
- Endo, S., Sugita, K., Sakai, M., Tanaka, H., Ebinuma, H., 2002. Single-step transformation for generating marker-free transgenic rice using the ipt-type MAT vector system. *Plant J.*, **30**: 115–122.
- Fukaki, H., Tameda, S., Masuda, H., Tasaka, M., 2002. Lateral root formation is blocked by a gain-of-function mutation in the *SOLITARY-ROOT/IAA14* gene of *Arabidopsis*. *Plant J.*, **29**: 153–168.
- Fukuoka, H., Ogawa, T., Mitsuhashi, I., Iwai, T., Isuzugawa, K., Nishizawa, Y., Gotoh, Y., Nishizawa, Y., Tagiri, A., Ugaki, M., Ohshima, M., Yano, H., Murai, N., Niwa, Y., Hibi, T., Ohashi, Y., 2000. *Agrobacterium*-mediated transformation of monocot and dicot plants using the NCR promoter derived from soybean chlorotic mottle virus. *Plant Cell Rep.*, **19**: 815–820.
- Gordon, C. L., Khalaj, V., Ram, A. F., Archer, D. B., Brookman, J. L., Trinci, A. P., Jeenes, D. J., Doonan, J. H., Wells, B., Punt, P. J., van den Hondel, C. A., Robson, G. D., 2000. Glucoamylase::green fluorescent protein fusions to monitor protein secretion in *Aspergillus niger*. *Microbiology*, **146**: 415–426.
- Grebenok, R. J., Pierson, E., Lambert, G. M., Gong, F. C., Afonso, C. L., Haldeman-Cahill, R., Carrington, J. C., Galbraith, D. W., 1997. Green-fluorescent protein fusions for efficient characterization of nuclear targeting. *Plant J.*, **11**: 573–586.
- Gubler, F., Chandler, P. M., White, R. G., Llewellyn, D. J., Jacobsen, J. V., 2002. Gibberellin signaling in barley aleurone cells. control of SLN1 and GAMYB expression. *Plant Physiol.*, **129**: 191–200.
- Hagenbeek, D., Ralph S. Quatrano, R. S., Christopher D. Rock, C. D., 2000. Trivalent ions activate abscisic acid-inducible promoters through an *ABI1*-dependent pathway in rice protoplasts. *Plant Physiol.*, **123**: 1553–1560.
- Hara, K., Sugita, M., Aoki, S., 2001. Cloning and characterization of the cDNA for a plastid sigma factor from the moss *Physcomitrella patens*. *Biochem. Biophys. Acta*, **1517**: 302–306.
- Haseloff, J., Siemering, K. R., Prasher, D. C., Hodge, S., 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. U. S. A.*, **94**: 2122–2127.
- Hashizume, F., Tsuchiya, T., Ugaki, M., Niwa, Y., Tachibana, N., Kowayama, Y., 1999. Efficient *Agrobacterium*-mediated transformation and the usefulness of a synthetic GFP reporter gene in leading varieties of Japonica rice. *Plant Biotechnol.*, **16**: 397–401.
- Hayashi, Y., Yamada, K., Shimada, T., Ryo Matsushima, R., Nishizawa, N. K., Nishimura, M., Hara-Nishimura, I., 2001. A proteinase-storing body that prepares for cell death or stresses in the epidermal cells of *Arabidopsis*. *Plant Cell Physiol.*, **42**: 894–899.
- Ho, C.-L., Noji, M., Saito, M., Yamazaki, M., Saito, K., 1998. Molecular characterization of plastidic phosphoserine aminotransferase in serine biosynthesis from *Arabidopsis*. *Plant J.*, **16**: 443–452.
- Hong, B., Ichida, A., Wang, Y., Gens, J. S., Pickard, B. G., Harper, J. F., 1999. Identification of a calmodulin-regulated Ca^{2+} -ATPase in the endoplasmic reticulum. *Plant Physiol.*, **119**: 1165–1176.
- Igarashi, D., Ishida, S., Fukazawa, J., Takahashi, Y., 2001. 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. *Plant Cell*, **13**: 2483–2497.
