**Green Fluorescent Protein in the Genetic Transformation of Plants**

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**ABSTRACT**

The genetic transformation of plants is a promising method for introducing and modifying various desirable traits. Several marker genes (β-glucuronidase, GUS; luciferase, LUC; or β-galactosidase, LacZ) have been successfully used in genetic transformation protocols. Relatively recently, green fluorescent protein (GFP) marker has become popular due to its many advantages in plant transformation studies. Most importantly, it does not require the addition of any interfering substances like exogenous substrates or enzymes. It also allows for the monitoring of transgenic expression from early stages of transformation through the recovery of living transgenic plants without the need to sacrifice valuable, sometimes sparse, transgenic material. However, the use of GFP is associated with some limitations, mostly related to its low levels of expression, and high toxicity when it is expressed at high levels in plants. This review aims to provide a broad overview of the use of GFP in a wide spectrum of plants.

**Keywords:** genetic engineering, GFP, marker gene

**Abbreviations:** ER, endoplasmic reticulum; GFP, green fluorescent protein; gfp, green fluorescent protein gene; GUS, β-glucuronidase; SE, somatic embryo; wt, wild-type

**INTRODUCTION**

Genetic transformation of plants is a popular tool for modifying and improving various desirable traits. Successful genetic transformation of plants with agronomic and/or horticultural traits is the primary objective of plant transgenesis (Aronen et al. 1995; Aronen 1997; Haggman and Aronen 1998; Haggman et al. 1997; Malabadi and Nataraja 2007a, 2007b, 2007c, 2007d, 2007e). This ultimately requires an efficient strategy for transferring genes into plant cells, and subsequent selection and regeneration of putative transgenic plants (Eady et al. 2000; Miki and McHugh 2004). It will also help in fundamental studies of genetics, cell biology and plant physiology (Haseloff 1999; Smirnoff and Wheeler 2000). The use of marker genes that would allow for critical assessment of each step in the procedure, are the most suitable for such fundamental studies. They can increase transformation efficiency by reducing the time and amount of material to be handled allowing a reproducible transformation protocol to be established. Many genes coding for various markers are now available, but these will not be covered in this review. 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 (Hraska et al. 2006). However, these require the addition of exogenous substrates or some other cofactors for their manifestation. These markers generally do not offer the possibility of determining the exact transgenic status of plants, while also monitoring the transgenic expression in real time and in living plants (Hraska et al. 2006). The ideal marker should possess some important character-

**REFERENCES**

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istics: it should be easily expressed; it should be easily visualized in plants; it should not be toxic or affect in any way the physiology of living intact plants (Reichel et al. 1996; Chytílova et al. 1999; Baumann et al. 1998; Bellucci et al. 2003). In stark contrast to most marker genes available, green fluorescent protein (GFP) does not require the addition of any interfering substances like exogenous substrates or enzymes. It allows for the monitoring of transgenic expression through observation of transfected cells. GFP facilitates the recovery of living transgenic plants (Zolotukhin et al. 1996; Chudakov et al. 2005). Another advantage of GFP is its relatively small size (26.9 kDa) which can tolerate both N- and C-terminal protein fusions, lending itself to studies of protein localization and intracellular protein trafficking (Park and Raines 1997). Detection of GFP in living cells thus only requires excitation by light at 395 or 475 nm (Cubitt et al. 1995). This represents the significant advantage for using GFP as a marker during genetic transformation of transgenic plants (Halflill et al. 2007). This paper briefly summarizes basic GFP properties and applications, limitations and improvement of transformation protocols for plants. This review also focuses on the utility of GFP as a visual selectable marker in aiding the plant transformation process, although it does not exhaustively cover every plant ever transformed with GFP; GFP has been observed in monocots in dicot transformation. Finally, the potential utility of new fluorescent proteins is speculated upon.

**BACKGROUND: STABILITY, STRUCTURE AND EXPRESSION**

GFP has been widely used in a variety of biological applications because of several unique features of the protein. GFP gene was isolated from the pacific jellyfish *Aequorea victoria* and first described by Prasher et al. (1992). Niedez et al. (1995) were the first to show that wild-type (wt) Aequorea GFP could be visualized in plant cells, specifically sweet orange (*Citrus sinensis*) protoplasts. *A. victoria* are brightly luminescent, with glowing points around the margin of the jellyfish umbrella (Shinomura 1979; Haseloff 1999). Light arises from yellow tissue masses that each consist of about 6000-7000 photogenic cells (Heim 1996; Haseloff 1999). The components required for bioluminescence in other bioluminescent coelenterates, with cells remaining as a highly fluorescent callus (Haseloff et al. 1999). Fluorescent protein has now been produced in a number of heterologous cell types and there appears to be little requirement for specific additional factors for post-translational modification of the protein, which may be autocatalytic or require ubiquitous factors (Heim et al. 1994, 1995; Haseloff et al. 1997; EI-Shemy et al. 2004).

In most cases, it is most likely the CaMV-35S was used as a promoter during the transformation studies with GFP. GFP transforms the luminous blue light emitted by another hydromedusus protein, *aequorin*, into green light. Hu and Chenge (1995) demonstrated that GFP could be synthesized in corn protoplasts. However, they failed to observe GFP in transformed *Arabidopsis thaliana* to tobacco cells, presumably the result of low expression of the wt gene. Using a stronger promoter (heat shock promoters), Sheen et al. (1995) were able to visualize wt GFP in corn and *Arabidopsis* cells. Both the latter two groups used heat shock promoters to attempt to drive GFP with inducible expression as well; Sheen et al. (1995) were successful, while Hu and Chenge (1995) were not. The differences in the promoters used for the experiments likely do not explain the disparate results – these are more likely due to the excitation source: a laser (Sheen et al. 1995; Nagatani et al. 1997) versus incandescent lamp with excitation filters (Hu and Chenge 1995; Stewart 2001). These studies show that successful GFP detection is highly dependent on the strength and source of the excitation source. Nonetheless, the experience with a low expression of wt GFP encouraged researchers to modify it to forms that could be more effectively synthesized in plants.

Haseloff et al. (1997) reported that a cryptic intron existed in the wt *Aequorea* GFP that caused aberrant splicing in plant cells between nucleotides 380 and 463, thereby creating an 84-nucleotide intron. When the cryptic splice sites were altered with silent mutations, a variant called mGFP4 was produced (Haseloff et al. 1997) that had essentially wt spectral characteristics: maximal excitation at 395 nm and maximal emission at 509 nm. It has proved necessary to destroy this cryptic intron to ensure proper expression in plants. Hence they have altered the codon usage for GFP, deliberately mutating recognition sequences at the putative 5’ splice site and branchpoint and decreasing the AU content of the intron. All of the sequence modifications affected only codon usage, and this modified gene, mgfp4, encodes a protein product which is identical to that of the jellyfish *Aequorea* which they expect arose from position-dependent modulation of gene expression in different transformants. It proved difficult to regenerate fertile plants from the brightest transformants, with cells remaining as a highly fluorescent callus

[87]
or mass of shoots after several months of culture. It is possible that high levels of GFP expression were mildly toxic or interfered with differentiation. This is of special concern with a fluorescent molecule such as GFP, which would be expected to generate free radicals upon excitation, and which undergoes oxidative modification and could possess catalytic properties. The conditions that they have used for plant regeneration should provide a stringent test for any decreased toxicity of GFP to transgenic cells.

The 35S promoter was used to drive expression of the protein at high levels throughout the plant, including meristematic cells, and regeneration took place under continued illumination, allowing the possibility for GFP mediated phototoxicity. Despite poor regeneration of the brightest transformants, they have managed to obtain over 50 separate transgenic Arabidopsis lines, most of which contained levels of GFP that were easily detectable by microscopy (Haseloff et al. 1997).

Using this modified gene (mgfp4-ER), it has been possible to regenerate intensely fluorescent and fertile plantlets consistently. Fluorescence within these plants could be readily observed by eye using a long wavelength UV lamp. The mgfp4-ER-expressing plants were examined by confocal microscopy, and fluorescent protein was found mainly within the endomembrane system. When the protein is excised from the nucleus, shows a perinuclear distribution, and is found associated with the ER which forms a characteristic reticulate network in highly vacuolate cells. In highly cytoplasmic meristematic cells, the nuclei and orientation of cell divisions can be clearly distinguished. Localization of the modified protein to cytoplasmic organelles was also evident, to what appear to be large leuco-plasts or proplasts. Such hypocotyl cells in mgfp4-ER-transformed seedlings appear to contain a spectrum of developing plastids that range from the brightly green fluorescent to those which take on a yellow, orange or red appearance in dual channel confocal micrographs. They also presume that this is due to increasing chlorophyll synthesis, and that the green fluorescent plastids may be the maturing precursors of chloroplasts in these cells. These green fluorescent plastids are also found within the chloroplast-free epidermal cells of leaves and cotyledons, but are not found within the underlying mesophyll cells that are packed with mature chloroplasts. It seems likely that these organelles are proplasts and are capable of developing into chloroplasts, but they cannot exclude the possibility that they are some specialised form of leucoplast (Haseloff et al. 1997).

The accumulation of mgfp4-ER protein within leucoplasts or developing proplasts, in addition to its entry into the secretory pathway and retention in the endoplasmic reticulum (ER), may indicate misrecognition of the N-terminal signal peptide. Proplastid accumulation of GFP is not seen in the 35S-mgfp4-transformed plants. If the mgfp4-ER encoded signal peptide is insufficiently recognised prior to docking and cotranslational transport of the protein into the lumen of the ER, a proportion of GFP bearing fused terminal sequences may be produced in the cytoplasm. If so, it is possible that the translocated signal may act as a target for an autocatalytic process or it requires only ubiquitous cellular components (Misteli and Spector 1997). It has been postulated that poor expression of the wt GFP gene in plants is due to its high AT content and/or a cryptic intron sequence (Haseloff and Ames 1995). A modified version of the GFP sequence has been constructed (GFP4) with altered codon usage, to mutate the cryptic splice sites and to decrease the AU content of the mRNA (Haseloff et al. 1997). The weak and often non-detectable level of expression and the relative toxicity of the GFP when expressed at very high levels are the two main problems limiting the wider use of GFP for fundamental and applied plant biology (Haseloff and Ames 1995; Leffel et al. 1997). GFP did not appear to have any adverse effects on plant (wheat, oat and barley) growth, development and fertility (Jordan 2000; Kaeppeler et al. 2000; Murray et al. 2004). Therefore, modifications in GFP have been made using various mutagenesis schemes. Mutants have been reported that improve fluorescence intensity, thermostability, folding and formation of the chromophore, codon usage, removal of cryptic intron sequences and spectral qualities (Yang et al. 1996; Haseloff et al. 1997).

GFP VISUALIZATION

The use of the appropriate observation and excitation system is a prerequisite for a successful GFP study. 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 being used for sub-cellular localization of GFP. For observations of GFP, both high-power and low-power microscopes and various hand-held UV or blue light sources may be used. GFP may act as an autoluminescent material, and can be visualized directly in living plant tissue, unlike commonly used markers such as β-glucuronidase, which require a prolonged and lethal histochemical staining procedure (Jefferson et al. 1987). GFP is therefore finding an application in three broad areas (1) for the dynamic visualization of labelled protein within the cells, and at a larger scale, (2) for the selective labelling and subcellular localization of whole plants, and (3) for the identification of individual transgenic plants expressing GFP (Haseloff and Ames 1995; Haseloff 1999). For example, different peptide domains can be fused to GFP to allow the decoration of particular structures within cells and/or to observe the subcellular distribution of the fusion protein. In addition, use of an active GFP marker gene allows transgenic cells to be scored by simple observation during a plant transformation experiment, throughout regeneration to the adult plant and its progeny. The use of tissue
specific promoters to drive expression of GFP also allows the selective labelling of particular cell types within intact transformed plants. In these cases it is beneficial to express GFP at high levels within the marked cells to aid detection, and to minimize any deleterious effects of GFP expression. Haseloff et al. (1997) have found the optimised mhp5-ER gene very useful for this type of experiment. The dynamic properties of labelled cells or subcellular features can be restored with fluorescence microscopy in whole tissues using fluorescence microscopy techniques; however the use of intact tissue imposes some additional constraints on the imaging process.

