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REVIEW

Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants

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Abstract Transformation of plants is a popular tool for modifying various desirable traits. Marker genes, like those encoding for bacterial *β*-glucuronidase (GUS), firefly luciferase (LUC) or jellyfish green fluorescent protein (GFP) have been shown to be very useful for establishing of efficient transformation protocols. Due to favourable properties such as no need of exogenous substrates and easy visualization, GFP has been found to be superior in to other markers in many cases. However, the use of GFP fluorescence is associated with some obstacles, mostly related to the diminishing of green fluorescence in older tissues, variation in fluorescence levels among different tissues and organs, and occasional interference with other fluorescing compounds in plants. This paper briefly summarizes basic GFP properties and applications, and describes in more detail the contribution of GFP to the establishment, evaluation and improvement of transformation procedures for plants. Moreover, features and possible obstacles associated with monitoring GFP fluorescence are discussed.

Keywords *Agrobacterium tumefaciens* · Green fluorescent protein · Particle bombardment · Plant transformation · Selection

Abbreviations

GFP	Green fluorescent protein
GUS	*β*-Glucuronidase
LUC	Firefly luciferase
RT-PCR	Reverse transcription polymerase chain reaction
gfp	Green fluorescent protein gene
uidA	*β*-Glucuronidase gene

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Introduction

Genetic transformation of plants is a promising method not only for improving various agronomic and/or horticultural traits, but also for fundamental studies of plant physiology (Bauchera et al. 1998; Smirnoff and Wheeler 2000), genetics,

molecular and cell biology (Kocábek et al. 1999), pathology (Franchea et al. 1998; Panstruga 2004) and other areas. The development of plants with new qualitative or quantitative traits is the primary objective of plant transgenesis. The successful introduction of new desirable traits usually requires the development of an efficient and reliable transformation protocols. Such protocols should contribute to the development of the most efficient strategy for transferring the genes into plant cells (Chilton et al. 1977; Klein et al. 1987), selection and regeneration of putative transgenic cells (Miki and McHugh 2004) and subsequent recovery of transgenic plant(s). Generally, model genes, which allow for the critical assessment of each step in the procedure, are the most suitable for such fundamental studies. The use of visual markers, which enable direct observation of transformation events, results in a more precise and easier evaluation of various treatments and procedures. They can increase transformation efficiency by reducing the time and amount of material to be handled and screened (Baranski et al. 2006) allowing the most efficient, reliable and reproducible transformation protocol to be established. The ideal marker should possess the following desirable traits. First, it should be readily expressed in plant cells or capable of being engineered for such expression by molecular biology methods. Second, its expression should be easily visualized, and finally, the marker should not be toxic or affect in any way the physiology of living intact plants. Many genes coding for various markers are available now. Markers such as β-glucuronidase (GUS) (Jefferson et al. 1987), luciferase (LUC) (Ow et al. 1986) or β-galactosidase (LacZ) (Helmer et al. 1984) have become very popular tools for monitoring gene expression in transgenic plants. However, these require either destructive assays of the studied sample or the addition of exogenous substrates or some other cofactors for their manifestation. These markers usually do not offer the possibility of determining the exact transgenic status of plants, while also monitoring the transgene expression in real time and in living plants. On the other hand, green fluorescent protein (GFP) marker, in principle, allows for the monitoring of transgene expression from early stages of the transformation procedure though the recovery of living transgenic plants. Moreover, GFP manifestation does not require the addition of any interfering substances like exogenous substrates or enzymes. Thus plants can continue their growth and development, and can be investigated repeatedly at any growth stage (Heim et al. 1995; Chiu et al. 1996). This represents a huge benefit for using GFP as a visual marker during genetic transformation and regeneration of transgenic plants.

Molecular structure, properties and use of GFP

GFP was isolated from the pacific jellyfish *Aequorea victoria* and first described by Prasher et al. (1992). GFP transforms the luminescent blue light emitted by another hydromedusas protein, *aequorin*, into green light. The fluorescing chromophore of GFP is formed by post-translational modification in which a tripeptide Ser65-Tyr66-Gly67 is cyclized and later oxidized. This chromophore is in the geometric centre of the protein to which it is covalently attached (Shinomura 1979; Cody et al. 1993). Eleven β sheets form a barrel structure that is capped with α-helices on the top and bottom of the protein. α-helices also form a scaffold for the centrally placed chromophore. GFP represents a new class of proteins called ''beta can''. Wild type GFP is a dimer consisting of two monomer units, each consisting of 238 amino acids with a relative molecular weight of 27 kDa. The diameter of the barrels is 30 Å and length is 40 Å (Yang et al. 1996a). This wild type GFP emits light after excitation by UV (λ = 360–400 nm) or blue (λ = 440–480 nm) light with emission spectra at λ = 509 nm and with a minor peak at λ = 540 nm. GFP does not require any endogenous cofactors and substrates or exogenous compounds for fluorescence manifestation, because the formation of the chromophore is either an autocatalytic process or it requires only ubiquitous cellular components (Heim et al. 1994; Misteli and Spector 1997). GFP possesses a rigid structure with a broad stability range in pH 5–11 at temperatures up to 65°C (Tsien 1998). It maintains its fluorescence even in the presence of strong

Table 1 List of various GFP variants that are the most common in plant transformation experiments

GFP variant	Excitation/ emission spectra (nm)	Modification/s	Effect of modification/s	References
Wild type	395*, 475/510	None	None	Chalfie et al. (1994)
mGFP4	395*, 475/509	ACU	Brighter fluorescence in plants	Haseloff and Amos (1995)
mGFP5	395, 473/509	ACU, V163A, I167T, S175G	Better protein folding at 37°C, increased fluorescence, dual excitation peaks-equal amplitude	Haseloff et al. (1997)
sGFP	490/511	Humanized codon usage	Improved expression and fluorescence	Haas et al. (1996)
SGFP S65T	489/511	ACU, S65T, humanized codon usage	Increased detection limits, enhanced brightness, faster chromophore formation, slower photobleaching	Chiu et al. (1996)
EGFP	488/507	ACU, F64L, S65T, Y145F, humanized codon usage	Brighter fluorescence, improvement solubility, more efficient protein folding in bacteria	Yang et al. (1996b), Clontech
mut3GFP	400*, 475/510	ACU, F100S (F99S), M154T (M153T), V164A (V163A)	Putatively improved folding at higher temperatures	Siemering et al. (1996)
smGFP	397*, 480/507	ACU, F99S, M153T, V163A	Improved solubility and brightness	Davis and Viestra (1998)
smRS-GFP	495/510	ACU, S65T, F99S, M153T, V163A	Improved solubility and brightness, red-shifted emission	Davis and Viestra (1998)
GFP	450–490/510	ACU, S65C, S65T	Increased fluorescence	Reichel et al. (1996)
smBFP	385/448	Y66H, M153T	Improved solubility, blue-shifted emission, increase of the fluorescence	Davis and Viestra (1998)
synGFP	395/509	ACU, increasing of CG content up to 48 %, potential polyadenilation sites removed	More efficient translation, increased expression	Rouwendal et al. (1997)
BFP	382/448	ACU, Y66H	Blue-shifted emission, weaker brightness, photobleaching	Reichel et al. (1996)
mYFP	514/527	S65G, S72A, V163A, I167T, S175G, T203Y	Yellow-shifted emission	Haseloff (1999)
mCFP	440/485	Y66W, V163A, S175G	Cyan-shifted emission	Haseloff (1999)

The amino acids mutations are given in a standard format, e.g. S65T, serine to threonine at 65th amino acid; ACU, altered codon usage according to Haseloff et al. (1997); *Major excitation peak in the case of two peaks are available

denaturing agents such as 6 M guanidine HCl, 8 M urea or 1% sodium dodecyl sulphate (Yang et al. 1996a).