- Imlau, A., Truernit, E., Norbert Sauer, N., 1999. Cell-to-cell and long-distance trafficking of the Green Fluorescent Protein in the phloem and symplastic unloading of the protein into sink tissues. *Plant Cell*, **11**: 309–322.
- Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., Okada, K., 2001. The *DEFECTIVE IN ANTER DEHISCENCE1* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. *Plant Cell*, **13**: 2191–2209.
- Isono, K., Shimizu, M., Yoshimoto, K., Niwa, Y., Sato, K., Yokota, A., Kobayashi, H., 1997. Leaf-specifically expressed genes for polypeptides destined for chloroplasts with domains for sigma 70 factors of bacterial RNA polymerases in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A.*, **94**: 14948–14953.
- Itoh, H., Ueguchi-Tanaka, M., Sato, Y., Ashikari, M., Matsuoka, M., 2002. The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. *Plant Cell*, **14**: 57–70.
- Iuchi, S., Kobayashi, M., Yamaguchi-Shinozaki, K., Shinozaki, K., 2000. A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. *Plant Physiol.*, **123**: 553–562.
- Ivanchenko, M., Vejlupkova, Z., Quatrano, R. S., Fowler, J. E., 2000. Maize ROP7 GTPase contains a unique, CaaX box-independent plasma membrane targeting signal. *Plant J.*, **24**: 79–90.
- Iwakawa, H., Ueno, Y., Semiarti, E., Onouchi, H., Kojima, S., Tsukaya, H., Hasebe, M., Soma, T., Ikezaki, M., Machida, C., Machida, Y., 2002. The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana*, required for formation of a symmetric flat leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. *Plant Cell Physiol.*, **43**: 467–478.
- Jang, I.-C., Choi, W.-B., Lee, K.-H., Song, S. I., Nahm, B. H., Kim, J.-K., 2002. High-level and ubiquitous expression of the rice cytochrome c gene *OsCc1* and its promoter activity in transgenic plants provides a useful promoter for transgenesis of monocots. *Plant Physiol.*, **129**: 1473–1481.
- Jefferson, R. A., 1987. Assaying chimeric genes in plants:

- the GUS gene fusion system. *Plant Mol. Biol. Rep.*, **5**: 387–405.
- Jefferson, R. A., Kavanagh, T. A., Bevan, M. W., 1987. GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.*, **6**: 3901–3907.
- Kabeya, Y., Hashimoto, K., Sato, N., 2002. Identification and characterization of two phage-type RNA polymerase cDNAs in the moss *Physcomitrella patens*: Implication of recent evolution of nuclear-encoded RNA polymerase of plastids in plants. *Plant Cell Physiol.*, **43**: 245–255.
- Kim, C. S., Woo, Y.-m., Clore, A. M., Burnett, R. J., Carneiro, N. P., Larkins, B. A., 2002. Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. *Plant Cell*, **14**: 655–672.
- Kitakura, S., Fujita, T., Ueno, Y., Terakura, S., Wabiko, H., Machida, Y., 2002. The protein encoded by oncogene *6b* from *Agrobacterium tumefaciens* interacts with a nuclear protein of tobacco. *Plant Cell* **14**: 451–463.
- Kobayashi, Y., Dokiya, Y., Sugiura, M., Niwa, Y., Sugita, M., 2001. Genomic organization and organ-specific expression of a nuclear gene encoding phage-type RNA polymerase in *Nicotiana sylvestris*. *Gene*, **279**: 33–40.
- Kohchi, T., Mukougawa, K., Frankenberg, N., Masuda, M., Yokota, A., Lagarias, J. C., 2001. The Arabidopsis *HY2* gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. *Plant Cell*, **13**: 425–436.
- Koizumi, N., Martinez, I. M., Kimata, Y., Kohno, K., Sano, H., Chrispeels, M. J., 2001. Molecular characterization of two Arabidopsis Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. *Plant Physiol.*, **127**: 949–962.
- Kosugi, S., Ohashi, Y., 2002. E2Ls, E2F-like repressors of Arabidopsis that bind to E2F sites in a monomeric form. *J. Biol. Chem.*, **277**: 16553–16558.