Direct visualization of GFP fluorescence does not require any fixation, staining or addition of substrates, and allows for the study of various events within the living cells such as cytoplasmic streaming without the sacrifice of important and often sparse plant material. Moreover, the activities of living cells, such as cytoplasmic streaming, are clearly evident during microscopy. Ordinarily, movement within a sample is a nuisance, placing constraints on the use of sometimes lengthy techniques for noise reduction during confocal microscopy, such as frame averaging. However, it was shown that it is also possible to monitor dynamic events by time-lapse confocal microscopy, and this combination of techniques peaks at 395 nm and an emission peak at 509 nm. This is a useful property for simple detection of the protein using a long wavelength UV source. UV illumination is not efficiently detected by the human eye and a suitable long wavelength UV lamp can be used to excite GFP for simple observation of transformed plant material without obscuring the green emission. However, efficient blue light excitation (around 470 nm) is essential for use with imaging devices such as confocal microscopes or cell sorters which are equipped with argon laser sources. For blue-excited GFPs, one can use the photons of a microscope light source with the proper cutoff or bandpass filters for visualizing GFP-transgenic plants. For UV-excited GFPs, others typically use a portable UV lamp (UVP 100 AP, Upland, Calif) with no emission filter or the lighter Spectroline BIB-395 produced by Spectroline or UVP lamps work well for UV excitation of GFP, they would be even more effective if they used a 400-nm filter instead of the 365-nm filter, since the former better matches GFP excitation. UV protective eye-wear should be used (Stewart 2001).

GENETIC TRANSFORMATION

Successful genetic transformation of plants can be achieved if proper signal genes(s) are used throughout the study (Snap6 1998; Sunil Kumar et al. 2002; Baranski et al. 2006). Transformation procedures should be fast and efficient, and then only is it easy for the insertion of exogenous DNA into plant cells via Agrobacterium-mediated transfer or particle bombardment (e.g. Malabadi and Nataraja 2007a, 2007b, 2007c for difficult-to-transform coniferous species). Further the transformation methods differ in their suitability for various purposes and plant species (Repellin et al. 2001), modification of gene gun settings (Richards et al. 2002; Tee et al. 2003), and type of transformation (e.g. Repellin et al. 2001) and evaluation of various promoters (Tee et al. 2003).

GFP has a unique advantage of a wide range of applications covering whole areas of transformation and regeneration procedures. GFP can be observed in each step of transformation by fluorescence microscopy. GFP-expressing cells and tissues can easily be distinguished from untransformed ones, without destroying the studied material (Kamata et al. 2000). The ratio between fluorescing and non-fluorescing cells, tissue and organs as a measure of trans-
formation efficiency has been successfully used to improve the different stages of transformation protocols. Recently, GFP fluorescence was applied for critical assessment of the whole transformation procedure of the Agrobacterium rhizogenes-mediated transformation of carrot (Baranski et al. 2006). Based on the green fluorescent intensity they selected the most virulent Agrobacterium strain, effective acetosyringone concentration and the most suitable carrot genotype. Infiltration was performed correctly, but the fluorescence signal remained relatively weak, possibly due to aggregation of the encoded protein (Haseloff et al. 1997). Haseloff et al. (1997) were able to improve the level of fluorescence obtained in vivo by adding an ER localization signal to their modified GFP. Increased fluorescence was also obtained by making codon alterations to increase solubility, giving rise to the modified soluble-modified form of GFP (Davis and Vierstra 1998). These forms can be used in dual localization studies. Photochemically excited GFP may generate free radicals such as nitroxide, hydroxyl and hydrogen peroxide that are cytotoxic in high doses (Leffel et al. 1997). This phototoxicity seems to be reduced when GFP is localized in the ER (Harper et al. 1999), but may still be important in certain contexts. On the other hand phototoxicity was not observed when transforming Arabidopsis by various methods (Kamath et al. 2000). We found that Arabidopsis and tobacco transformants having the same efficiency as control (luciferase) transformants lacking GFP. However, a reduction in the efficiency of root transformation procedures was observed (Mankin and Thompson 2001). Regeneration of GFP containing transformants was improved by shading them with white paper disks, but even shaded transformants regenerated more slowly than luciferase transformants in Arabidopsis (Mankin and Thompson 2001).

The gfp gene was also introduced into conifer tissues by microprojectile bombardement and its transient expression was detected in black spruce (Picea mariana), white spruce (Picea glauca) and pine (Pinus strobus) embryonal masses, suspension culture, somatic embryos, and pollen (Tian et al. 1997). The successful expression of GFP gene in various tissues suggests that it is a useful reporter/marker gene for conifers. GFP transgene was stable over multiple subcultures (Tian et al. 1999). The GFP gene and the gene conferring resistance to kanamycin (nptII) were introduced in black and white spruce, and white pine by biolistic or Agrobacterium method technology (Tian et al. 1999). GFP has become a powerful reporter gene to complement selectable marker genes and can be used to select for transformed material alone. The great advantage of GFP as a non-conditonal reporter is the direct visualization of GFP fluorescence in vivo without immunodetection, such as the application or penetration of cells with substrate and products that may diffuse within or among cells. Both considerations provide a significant improvement over GUS and LUC as reporter genes (Tian et al. 1997, 1999).

Moseyko and Feldman (2001) for the first time reported on the use of GFP as a pH reporter in plants. Protein fluxes and pH regulation play important roles in plant cellular activity and therefore, it is essential to have plant gene reporter system for rapid, non-invasive visualization of intracellular pH changes. In order to develop such a system three vectors for transient and stable transformation of plant cells with a pH-sensitive derivative of GFP were developed (Moseyko and Feldman 2001). Using these vectors transgenic A. thaliana and tobacco plants were produced and, for the first time pH gradients between different developmental compartments could be extected without the help of vectors. The pH-sensitive GFPs in A. thaliana were visualized. The utility of pH-sensitive GFP in revealing rapid, environmentally-induced changes in cytoplasmic pH in roots was also demonstrated (Moseyko and Feldman 2001). On the other hand corn and tobacco protoplast transient assays showed that pgpo gave about 20-fold brighter fluorescence than the wt gene gfp. Replacement of serine at position 65 with a threonine or cysteine yielded 100- to 120-fold brighter fluorescence than wt gfp upon excitation with 490-nm light (Casper and Holt 1996).
Bright-green fluorescence was observed with a fluorescence microscope in virtually all examined tissues of transgenic monocots and dicots and, green fluorescence that was readily detectable by eye using a hand-held, long-wave ultraviolet lamp and/or black-light source. GFP is mainly localized within the nucleoplasm and cytoplasm of transformed Arabidopsis cells and can give rise to high levels of fluorescence, but it proved difficult to efficiently regenerate transgenic plants from such highly fluorescent cells. How-ever, when GFP is targeted to the ER, transformed cells regenerate routinely to give highly fluorescent plants. These modified forms of the gfp gene are useful for directly monitoring gene expression and protein localization and dynamics at high resolution, and as a simply scored genetic marker in living plants (Haseloff et al. 1997). GFP has also been successfully expressed at high levels in tobacco plants using the cytoplasmic RNA virus's potato mosaic virus (Baulcombe et al. 1995). In all these experiments, the gene was directly expressed as a viral mRNA in infected cells, and very high levels of GFP fluorescence were seen. However, poor or no fluorescence was seen when the gfp cdNA was transformed into isolated cells or transformed plants of A. thaliana (Haseloff et al. 1997).

Pollen flow is a prominent mode for transgene movement and it was used more to track the transgene movement under field conditions to assess potential ecological risks such as interspecific hybridization with weedy relatives and potential impact on non-target organisms. GFP expressed in pollen grains was used as a marker to directly measure the pollen movement under environmental conditions of interest and, was noticed in Brassica napus (Moon et al. 2006). Plastid transformation has the desirable characteristics of an increased expression of bacterial (and bacteria-like) genes and, in most cases, the material inheritance of chloroplasts, which could help limit transgene escape via pollen. There are several problems associated with plastid transformation. It is very inefficient and can be performed on relatively few species. There is also a problem with homoplasmy, the need for every plastid in putatively transformed cells to be transgenic to avoid rever-sion to the non-transgenic state over time. GFP, in conjunction with antibiotic selection, is promising in partially addressing these problems. Siderov et al. (1999) transformed potato plastids using the S65T GFP and found that GFP did indeed help to confirm that homoplastic status was achieved. High expression levels of GFP were noticed in potato (Siderov et al. 1999). SGFP-S65T was used to produce fertile transgenic rice in which a nuclear transgene was targeted for expression in chloroplasts. In another study, transgenic rice were produced using the mGFP5-ER (Siderov et al. 1999) and/or is opaque to the excitation signal, represents an obstacle which could complicate the monitoring of the fluorescence signal in older tissues, especially leaves, usually occurs (Hasraska et al. 2008). The presence of some agent(s), namely chlorophyll, which can mask the GFP fluorescence and/or is opaque to the excitation signal, represents an obstacle, which could complicate the monitoring of the flu-

WHICH GFP IS BEST IN PLANTS?

The performance of GFP variants has seldom been compared in plants other than trivial comparisons (wt GFP versus sGFP-S65T). However, some directed experiments have been performed. Elliot et al. (1999) reported that SGFP-S65T had brighter fluorescence in sugarcane callus than mGFP-ER. The S65T mutation variant and derivatives have been used more to track the transf-

EXPRESSION OF GFP IN PLANTS: CASE STUDIES

**Model plants: Tobacco, Arabidopsis and carrot**

Hasraska et al. (2008) reported the study of GFP fluorescence intensity using the T1 generation of transgenic tobacco (Nicotiana tabacum L. cv. ‘Petit Havana’, ‘SR1 WT’) expressing the m-gfp5-ER gene. This study revealed the significant differences in the fluorescence intensity between the abaxial and adaxial side of the leaf surface in transgenic tobacco plants. Stronger signal was detected on the abaxial side of leaf surface in transgenic tobacco (Table 1). Subsequently, the effect of the tissue location within the leaf surface was also investigated and higher fluorescence was detected on the samples detached from leaf tips. Surprisingly, the variability of the fluorescence within the clones of studied genotype was high enough to conclude, that the fluorescence of each individual is unique and affected by particu-

Table 1

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<th>Model Plant</th>
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<td>Stronger signal was detected on the abaxial side of leaf surface in transgenic tobacco.</td>
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<td>The presence of some agent(s), namely chlorophyll, which can mask the GFP fluorescence and/or is opaque to the excitation signal, represents an obstacle, which could complicate the monitoring of the fluor.</td>
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### Table 1 Examples of genetic transformation experiments using various variants of gfp gene.

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<td>Pinus densiflora</td>
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<td>Terakami et al. 2007</td>
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<td>Coleoptiles, cali, leaf explants</td>
<td>Elliot et al. 1999</td>
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<td>Li et al. 2001</td>
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<td>Leaf tissues</td>
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orecurrence emitted from a particular tissue. It has been reported many times that chlorophyll content could negatively interfere with the GFP fluorescence (Ponappa et al. 1999). Moreover, Zhou et al. (2005) reported reconstruction of once diminished fluorescence in the Medicago leaves after the chlorophyll removal. Hraska et al. (2008) confirmed some role of the chlorophyll content in the decrease of GFP fluorescence intensity; nevertheless the negative correlation was not so strong to conclude that chlorophyll only. Therefore, it seems that the GFP fluorescence intensity may be affected by synergic incidence/activation of several factors, including various physiological aspects, particular plant species, may be the genotype and even environment, while another reason could be the different cytoplasmic density of cells in young and older leaves, leading to the dilution of GFP in older tissues and thus weaker fluorescence (Hraska et al. 2008). Moreover, the fluorescence in older leaves tends to display higher variability. Second, the variability of the fluorescence among leaves situated in various positions within the plant body was previously reported (Halhill et al. 2001, 2003). Another frequently discussed reason of observed fluctuations in transgenic expression could be the specific tissue and developmental expression patterns of the promoters used, mostly the constitutive CaMV 35S (Halfhill et al. 2001; Escobar et al. 2005). The CaMV 35S is generally considered to be a constitutive promoter, the differential expression patterns of transgenes driven by this promoter have been described previously for various plant species (Hraska et al. 2008).