Due to its favourable features, GFP rapidly became a popular tool in various applications in biology research. During the last decade, it has been introduced into a wide range of organisms, including bacteria, yeasts (Morschhäuser et al. 1998), nematodes (Chalfie et al. 1994), insects (Wang and Hazelrigg 1994), fish (Kinoshita 2004), mammals (Zolotukhin et al. 1996) and plants (Chiu et al. 1996). Its suitability for plant transformation was first demonstrated by Niedz et al. (1995), who successfully inserted wild type GFP into sweet orange (*Citrus sinensis*) protoplasts. Transformation of other plant species soon followed, but complications with low expression and quenching of fluorescence occurred (Hu and Cheng 1995). Detail sequence analysis performed by Haseloff et al. (1997) revealed the existence of a cryptic intron in the wild type GFP gene sequence. Its presence resulted in aberrant splicing between nucleotides 380–463 during processing in plant cells and finally the loss of the 84-nucleotide region. A new variant, denoted mGFP4, was derived by altered codon usage, maintaining the same spectral characteristics as wild type GFP, but resulting in enhanced protein fluorescence (Haseloff et al. 1997). Subsequently, many other GFP variants have been developed, differing in their spectral characteristics, fluorescence intensity or cell targets, e.g. nucleus, endoplasmic reticulum, plastids (reviewed by Stewart 2001). Different colour GFP variants offer simultaneous tracking and study of various biological events (Baumann et al. 1998; Haseloff 1999). GFP has been used for various purposes in plant research, e.g. for the study of the expression patterns of promoters (Sheen et al. 1995; Nagatani et al. 1997), protein tagging (Chytilova et al. 1999; Shiina et al. 2000), disease tracking (Itaya et al. 1997), developmental studies (Misteli and Spector 1997), expression studies and ecological monitoring of transgene spread (Halfhill et al. 2001). GFP is being increasingly used for various purposes associated with the transformation of plants (Baranski et al. 2006; Yong et al. 2006). Nowadays, many GFP homologues originating from various organisms are available, allowing for broad range of use in biology (Chudakov et al. 2005) (Table 1).

Although some concerns about the possible toxicity of GFP to plants were raised, these have not been confirmed (reviewed by Stewart 2001). GFP did not appear to have any adverse effects on plant growth, development and fertility (Maximova et al. 1998; Ghorbel et al. 1999; Harper et al. 1999; Jordan 2000; Kaeppler et al. 2000; Murray et al. 2004). Moreover GFP has been found to be non-toxic to rats when ingested in purified form or in transgenic plants (Richards et al. 2003a).

GFP as a tool for evaluation of transformation parameters

Although many different approaches to plant transformation are available, most of them involve the insertion of exogenous DNA into plant nucleus via *Agrobacterium*-mediated transfer (Chilton et al. 1977) or particle bombardment (Klein et al. 1987). Transformation methods differ in their suitability for various purposes and plant species (Finer et al. 1999; Repellin et al. 2001), DNA integration patterns (Christou 1995; Birch 1997; Christou 1997) and their efficiency (Snape 1998). It has been shown by many authors that the development of any of transformation procedures may be much faster and more efficient if proper signal gene(s) are used throughout the study (Birch 1997; Baranski et al. 2006).

Compared to other signal genes, GFP has an advantage of wide range of applications covering whole areas of transformation and regeneration procedures. The transformation events, formation of calli followed by the emergence of fluorescing shoots can all be observed sequentially in each step of transformation and during different phases of development by fluorescence microscopy. GFP-expressing cells and tissues can easily be distinguished from untransformed ones, without destroying the studied material (Kamaté et al. 2000). The ratio between fluorescing and non-fluorescing cells, shoots and various organs as a measure of transformation efficiency has been successfully used to improve the various stages and procedures in transformation protocols.

Steps, such as the selection of the most suitable *Agrobacterium* strain for transient and stable expression studies (Galperin et al. 2003; Tang and Newton 2005), determination of the suitable acetosyringone concentration in co-cultivation medium (Jeoung et al. 2002; Tang and Newton 2005; Wang and Ge 2005), or the optimisation of other various pre-cultivation, co-cultivation (Zhou et al. 2004) and post-transformation steps (Eady et al. 2000; Cardoza and Stewart 2003), including e. g. the effect of the antibiotic treatment on explant viability, were critically assessed using the GFP marker (Tang and Newton 2005). Based on differences in GFP fluorescence, the effect of desiccation of co-cultivated explants on efficacy of transformation has also been analysed (Polin et al. 2006). For example, Baranski et al. (2006) successfully employed the GFP fluorescence for critical assessment of the whole transformation procedure of the *Agrobacterium rhizogenes*-mediated transformation of carrot. Based on the green fluorescence intensity they selected the most virulent *Agrobacterium* strain, effective acetosyringone concentration and the most suitable carrot genotype for transformation. Moreover, they were able to assess other parameters, such as the effect of delayed inoculation on the number of adventitious roots production.

In order to achieve higher efficacy of direct transformation, the *gfp* expression has been successfully used as an efficient tool for evaluation and subsequent modification of various parameters and procedures associated with particle bombardment transformation, such as the selection of appropriate tissue to be bombarded (Huber et al. 2002; Tee et al. 2003), modification of gene gun settings (Richards et al. 2001), optimisation of bombardment parameters (Jordan 2000), and evaluation of various promoters (Cho et al. 2002; Tee et al. 2003).

Monitoring the *gfp* expression in primary transformed tissues

At the beginning of the tissue transformation the GFP fluorescence is usually visible in the cuts or other wounded sectors (Zhou et al. 2004), but sometimes it can be confused with a false autofluorescence of wounded tissues (Molinier et al. 2000). For example, high levels of background green fluorescence were observed in both, untransformed (control) and transformed flax hypocotyls. This precluded their use in GFP studies and therefore different plant organs were chosen for this purpose. Moreover, in some cases, transformed tissue could possess so strong autofluorescence, that green fluorescence could not be easily distinguished (Hraška and Rakouský 2005).

Low levels of background fluorescence of various compounds in intact, wounded and untransformed tissues and/or in *Agrobacterium* strains do not usually impede the successful detection of GFP fluorescence and can be restricted by implementation of suitable filter systems (Maximova et al. 1998; Elliott et al. 1999).

A strong GFP fluorescence signal is usually visible within a few hours after co-cultivation, indicating high levels of transient *gfp* expression, which usually decrease within a few days (Elliott et al. 1999; Mercuri et al. 2001; Jeoung et al 2002; Pishak et al. 2003). This has also been reported if other marker genes, (e.g. GUS) were used (Rakouský et al. 1997). Detailed study of *gfp* transient expression in transformed apple leaf explants showed an increase in GFP fluorescence after 9 days of bacterial infection, followed by decrease and stabilization of fluorescence between 11th and 15th day. This was most probably the result of degradation of non-integrated T-DNA or gene silencing of integrated T-DNA. The fluorescence increased at 15 days after transformation, indicating growth of stably transformed cells and transgenic calli formation. Another observed event was the high number of fluorescing cells associated with the cut vascular tissues. This was explained as being due to a higher cell number and density in vascular tissue or due to vascular tissues being more susceptible to *Agrobacterium* infection (Maximova et al. 1998).