- Kumagai, F., Yoneda, A., Tomida, T., Sano, T., Nagata, T., Hasezawa, S., 2001. Fate of nascent microtubules organized at the M/G1 interface, as visualized by synchronized tobacco BY-2 cells stably expressing GFP-tubulin: Time-sequence observations of the reorganization of cortical microtubules in living plant cells. *Plant Cell Physiol.*, **42**: 723–732.
- Kuruto-Niwa, R., Nakamura, M., Takeishi, K., Nozawa, R., 1998. Transcriptional regulation by C/EBP α and β in the expression of the gene for the MRP14 myeloid calcium binding protein. *Cell Struct Funct.*, **23**: 109–118.
- Li, L., Tutone, A. F., Drummond, R. S. M., Gardner, R. C., Sheng Luan, S., 2001. A novel family of magnesium transport genes in Arabidopsis. *Plant Cell*, **13**: 2761–2775.
- Mano, S., Hayashi, M., Nishimura, M., 1999. Light regulates alternative splicing of hydroxypyruvate reductase in pumpkin. *Plant J.*, **17**: 309–320.
- Mano, S., Nakamori, C., Hayashi, M., Kato, A., Kondo, M., Nishimura, M., 2002. Distribution and characterization of peroxisomes in Arabidopsis by visualization with GFP: Dynamic morphology and actin-dependent movement. *Plant Cell Physiol.*, **43**: 331–341.
- Marc, J., Granger, C. L., Brincat, J., Fisher, D. D., Kao, T.-h., McCubbin, A. G., Richard J. Cyr, R. J., 1998. A *GFP-MAP4* reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. *Plant Cell*, **10**: 1927–1940.
- Matsuhara, S., Jingu, F., Takahashi, T., Komeda, Y., 2000. Heat-shock tagging: a simple method for expression and isolation of plant genome DNA flanked by T-DNA insertions. *Plant J.*, **22**: 79–86.
- Mitsunashi, N., Shimada, T., Mano, S., Nishimura, M., Hara-Nishimura, I., 2000. Characterization of organelles in the vacuolar-sorting pathway by visualization with GFP in tobacco BY-2 cells. *Plant Cell Physiol.*, **41**: 993–1001.
- Morita, M. T., Kato, T., Nagafusa, K., Saito, C., Ueda, T., Nakano, A., Tasaka, M., 2002. Involvement of the vacuoles of the endodermis in the early process of shoot gravitropism in Arabidopsis. *Plant Cell*, **14**: 47–56.
- Muramoto, T., Kohchi, T., Yokota, A., Hwang, I., Goodman, H. M., 1999. The Arabidopsis photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase. *Plant Cell*, **11**: 335–348.
- Nakamura, N., Hirata, A., Ohsumi, Y., Wada, Y., 1997. Vam2/Vps41p and Vam6/Vps39p are components of a protein complex on the vacuolar membranes and involved in the vacuolar assembly in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **272**: 11344–11349.
- Nakazono, M., Tsuji, H., Li, Y., Saisho, D., Arimura, S., Tsutsumi, N., Hirai, A., 2000. Expression of a gene encoding mitochondrial aldehyde dehydrogenase in rice increases under submerged conditions. *Plant Physiol.*, **124**: 587–598.
- Nebenfuhr, A., Gallagher, L. A., Dunahay, T. G., Frohlick, J. A., Mazurkiewicz, A. M., Meehl, J. B., Staehelin, L. A., 1999. Stop-and-go movements of plant Golgi stacks are mediated by the acto-myosin system. *Plant Physiol.*, **121**: 1127–1141.
- Nishihama, R., Soyano, T., Ishikawa, M., Araki, S., Tanaka, H., Asada, T., Irie, K., Ito, M., Terada, M., Banno, H., Yamazaki, Y., Machida, Y., 2002. Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. *Cell*, **109**: 87–99.
- Niwa, Y., Muranaka, T., Baba, A., Machida, Y., 1994. Organ-specific and auxin-inducible expression of two tobacco *parA*-related genes in transgenic plants. *DNA Res.*, **1**: 213–221.
- Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M., Kobayashi, H., 1999. Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant J.*, **18**: 455–463.
- Noji, M., Takagi, Y., Kimura, N., Inoue, K., Saito, M., Horikoshi, M., Saito, F., Takahashi, H., Saito, K., 2001. Serine acetyltransferase involved in cysteine biosyn-

- thesis from spinach: Molecular cloning, characterization and expression analysis of cDNA encoding a plastidic isoform. *Plant Cell Physiol.*, **42**: 627–634.
- Obara, K., Sumi, K., Fukuda, H., 2002. The use of multiple transcription starts causes the dual targeting of *Arabidopsis* putative monodehydroascorbate reductase to both mitochondria and chloroplasts. *Plant Cell Physiol.*, **43**: 697–705.
- Ohta, S., Mita, S., Hattori, T., Nakamura, K., 1990. Construction and expression in tobacco of a β -glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol.*, **31**: 805–813.
- Ohta, M., Ohme-Takagi, M., Shinshi, H., 2000. Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. *Plant J.*, **22**: 29–38.
- Okada, K., Saito, T., Nakagawa, T., Kawamukai, M., Yuji Kamiya, Y., 2000. Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in *Arabidopsis*. *Plant Physiol.*, **122**: 1045–1056.
- Okada, T., Toriyama, K., 2001. Pollen vegetative cell-specific expression of *Bra r 1*: useful tool for observation of the vegetative nucleus and identification of transgenic pollen by nuclear-targeted GFP. *Sex. Plant Reprod.*, **13**: 301–307.
- Okushima, Y., Koizumi, N., Yamaguchi, Y., Kimata, Y., Kohno, K., Sano, H., 2002. Isolation and characterization of a putative transducer of endoplasmic reticulum stress in *Oryza sativa*. *Plant Cell Physiol.*, **43**: 532–539.
- Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergrast, F. G., Cormier, M. J., 1992. Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene*, **111**: 229–233.
- Saito, C., Ueda, T., Abe, H., Wada, Y., Kuroiwa, T., Hisada, A., Furuya, M., Nakano, A., 2002. A complex and mobile structure forms a distinct subregion within the continuous vacuolar membrane in young cotyledons of *Arabidopsis*. *Plant J.*, **29**: 245–255.
- Saito, T., Niwa, Y., Ashida, H., Tanaka, K., Kawamukai, M., Matsuda, H., Nakagawa, T., 1999. Expression of a gene for cyclophilin which contains an amino-terminal endoplasmic reticulum-targeting signal. *Plant Cell Physiol.*, **40**: 77–87.
- Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., Galbraith, D. W., 1995. Green-fluorescent protein as a new vital marker in plant cells. *Plant J.*, **8**: 777–785.
- Shimomura, O., Johnson, F. H., Saiga, Y., 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusa, *Aequorea*. *J. Cell Comp. Physiol.*, **59**: 223–239.
- Siemering, K., Golbik, R., Sever, R., Haseloff, J., 1996. Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.*, **6**: 1653–1663.
- Sitthithaworn, W., Kojima, N., Viroonchatapan, E., Suh, D.-Y., Iwanami, N., Hayashi, T., Noji, M., Saito, K., Niwa, Y., Sankawa, U., 2001. Geranylgeranyl diphosphate synthase from *Scoparia dulcis* and *Croton sublyratus*. Plastid localization and conversion to a farnesyl diphosphate synthase by mutagenesis. *Chem. Pharm. Bull.*, **49**: 197–202.
- Sugita, K., Matsunaga, E., Kasahara, T., Ebinuma, H., 2000. Transgene stacking in plants in the absence of sexual crossing. *Mol. Breed.*, **6**: 529–536.
- Takahashi, H., Watanabe-Takahashi, A., Smith, F. W., Blake-Kalff, M., Hawkesford, M. J., Saito, K., 2000. The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. *Plant J.*, **23**: 171–182.
- Takechi, K., Sodmergen, Murata, M., Motoyoshi, F., Sakamoto, W., 2000. The *YELLOW VARIEGATED (VAR2)* locus encodes a homologue of FtsH, an ATP-dependent protease in *Arabidopsis*. *Plant Cell Physiol.*, **41**: 1334–1346.