Analysis of fluorescence intensity could be easily used as a powerful toolkit for the definition, measurement and comparison of transformation events among various transgenic plants. Hraska et al. (2008) also reported the GFP fluorescence pattern within the mature plants of tobacco (Table 1). High variability in the GFP fluorescence was revealed among the plant clones within the same genotype, nevertheless this was not higher than the variability between the genotypes. Data obtained from the three leaves from each mature plant showed the decrease of the fluorescence towards the plant top therefore, to youngest leaves. The highest intensity of the fluorescence was retained by the youngest leaves during the whole oilseed rape life cycle. Moreover, the decline in the fluorescence intensity towards the plant base and thus in the older leaves was reported in tobacco plants. Moreover, when evaluating the fluorescence profile of a group of plants, the individual variability, most probably affected by the environment, should be taken into account and such studies should be provided with wider numbers of individuals, clones or populations.

Chen et al. (2005) investigated the potential of a novel double T-DNA vector for generating marker-free transgenic plants. Co-transformation methods using a double T-DNA vector was then constructed by assembling the nptII gene driven by this promoter have been described previously for various plant species (Hraska et al. 2008). Moreover, the fluorescence in older leaves tends to display higher variability. Second, the variability of the fluorescence among leaves situated in various positions within the plant body was previously reported (Halhill et al. 2001, 2003). Another frequently discussed reason of observed fluctuations in transgenic expression could be the specific tissue and developmental expression patterns of the promoters used, mostly the constitutive CaMV 35S (Halfhill et al. 2001; Escobar et al. 2005). The CaMV 35S is generally considered to be a constitutive promoter, the differential expression patterns of transgenes driven by this promoter have been described previously for various plant species (Hraska et al. 2008).

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cells expressing GFP in the cotyledonary epidermis of intermediate stage embryos. These authors suggest that in the future, different pathways for vacuolar targeting could be compared in the same live plants by using differently colored GFP derivatives, and that vacuolar pH could be studied with similar constructs in live embryos using pH-sensitive GFP derivatives. GFP was also used by these authors as a marker to identify components of subcellular structure on a genetic and tissue-specific level. A library of the brightest GFP-expressing calli in Arabidopsis transformation experiments, Molinier et al. (2000) observed no difference in the regeneration process, in quantity, quality or duration, between tobacco leaf discs treated with agrobacteria containing plasmid pHB2892 coding for GFP and those treated with agrobacteria containing a control construct devoid of GFP. Seeds of control plants (wt for GFP) showed strong yellow seed coat fluorescence in tobacco (Molinier et al. 2000). However, this autofluorescence was clearly different from that characteristic for GFP. After germination, only the red chlorophyll auto-fluorescence was detectable in aerial tissues. No green fluorescence was found in healthy and unwounded tissues of wt seedlings. In contrast, green fluorescence visually indistinguishable from GFP fluorescence was observed in and below the crown region and throughout the hypocotyls and cotyledons of GFP-segregating seedlings (Molinier et al. 1997). The natural presence of green-fluorescing compounds makes the detection of GFP in tobacco roots delicate, requiring carefully controlled observations. As development progressed, chlorophyll autofluorescence increasingly masked the GFP fluorescence which thus became more difficult to detect in tissues comprising more differentiated cells, exactly as previously observed with the primary transformants (Molinier et al. 2000). According to Molinier et al. (2000) high-level fluorescence was observed in 25%, low-level fluorescence in 50% of the progeny, while 25% of the seedlings showed no fluorescence in tobacco (Molinier et al. 2000). High and low levels of GFP fluorescence were always correlated with kanamycin resistance and the presence of T-DNA, while the absence of GFP fluorescence was always correlated with kanamycin sensitivity and absence of the T-DNA. This 1:2:1 segregation is consistent with the assumption that the progeny segregated for a single dominant locus, i.e., a single-copy transgene. Progeny of plant 145–4 which harbored the T-DNA in two loci fell into at least four different classes of fluorescence intensity. Although easily detectable in the dissecting microscope, these different levels of GFP fluorescence were difficult to group into individual classes of fluorescence intensity at a low magnification. Nevertheless, the ratio of fluorescence to non-fluorescing plants (15:1) was in agreement with the hypothesis of two independent dominant loci (Molinier et al. 2000). These authors also noticed that progeny of T1 plants belonging to the high-fluorescence class were uniform and 100% of the analyzed seedlings showed a high level of fluorescence. Such T1 and T2 tobacco plants thus appeared to be homozygous for the T-DNA marker. The progeny of the T1 plants belonging to the low-fluorescence class were uniformly lacking the fluorescence characteristic of GFP. The non-fluorescing class therefore, corresponds to wt segregants. T1 plants from the low-fluorescence class produced offspring of heterogenous phenotypes which fell into the three classes of high, low, and no fluorescence. This result confirms the hypothesis that low level fluorescence is indicative of homozygous plants. Thus the expression pattern of GFP fluorescence in an epifluorescence dissecting microscope not only permitted the identification of transformed tobacco plants but also enabled separation of homozygous from hemizygous plants. Artefacts caused by endogenous fluorescence could be recognized in appropriate control experiments and were not a serious obstacle. For progeny of several independent events analyzed, the quantitative nature of GFP fluorescence allowed discrimination of homozygous and hemizygous seeds and seedlings using a simple visual all conditions, GFP-positive tissue was more readily identified in calli and young leaf primordia of shoot tips than in older, more developed parts of regenerating shoots of tobacco (Molinier et al. 2000). The higher cytoplasmic density in young tissues may explain why GFP was detected more easily there than in older leaves where the vacuole, devoid of GFP, constitutes the largest part of the cell.
Effectively in carrot (combined with a visual selection for GFP has been applied). The highest transformation rate was found for A4T, formed adventitious roots and the frequency of discs produced adventitious roots can be visually selected solely. All of the 12 used carrot cultivars produced transformation, as it works effectively in a wide range of genotypes. Transgenic carrot and other Apiaceae are discussed by Baranski in detail elsewhere (Baranski 2008).

**Fruit species**

Recently grapevine (Vitis vinifera L.) leaf tissues were transformed with fluorescent markers targeted to cytoplasm (free GFP and mRFP1) (mRFP1=red fluorescent protein), ER (GFP::HDEL), chloroplast (GAPAL1::YFP) (YFP=yellow fluorescent protein) and mitochondria (GFP) (Zottini et al. 2006). Carrot root discs were inoculated with Agrobacterium rhizogenes (Haseloff et al. 1995). A comparison of conventional selection using antibiotics and visual selection of fluorescent cells revealed that the latter process is less efficient and more laborious, and therefore not preferred for routine production of transgenic plants (Haseloff et al. 1999).

This methodology may however prove advantageous where retransformation (or gene stacking) is to be applied. Also, visual monitoring of regenerating plants would be advantageous when meristems or apices are targeted for transformation to reduce the frequency of chimeric transformant recovery (Elliot et al. 1999). GFP has also enabled the identification of nonproliferating, green-fluorescent sugarcane calli, which showed renewed growth after transfer to fresh medium, thus implicating its use in maximizing the recovery of transformants. Nuclear localization also aided detection due to the concentration of GFP in the nucleus. However, mGFP5-ER is targeted to the ER due to reported toxic effects of GFP nuclear localization in regenerating transformed cells (Haseloff et al. 1997). On the basis of Elliot et al.’s (1999) study, no signs of toxicity or reduced regenerability were observed in highly green-fluorescent sugarcane calli or plants. Rather, their growth was as rapid as calli bombarded with the gene for selection alone (Elliot et al. 1999).

Very low levels of endogenous green fluorescence did not impede the detection of bright GFP fluorescence in the four species examined. All control treatments including bombardment with tungsten or gold particles coated with plasmid DNA containing the selectable gene (pEmuK), physical wounding, or the presence of Agrobacterium failed to induce bright-green fluorescence (Elliot et al. 1999). Wounding frequently initiated the production of pale yellow fluorescent compounds, in localized areas on calli; however, this did not impede detection of green GFP fluorescence in the four species examined (Elliot et al. 1999).

On the other hand Li and Yang (2000) reported that introduced GFP gene constructs showed transient expression in about 2.6% of the electroporated tobacco zygotes. Expression was visualized 3 days after destination of the zygotes of tobacco after 2 days in culture and produced a green fluorescence. In the millicell, however, the detection of GFP fluorescence of the transgenic tobacco zygotes was complicated by the strong autofluorescence of the feeder cells. For this reason, they removed the zygotes from the millicell for GFP detection. Of the 303 electroporated zygotes examined for transgene expression 2 days after culture, 2.6% showed fluorescence – a frequency of 2.6% (Li and Yang 2000).