The level of GFP fluorescence differs depending on target genotype and tissue, *gfp* variant and the promoter used. For example, if barley immature embryos were transformed with *gfp* gene driven by either rice actin gene (*Act1*) promoter or endosperm-specific hordein promoter, they exhibited stronger transient *gfp* expression

when driven by *Act1* promoter than by the second one. On the other hand, endosperm-specific-hordein-promoter-driven *gfp* possesses more stable expression in T_1 progeny than *Act1* driven *gfp* (Cho et al. 2002).

Following the transformation and subsequent regeneration, only a small number of fluorescing foci stay fluorescent for periods, long enough to indicate stable genetic transformation. This event has been reported for many plant species such as wheat (Jordan 2000), barley (Ahlandsberg et al. 1999; Carlson et al. 2001), oat (Cho et al. 2003), soybean (Ponappa et al. 1999), papaya (Zhu et al. 2004), *Dendrobium* orchid (Tee et al. 2003) and tobacco (Li and Yang 2000). No correlation between the level of transient expression and the subsequent level of stable transformation has been observed (Huber et al. 2002).

Monitoring of the *gfp* expression in transformed tissue can be used to improve the selection efficiency during the subsequent plant regeneration. For example, if the GFP fluorescence was observed during the regeneration of explants cultivated on media supplemented with hygromycin as a selective agent, it resulted in stringent, 4.5% transformation efficiency of red fescue and 82% regenerability, giving an effective transformation frequency 3.7% (Cho et al. 2000). The following example presents quite a different story: monitoring of green fluorescence was used as a tool for critical comparison of the efficacy of two strategies for rhododendron transformation, *Agrobacterium*-mediated and direct transformation. Successful *Agrobacterium*-mediated transformation of *Rhododendron* was previously reported by many authors (Ueno et al. 1996; Pavingerová et al. 1997; Tripepi et al. 1999). Knapp et al. (2001) reported a surprisingly low transformation efficacy (0.2%) after using the particle bombardment of rhododendron leaves as compared with the *Agrobacterium*-mediated transformation efficacy of 5% reported by Ueno et al. (1996). Based on these findings some possible reasons such as the difficulties of penetration of hard and waxy leave cells by gold particles, cell death caused by wounding by gold particles or degradation of naked DNA, were hypothesised and subsequently the transformation protocol was refined (Table 2).

GFP manifestation in regenerating shoots and mature plants

Following the recovery of a new transgenic plant, GFP fluorescence is usually visible in new emerging shoots and young tissues or organs, whereas it declines to give a weak signal in older ones (Kamaté et al. 2000; Tamura et al. 2003; Zhou et al. 2004). On the other hand, the variability in green fluorescence in early transformation stages was reported by some authors (Eady et al. 2000; Taniguchi et al. 2005). During subsequent regeneration the fluorescence normally declines to the extent that it is not visible in older tissues or organs. Weak or no fluorescence has normally been observed in mature leaves (Kamaté et al. 2000; Cho and Widholm 2002; Cui et al. 2003; Zhou et al. 2004; Taniguchi et al. 2005), with the occasional occurrence of small fluorescing regions in some cells (Eady et al. 2000) or organs, e.g. trichomes (Mercuri et al. 2001; Han et al. 2005) or stomatal guard cells (Kim et al. 2004). On the other hand, GFP fluorescence was normally visible in inflorescences, petals, stamens and pistils (Cui et al. 2003; Zhou et al. 2004), roots (Elliot et al. 1999; Zhou et al. 2004), whole flowers, plantlets and seedlings (Kamaté et al. 2000; Zhou et al. 2004), suggesting that the reason for low levels of GFP fluorescence in older leaves is associated with the increasing content of chlorophyll, which possess strong red autofluorescence, or other flourescing compounds.

Lowering of overall *gfp* expression level during the growth and development of organs may not be the sole reason for diminishing of fluorescence. Some authors studied this event in a more detailed way. For instance, Zhou et al. (2004) also reported high GFP fluorescence in young *Medicago truncatula*, var. A17 leaves and lowering of the fluorescence in older leaves. Based on it they decided to study mRNA levels in leaves of different age. Semi-quantitative RT-PCR showed similar RNA transcript spectra in all samples, indicating that the lack of expression is not the reason. An important fact is that, for many studies *gfp* driven by constitutive promoters such as CaMV 35S or *Act1* were mainly used, and although their constitutive features in transgenic

Table 2 Examples of transformation experiments using various variants of *gfp* gene

Plant species	*gfp* gene variant/s	Promoter/s	Transformed tissue	Selectable marker gene	Transformation method	References
Abies fraseri (Pursh) Poir, *Abies nordmanniana* (Stevens) Spach, *Pinus virginiana* Mill.	*mgfp5*-ER	CaMV 35S	Mature zygotic embryos	*nptII*	At	Tang and Newton (2005)
Aliium cepa L.	*mgfp5*-ER	CaMV 35S	Immature embryos	*nptII*	At	Eady et al. (2000)
Antirrhinum majus L.	*sgfp*(S65T)	CaMV 35S	Hypocotyls	*nptII*	At	Cui et al. (2003)
Arabidopsis thaliana (L.) Heynh.	*mgfp4*	CaMV 35S	Roots	*gfp*	PB	Haseloff et al. (1997)
Artemisia annua L.	*gfp*	CaMV 35S	Leaf explants	*nptII*	At	Han et al. (2005)
Astragalus sinicus L.	*mgfp5*-ER	CaMV 35S	Cotyledons	*nptII*	At	Cho and Widholm (2002)
Avena sativa L.	*sgfp*(S65T)	*Act1*	Shoot meristematic culture	*bar*, *hpt, nptII*	PB	Cho et al. (2003)
Avena sativa L.	*sgfp*	*ubi1*	Calli	*gfp*	PB	Goldman et al. (2003)
Brassica campestris L.	*mgfp5*-ER	CaMV 35S	Cotyledons	*nptII*	At	Malyshenko et al. (2003)
Brassica napus L.	*mgfp5*-ER, *egfp*	CaMV 35S	Hypocotyls	*nptII*	At	Cordoza and Stewart (2003)
Brassica rapa L.	*egfp*	CaMV 35S	Cotyledons	*nptII*	At	Wahlroos et al. (2003)
Cajanus cajan (L.) Millsp.	*mgfp5*-ER	CaMV 35S	Decapitated mature embryo axis	*nptII*	At	Mohan and Krishnamurthy (2003)
Carica papaya L.	*mgfp5*	CaMV 35S	Embryogenic calli	*nptII*	PB	Zhu et al. (2004)
Castanea dentata (Marsh.) Borkh.	*mgfp5*-ER	CaMV 35S	Somatic embryos	*nptII*	At	Polin et al. (2006)
Chamaecyparis obtusa Sieb. *et* Zucc.	*sgfp*(S65T)	CaMV 35S	Embryogenic tissues	*nptII*	At	Taniguchi at al. (2005)
Citrus aurantium L.	*sgfp*	CaMV 35S	Internodal stems, epicotyls	*nptII, gfp*	At	Ghorbel et al. (1999)
Citrus sinensis L. Osbeck	*egfp*	Double CaMV 35S	Protoplasts	*gfp*	E	Guo et al. (2005)
Cucumis melo L.	*mgfp5*	CaMV 35S	Cotyledons	*nptII*	At	Galperin et al. (2003)
Daucus carota L.	*mgfp5*-ER	CaMV 35S	Root discs	*gfp*	Ar	Baranski et al. (2006)
Dendrobium spp.	*sgfp*(S65T)	*Ubi1*, CaMV 35S, HBT	Tips of inflorescences, various types of calli	*gfp*	PB	Tee et al. (2003)
Festuca arundinacea Schreb.	*mgfp*	CaMV 35S	Embryogenic calli	*hph*	At	Wang and Ge (2005)
Festuca rubra L.	*sgfp*(S65T)	*Act1*	Highly regenerative tissue	*hpt*	PB	Cho et al. (2002)
Glycine max L.	SGFP-TYG, *mgfp4*, *mgfp5*-ER, *smgfp*, *sm*RS-*gfp*	CaMV 35S	Embryogenic tissue	*hpt*	PB	Ponappa et al. (1999)
Helianthus annuus L.	*mgfp*	CaMV 35S	Shoot apices	*nptII*	At	Weber et al. (2003)
Hordeum vulgare L.	*sgfp*(S65T)	*Act1*/ES-hordein	Immature embryos	*bar*	PB	Cho et al. (2002)
Hordeum vulgare L.	*sgfp*(S65T), *pgfp*	*Act1*, CaMV 35S	Microspore culture	*gfp*	PB	Carlson et al. (2001)
Hordeum vulgare L.	*pgfp*	*Act1*	Embryogenic calli	*gfp*	PB	Ahlandsberg et al. (1999)
Ipomoea batatas (L.) Lam.	*mgfp4*	CaMV 34S	Protoplasts, somatic embryos	*gfp*	E, PB	Lawton et al. (2000)
Juglans regia L.	*sgfp*(S65T)	CaMV 35S	Embryos	*nptII*	At	Escobar et al. (2000)