- Takeuchi, M., Ueda, T., Sato, K., Abe, H., Nagata, T., Nakano, N., 2000. A dominant negative mutant of Sar1 GTPase inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus in tobacco and *Arabidopsis* cultured cells. *Plant J.*, **23**: 517–525.
- Takeuchi, M., Ueda, T., Yahara, N., Nakano, N., 2002. Arf1 GTPase plays roles in the protein traffic between the endoplasmic reticulum and the Golgi apparatus in tobacco and *Arabidopsis* cultured cells. *Plant J.*, **31**: 499–515.
- Tamura, K., Mitsuhashi, N., Hara-Nishimura, I., Imai, H., 2001. Characterization of an *Arabidopsis* cDNA encoding a subunit of serine palmitoyltransferase, the initial enzyme in sphingolipid biosynthesis. *Plant Cell Physiol.*, **42**: 1274–1281.
- Tamura, K., Adachi, Y., Chiba, K., Oguchi, K., Takahashi, H., 2002. Identification of Ku70 and Ku80 homologues in *Arabidopsis thaliana*: evidence for a role in the repair of DNA double-strand breaks. *Plant J.*, **29**: 771–781.
- Tamura, M., Togami, J., Ishiguro, K., Nakamura, N., Katsumoto, Y., Suzuki, K., Kusumi, T., Tanaka, Y., 2002. Regeneration of transformed verbena (*Verbena X hybrida*) by *Agrobacterium tumefaciens*. *Plant Cell Rep.*, in press.
- Tsien, R. Y., 1998. The green fluorescent protein. *Annu. Rev. Biochem.*, **67**: 509–544.
- Ueoka-Nakanishi, H., Tsuchiya, T., Sasaki, M., Nakanishi, Y., Cunningham, K. W., Maeshima, M., 2000. Functional expression of mung bean $\text{Ca}^{2+}/\text{H}^{+}$ antiporter in yeast and its intracellular localization in the hypocotyl and tobacco cells. *Eur. J. Biochem.*, **267**: 3090–3098.
- Valdez-Taubas, J., Diallinas, G., Scazzocchio, C., Rosa, A. L., 2000. Protein expression and subcellular localization of the general purine transporter UapC from *Aspergillus nidulans*. *Fungal Genet. Biol.*, **30**: 105–113.
- Versaw W. K., Harrison, M. J., 2002. A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses. *Plant Cell*, **14**: 1751–1766.
- Yadegari, R., Kinoshita, T., Lotan, O., Cohen, G., Katz, A., Choi, Y., Katz, A., Nakashima, K., Harada, J. J., Goldberg, R. B., Fischer, R. L., Ohad, N., 2000. Mutations in the *FIE* and *MEA* genes that encode

- interacting polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. *Plant Cell*, **12**: 2367–2382.
- Yanagisawa, S., 2001. The transcriptional activation domain of the plant-specific Dof1 factor functions in plant, animal, and yeast cells. *Plant Cell Physiol.*, **42**: 813–822.
- Yang, S., Sweetman, J. P., Amirsadeghi, S., Barghchi, M., Huttly, A. K., Chung, W.-I., Twell, D., 2001. Novel anther-specific *myb* genes from tobacco as putative regulators of phenylalanine ammonia-lyase expression. *Plant Physiol.*, **126**: 1738–1753.
- Yokoyama, R., Nishitani, K., 2001. Endoxyloglucan transferase is localized both in the cell plate and in the secretory pathway destined for the apoplast in tobacco cells. *Plant Cell Physiol.*, **42**: 292–300.
- Yoshimoto, N., Takahashi, H., Smith, F. W., Yamaya, T., Saito, K., 2002. Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. *Plant J.*, **29**: 465–473.
- Zhang, C. L., Chen, D. F., McCormac, A. C., Scott, N. W., Elliott, M. C., Slater, A., 2001. Use of the GFP reporter as a vital marker for *Agrobacterium*-mediated transformation of sugar beet (*Beta vulgaris* L.). *Mol. Biotechnol.*, **17**: 109–117.