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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 (GPF) 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

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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-

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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; Chytilova *et al*. 1999; Baumann *et al.* 1998; Bellucci *et al.* 2003). In stark contrast to most marker genes available, green fluorescent protein (GPF) does not require the addition of any interfering substances like exogenous substrates or enzymes. It allows for the monitoring of transgenic expression from early stages of transformation through the recovery of living transgenic plants (Zolotukhin *et al.* 1996; Chudakov *et al.* 2005). Another advantage of GFP is its relatively small (26.9 kD) size 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 (Halfhill *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 more important in monocot than 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). Niedz *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 *et al.* 1994). The cytoplasm of these cells is densely packed with fine granules that contain the components necessary for bioluminescence (Haseloff and Amos 1995). In other bioluminescent coelenterates these have been characterised as 0.2 μm diameter particles enclosed by a unit membrane, and have been termed lumisomes (Haseloff 1999). The components required for bioluminescence include a Ca^{++}-activated photoprotein, aequorin, that emits blue-green light, and an accessory green fluorescent protein (GFP), which accepts energy from aequorin and re-emits it as green light (Haseloff 1999). GFP is an extremely stable protein of 238 amino acids (Haseloff 1999). The fluorescent properties of the protein are unaffected by prolonged treatment with 6M guanidine HCl, 8M urea or 1% SDS, and two day treatment with various proteases such as trypsin, chymotrypsin, papain, subtilisin, thermolysin and pancreatin at concentrations up to 1 mg/ml fail to alter the intensity of GFP fluorescence (Haseloff *et al*. 1997). GFP is stable in neutral buffers up to 65°C, and displays a broad range of pH stability from 5.5 to 12. The protein is intensely fluorescent, with a quantum efficiency of approximately 80% and molar extinction coefficient of 2.2×10^4 cm^{-1} M^{-1} (after correction for the known molecular weight). GFP fluoresces maximally when excited at 400 nm with a lesser peak at 475 nm, and fluorescence emission peaks at 509 nm (Heim *et al*. 1994; Siemering *et al.* 1996; Haseloff *et al.* 1997).

The intrinsic fluorescence of the protein is due to a unique covalently-attached chromophore which is formed post-translationally within the protein upon cyclisation and oxidation of residues 65-67, Ser-Tyr-Gly (Haseloff *et al.* 1997). Several genomic and cDNA clones of *gfp* have been obtained from a population of *A. Victoria* (Heim *et al.* 1995). The *gfp* gene contains at least three introns, and the coding sequence derived from one of the cDNA clones, pGFP10.1 has been used for protein expression, first in *Escherichia coli*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Haseloff 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 luminescent blue light emitted by another hydromedusas 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 Cheng (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: laser (Sheen *et al*. 1995; Nagatani *et al.* 1997) *versus* incandescent lamp with excitation filters (Hu and Cheng 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 (Haseloff *et al.* 1997; Millwood *et al.* 2003). When the *mgfp4* sequence was inserted behind the 35S promoter and introduced into *Arabidopsis* using the root transformation technique, bright green fluorescent plant cells were detected within 2-3 days of cocultivation. As cell proliferation continued, the brightest clumps of callus and developing shoot tissue were so intensely fluorescent that they were clearly visible by eye, using a 100 Watt long wavelength hand-held UV lamp (UV Products, B100AP). They have also adapted an inverted fluorescence microscope (Leitz DM-IL) to allow more sensitive, higher magnification observation of cells in sterile culture during transformation and regeneration. The microscope was fitted with a filter set (Leitz-D excitation BP355-425, dichroic 455, emission LP460) suitable for the main 395 nm excitation and 509 nm emission peaks of GFP, and they have used a 7 mm threaded extension tube with a 4X objective (EF 4/0.12) to give a greater working distance above the microscope stage. This allows the convenient direct observation of transformed tissues and plantlets within sealed inverted petri dishes (Heim *et al.* 1995; Haseloff *et al.* 1997).