Table 2 continued

Plant species	*gfp* gene variant/s	Promoter/s	Transformed tissue	Selectable marker gene	Transformation method	References
Eustona grandiflorum (Raf.) Shinners, *Osteospermum ecklonis* (DC.) Norlindh	*mgfp4*, *mgfp5*-ER, *sgfp* (S65T/49), *sgfp* (S65T/63), *sgfp* S65C	CaMV 35S, enhanced CaMV 35S	Leaf explants	*nptII*	At	Mercuri et al. (2001)
Malus domestica Borkh.	*sgfp*-TYG	CaMV 35S	Leaf explants	*nptII*	At	Maximova et al. (1998)
Medicago truncatula Gaertn.	*sgfp*	CaMV 35S	Cotyledons, embryogenic axis	*bar*	At	Zhou et al. (2004)
Medicago truncatula Gaertn.	*gfp*	CaMV 35S	Floral organs	*nptII*	At	Kamaté et al. (2000)
Medicago truncatula Gaertn.	*mgfp5*-ER	CaMV 35S	Leaflets form 2–3 week-old plantlets	*nptII*	At	Chabaud et al. (2003)
Melastoma malabathricum L., *Tibouchina semidecandra* Cogn.	*mgfp*	CaMV 35S	Shoot, node explants	*gfp*	At	Yong et al. (2006)
Nemesia strumosa Benth.	*sgfp*(S65T)	CaMV 35S	Stem cuts	*nptII*	At	Cui and Ezura (2003)
Nicotiana tabacum L.	*gfp*	Not reported	Zygote	*gfp*	E	Li and Yang (2000)
Nicotiana tabacum L.	*mgfp5*-ER	CaMV 35S	Leaf discs	*nptII*	At	Chen et al. (2005)
Nicotiana tabacum L.	*s-gfp*	CaMV 35S	Leaf discs	*nptII*	At	Molinier et al. (2000)
Oryza sativa L.	*mgfp4*	CaMV 35S	Immature embryos	*aphIV, gfp*	PB	Vain et al. (1998)
Oryza sativa L.	*sgfp*(S65T)	CaMV 35S	Embryogenic calli	*hpt*	At	Sallaud et al. (2003)
Panicum virgatum L.	*sgfp*	*Act1*	Calli	*bar*	PB	Richards et al. (2001)
Pennisetum glaucum L.	*egfp*	CaMV 35S	Embryogenic calli	*bar*	PB	Goldman et al. (2003)
Pyrus communis L.	*gfp*	Not reported	Leaf explants	*nptII*	At	Yancheva et al. (2006)
Prunus persica L.	*sgfp*	CaMV 35S	Embryo sections	*nptII*	At	Pérez-Clemente et al. (2004)
Rhododendron spp.	sm*GFP*	CaMV 35S	Leaves	*nptII*	PB	Knapp et al. (2001)
Rosa hybrida L.	*mgfp5*-ER	CaMV 35S	Embryogenic calli	*nptII*	At	Kim et al. (2004)
Saccharum spp., *Zea mays* L., *Lactuca sativa* L., *Nicotiana tabacum* L.	*sgfp* (S65T), *mgfp5*-ER	CaMV 35S, *Ubi1*	Coleoptyles, calli, cotyledons, leaf explants	*aphA*, *gfp*	At, PB	Elliot et al. (1999)
Sorghum spp.	smRS-*gfp*	CaMV 35S, *Ubi1*, HBT	Embryogenic calli, immature embryos, leaves	Not reported	At, PB	Jeoung et al. (2002)
Spinacia oleracea L.	*smgfp*	CaMV 35S	Cotyledons	*nptII*	At	Zhang and Zeevaart (1999)
Theobroma cacao L.	*egfp*	CaMV 35S	Cotyledons	*nptII*	At	Pishak et al. (2003)
Triticum aestivum L.	S65T*gfp*	CaMV 35S	Immature embryos	*bar*	PB	Huber et al. (2002)
Triticum aestivum L	*sgfp*(S65T)	*Act1*	Embryos	*gfp*	PB	Jordan (2000)
Verbena x hybrida	*sgfp*(S65T)	CaMV 35S	Shoots	*nptII*	At	Tamura et al. (2003)
Vigna angularis (Willd.) Ohwi et Ohashi	*sgfp*(S65T)	CaMV 35S	Epicotyls	*nptII*	At	Yamada et al. (2001)
Vitis vinifera L.	*egfp*	Enhaced CaMV 35S, CsVMV, double CsVMV, ACT2	Somatic embryos	*nptII*	At	Li et al. (2001)
Zea mays L.	*mgfp*	*Ubi1*	Embryogenic calli	*bar*	PB	van der Geest and Petolino (1998)

Table 2 continued

Following abbreviations are used throughout the table:

At, *Agrobacterium tumefaciens*-mediated transfer; Ar, *A. rhizogenes*-mediated transfer; PB, particle bombardment transformation; E, electroporation; *aph*, aminoglykosid phosphotransferase gene; *bar*, phosphinotricin acetyltransferase gene; *hph*, hygromycin phosphotransferase gene; *nptII*, neomycin phosphotransferese gene; *egfp*, engineered *gfp* gene; *mgfp5*-ER, modified *gfp* targeted to the endoplasmatic reticulum; *pgfp*, plant optimized *gfp* gene; *sgfp*, synthetic green fluorescent gene; *sm-gfp*, soluble-modified *gfp* gene

plants have been reported (Benfey et al. 1989; Battraw and Hall 1990), recent studies revealed that some differences in expression can occur (Williamson et al. 1989; Malik et al. 2002; Sunilkumar et al. 2002).

Therefore, some spatial or other fluctuations are possible. Such presumption was confirmed by Zhou et al. (2004), who reported different *gfp* expression patterns in *Medicago truncatula* plants. It is not clear whether an aberrant activity of CaMV 35S promoter is the reason for fluorescence quenching, and/or production of a quenching substance such as protease could also be involved (Zhou et al. 2004). Finally, the expression of *gfp* might be influenced by the positional effect of inserted transgenes or by co-suppression due to the higher transgene copy number (Tamura et al. 2003).