Agrobacterium rhizogenes-mediated transformation combined with a visual selection for GFP has been applied effectively in carrot (Daucus carota L.) transformation (Baranski et al. 2006). Carrot root discs were inoculated with A4, A4T, LBA1334 and LBA9402 strains, all bearing gfp gene in pBIN-m-gfp5-ER. The results indicate that transformed adventitious roots can be visually selected solely based on GFP fluorescence with a very high accuracy. The method requires no selection agents like antibiotics or herbicides and enables a reduction of labour and time necessary for tissue culture. Moreover, individual transformants can be easily excised from the host tissue and cultured separately. All of the 12 used carrot cultivars produced transformed adventitious roots and the frequency of discs producing GFP expressing adventitious roots varied from 13 to 85%. The highest transformation rate was found for A4T and LBA1334 strains possessing chromosomal background of A. tumefaciens C58. The results of Baranski et al. (2006) encourage that visual selection of transformed, fluorescing adventitious roots can be highly effective and applied routinely for the production of carrot transgenic plants. These results suggest that A. rhizogenes mediated transformation of carrot root disc can be a valuable tool for the production of transgenic plants. Deployment of vector constructs containing the gfp gene enables simple and reliable identification of transformed adventitious roots using a portable UV lamp and then culture of individual clones. Therefore, the selection with antibiotics or herbicides can be omitted, and the described method was applied for routine carrot transformation, as it works effectively in a wide range of genotypes. Transgenic carrot and other Apiaceae are discussed by Baranski in detail elsewhere (Baranski 2008).
a promoter isolated from *Cassava vein mosaic virus* (CsVMV) supported high levels of transgene expression equivalent to those achieved using an enhanced double CaMV 35S promoter. Duplication of the 5%-upstream enhancer region of the CsVMV promoter further enhanced its ability to increase transgene expression. However, the pattern of transgene expression driven by these two viral promoters was significantly different at the whole plant level. The CaMV 35S maximum transgene expression was relatively high and active in most tissues and organs including roots, mature leaves, shoot apices and lateral buds. In contrast, the CsVMV promoter and its double enhancer derivative induced relatively weak expression in these tissues. The results of Li et al. (2001) suggest that activity of the CsVMV promoter, in contrast to the CaMV 35S promoter, was under developmental regulation in transgenic grape plants unlike the CaMV 35S promoter. However, this study indicated that the ACT2 promoter from *A. thaliana* failed to support high levels of transgene expression in cells of grape SE. Although transgenic calli were recovered after transformation using the bifunctional fusion marker gene under the control of the ACT2 promoter, these calli were mostly chimeric and expressed a low level of GFP fluorescence and kanamycin resistance. Such low levels of transgene expression provided a low level of GFP fluorescence and kanamycin resistance. Such low levels of transgene expression were produced relatively low levels of expression in roots of other species, with the strongest expression activity conferred by the CaMV 35S promoter. The CsVMV promoter and its single and double enhancer versions, also generated a relatively low level of expression in mesophyll cells of *Nicotiana tabacum* cv. ‘Dynes’ in which high levels of promoter activity were mainly associated with vegetative tissues. That is, regulatory cis-elements within the CsVMV promoter may not be recognizable by trans-acting factors present in certain types of cells, including the cells of grape SE and callus. Actins in eukaryotic organisms are encoded by a multigene family. The number of gene members in the actin multigene family may vary among different species (Yang et al. 2003). Although actin genes are relatively conserved at the DNA level, distinct patterns of expression regulation and protein functions occur among different actin gene members within a species. Nonetheless, Li et al. (2001) suggested that caution be exercised in the use of the ACT2 promoter to provide expression of marker genes for the purpose of transformant recovery in genetic transformation processes that utilize SEs. In spite of the strong expression activity conferred by the CsVMV promoter in transgenic SE tissue, this promoter produced relatively low levels of expression in roots of transgenic grape plants as compared to the CaMV 35S promoter. Based on the observations that transgene expression remained high in cells on the surface of the root, and in root-derived callus cells, Li et al. (2001) suggested that the lack of expression in transgenic grape roots may have been the result of transcriptional regulation, and not the result of gene silencing phenomenon. The CsVMV promoter, in both its single and double enhancer versions, also generated a relatively low level of expression in roots of transgenic *Nicotiana tabacum* cv. ‘Dynes’. Increased expression of expression could be achieved in root tissues of other species, including tobacco and rice, using the CsVMV promoter (Li and Yang 2000; Richards et al. 2003; Sallaud et al. 2003). Promoter analysis using deletion mutations suggested that expression in tobacco mesophyll cells was associated with a GATA motif located downstream of the as1 motif and the synergistic interactions between these elements in the CsVMV promoter, as in the case of the CaMV 35S cells of ter (Li et al. 2001). However, the number and the relative position of both as1 and GATA elements are significantly different between the CsVMV and the CaMV 35S promoter. Further, the study of Li et al. (2001) speculated that the low activity of the CsVMV promoter in various tissues of grape plants may have been influenced by unique structural characteristics of this promoter. Terakami et al. (2007) reported an Agrobacterium-mediated transformation system for expression of GFP in the dwarf pomegranate (*Punica granatum* L. var. *nana*). Adventitious shoots regenerated from leaf segments were inoculated with *A. tumefaciens* strains LBA4404 and EHA 105 harbouring the binary vector pBin19-sgfp, which contains *nptII* driven by the *nos* promoter and *gfp* gene driven by the CaMV 35S promoter, as a selectable and visual marker, respectively in pomegranate. Among these two *Agrobacterium* strains, they found EHA 105 was more suitable and resulted in a transformation rate of 25% and 39%, respectively in pomegranate. They also concluded that, the leaves, shoots of *in vitro* plants were inoculated with *A. tumefaciens*, but no GFP activity was observed. This was observed in adventitious shoots inoculated with *A. tumefaciens* EHA 105; therefore, adventitious shoots were used as explants to examine the stable transformation rate. During their investigation GFP expression was stably detected in the transgenic grapes. Based on their data, it was concluded that the CsVMV promoter induced relatively low level of expression in transgenic grape roots, and that the plant development and induction of GFP expression was inefficient in pomegranate. GFP expression was observed in shoot apices and lateral buds. In contrast, the CsVMV promoter was under developmental regulation in transgenic grape plants unlike the CaMV 35S promoter, was under developmental regulation in transgenic grape plants. The transformed pomegranate plants showed GFP fluorescence in all organs, and there were no growth and phenotypic alterations between transformants and non-transformants. During the transformation events, Terakami et al. (2007) also found that a regeneration medium containing 50 mg/L kanamycin and 10 mg/L meropenem was found to be the most suitable medium for selecting the transformed cells of pomegranate expressing GFP. The copy number of the transgene integrated into the plant genome was estimated by the progeny segregation test. The seeds (T1 generation) were obtained from four self-pollinated T0 plants, and the GFP assay was performed for the investigation of transgene segregation. However, Terakami et al. (2007) showed GFP fluorescence in T1 plantlets. The inheritance of *gfp* gene indicated that T0 plants were stable transformants and the transgene was integrated into plant genome of pomegranate. Yancheva et al. (2006) introduced GFP into the pear (*Pyrus communis* L.) cv. ‘Spadona’ using the plasmid PZP carrying the nuclear-targeted GFP and *nptII* genes. High expression levels of GFP were detected in transgenic cells as early as 7 days after transformation. GFP marked calli and transformed plants were observed after 14 and 24 days, respectively. Fluorescence microscopy screening of transformed plant material, under the selection of kanamycin and gentamicin, increased the transformation efficiency from 0.4% to 3.0–4.0%, thus concluding that the introduction of GFP improves the selection of transformed plants of ‘Spadona’ pear. Putative transgenic plants were selected on the basis of a newly developed efficient regeneration system. Transgenic status of the selected clones regenerated after inoculation with pME504 was confirmed by GUS histochemical assay, positive PCR, and Southern Blot analysis. In the transgenic *Spadona* plants, the pME504 with *gfp* gene was detected in leaves of the putative transgenic plants by PCR and Southern Blot analyses and by microscopic detection of GFP. GFP was expressed strictly in nuclei of the epidermis and guard cells transformed ‘Spadona’ pear. Zhu et al. (2004) used GFP as a visual selectable marker to produce transformed papaya (*Carica papaya*) plants. Following transformation of *Carica papaya* plants harboring a transgenic binary vector with a GFP reporter gene into an embryogenic callus (Table 1), GFP selection reduced the selection time from 3 months on a geneticin (G418) antibiotic containing medium to 3–4 weeks. Moreover, GFP selection increased the number of transformed papaya plants by five-to eight-fold compared to selection in the presence of antibiotics. Overall, the use of GFP for selecting transgenic papaya lines improved the throughput for transformation by 15- to 24-fold while avoiding the drawbacks associated with the use of antibiotic resistance based selection markers. Plas-
mid pCAMBIA1303 containing gusA and mgfp5 genes for the production of fusion GUS and GFP (mgfp5), respectively, driven by the CaMV 35S promoter have been used during the transformation of papaya. Zhu et al. (2004) reported that selection on the basis of GFP is not only possible but that it also appears to be more efficient for papaya than selection based on antibiotics. The green fluorescence produced in papaya cell cultures containing the functioning gfp is both normally bright under UV light. The fluorescence interacted with the green fluorescence of GFP to make the plants appear yellow under UV light. The use of an appropriate yellow or orange filter blocked the emitted red fluorescence to reveal the transformed plants as expressing green fluorescence (Elliott et al. 1999; Zhu et al. 2004). These investigators compared transformation efficiency in rice based on either GFP or hygromycin selection and reported that GFP selection produced a fourfold increase in recovery of transformed plants in half the time required for selection on hygromycin. A further 3-fold decrease in time for growth and development was associated with an increase in the regeneration capacity of the GFP cultures as these cultures produced either 7.8 or 5.1 plants per bombarded plate versus 0.9 plants for the G418 selection system in case of papaya (Zhu et al. 2004). However, Zhu et al. (2004) also noticed more rapid tissue expansion and earlier regeneration of a greater number of plants in the GFP lines cultured without chemical selection than in the transformed lines selected on G418 in papaya. GFP selection time was reduced to 4–5 weeks compared to the G418 selection time of about 12 weeks before there was sufficient tissue to transfer for regeneration culture of papaya.

A. tumefaciens strain C58 pMP90 containing the binary plasmid pBin19 was used as vector system for transformation in peach Prunus persica L. (Perez-Clemente et al. 2004) (Table 1). They used the Nospro-nptII-Nosters cassette as a selectable marker and the CaMV35Spro-sgfpc-MV35Stter cassette as a vital reporter gene coding for an improved version of the green fluorescent protein sGFP. In vitro cultured embryo sections were Agrobacterium-cocultivated with A. tumefaciens strain C58 pMP90 following the protocol of Schilperoot et al. (2001) and Transformation efficiency was improved by evaluating and manipulating the age of the plant material, the concentration of kanamycin in the medium, GFP-positive shoots were obtained in all four of their host plants. The lines with a single copy had stronger differences in fluorescence intensity could be observed associ-
have been influenced by the positional effect of the transgene or by co-suppression due to the integration of multycopies. In GFP-positive shoots, green fluorescence was expressed in all of the plant tissues, with especially strong expression observed in new leaves, vascular tissues, and stomata. All GFP-positive shoots successfully rooted with strong GFP expression (Tamura et al. 2003).

A high frequency of embryogenesis and transformation from hypocotyl explants of two lines of Medicago trunculata R-108–1 and Jemalong J5 were obtained (Kamate et al. 2000). Using this flower system, they obtained transgenic plants expressing promoter-uidA gene fusions as well as the gfp living cell color reporter gene. Moreover, this method allows the authors to save time and to use a smaller greenhouse surface for the culture of donor plants. Southern hybridization showed that the internal gfp fragment had the expected size and the number of T-DNA copies integrated in the plant genome varied between one and three. These data suggest that the presence of the GFP protein has no toxic effects, since no rearrangement of the gfp reporter gene was detected in the regenerated plants. Plasmid pPR89 containing the M. sativa EnodIIA promoter-uidA reporter gene fusion and plasmid pLP35gfp containing a CaMV35S promoter-gfp reporter gene fusion derived from pMon30049 were transformed into A. tumefaciens strain EHA105. In cases the binary vector was derived from pLP100 and contained a chimeric nos promoter-nptII-nos polyA gene for in planta selection. During transformation of M. truncatula, after co-cultivation with A. tumefaciens for 48 h, gfp expression was observed in a few cells with nuclear fluorescence. One week after transferring the calli onto selective medium, division of cells with nuclear fluorescence was observed on the side of the explant which was in contact with the medium. Despite M. truncatula calli being autofluorescent, they were able to distinguish untransformed calli from the gfp-expressing calli. At later stages, at the initiation of embryos, gfp expression was weaker or ceased. However, in the transgenic plantlets, GFP activity was detectable again in the flowers and in the roots. Southern blot analysis of transgenic plant tissues revealed the internal gfp fragment of the expected size. The number of T-DNA copies integrated into the plant genome varied between one to probably three.

Two plants, probably arising from the same explant and representing the same transformed cell-line, showed the same hybridization pattern, while the other plants, originating from different explants exhibited distinct patterns. On the basis of data they concluded that the expression of the gfp reporter gene has no toxic effects, since no abnormal phenotype or rearrangement of the transgenes have taken place in the genome.

Efficient Agrobacterium-mediated transformation of Antirrhinum majus L. was achieved via indirect shoot organogenesis from hypocotyl explants of seedlings (Cui et al. 2003). Stable transformants were obtained by inoculating explants with A. tumefaciens strain LBA4404 and GV2260 harboring the binary vector pBIGFP121, which contains the nptII gene as a selectable marker and the gfp gene as a visual marker to the CaMV 35S promoter. Transformants were identified by selection for kanamycin resistance and by examining the shoots using fluorescence microscopy. PCR and Southern analyses confirmed integration of the GFP gene into the genomes of the transformants of A. majus. The transformants had an intron-containing GUS gene driven by the CaMV 35S promoter and an nptII gene, conferring kanamycin tolerance, under regulatory control of the nos promoter (Yang et al. 2003). The presence of sGFP(S65T) was detected by blue light excitation (Tamura et al. 2001). Root tips of regenerated plants and progeny were observed with a fluorescent microscope with a filter set providing 455–490 nm excitation and emission above 515 nm (Yamada et al. 2001).

An A. tumefaciens strain LBA4404 containing the binary vector pBIN-m-gfp-ER was used for the genetic transformation of onions (A. cepa) using immature embryos as the ex-
plant source (Eady et al. 2000). Fluorescing shoots were never observed to be chimeric, suggesting that individual cells in the initial embryogenic tissue are, with the initial support of surrounding cells, totipotent. Chromosome counts in the two primary transformants tested showed a diploid (2n=16) chromosome complement in A. cepa (Eady et al. 2000). Southern analyses, probing with the gfp gene showed that 10 of the 13 transformants had single copies, while the remaining three had multiple copies. On this basis, only the transgenic cDNA clone (Triticum porrum) and garlic (Allium sativum) plants have been recovered by the selective culturing of immature leaf and garlic embryos via Agrobacterium-mediated transformation (Eady et al. 2005). This method involved the use of a binary vector containing the m-gfp-ER reporter gene and nptII selectable marker. Transgenic cultures were selected for their ability to express the m-gfp-ER reporter gene and grown in the presence of geneticin (20 mg/l). The presence of transgenes in the genome of the plants was confirmed using TAIL-PCR and Southern analysis. A. tumefaciens strain LBA4404 containing the binary vector pBIN m-gfp-ER, which includes the m-gfp reporter gene that is targeted to the ER (m-gfp-ER) and the nptII antibiotic selectable marker gene, was used (Eady et al. 2005).