Instrumentation and approaches for GFP visualization, occurrence of interfering factors and diminishing of green fluorescence

The fluorescence properties of GFP allow for detection of gene expression in whole living plants with some simple UV lamp or more precise visualization of various events in living cells using fluorescence microscopy (Haseloff 1999).

Various observation systems are being used to study the GFP fluorescence. These usually consist of an excitation source, detection or observation device and usually appropriate filter sets. Previous investigations of GFP fluorescence mostly utilised high-power microscopes, but recent studies usually found that low-power microscopes and various hand-held UV or blue light sources could be sufficient too (Elliot et al. 1999; Li et al. 2001; Cui et al. 2003). Some instruments, because they exhibit a wide range of broad-wavelengths and wide light-diffusion angles, and therefore possess only limited energy in the wavelengths required for GFP excitation, can be used in situations of high *gfp* expression levels only (Vain et al. 1998). In addition, various confocal laser scanning microscopes are used for more detailed studies, e.g. of the precise sub-cellular GFP localization, allowing for the reconstruction for three-dimensional structures (Haseloff 1999; Belluci et al. 2003;

Pérez-Clemente et al. 2004). The use of the appropriate observation and excitation system is a prerequisite for successful GFP study. For example, Ponappa et al. (1999) reported weaker fluorescent signals after excitation of soybean embryogenic cultures when 50 W mercury lamp was used instead of stronger 100 W source.

Intact plant tissue represents a complicated subject for common fluorescence microscopy. Deep layers of highly refractile walls and aqueous cytosol coupled with the occurrence of various autofluorescence and light scattering compounds also make confocal microscopy a difficult mission. To circumvent these obstacles, fixing and clarification of studied samples in a high refractive index medium (1) or the use of suitable optic set (2) is recommended (Haseloff 1999). Nevertheles, in the case of *Arabidopsis* wholemounts, the first approach was associated with the loss of GFP fluorescence (Haseloff and Amos 1995). It should be noted in this context that direct visualization of GFP fluorescence does not require any fixation, staining or addition of some substrates, and allows for study of various events within the living cells such as cytoplasmatic streaming. Moreover, the presence of various autofluorescent organelles and compounds can be employed as a useful counter staining tool. This can be enhanced by addition of some exogenous substrates (Haseloff 1999).

On the other hand, loss of, or lack of GFP fluorescence is not always associated with the interference of various undesirable signals co-emitted along with the GFP signal. It can also be caused by pigment, which is opaque to exciting UV or blue light and thus negatively affects the effect of exciting light. Mercuri et al. (2001), who detected sufficient levels of GFP protein in transgenic *Limonium* flowers, failed to detect macroscopic green fluorescence due to the presence of various floral pigments. Another cause of the GFP fluorescence quenching in older leaves may be a change in cytoplasmic density of cells. This may explain, why the GFP manifestation is visible better in young cells and organs, than in older ones, especially leaves, since the vacuoles devoid of GFP constitute the largest part of the cell and finally ''dilute'' the GFP content (Maximova et al. 1998; Molinier et al. 2000; Cho and Widholm 2002). As can be seen from the above discussion, the quenching of GFP signal in mature or older transformed tissues and organs commonly occurs.

However, the most important cause seems to be the chlorophyll red autofluorescence interfering with the GFP green fluorescence, which finally obscures the GFP manifestation, so that it is often only visible in albino tissues lacking the chlorophyll such as roots (Cho et al. 2000; Carlson et al. 2001; Huber et al. 2002). The same observations were published by many other authors (van der Geest and Petolino 1998; Vain et al. 1998; Ponappa et al. 1999; Kaeppler et al. 2000; Jordan 2000; Cho et al. 2002; Zhou et al. 2005), suggesting that the chlorophyll autofluorescence precludes the GFP visualization in tissues with high chlorophyll content. In some cases the GFP fluorescence is visible through the chlorophyll background (Goldman et al. 2003). This can be effectively enhanced by using appropriate filter sets cutting off the undesirable autofluorescence (Ahlandsberg et al. 1999; Jordan 2000; Kamaté et al. 2000; Molinier et al. 2000; Richards et al. 2001; Taniguchi et al. 2005). A brief list of various observation devices coupled with suitable filters is given in Table 3. A different approach was reported by Wahlroos et al. (2003), who used laser-scanning microscopy for study of putative *Brassica rapa* plants, which possess a strong background fluorescence after the illumination with a hand-held long-wave UV lamp to confirm the transgene expression and transgenic status of plants.

Other possible reasons for the poor expression are developmental or cell specific expression of 35S promoter (Ponappa et al. 1999; Zhou et al. 2004), dilution of GFP content in dividing and growing cells (Zhou et al. 2004) or gene silencing (Voinnet and Baulcombe 1997).

Attempts to use GFP as an alternative selection tool in plant transformations

The early visualization and identification of transgenic events using GFP fluorescence allows the regeneration of transgenic cells without any selective (either negative or positive) pressure. GFP fluorescence can serve as a tool for rapid discrimination of transformed and non-

transformed cells, calli and shoots and hence help to eliminate untransformed cells and shoots from further cultivation. Unfortunately, this approach depends on high transformation frequencies, resulting in the development of sufficiently large clusters of cells or organs that can be relatively easily handled. This requires continuous suppression or removal of untransformed cells, followed by sub-culturing of transformed cells. Such approaches have been found to be labour and time consuming (Ghorbel et al. 1999). Elliott et al. (1999) tested the use of visual selection based on GFP fluorescence in comparison with conventional antibiotic selection. They bombarded sugarcane calli and isolated regenerating green fluorescent calli. However, it was difficult to maintain preferential growth of transformed cells, despite the fact that non-fluorescing cells were removed. Furthermore the sectioning of calli was reported to alter the direction rate of growth within individual clusters of cells. After 12 weeks they obtained 2.4 ± 0.9 (SE) green fluorescent calli that reached at least 5 mm in diameter. This was less than average callus formation on geneticin (29.6 ± 1.6). They suggested that the conventional selection is more suitable for routine production of transgenic plants. Quite similar conclusions were reported by Jordan (2000), who cultivated bombarded wheat embryos for the first 4 weeks on a medium without antibiotics, but additional application of antibiotics led to stringent selection of transgenic plants among regenerants. On the other hand, Baranski et al. (2006) were more successful when they screened *A. rhizogenes*-transformed adventitious roots emerged from co-cultivated carrot root discs for GFP fluorescence. Roots positive for green fluorescence were selected for further regeneration and it has been shown that such approach can be an efficient method for the production of transgenic carrot. Although possibilities for selection exclusively based on a screening for GFP fluorescence are limited, due to difficulties in identification of fluorescent tissues and plants among large masses of cells or shoots, some recent reports have confirmed that such an approach is promising for transformation of some objects, and represents a new alternative to current selection schemes (Jordan 2000; Baranski et al. 2006).

Conclusion remarks and further prospects

Green fluorescent protein offers a wide range of applications in plant biology (Leffel et al. 1997; Stewart 2001). Although the study of green fluorescence in plants embodies its own obstacles, it possesses many advantages compared with other marker genes. Monitoring of GFP green fluorescence allows for the rapid non-invasive identification of transformed cells and, therefore, early elimination of non-transformed cells. It has been shown in many cases that GFP fluorescence has been successfully used for the critical evaluation of various transformation parameters resulting in subsequent modifications of transformation protocols. Therefore, plant transformation could be faster and less labour intensive and thus cheaper. Moreover, it may help to identify and therefore to reduce negative events associated with plant transformation (e.g. gene silencing) and to facilitate the successful recovery of transgenic plant tissues, which stably express the gene of interest (El-Shemy et al. 2004). Additionally, various attempts at quantitative or semi-quantitative detection of GFP fluorescence have been reported recently (Millwood et al. 2003; Hraška et al. 2005), allowing for the early identification of homozygotes (Molinier et al. 2000) or estimation of recombinant protein content in transgenic plants (Halfhill et al. 2003; Richards et al. 2003a, b). Such new methods represent an additional asset of GFP use to plant transgenesis.

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References

Ahlandsberg S, Sathish P, Sun Ch, Jansson Ch (1999) Green fluorescent protein as a reporter system in the transformation of barley cultivars. Physiol Plant 107:194–200

Bauchera M, Montiesb B, van Montagua M, Boerjana W (1998) Biosynthesis and genetic engineering of lignin. Crit Rev Plant Sci 17:125–197

Baranski R, Klocke E, Schumann G (2006) Green fluorescent protein as an efficient selection marker for *Agrobacterium rhizogenes* mediated carrot transformation. Plant Cell Rep 25:190–197

Battraw MJ, Hall TC (1990) Histochemical analysis of CaMV 35S promoter-*ß*-glucuronidase gene expression in transgenic rice plants. Plant Mol Biol 15:527–538

Baumann CT, Lim CS, Hager GL (1998) Simultaneous visualization of the yellow and green forms of the green fluorescent protein in living cells. J Histochem Cytochem 46:1073–1076

Bellucci M, De Marchis F, Mannucci R, Arcioni S (2003) Jellyfish green fluorescent protein as a useful reporter for transient expression and stable transformation in *Medicago sativa* L. Plant Cell Rep 22:328–337

Benfey PN, Ren L, Chua N (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8:2195–2202

Birch RG (1997) Plant transformation: problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol 48:297–326

Cardoza V, Stewart CN (2003) Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola (*Brassica napus* L.) from hypocotyl segment explants. Plant Cell Rep 21:599–604

Carlson AR, Letarte J, Chen J, Kasha KJ (2001) Visual screening of microspore-derived transgenic barley (*Hordeum vulgare* L.) with green-fluorescent protein. Plant Cell Rep 20:331–337

Chabaud M, de Carvalho-Niebel F, Barker DG (2003) Efficient transformation of *Medicago truncatula* cv. Jemalong using the hypervirulent *Agrobacterium tumefaciens* strain AGL1. Plant Cell Rep 22:46–51

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263:802–805

Chen S, Li X, Liu X, Xu H, Meng K, Xiao G, Wei X, Wang F, Zhu Z (2005) Green fluorescent protein as a vital elimination marker to easily screen marker-free transgenic progeny derived form plants co-transformed with double T-DNA binary vector system. Plant Cell Rep 23:625–631

Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant-cells-the molecular-basis of crown gall tumorigenesis. Cell 11:263–271

Chiu W, Niva Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6:325–330

Cho H-J, Widholm JM (2002) *Agrobacterium tumefaciens*-mediated transformation of the legume *Astralagus sinicus* using kanamycin resistance selection and green fluorescent protein expression. Plant Cell Tissue Organ Cult 69:251–258

Cho M-J, Ha CD, Lemaux PG (2000) Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly regenerative tissues. Plant Cell Rep 19:1084–1089

Cho M-J, Choi H-W, Jiang W, Ha ChD, Lemaux PG (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

Cho M-J, Choi HW, Okamoto D, Zhang S (2003) Expression of green fluorescent protein and its inheritance in transgenic oat plants generated from shoot meristematic cultures. Plant Cell Rep 21:467–474

Christou P (1995) Strategies for variety-independent genetic transformation of important cereal, legumes and woody species utilizing particle bombardment. Euphytica 85:13–27

Christou P (1997) Rice transformation: bombardment. Plant Mol Biol 35:197–203

Chudakov DM, Lukyanov S, Lukyanov KA (2005) Fluorescent proteins as a toolkit for *in vivo* imaging. Trends Biotechnol 23:605–613

Chytilova E, Macas J, Galbraith DW (1999) Green fluorescent protein targeted to the nucleus, a transgenic phenotype useful for studies in plant biology. Ann Bot 83:645–654

Cody CW, Prasher DC, Westler WM, Prendergast FG, Ward WW (1993) Chemical structure of the hexapeptide chromophore of *Aeqourea*-green fluorescent protein. Biochemistry 32:1212–1218

Cui M-L, Ezura H (2003) *Agrobacterium*-mediated transformation of *Nemesia strumosa* Benth, a model plant for asymmetric floral development. Plant Sci 165:863–870

Cui M-L, Handa T, Ezura H (2003) An improved protocol for *Agrobacterium*-mediated transformation of *Antirrhinum majus* L. Mol Genet Genomics 270:296–302

Davis SJ, Viestra RD (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol 36:521–528

Eady CC, Weld RJ, Lister CE (2000) *Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (*Allium cepa* L.). Plant Cell Rep 19:376–381

Elliott AR, Campbell JA, Dugdale B, Brettell RISL, Grof CP (1999) Green-fluorescent protein facilitates rapid *in vivo* detection of genetically transformed plant cells. Plant Cell Rep 18:707–714

El-Shemy HA, Teraishi M, Khalafalla MM, Katsube-Tanaka T, Utsumi S, Ishimoto M (2004) Isolation of soybean plants with stable transgene expression by visual selection based on green fluorescent protein. Mol Breed 14:227–238

Escobar MA, Park J-I, Polito VS, Leslie Ch A, Uratsu SL, McGranahan GH, Dandekar AM (2000) Using GFP as a scorable marker in walnut somatic embryo transformation. Ann Bot 85:831–835

Finer JJ, Finer KR, Ponappa T (1999) Particle bombardement mediated transformation. Curr Topics Microbiol Immunol 240:59–80

Franchea C, Laplazea L, Duhouxa E, Bogusza D (1998) Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. Crit Rev Plant Sci 17:1–28

Galperin M, Patlis L, Ovadia A, Wolf D, Zelcer A, Kenigsbuch D (2003) A melon genotype with superior competence for regeneration and transformation. Plant Breed 122:66–69

Ghorbel R, Juárez J, Navarro L, Pena L (1999) Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants. Theor Appl Genet 99:350–358

Goldman JJ, Hanna WW, Fleming G, Ozias-Akins P (2003) Fertile transgenic pearl millet [*Pennisetum glaucum* (L.) R. Br.] plants recovered though microprojectile bombardment and phosphinothricin selection of apical meristem-, inflorescence-, and immature embryo-derived embryogenic tissues. Plant Cell Rep 21:999–1009

Guo W, Duan Y, Olivares-Fuster O, Wu Z, Aris CR, Burns JK, Grosser JW (2005) Protoplast transformation and regeneration of transgenic Valencia sweeet orange plants containing a juice duality-related pectin methylesterase gene. Plant Cell Rep 24:482–486

Haas J, Park E-C, Seed B (1996) Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr Biol 6:315–324

Halfhill MD, Millwood RJ, Rufty TW, Weissinger AK, Stewart CN (2003) Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during leaf canopy development in transgenic oilseed rape, *Brassica napus* L. Plant Cell Rep 22:338–343

Halfhill MD, Richards HA, Mabon SA, Stewart Jr CN (2001) Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa* Theor. Appl Genet 130:659–667