Huber et al. (2002) reported transformation for the expression of immature ovary of wheat (Triticum aestivum L. cv. ‘Combi’), and noticed the mean transformation frequency (1.06%) was 8-fold higher than the previous study. In addition, embryo techniques were over 2 weeks faster than scutellar callus procedures. Introducing gfp as a vital marker led to an improvement of embryo-based techniques. In a first screening, transient gfp-expressing embryos were transferred to phosphinothricin (PPT)-containing callus medium. Only gfp-expressing calli which developed on it were cultured further on PPT-containing regeneration medium. Shoots obtained from gfp-expressing calli were rooted on PPT-free medium, and cultured ex vitro. Average transformation frequency (4.93%) was 38-fold higher than with scutellar callus techniques. Differences between the transformation strategies used were were of high statistical significance. Combining green fluorescent protein screening with PPT selection in embryobased techniques offers a promising system to obtain high wheat transformation frequencies (Huber et al. 2002). Transient gfp-expression was determined 24 hours after bombardment by counting fluorescent spots. On average, 20% of the gfp-bombarded wheat embryos showed transient gfp-expression. After 192 hours after bombardment, transient expression had been gradually reduced to zero. By 4 weeks after bombardment, about 8% of the calli showed GFP-fluorescent clusters again, however, there was no clear evidence for stable transgenic plants. Only gfp-transgenic plantlets, GFP fluorescence was masked by chlorophyll fluorescence, but could be detected easily in roots. GFP fluorescence was also found in young leaves of transgenic plants grown under greenhouse conditions. In addition, GFP preparations from such leaves were fluorescent. In older leaves, however, regardless of whether the plants were kept in vitro or in greenhouses, the detection of gfp was more difficult. Antifungal antibiotics were more effective for retaining amounts of GFP disappeared behind the bulk of photosynthetic proteins, and additionally were masked by chlorophyll fluorescence. Analysis of all floral organs showed GFP in ovary and filaments, but not in stigma or anther walls. Furthermore, no GFP was detected from pollen of transgenic Tg-plants when gfp was controlled by the 35S promoter. Young (10 to 15 days after anthesis) transgenic grains obtained by selfing T0-transformants sometimes contained high levels of GFP. Bright green fluorescence was observed in both endosperm and embryos. Coats masked fluorescence of embryos and endosperms, so that they had to be removed at least partly to detect non-transgenic endosperm and embryos segregating following Mendelian rules. Embryos from such cut grains were saved by embryo rescue. Therefore, an easy and early screening method for transgenic Tg embryos and plants was possible. The same was true with T2 progenies. Protein extracts from young grains also showed GFP-fluorescence. In older grains, however, due to other fluorescent grain components, unequivocal GFP detection was no longer possible. Combining GFP screening with PPT selection proved to be another, practical way to save labour as the number of regenerants were reduced. Screening for transient gfp-expressing embryos was facilitated by cultivating these embryos on PPT-containing callus medium. Only gfp-expressing calli which had developed on it were transformed by bombardment in regeneration medium. Rooted transgenic plantlets, detectable by GFP-fluorescent roots, were cultured ex vitro.

Jordan (2000) reported the expression of GFP to act as a marker for detecting transformed cells and tissues of wheat (T. aestivum cv. ‘Fielder’). Multicellular clusters emitting green fluorescence were observed 14 days after particle bombardment with a sgFPlS65T gene construct, and gfp-expressing shoots (often with expressing roots) were observed as early as 21 days after bombardment. Transgenic wheat plants were selected on the basis of gfp expression alone although the inclusion of antibiotic resistance as a selectable marker could improve the efficiency. Using sgFPlS65T as a marker gene in an experiment comparing bombardment parameters allowed the rapid identification of variables that were targeted for optimization. Expression of GFP was observed transiently beginning 24 h post-bombardment, but after 2 weeks of culture on callus induction medium in the dark, embryogenic areas expressing GFP were observed; as little as 3 weeks following bombardment, shoots and entire plantlets (shoots plus roots) expressing GFP were observed. The use of the GFP-Plant (Leica) filter set eliminated background chlorophyll fluorescence that has been observed with other filter sets (Elliott et al. 1999) and allowed GFP-expressing green leaf tissue to fluoresce bright green. Non-expressing leaf tissue appeared dark and did not fluoresce. Plantlets expressing GFP were separated at this point and grown until they were large enough for establishment in soil. No selection aside from visual GFP-based selection was used up to 4 weeks post-bombardment. Application of antibiotic selection after 4 weeks post bombardment increased the number of transgenic shoots obtained. The selected Tg plants were grown to maturity, and in some cases immature seeds were collected 20 days after anthesis. Southern blots confirmed transformation in the Tg plants and showed that all shoots which were selected solely on the basis of GFP expression carried both the gfp and nptII genes. Segregation was observed for expression of GFP in the progeny embryos providing further evidence of stable transformation. Dissection of the embryos was necessary to observe segregation as embryo expression (or lack of it) was masked in whole seeds by GFP fluorescence from transformed tissue (Jordan 2000). Segregation data on the T1 progeny of four plants showed that for three of the four Tg plants, GFP expression was inherited in a 3:1 ratio consistent with a single transgene locus. The optimum time for GFP visual selection was shortly after the somatic embryos of wheat germinated (~3 weeks after bombardment). These shoots can be followed through root formation and then separated. Application of antifungal antibiotic can increase the number of transgenic obtained. This is likely due to being able to easily identify later-germinating transgenic embryos which otherwise are masked due to massive overgrowth of surrounding non-transgenic shoots.

Elliott et al. (1999) used GFP expression for the early identification of transformed sugar cane (Saccharum L. hybrid cv. ‘Q117’), cells under selection. This enabled the removal of untransformed tissue at an early age of transgenic clones. They also reported the identification of transformed callus clusters using visual selection alone but concluded that this was more laborious than combining visual and antibiotic selection. In the sugar cane transformation protocol, the continuous selection and isolation of transformed cell clusters over an extended period of time used for sugar cane is not necessary; selection can simply be applied to screen germinating somatic embryos and small shoots over a 2-week period (2-
4 weeks after bombardment), thus reducing labour input (Jordan 2000). However, combining antibiotic selection with GFP selection can result in the production of more transformed plants than GFP alone because large masses of shoots make identification of GFP-expressing plants difficult after 4 weeks post bombardment. While the use of GFP may not directly improve transformation frequency (which depends on many factors) its use would allow for the identification of the T-DNA integration pattern per given time period with a given amount of labour. This would be due to the elimination of escapes by transferring to soil only healthy shoots growing on selection medium and also expressing GFP as well as to the rapid optimization of experimental conditions which the use of GFP allows. Using GFP and observing expression very early on in the transformation process may reduce chimerics by allowing the identification and manual elimination of chimeric cell clusters, embryos and shoots as they develop. GFP would also assist in optimizing conditions to reduce the frequency of chimerics (Jordan 2000).

An engineered GFP was used to develop a facile and rapid rice transformation system using particle bombardment of immature rice embryos (Oryza sativa indica cv. "TN1") (Vain et al. 1998). The mgfp4 gene under the control of the CaMV 35S promoter positively selected transgene expression easily detectable and screenable in rice tissue after 12 to 22 days after bombardment. Visual screening of transformed rice tissue, associated with a low level of antibiotic selection, drastically reduced the quantity of tissue to be handled and the time required for the recovery of transformed plants. GFP expression was observed in primary transformed rice plants (T₀) and their progeny (T₁). Following bombardment very little fluorescence was observed during the first 10 days of culture during rice transformation, other than pale-yellow autofluorescence, orange fluorescence in necrotic tissues, or GFP transient expression. Twenty two days after bombardment, large GFP fluorescent sectors were observed and dissected from rice calli grown at all levels of hygromycin tested (0, 25 and 50 mg/l) using a simple hand-held ultraviolet lamp for GFP excitation. Visually selected fluorescent rice tissue was used for subsequent clonal propagation or direct regeneration of transformed plants. Transgenic rice plants were regenerated from visually selected GFP-positive calli (Vain et al. 1998). Mgfp4 expression did not appear to interfere with plant regeneration, the development or the fertility of transformed plants. High levels of GFP fluorescence was observed primarily in the root system. Expression was limited in chlorophytic tissues, such as developed leaves, but could be easily detected in the root system using the bright-field microscope. Twenty-five percent of these plants would be consequently useless to screen. Moreover, multiple T-DNA insertions are often located at the same locus and subject to T-DNA rearrangement. The T-DNA carries at least one promoter to direct the expression of the gene coding for antibiotic resistance to select the transformed cells. Promoters such as CaMV 35S often carry enhancer elements which then could interact positively or negatively with the gene detection system, but this silencing phenomenon, the fundamental mechanism of which has been extensively studied in the past few years, is not often taken into account when a gene detection system is used in a functional genomics project.

GFP was used as a screenable marker in the production of transgenic barley (Hordeum vulgare cvs. "Igri" and "Trinity") plants (Carlson et al. 2001). Isolated barley mesophore cultures were bombarded with a mixture of plasmids containing the synthetic GFP gene, sgfp and pgfp. Thirty-seven fluorescent multicellular structures were isolated using epifluorescent microscopy. Sixteen structures developed shoots, but only five regenerated into green plants. Three events had been co-bomarded with gus and assayed positive for gus expression in the leaves, and all five events were positive for gfp expression. GFP-based visual screening provides a viable alternative to the chemical selection of transgenic plants from barley mesophore culture.

The expression of GFP and its inheritance were studied in transgenic oat (Avena sativa L.) plants transformed with a synthetic GFP gene [sgfp(S65T)] driven by a rice actin promoter (Cho et al. 2003). In vitro shoot meristematic cultures (SMCs) induced from shoot apices of germinating mature seeds of a commercial oat cv. "Garry", were used as a transformation target. Replicate SMCs were bombarded with a mixture of plasmids containing the sgfp(S65T) gene and one of three selectable marker genes, phosphinothricin acetyltransferase (bar), hpt and nptII. Cultures were selected with bialaphos, hygromycin B and gentamicin (G418), respectively, to identify transgenic tissues. From 289 individual explants bombarded with the sgfp(S65T) gene and one of the three selectable marker genes, 23 independent transgenic events were obtained, giving an 8.0% transformation frequency. All 23
transgenic events were regenerable, and 64% produced fertile plants. Strong GFP expression driven by the rice actin promoter was observed in a variety of tissues of the T₀ plants and their progeny in 13 out of 23 independent transgenic lines. Stable GFP expression was observed in T₂ progeny from five independent GFP-expressing lines tested, and homozygous plants of A. sativa from two lines were obtained. This study also noticed transgene silencing in T₀ plants and the progeny of some sugarcane (Saccharum officinarum L.), maize (Zea mays L.), lettuce (Lactuca sativa L. cv. 'Crystal'), and tobacco (Nicotiana tabacum cv. 'Wisconsin 38').