Han J, Wang H, Ye H, Liu Y, Li Z, Zhang Y, Zhang Y, Yan F, Li G (2005) High efficiency of genetic transformation and regeneration of *Artemisia annua* L. via *Agrobacterium tumefaciens*-mediated procedure. Plant Sci 168:73–80

Harper BK, Mabon SA, Leffel SM, Halfhill MD, Richards HA, Moyer KA, Stewart Jr CN (1999) Green fluorescent protein as a marker for expression of a second gene in transgenic plants. Nature Biotech 17:1125–1129

Haseloff J (1999) GFP variants for multispectral imaging of living cells. Methods Cell Biol 58:139–151

Haseloff J, Amos B (1995) GFP in plants. Trends Genet 11:328–329

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci USA 94:2122–2127

Heim R, Cubitt AE, Tsien RY (1995) Improved green fluorescence. Nature 373:663–664

Heim R, Prasher DC, Tsien RY (1994) Wavelenght mutations and posttranslational autooxidation of green fluorescent protein. Proc Natl Acad Sci USA 91:12501–12504

Helmer G, Casadaban M, Bevan M, Kayers L, Chilton MD (1984) A new chimeric gene as a marker for plant transformation: the expression of *Escherichia coli* ß-galactosidase in sunflover and tobacco cells. Biotechnology (NY) 2:520–527

Hraška M, Rakouský S (2005) GFP detection in low level signal/noise ratio plant samples. J Appl Biomed 3:S19

Hraška M, Rakouský S, Kocábek T (2005) Use of simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants. Biol Plant 49:313–316

Hu W, Cheng CL (1995) Expression of *Aequorea* green fluorescent protein in plant cells. FEBS Lett 369:331–334

Huber M, Hahn R, Hess D (2002) High transformation frequencies obtained from a commercial wheat (*Triticum aestivum* L. cv. ''Combi'') by microbombardment of immature embryos followed by GFP screening combined with PPT selection. Mol Breed 10:19–30

Itaya A, Hickman H, Bao Y, Nelson R, Ding B (1997) Cell-to-cell trafficking of cucumber mosaic virus movement protein:green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. Plant J 12:1223–1230

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jeoung JM, Krishnaveni S, Muthukrishnan S, Trick HN, Liang GH (2002) Optimization of sorghum transformation parameters using genes for green fluorescent protein and ß-glucuronidase as visual markers. Hereditas 137:20–28

Jordan MC (2000) Green fluorescent protein as a visual marker for wheat transformation. Plant Cell Rep 19:1069–1075

Kaeppler HF, Menon GK, Skadsen RW, Nuutila AM, Carlson AR (2000) Transgenic oat plants via visual selection of cells expressing green fluorescent protein. Plant Cell Rep. 19:661–666

Kamaté K, Rodriguez-Llorente ID, Scholte M, Durand P, Ratet P, Kondorosi E, Kondorosi A, Trinh TH (2000) Transformation of floral organs with GFP in *Medicago truncatula*. Plant Cell Rep 19:647–653

Kim CK, Chung JD, Park SH, Burrell AM, Kamo KK, Byrne DH (2004) *Agrobacterium tumefaciens*-mediated transformation of *Rosa hybrida* using green fluorescent protein (GFP) gene. Plant Cell Tissue Organ Cult 78:107–111

Kinoshita M (2004) Transgenic medaka with brilliant fluorescence in skeletal muscle under normal light. Fish Sci 70:645–649

Klein TM, Wolf ED, Wu R, Stanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70–73

Knapp JE, Kausch AP, Auer C, Brand MH (2001) Transformation of *Rhododendron* through microprojectile bombardment. Plant Cell Rep 20:749–754

Kocábek T, Rakouský S, Ondřej M, Řepková J, Relichová J (1999) Identification and mapping of a T-DNA induced flower mutation in *Arabidopsis thaliana*. Biol Plant 42:349–359

Lawton R, Winfield S, Daniell H, Bhagsari AS, Dhir SK (2000) Expression of green-fluorescent protein gene in sweet potato tissues. Plant Mol Biol Rep 18:139a–139i

Leffel SM, Mabon SA, Stewart Jr CN (1997) Applications of green fluorescent protein in plants. Biotechniques (NY) 23:912–918

Li ST, Yang HY (2000) Gene transfer into isolated and cultured tobacco zygotes by a specially designed device for electroporation. Plant Cell Rep 19:1184–1187

Li Z, Jayasankar S, Gray DJ (2001) Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). Plant Sci 160:877–887

Malik K, Wu K, Li X-Q, Martin-Heller T, Hu M, Foster E, Tian L, Wang C, Ward K, Jordan M, Brown D, Gleddie S, Simmonds D, Zheng S, Simmonds J, Miki B (2002) A constitutive gene expression system derived from the *tCUP* cryptic promoter elements. Theor Appl Genet 105:505–514

Malyshenko SI, Tyuĺkina LG, Zvereva SD, Raldugina GN (2003) Transgenic *Brassica campestris* plants expressing the gfp gene. Russ J Plant Physiol 50:276–281

Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant Mol Biol 37:549–559

Mercuri A, Sacchetti A, De Benedetti L, Schiva T, Alberti S (2001) Green fluorescent flowers. Plant Sci 161:961–968

Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J Biotechnol 107:193–232

Millwood RJ, Halfhill MD, Harkins D, Russotti R, Stewart Jr CN (2003) Instrumentation and methodology for quantitative GFP fluorescence in intact plant organs. Biotechniques (NY) 24:638–643

Misteli T, Spector DL (1997) Applications of the green fluorescent protein in cell biology and biotechnology. Nature Biotech. 15:961–963

Mohan ML, Krishnamurthy KV (2003) Plant regeneration form decapitated mature embryo axis and *Agrobacterium* mediated genetic transformation of pigeonpea. Biol Plant 46:519–527

Molinier J, Himber C, Hahne G (2000) Use of green fluorescent protein for detection of transformed shoots and homozygous offspring. Plant Cell Rep 19:219–223

Morschhäuser J, Michel S, Hacker J (1998) Expression of a chromosomally integrated, single-copy GFP gene in *Candida albicans*, and its use as a reporter of gene regulation. Mol Gen Genet 257:412–420

Murray F, Brettell R, Matthewes P, Bishop D, Jacobsen J (2004) Comparison of *Agrobacterium*-mediated transformation of four barley cultivars using the GFP and GUS reporter genes. Plant Cell Rep 22:397–402

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

Niedz RP, Sussman MR, Satterlee JS (1995) Green fluorescent protein: an *in vivo* reporter of plant gene expression. Plant Cell Rep 14:403–406

Ow DW, Wood KV, Deluca M, Dewet JR, Helinski DR, Howell SH (1986) Transient and stable expression of firefly luciferase gene in plant cells and transgenic plants. Science 234:856–859

Panstruga R (2004) A golden shot: how ballistic single cell tarnsformation boosts the molecular analyse of cereal-mildew interactions. Mol Plant Pathol 5:141–148

Pavingerová D, Bříza J, Kodýtek K, Niedermeierová H (1997) Transformation of *Rhododendron* spp. using *Agrobacterium tumefaciens* with a GUS-intron chimeric gene. Plant Sci 122:165–171

Pérez-Clemente RS, Pérez-Sanjuán A, García-Férriz L, Beltrán J-P, Canas LA (2004) Transgenic peach plants (*Prunus persica* L.) produced by genetic transformation of embryo sections using the green fluorescent protein (GFP) as an *in vivo* marker. Mol Breed 14:419–427

Pishak S, Young A, Guiltinan MJ (2003) Stable transformation of *Theobroma cacao* L. and influence of matrix attached regions on GFP expression. Plant Cell Rep 21:872–883

Polin LD, Liang H, Rothrock RE, Nishii M, Diehl DL, Newhouse AE, Nairn CJ, Powell WA, Maynard ChA (2006) *Agrobacterium*-mediated transformation of American chestnut (*Castanea dentata* (Marsh.) Borkh.) somatic embryos. Plant Cell Tissue Organ Cult 84:69–79

Ponappa T, Brzozowski AE, Finer JJ (1999) Transient expression and stable transformation of soybean using the jellyfish green fluorescent protein. Plant Cell Rep 19:6–12

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111:229–233

Rakouský S, Kocábek T, Vincenciová R, Ondřej M (1997/98) Transient ß-glucuronidase activity after infiltration of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*. Biol Plant 40:33–41

Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, Shell J, Reiss B, Mass C (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

Repellin A, Baga M, Jauhar PP, Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: new challenges. Plant Cell Tissue Organ Cult 64:159–183

Richards HA, Han CT, Hopkins RG, Failla ML, Ward WW, Stewart CN (2003a) Safety assessment of recombinant green fluorescent protein orally administered to weaned rats. J Nutr 133:1909–1912

Richards HA, Halfhill MD, Millwood RJ, Stewart CN (2003b) Quantitative GFP fluorescence as an indicator of recombinant protein synthesis in transgenic plants. Plant Cell Rep 22:117–121

Richards HA, Rudas VA, Sun H, McDaniel JK, Tomaszewski Z, Conger BV (2001) Construction of a GFP-BAR plasmid and its use for switchgrass transformation. Plant Cell Rep 20:48–54

Rouwendal GJA, Mendes O, Wolbert EJH, deBoer AD (1997) Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. Plant Mol Biol 33:989–999

Sallaud C, Meynard D, van Boxtel J, Gay C, Bes M, Brizard JP, Larmande P, Ortega D, Raynal M, Portefaix M, Ouwerkerk PBF, Rueb S, Delseny M, Guiderdoni E (2003) Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theor Appl Genet 106:1396–1408

Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DW (1995) Green-fluorescent protein as a new vital marker in plant cells. Plant J 8:777–784

Shiina T, Hayashi K, Ishii N, Morikawa K, Toyoshima Y (2000) Chloroplast tubules visualized in transplastomic plants expressing green fluorescent protein. Plant Cell Physiol 41:367–371

Shinomura O (1979) Structure of the chromophore of *Aeqourea* green fluorescent protein. FEBS Lett 104:220–222

Siemering KR, Golbik R, Sever R, Haselloff (1996) Mutations that supress the thermosensitivity of green fluorescent protein. Curr Biol 6:1653–1663

Smirnoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. Crit Rev Plant Sci 19:267–290

Snape JW (1998) Golden calves or white elephants-biotechnologies for wheat improvement. Euphytica 100:207–217

Stewart CN Jr (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep 20:376–382

Sunilkumar G, Mohr L, Lopata-Finch E, Emani C, Kathore KS (2002) Developmental and tissue-specific expression of a CaMV 35S promoter in cotton as revealed by GFP. Plant Mol Biol 50:463–474

Tamura M, Togami J, Ishiguro K, Nakamura N, Katsumoto Y, Suzuki K, Kusumi T, Tanaka Y (2003) Regeneration of verbena (*Verbena x hybrida*) by *Agrobacterium tumefaciens*. Plant Cell Rep 21:459–466

Tang W, Newton RJ (2005) Transgenic Christmas trees regenerated from *Agrobacterium tumefaciens*-mediated transformation of zygotic embryos using the green fluorescence protein as a reporter. Mol Breed 16:235–246

Taniguchi T, Kurita M, Ohmiya Y, Kondo T (2005) *Agrobacterium tumefaciens*-mediated transformation of embryogenic tissue and transgenic plant regeneration in *Chamaecyparis obtusa* Sieb. et Zucc. Plant Cell Rep 23:796–802

Tee CS, Marziah M, Tan CS, Abdullah MP (2003) Evaluation of different promoters driving the GFP reporter gene and selected target tissues for particle bombardment of *Dendrobium Sonia* 17. Plant Cell Rep 21:452–458

Tripepi RR, George MW, Sripo T, Johnsen SA, Caplan AB (1999) Infection and transformation of *Rhododendron* by *Agrobacterium tumefaciens* strain B6. HortScience 34:455

Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67:509–544

Ueno K, Fukunaga Y, Arusumi K (1996) Genetic transformation of *Rhododendron* by *Agrobacterium tumefaciens*. Plant Cell Rep 16:38–40

Vain P, Worland B, Kohli A, Snape JW, Christou P (1998) The green fluorescent protein (GFP) as a vital screenable marker in rice transformation. Theor Appl Genet 96:164–169

van der Geest AHM, Petolino JF (1998) Expression of modified green fluorescent protein gene in transgenic maize plants and progeny. Plant Cell Rep 17:760–764

Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing Nature 389:553

Wahlroos T, Susi P, Tylkina L, Malyshenko S, Zvereva S, Korpela T (2003) *Agrobacterium*-mediated transformation and stable expression of the green fluorescent protein in *Brassica rapa*. Plant Physiol Biochem 41:773–778

Wang S, Hazelrigg T (1994) Implications for *bdc* mRNA localization from the spatial distribution of *exu* protein in *Drosophila* oogenesis. Nature 369:400–403

Wang Z-Y, Ge Y (2005) *Agrobacterium*-mediated high efficiency transformation of tall fescue (*Festuca arundinacea*). J Plant Physiol 162:103–113

Weber S, Friedt W, Landes N, Molinier J, Himber C, Rousselin P, Hahne G, Horn R (2003) Improved *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. Plant Cell Rep 21:475–482

Williamson JD, Hirsch-Wyncott ME, Larkins BA, Gelvin SB (1989) Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiol 90:1570–1576

Yamada T, Teraishi M, Hattori K, Ishimoto M (2001) Transformation of azuki bean by *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult 64:47–54

Yancheva SD, Schlizerman LA, Golubowicz S, Ybloviz Z, Perl A, Hanynka U, Flaishman MA (2006) The use of green fluorescent protein (GFP) improves *Agrobacterium*-mediated transformation of ''Spadona'' pear (*Pyrus communis* L.). Plant Cell Rep 25:183–189

Yang F, Moss LG, Phillips Jr GN (1996a) The molecular structure of green fluorescent protein. Nature Biotech 14:1246–1251

Yang T-T, Cheng L, Kain SR (1996b) Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acids Res 24:4592–4593

Yong WTL, Abdullah JO, Mahmood M (2006) Optimization of *Agrobacterium*-mediated transformation parameters for *Melastomataceae* spp. using green fluorescent protein (GFP) as a reporter. Sci Hortic DOI: 10.1016/j.scienta.2006.03.005

Zhang H-X, Zeevaart JAD (1999) An efficient *Agrobacterium tumefaciens*-mediated transformation and regeneration system for cotyledons of spinach (*Spinacia oleracea* L.). Plant Cell Rep 18:640–645

Zhou X, Carranco R, Vitha S, Hall TC (2005) The dark side of green fluorescent protein. New Phytol 168:313–322

Zhou X, Chandrasekharan MB, Hall TC (2004) High rooting frequency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17. New Phytol 162:813–822

Zhu YJ, Agbayani R, Moore PH (2004) Green fluorescent protein as a visual marker for papaya (*Carica papaya* L.) transformation. Plant Cell Rep 22:660–667

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