A. sativa. Different tissues formed from stably transformed shoot meristem cultures. T₀ plants and their progeny were tested for GFP activity. GFP driven by the rice actin promoter was strongly expressed in meristematic tissues, anther, ovary and stigma, root, and immature embryo and endosperm tissues of A. sativa. GFP expression in leaf tissues was obscured by chlorophyll fluorescence. Copy numbers per genome of the intact sgfp(S65T) gene ranged from three to seven. All 23 transgenic lines of A. sativa were regenerable, and 64% of them were fertile. In addition, the sgfp (S65T) gene could be used as a vital reporter gene for stable oat transformation, although there appeared to be transcriptional silencing in some transgenic plants. Elliot et al. (1999) assessed modified versions of GFP using a dissecting fluorescence microscope with appropriate filters. GFP-expressing cells from four different plant species sugarcane (Saccharum hybrid cv. 'Q117'), maize (Zea mays L.), lettuce (Lactuca sativa L. cv. 'Crystal'), and tobacco (Nicotiana tabacum cv. 'Wisconsin 38') were readily distinguished, following either Agrobacterium-mediated or particle bombardment-mediated transformation. The identification of gfp-expressing sugarcane cells allowed for the elimination of a high proportion of non-expressing explants and also enabled visual selection of dividing transgenic cells, an early step in the generation of transgenic organisms. The recovery of transgenic cell clusters was streamlined by the ability to visualize gfp-expressing tissues in vitro. The use of GFP as a non-toxic marker to identify transgenic cells after transformation is an effective procedure for discriminating transgenic cells and removing untransformed or non-expressing tissue (Elliot et al. 1999). Green-fluorescent cells of tobacco and lettuce were observed after cocultivation with Agrobacterium containing pBin.35S-mgsf5-ER. However, in this study, pBin.35S-mgsf5-ER did not confer increased green fluorescence in Agrobacterium. The reasons for this were unclear, but low-level gfp expression in Agrobacterium may have been masked by endogenous fluorescence of Agrobacterium cells. GFP fluorescence in cells was a good indicator of transformation with further indication provided by the increased level of green fluorescence in nuclei of green-fluorescent cells, which is consistent with the use of a non-targeted gfp version. Continued growth and division of the fluorescent cells further supports their transformed nature.

Several modifications of a wt GFP gene were combined into a single construct, driven by the ubi1-1 promoter and intron region, and transformed into maize (van der Geest and Petulino 1998). Green fluorescence, indicative of GFP expression, was observed in stably transformed callus as well as in leaves and roots of regenerated plants and their progeny. Cell wall autofluorescence made GFP expression difficult to observe in sections of leaves and roots. However, staining sections with toluidine blue allowed detection of GFP in transgenic tissue. Bright GFP fluorescence was observed in approximately 50% of the pollen of transgenic plants. These results suggest that GFP can be used as a reporter gene in transgenic maize; however, further modification, i.e., to alter the emission spectra, would increase its utility. Microscopic analysis of transgenic callus and young roots of tobacco was not impeded by endogenous fluorescence of Agrobacterium cells. GFP fluorescence in cells was a good indicator of transformation with further indication provided by the increased level of green fluorescence in nuclei of green-fluorescent cells, which is consistent with the use of a non-targeted gfp version. Continued growth and division of the fluorescent cells further supports their transformed nature.

ently observed in tissues of consecutive sections taken from the same sample, expression was not necessarily seen in cells occupying the same location within the tissue. Either all cells within a tissue were not accumulating GFP to observable levels, or this was an artifact of tissue preparation. The spotty nature of fluorescence in these sections may relate to the fact that they were cut from fresh tissue without any type of fixation prior to staining. Perhaps only those embryos in combination with antibiotic selection during processing still contained enough GFP to fluoresce to observed observable levels. Prolonged fixation of tissues (i.e., 16 h in formaldehyde: acetic acid: alcohol) resulted in loss of visible GFP fluorescence while bright GFP fluorescence was observed in approximately 50% of the pollen of those cells that remained intact within a given section without any type of fixation prior to staining. Perhaps only may relate to the fact that they were cut from fresh tissue or this was an artifact of tissue preparation.

Nishizawa et al. (2006) evaluated the red fluorescent protein mDsRed2 in somatic embryos of soybean (Glycine max). Transient and stable expression of mDsRed2 in somatic embryos of soybean was readily detected by fluorescence microscopy, allowing easy confirmation of gene introduction. mDsRed2 is a modified form of DsRed from Discosoma sp. for its suitability as a visual marker in combination of soybean (Glycine max). Transient and stable expression of mDsRed2 was detected by fluorescence microscopy without background fluorescence in both leaves and seeds of the transgenic soybean plants. Furthermore, in contrast to seeds expressing GFP, those expressing mDsRed2 were readily identifiable even under white light by the color conferred by the transgene product. The protein composition of seeds was not affected by the introduction of mDsRed2, with the exception of the accumulation of DsRed2 itself, which was detectable as an additional band on electrophoresis during transformation events of soybean. The CaMV 35S promoter and the nos terminator were used to control the expression of mDsRed2 with the plasmid vectors such as pUHR and pUHG during the genetic transformation of soybean by particle bombardment. Polin et al. (2006) recently noticed two days of gradual desiccation was found to significantly enhance transient GFP expression frequency in American chestnut (Castanea dentata). Phenotypically normal transgenic shoots of American chestnut were regenerated and rooted (Polin et al. 2006).

In the Juglans regia L. (Persian walnut) somatic embryogenesis system, a major limiting factor is the selection of non-chimeric transgenic embryos in tissue culture (Escobar et al. 2000). They transformed Persian walnut SEs with the S65T synthetic GFP gene in order to assess the effect of this visual marker gene on SE viability and the selection of transgenic SEs. Following a 10-d period of transient GFP expression in all inoculated SEs, stable fluorescent sectors were apparent in several SEs, allowing rapid and visual selection of transgenic SEs. Two chimeric SEs were selected 40 d after transformation and, these gave rise to 13 stable transgenic SE lines and 44 whole plants. GFP-expressing walnut plants and SEs developed normally and transformation was verified by GFP analysis. Faint green fluorescence was visible in epidermal cells of walnut SEs as early as 22 h after inoculation with A. tumefaciens EHA101/pPDM96.0501. Un-inoculated- and control cultures of primary/pPDM96.0501 were non-fluorescent.Transient GFP expression was apparent on the surface of all inoculated embryos, with maximum brightness at 4 d after inoculation. Transient expression was completely extinguished after 10 d, leaving small, dimly fluorescent sectors of cells on a small number of SEs. These results suggest a highly efficient delivery of T-DNA into the SE cell nuclei, but a low efficiency of stable T-DNA integration into the genome. There were no observable developmental differences between the chimeric (partially fluorescent) SEs and the non-transformed SEs during the 40 d observation phase. All inoculated SEs remained alive during 40 d of selection on kanamycin-containing media, but SEs expressing the GFP transgene were readily identified when examined in culture with a dissecting fluorescence microscope. Thus, GFP expression allowed rapid, non-invasive visual selection of chimeric SEs with 100% efficiency as compared to selection by kanamycin resistance alone. Therefore, it was of great interest to investigate whether the bryogenically fated epidermal cell was transformed, a wholly fluorescent secondary SE would be expected. Alternatively, if a single cell of an already multicellular secondary SE was transformed, a chimeric secondary SE would be expected. Because GFP expression could be detected in globular-stage secondary SEs, selection of transgenic E, generation SEs could be made by visual assay several weeks earlier than was possible. Whole mounts of transgenic SEs and roots demonstrate a global pattern of GFP expression with no discernible tissue specificity, as is expected from a transgene driven by the constitutive CaMV 35S promoter. Likewise, a non-tissue specific pattern of fluorescence was observed in stem and root tissues in longitudinal and transverse sections. These results suggested that S65T synthetic GFP is an efficient selectable marker in walnut SE culture and was subsequently validated in tissue culture or in the greenhouse. GFP has the potential to significantly decrease labour, time, and cost constraints in walnut SE culture and represents a significant improvement over existing kanamycin and GUS-based selection systems.

Malyshenko et al. (2003) reported an efficient regeneration and genetic transformation of summer rape (Brassica campestris L. var. oleifera) (Table 1). Cotyledons of 5-day-old seedlings were transformed with A. tumefaciens strain AGL1 comprising a binary vector pBinn-gfp5-ER with a selectable nptII gene and the gfp gene under the CaMV 35S promoter. Transgenic plants were identified by GFP fluorescence and by polymerase chain reaction and Western blotting analysis. The transformation efficiency was as high as 75% of the total number of regenerated shoots of summer rape. An efficient Agrobacterium-mediated method for transformation, regeneration and screening of Brassica rapa subsp. oleifera (syn. B. campestris) was developed by Wahlroos et al. (2003). For transformation of B. rapa, 5-d-old cotyledons were co-cultivated for 2 d with Agrobacteria (strain AGL1) harbouring a binary vector carrying a gene GFP. This study also indicated that GFP did not prove to be very useful in regeneration steps but reduced the time and number of plants to be handled in screening process. Using the method, up to 9% of fluorescing transformants (T0 generation) were obtained. Transformation was further confirmed by Western blotting and fluorescence/confocal microscopy (Wahlroos et al. 2003). Bright fluorescent sections were occasionally detected indicating unequal expression of GFP, but in most cases fluorescence was uniform covering the whole leaf area. Occasionally, some background fluorescence was detected from young wt plants when illuminated with hand-held UV-light, and, therefore, tissue culture conditions were optimized to further remove the background using confocal laser scanning microscopy to further confirm GFP-fluorescence. It must be noted that visualization of GFP-positive B. rapa ssp. oleifera plants with a UV-lamp was not trivial due to high background autofluorescence (Wahlroos et al. 2003). It was shown that the use of GFP drastically reduced the quantity of tissue to be handled in regeneration processes of transgenic rice (Vain et al. 1998). Arabidopsis plants (GFP-plants) were studied in detail using confocal laser scanning microscopy to further confirm GFP-fluorescence. It must be noted that visualization of GFP-positive B. rapa ssp. oleifera plants with a UV-lamp was not trivial due to high background autofluorescence (Wahlroos et al. 2003). It was shown that the use of GFP drastically reduced the quantity of tissue to be handled in regeneration processes of transgenic rice (Vain et al. 1998). Therefore, GFP can prove to be valuable to directly detect and facilitate the screening process of plants that are potentially transgenic and express functional protein products from transgenes, at least if they are fused to target gene. This may be important especially when kanamycin is used for selection since it was recently shown that the pro-
portion of “escapes” may be even 90% under kanamycin-selection (Wahlroos et al. 2003). Mohan and Krishnamurthy (2003) reported the expression of GFP in pigeonpea using Agrobacterium strain LBA4404 harbouring a binary plasmid pBIN 35S-mgfp-ER was used as a vector for transformation. The elongated shoots of pigeonpea (Cajanus cajan) expressing GFP reporter gene were rooted and indicated normal growth in the greenhouse (Mohan and Krishnamurthy 2003). In agreement, suggesting that GFP expression in these plants is not toxic to the plant and may be useful to simplify the characterization of transgene introgression into unintended hosts; however, GFP is the best candidate for this application (Mohan and Krishnamurthy 2003).

Agrobacterium-mediated transformation of shoot apices of sunflower (Helianthus annuus L.) was evaluated following wounding by cell-wall-digesting enzymes and sonication (Weber et al. 2003). The frequency of explants with regenerated shoots expressing GUS or GFP increased following treatment with the macerating enzymes cellulase Onozuka R-10 and pectinase Boerozym M5, whereas treatment with macerating enzyme R-10 had no transient expression of reporter genes was also enhanced using sonication (50 MHz; 2, 4 and 6 s), but stable expression in regenerated shoots following 4 weeks of selection did not increase with this treatment. Enzyme treatment alone (0.1% cellulase and 0.05% pectinase) was superior to a combined treatment of sonication and enzymes with respect to stable transformation of sunflower. Regenerated plants were fertile and showed normal growth. It was also assumed that the beneficial effect of the macerating enzymes cellulose and pectinase mainly results from the enlargement of the area where Agrobacterium can attach to the meristematic cells (Weber et al. 2003). Sonication with or without enzyme treatment had very different effect on transient and stable expression of GFP in sunflower. Sonication was very effective in increasing transient GFP expression. The number of transient events increased with prolongation of the sonication time up to a maximum of 18.0 GFP-expressing spots per explant (average of three independent experiments) when the shoot tips were treated with 50 MHz for 4 s prior to infiltration with Agrobacterium omitting other treatment. The other treatment conditions gave a lower number of transient expression events compared to the non-enzyme treated control and sonication treatments. Further stable GFP expression was scored 4 weeks post-treatment of control as frequency of explants with shoots displaying either chimaeric GFP or uniform expression. In contrast to the transient expression results, enzyme treatment of explants was superior to sonication with respect to stable transformation. Although ultrasound treatment (50 MHz) slightly elevated the proportion of explants regenerating shoots expressing GFP, and non-sonicated but treated explants delivered shoots expressing GFP (as an in vivo marker to monitor transgene spread in the environment. Large-scale releases of transgenic may lead to invasiveness and competition of transgenic weeds containing a transgene conferring an increment of fitness. Currently no tracking system is in place to monitor transgene introgression into unintended hosts; however, GFP is the best candidate for this application (Mohan and Krishnamurthy 2003).

A fluorescence-based method was developed to quantify GFP levels in transgenic canola plants and protein extracts (Richards et al. 2003). Fluorescence intensity was linear with increasing levels of GFP over a range that encompasses transgene expression in plants by the CaMV 35S promoter. Standard curves were used to estimate GFP concentration in plants and in protein extracts. These values were consistent with ELISA measurements of GFP in protein extracts from transgenic plants, indicating that the technique is a reliable measure of recombinant GFP expression. The levels of in planta GFP expression in both homozygous and hemizygous plants was then estimated. Homozygous transgenic plants expressed twice the amount of GFP than hemizygous plants, suggesting additive transgene expression in canola (Richards et al. 2003). This methodology may be useful to simplify the characterization of transgene expression in plants (Richards et al. 2003). Richards et al. (2003) indicated that GFP fluorescence is an accurate tool for protein quantification. Fluorescence intensity increases linearly as the quantity of GFP increases; therefore, standard curves can be used to estimate the amount of GFP in an unknown sample. Data from ELISA support the reliability of the fluorescence-based estimates. In measuring GFP in plant protein extracts, both techniques were in agreement. However, the fluorescent protein extracts included 30% to 70% of the GFP detected in vivo, which is a valid alternative. While there is a strong association between the leaf-surface fluorescence estimates of GFP and the extrapolated estimates from the extracts, there is a discrepancy between the scale of the values. It remains unclear why fluorescence measurements from the leaf surface indicate 4-fold less GFP per gram leaf tissue than the ELISA and fluorescence-based estimates from soluble protein extracts in canola (Richards et al. 2003). It is possible that differential expression of GFP in the tissues is a factor. It was also concluded that GFP expression driven by the CaMV 35S promoter in tobacco tissues resulted in heterologous expression in tissue sub-types. This effect was measured by protein blot analysis and fluorescence intensity (Richards et al. 2003). The leaf surface estimate measured the fluorescence in a 0.78-cm2 disk (approximately 10 mg tissue) and the plant extracts were pooled from 200 mg tissue surrounding that location. This extra material may contain tissue types that have a higher expression (such as vasculature) than the disk measured by the fiber optic probe (Richards et al. 2003). It is also possible that there is interference from plant or fluorescently absorbent compounds that inhibit GFP fluorescence. They observed that known quantities of GFP in protein extracts resulted in lower fluorescence than equivalent quantities in the control buffer. This effect was evident when UV light was used for excitation but was not evident when blue light was used. They hypothesized that compounds in the plant extract may be absorbing the UV excitation wavelength. In the data presented here, this effect was found to be more pronounced and directly proportional to the density of protein in the extract. If the hypothesis is accurate, then those same compounds may limit GFP excitation in planta (Richards et al. 2003). If that is the case, then to bring the leaf surface estimates in line with the extrapolated extract-based estimates, the values could be multiplied by a factor of four (Richards et al. 2003). The three-dimensional structure of the leaf, such as cuticle thickness or cell-wall shape, may also play a role in the attenuation of measurable GFP fluorescence. Such structural features may refract GFP from excitation or block the emission wavelengths from the detector (Richards et al. 2003). These factors do not indicate that a limitation of GFP fluorescence is not a result of interference from the spectral qualities of the leaf itself (such as autofluorescence of chlorophyll) because purified GFP placed on the leaf surface was not inhibited (Richards et al. 2003).

Using this methodology it was possible to determine the difference in recombinant protein expression between the homozygous and hemizygous transgenic canola lines (Richards et al. 2003). The homozygous exhibited twice the level of recombinant protein expression, which supports the additive transgene hypothesis. In these experiments, only one line of transgenic canola was used, and this application must be studied in other lines and species before its effectiveness can be evaluated. However, this methodology may provide a useful tool for transgenic research. Several questions remain as to how transgene copy number affects expression, and questions pertaining to multiple loci and transgene stacking should play an important role in risk assessment of genetically engineered crops (Richards et al. 2003). As the fluorescence-based quantification technique is refined, it may be possible to use GFP-protein fusions to study these questions. This research extends the utility of fluorescence-base quantification by developing an in planta estimation protocol. Using a FluoroMax-2 spectrophotometer with a fiber optic cable probe, we were able to measure fluores-
cence directly from the leaf surface, which can then be converted into an estimate of GFP per unit of leaf material (Richards et al. 2003). At present, the probe is 1 cm in diameter, which prohibits tissue-specific estimations, and this methodology will be limited to only fluorescently active molecules. However, it is still an effective tool to rapidly and reliably quantify GFP in planta (Richards et al. 2003).

**Forest and timber plants**

The *gfp* gene was introduced into conifer tissues by microprojectile bombardment and its transient expression was detected (Tian et al. 1997). Two versions of the *gfp* gene, wt *gfp* and modified *gfp* with a cryptic intron removed were directly compared for their expression in black spruce pollen. While the wt *gfp* gene resulted in a low level of expression, the modified *gfp* gene resulted in a dramatic increase in the amount of expression (>100 times) (Tian et al. 1997), which was detected in all the tissues tested: pollen, embryonal masses, suspension culture, and SEs. Also, the *gfp* gene was introduced and expressed in three different conifer species (black and white spruce, and white pine). The successful expression of the *gfp* gene in various tissues and different species suggests that it is a useful reporter marker gene for conifers (Tian et al. 1997). During this study, the wt *gfp* gene gave rise to a low amount of transient expression in black spruce pollen following microprojectile bombardment, whereas a high degree of expression was detected with a modified *gfp* sequence (mgfp4) from which a cryptic intron had been mutated (Haseloff et al. 1997). Expression with the modified *gfp* gene has been observed in various types of tissues of several conifer plants, including SEs of black spruce (Tian et al. 1997).

The gene coding for GFP was successfully used as a vital marker for the transformation of three woody plant species, black spruce (*Picea mariana* (Mill.) BSP), white pine (*Pinus strobus* L.) and poplar (*Populus* spp.) (Tian et al. 1999). The *gfp* and *nptII* genes were introduced by microprojectile bombardment or *A. tumefaciens*-mediated transformation (Tian et al. 1999). Screening by fluorescence microscopy of the transformed plant material, under the selection of kanamycin, identified five to eight cell lines from each tree species that clearly expressed GFP. Expression of GFP was observed in somatic embryonal cells of the coniferous species and in stem sections of poplar. For all species, GFP transgene expression was stable over multiple subcultures. Stable integration of the *gfp* gene into plant genomes was confirmed by Southern hybridization or polymerase chain reaction (PCR) analysis. Tian et al. (1999) expressed two versions of the *gfp* gene, wild type and a modified *gfp* construct, mgfp5, which targets the recombinant protein to the ER, resulting in *in vivo* expression in plants. GFP was easily detected in white pine transformed with the mgfp5 construct. Transformation with this vector resulted in a uniformly distributed fluorescence, indicating that the chitinase N-terminal signal peptide sequence from *A. thaliana* is recognized properly in gymnosperm cells. A similar construct, when introduced in *A. thaliana*, displayed clear perinuclear partitioning of the GFP and associated with the endoplasmic reticulum in a distinctive reticulate network (Haseloff et al. 1997). Expression of the *gfp* gene was also observed in transformed cells resulting from bombardment of SEs of black spruce (Tian et al. 1999). The mgfp5 construct had a different pattern of accumulation of GFP compared with the mgfp5 construct, and was observed in transient expression with mgfp4 (Tian et al. 1997); GFP appeared to accumulate in the nucleus with some weak distribution in the cytoplasm (Tian et al. 1999). Further, no *gfp* expression was detected in the vacuoles of the suspensor cells. Similar localizations of GFP have been observed in different types of transformed *Arabidopsis* cells (Haseloff et al. 1997). The same pattern of GFP accumulation in both angiosperm and gymnosperm cells suggests that GFP has no obvious affinity for subcellular structures in plant cells and should be distributed freely (Tian et al. 1999). Such a characteristic makes it possible to target GFP to particular subcellular locations by making fusion protein with specific targeting sequences, thereby facilitating the study of organelles throughout plant development (Moliner et al. 2000; Zha et al. 2004; Chen et al. 2005). Tian et al. (1999) noticed that photographs of the non-transformed tissues of white pine and black spruce were essentially black indicating no auto-fluorescence. In transgenic poplar, GFP expression was visible from primary transformants. Because poplar transformation was performed with the mgfp5 construct, uniform distribution of GFP was observed. However the GFP specific fluorescence was masked by endogenous red fluorescence, making high magnification photography difficult. Expression was more easily visualized in stems, probably because of the low content of fluorescent chlorophyll in this tissue.

In black spruce and white pine, embryonal masses of GFP lines grew and proliferated normally when compared with untransformed cultures (Tian et al. 1999). Morphology of the embryonal masses was also normal when compared with untransformed controls. Furthermore, *gfp* expression in selected coniferous cell lines was maintained over multiple subcultures. To test the embryogenic capacity of transformed cells, *gfp* expression in white pine lines and ten black spruce lines were subjected to the maturation process. All tested lines produced normal SEs. Haseloff et al. (1997) also described the normal recovery of transgenic *Abies grandis* plants with mgfp4, although plant recovery was higher with the mgfp5 gene. The constitutive and stable expression of *gfp* in secondary embryonal masses in conifers has greatly facilitated identification of transformed cells at early stages without destruction and many transformants can be rescued (Tian et al. 1999) indicating that expression of *gfp* can also be useful in monitoring and studying initiation and development of transformed cells both temporally and spatially. *gfp* gene expression can also be used for histological studies of somatic embryogenesis in conifers, including tracking specific cell types from their origins to their final position in the organized tissues (Tian et al. 1999). In addition, stable expression of *gfp* in trees provides a means to monitor gene expression and protein localization in living cells (Tian et al. 1999; Richards et al. 2000, 2001).

*Malus* x *domestica* Borkh. (Malus domestica) has been a subject of extensive investigation for the last two decades. The first generation of transgenic *Malus* x *domestica* Borkh. (Malus domestica) has been a subject of extensive investigation for the last two decades. The first generation of transgenic *Malus* x *domestica* Borkh. (Malus domestica) has been a subject of extensive investigation for the last two decades. The first generation of transgenic *Malus* x *domestica* Borkh. (Malus domestica) has been a subject of extensive investigation for the last two decades.
mann firs, and Virginia pine. Further expression of m-gfp5.ER was clearly visible in the needles of transgenic plantlets of Virginia pine established in soil in the greenhouse for 5 weeks and for 5 months. GFP is still a relatively new scorable marker in plants, and very little is known about the timing of expression that could influence its detection in coniferous species.

A genetic transformation protocol for Chamaecyparis obtusa has been developed after cocultivation of embryogenic tissues with disarmed A. tumefaciens strain C58:pMP90, which harbours the sgfp (synthetic green fluorescent protein) as visual marker and nptII as the selectable marker genes (Taniguchi et al. 2005). C. obtusa is one of the most economically important conifers grown in plantations in Japan because its wood is of good quality and very suitable for timber. The intensity of fluorescence during genetic transformation of C. obtusa was driven by a CaMV 35S promoter and a NOS promoter respectively were used during genetic transformation of C. obtusa. During the transformation events, however, the fluorescence was often not clear in many tissues of the transgenic lines, which indicated a low expression of GFP. The sGFP (S66T) was shown to be available as a reporter in C. obtusa. However, the transient expression of GFP after particle bombardment was clearer in cotyledonalary somatic embryos of Larix kaempferi and zygoic embryos of Cryptomeria japonica (Taniguchi et al. 2004). Further many copies of sgfp might be introduced to single cells by particle bombardment, resulting in a strong intensity of fluorescence during genetic transformation of C. obtusa (Taniguchi et al. 2004). The low expression of sgfp in C. obtusa might be due to the low activity of the 35S promoter. More active promoters than 35S promoter might express sgfp more actively in transformed C. obtusa (Taniguchi et al. 2005). Moreover, the fluorescence of GFP could not be visualized in leaves, except for the youngest leaves of the shoot tips, and was detected in the roots of all transgenic C. obtusa, which might depend on a very low activity of the 35S promoter in leaves of C. obtusa. The introduced gene sgfp, was available as a reporter gene in C. obtusa, although gene expression driven by the 35S promoter was not strong.

**Other plants**

GFP seemed to reliably yield a scorable green-fluorescent trait enabling selection of transgenic tissue and plants of horseweed (Conyza canadensis) hybrids (Halfhill et al. 2007). At 45-day post-incubation 219 explants contained GFP callus sectors, demonstrating 90% transformation efficiency, whereas non-transgenic callus and regenerated plants appeared red under epifluorescence microscopy. Calli were found heterogenous for green fluorescence, but segments of callus were homogeneously green fluorescent, i.e. it appears that GFP in transgenic cells is expressed from definite loci. The GFP expressing shoots appeared morphologically normal when visualized under white light and fluorescence green when visualized under epifluorescent conditions in contrast with non-transgenic plants. The roots of the transgenic plants exhibited the same pattern of green-fluorescence. Meristic regions of intact transgenic plants of horseweed hybrids in pots were fluorescent green, suggesting that the specificity of genotype is related to the cell physiology, which indicate cell physiological reaction after wounded, concentrations of cell internal hormone, structure of cell wall, etc. (Han et al. 2005). Among leaves, stem fragments, and root fragments, the optimum explants used in transformation of A. annua were leaves, which indicated a high frequency of shoot induction of leaves is the highest and since A. tumefaciens has the highest ability to infect leaves. When the cotyledons or hypocotyls from 8-day-old seedlings of A. annua were used as transformation explants, the transformation efficiency was very low, and when the seeds were used as explants, the transformed seeds were merely able to germinate but was not able to root on selection medium.

A. tumefaciens strains EHA105 and LBA4404 were used for the transformation experiments of tall fescue (Festuca arundinacea) (Wang and Ge 2005). Binary vectors bearing chimeric hygromycin phosphotransferase gene (hph), GUS gene or m-gfp were tested in combination with EHA105 or LBA4404 strains. The pCAMBIA vectors tested were: pCAMBIA 1201 carrying hph and gusA, pCAMBIA 1304 bearing hph, mgfp and gusA, pCAMBIA 1305.1 carrying hph and GUSPlus, and pCAMBIA 1305.2 bearing hph and GUSPlus. All these chimeric genes were under control of the CaMV 35S promoter and all the gusA or GUS-Plus constructs contained a catalase intron for eukaryote-specific expression (Wang and Ge 2005).

Maximova et al. (2003) described a protocol for Agrobacterium-mediated genetic transformation of Theobroma cacao L. using cotyledonalary explants from primary somatic embryos (SEs) and A. tumefaciens strain AGL1. Transgenic plants carrying the visible marker gene EGF, the selectable marker nptII (NPTII), the class I chitinase gene from cacao pod endosperm and tobacco nuclear matrix attachment regions (MARs) in different combinations were successfully produced via regeneration of secondary SEs. The presence of the chi gene or MARs did not influence the number of transgenic plants produced compared to the marker genes alone. However, the inclusion of the chi gene contributed to increased mean GFP expression in the population of transgenics. Additionally, the presence of chi gene(s) or MARs reduced the occurrence of gene silencing and stabilized gene expression in the transgenic plants. The transgenic plants were multiplied via reiterative somatic embryogenesis. Ninety-four transgenic plants were acclimated in a greenhouse and grown to maturity. Detailed growth analysis indicated that there were no differences in various growth parameters between transgenic and non-transgenic SE-derived plants. Seeds produced from two genetic crosses with one of the transgenic lines were analyzed for EGF expression – a near perfect 1:1 segregation was observed for fertile plants that this line resulted from the insertion of a single locus of T-DNA. All maternal tissue in the cacao pods produced from the first cross exhibited high levels of GFP expression, including the pod surface, exocarp, placental tissues, and seed coats. Segregation of GFP expression was clearly observed after removal of the seed coats, and very high levels of GFP accumulation occurred in the cotyledons and embryos of the transgenic seeds. From all the crosses, 143 seeds scored positive and 139 negative for GFP expression in the cotyle-
donors. All seeds were germinated and grown in the greenhouse. Leaf and root samples from all of the seedlings were observed for EGFP expression at 6 months after germination. All of the originally GFP-positive seeds produced transgenic spinach (Colocasia esculenta) (Yang et al. 2003). A region of the TaBV genome spanning nucleotides 6,281 to 12 (T1200), including the 30 end of open reading frame 3 (ORF 3) and the intergenic region to the end of the tRNAmet-binding site, was tested for promoter activity along with the other 10 deletion fragments (T600, T500, T250 and T100) (Yang et al. 2003). In transient assays, only the T500 fragment showed activity in the promoter activity in tobacco leaves and roots, with strongest expression observed in the vascular tissue. In transgenic banana leaves, the T600 promoter directed stronger GUS activity than that of the TaBV-derived promoters may be useful for the high-level constitutive expression of transgenes in either monocotyledonous or dicotyledonous species. In transgenic tobacco, the pattern of expression directed by the three promoters was between 4- and 10-fold lower than that of the double CaMV 35S promoter. These results indicate that the TaBV-derived promoters may be useful for the high-level constitutive expression of transgenes in either monocotyledonous or dicotyledonous species. In transgenic tobacco, the pattern of expression directed by the TaBV-derived promoter fragments was assessed via the histochemical GUS staining of plant tissue. A total of six, five, and five tobacco plantlets stably transformed with the T500, T600, T1200, and double CaMV 35S (D35S)-GUS expression vectors, respectively, were examined. The D35S promoter was used as a control in tobacco because it had been shown to be between two and tenfold more active than the unmodified 35S promoter in stably transformed tobacco and was thus considered to be a good comparative marker for TaBV promoter strength. In transgenic tobacco, the D35S, T500, T600 and T1200 promoter fragments all directed GUS expression in leaves, stems and roots of all plants, with more intense blue staining being observed in the vascular-associated cells of the stem and in the root tips. An efficient transformation and regeneration system was established for the production of transgenic spinach (Spinacia oleracea L.) plants (Zhang and Zeevaart 1999). Cotyledon explants were infected with Agrobacterium tumefaciens strain LBA4404 carrying pNPTI, and the reporter gene smgfp, encoding soluble-modified green-fluorescent protein, driven by the CaMV 35S promoter. Southern blot analysis indicated that the smgfp gene was integrated (multiple copies) into the spinach genome while Northern and Western blots showed that the smgfp gene was expressed in progeny plants.

**TOXICITY OF GFP**

Many workers have claimed that GFP is cytotoxic to plant cells. Haseloff et al. (1997) reported that the mgfp4 gene was proving useful as a marker in transgenic Arabidopsis, and it was clear from the initial studies that it could bear improvement. They were able to generate 35S-mgfp4 transformed cells that were intensely fluorescent, and easily detectable by eye under long wavelength UV illumination, which proved difficult to regenerate fertile plants from the brightest transformants. It is possible that very high levels of GFP expression are mildly toxic or interfere with germin-ation. Perhaps the fluorescence is obscuring the fluorescence of other proteins. In addition, the protein is found sequestered in cytoplasmic granules. In contrast, the mature protein is found throughout the cytoplasm and accumulates within the nucleoplasm of transformed Arabidopsis cells. If GFP is a source of fluorescence-related free radicals, for example, it might be advisable to target the protein to a more localised compartment within the plant cell (Heim et al. 1995; Haseloff et al. 1997). This opinion was propagated primarily from anecdotal evidence that Arabidopsis transgenic lines that were the brightest expressers of GFP could not be converted into plants (Haseloff et al. 1997). It was reasoned that photonic disturbance from fluorescence could create free radicals and oxidative damage. This belief was the driving force for the serendipitous targeting of GFP to the ER (Haseloff et al. 1997). In fact this targeting does seem to enhance expression. Many researchers have failed to observe this apparent toxicity in plants (Chiu et al. 1996; Pang et al. 1996; Haseloff et al. 1997; Quaedvlieg et al. 1998).

To specifically address this issue some scientists tested plants for yield drag and biomass decrease associated with GFP synthesis and fluorescence in the field for two growing seasons with three GFP variants; no associations were found (Elliott et al. 1999; Lawton et al. 2000; Li et al. 2001; Zhou et al. 2004). ER targeting was not a factor in toxicity amelioration in the field experiments. Despite the evidence that GFP is not toxic, a recent report showed an association between GFP and apoptosis in mammalian cells (Liu et al. 1999), and the researchers consequently called for more research into GFP toxicity. However, adaptations between animal and plant cells are different. Evidence indicate that plants have a suite of morphological and physiological characters that enable them to deal with light that would sunburn and damage animal cells. GFP is not cytotoxic to plants (Stewart 2001), although some forms of GFP have been reported to be toxic to plant cells either by accumulation in the nucleus (Haseloff et al. 1997) or by protein insolubility (Davis and Vierstra 1998). Various modified forms of GFP – targeted to the ER (Haseloff et al. 1997) or with increased solubility (Davis and Vierstra 1998) – have been developed, and these have showed increased brightness and lower toxicity in Arabidopsis. Using a non-targeted form with normal solubility, Jordan et al. (2000) have seen no evidence of toxicity, with highly fluorescing shoots developing into normal, fertile plants in the case of wheat. A lack of toxicity of the sgfpS637 form of the gene has also been observed in sugarcane by Elliott et al. (1999). This may indicate a difference between monocots and dicots in their ability to tolerate the GFP protein.

**LIMITATIONS OF GFP**

GFP represents many advantages in plant biology, although the study of GFP in plants embodies its own limitations. Among them is the poor expression of GFP in plants. This is the main problem of using GFP as a marker during gene transfection. On the other hand, the high levels of GFP expression can also lead to relatively high toxicity in plants, a factor limiting its wider use of GFP for fundamental and applied plant biology (Haseloff and Amos 1995; Jeffel et al. 1997). The early visualization and identification of transgenic events using GFP fluorescence is allowed without any selection pressure. However, it is difficult to maintain preferential growth of transformed cells, despite the fact that non-fluorescing cells are removed. Many workers suggested that conventional selection is more suitable.
for routine production of transgenic plants (Jordan 2000; Baranski et al. 2006). Other problems of using GFP are the high levels of background green fluorescence which can sometimes be observed in both untransformed (control) and transformed tissues. Further auto-fluorescence of wounded tissues has been found to always be misleading and confusing during genetic transformation (Elliott et al. 1999; Zhou et al. 2008). The use of GFP in higher plants was initially limited by aberrant mRNA splicing (Haseloff et al. 1997) and by protein insolubility (Davis and Vierstra 1998). In addition, some GFP mutants exhibit a more rapid formation of the chromophore and higher excitation peaks at 475 to 490 nm than does the wt GFP protein, which results in increased detection sensitivity (Heim et al. 1995).

CONCLUSION

Green fluorescent protein is increasingly being used in plant biology from the cellular level to whole plant level. At the cellular level, GFP is being used as an in vivo reporter to assess frequency of transient and stable transformation. GFP has also proven to be an invaluable tool to monitor trafficking and subcellular localization of protein. At the organ level and up, many exciting applications are rapidly emerging. GFP is gaining many significant applications and excellent tool in plant biology. Monitoring of GFP during early developmental stages of plant regeneration during genetic transformation allows for the rapid non-invasive identification of transformed cells, therefore, early elimination of non-transformed cells. Hence plant transformation could be faster and less labour intensive and thus cheaper. GFP was also used for the identification of homeoygote or estimation of recombinant protein content in transgenic plants. One interesting example has been the use of GFP to monitor virus movement in and among whole plants. GFP is also emerging as a powerful tool to monitor transgenic movement and transgenic plants in the field. GFP is the first truly in vivo reporter system useful in whole plants. Such new methods represent an additional asset of GFP use to plant transgenisis. GFP protein has been targeted to the endoplasmic reticulum which solves its potential problem by protein insolubility (Davis and Vierstra 1998). In addition, some GFP mutants exhibit a more rapid formation of the chromophore and higher excitation peaks at 475 to 490 nm than does the wt GFP protein, which results in increased detection sensitivity (Heim et al. 1995).

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