Haseloff *et al.* (1997) observed a wide range of GFP fluorescence intensities in 35S-*mgfp4* transformed plantlets, 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

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 deleterious effect due to GFP (Ghorbel *et al.* 1999; Galperin *et al*. 2003). 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 continual 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. The protein is excluded 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. Localisation of the modified protein to cytoplasmic organelles was also evident, to what appear to be large leucoplasts or proplastids. 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 proplastids 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 proplastids, 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 inefficiently 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 neglected signal peptide may act as a transit sequence for plastid entry. Alternatively, there may be some direct exchange between developing plastids and the endomembrane system. Haseloff (1999) also noticed no free cytoplasmic fluorescence, and the protein is sorted very efficiently to the ER or to plastids.

However, several researchers reported that mGFP4 was not very stable in its fluorescence, especially under field conditions, even though it was expressed in the plant at levels that should have yielded visible green fluorescence. A similar synthetic human codon-optimized GFP with a wt chromophore was created by Haas *et al.* (1996) also in which the crypton intron was eliminated. Since humans and corn have a very similar codon usage, the gene proved to express well in plants. When it was expressed in plants it yielded 20 times more fluorescence than the wt gene (Chiu *et al*. 1996). GFP possesses a rigid structure with a broad stability range in pH 5-11 at temperatures up to 65°C (Tsien 1998). It maintains its fluorescence even in the presence of strong denaturing agents such as 6 M guanidine HCl, 8 M urea or 1% sodium dodecyl sulphate (Yang *et al.* 1996). GFP is increasingly a popular reporter gene in plant biology, which includes the study of the expression patterns of promoters (Sheen *et al*. 1995; Shiina *et al*. 2000), disease tracking (Itaya *et al*. 1997), developmental studies (Misteli and Spector 1997), expression studies and ecological monitoring of transgene spread (Halfhill *et al*. 2001).

The transient and stable expression of wt and various modified versions of GFP have been reported for different plant species including monocots and dicots (Haseloff and Amos 1995; Hu and Chenge 1995; Sheen *et al*. 1995; Chiu *et al*. 1996; Davis and Viestra 1998; Vain *et al*. 1998). The fluorescing chromophore of GFP is formed by post translational modification in which a tripeptide Ser65-Tyr66-Gly67 is cyclized and later oxidized. This chromophore is in the geometric centre of the protein to which it is covalently attached (Shinomura 1979; Cody *et al*. 1993). GFP represents a new class of proteins called 'beta can'. Wt GFP is a dimer consisting of two monomer units, each consisting of 238 amino acids with a relative molecular weight of 27 kDa. GFP does not require any endogenous cofactors and substrates or exogenous compounds for fluorescence manifestation because the formation of the chromophore is either an autocatalytic process or it requires only ubiquitous cellular components (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 Amos 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 Amos 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; Kaeppler *et al*. 2000; Murray *et al*. 2004). Therefore, modifications in GFP have been made using various mutagenesis schemes. Mutants have been reported that improve fluorescent 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 could also be sufficient (Elliot *et al*. 1999; Li *et al.* 2001). GFP can be visualised 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 monitoring of whole plant cells within growing plant tissue, and (3) for the identification of individual transgenic plants expressing GFP (Haseloff and Amos 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 minimise any deleterious effects of GFP expression. Haseloff *et al.* (1997) have found the optimised *mgfp5-ER* gene very useful for this type of experiment. The dynamic properties of labelled cells or subcellular features can be resolved at high resolution in whole plant 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 a vital fluorescent reporter with high resolution optical techniques shows much promise for use in cell biological and physiological experiments.

On the other hand in case of other gene markers such as the bacterial enzyme β-glucuronidase, which is coded by the *E. coli uidA* (*gusA*) gene is the most widely used reporter in plants. The enzyme utilizes the external substrates 4-methyl umbelliferyl glucuronide (MUG) for measurements of specific activity and 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for histological localization (Jefferson *et al*. 1987). It is therefore a conditional non-selectable marker gene. Luciferase as a reporter, showed the capability of monitoring gene expression patterns non-destructively in real time with great sensitivity. For example, this allows the continuous monitoring of gene activity during development. The firefly (*Photinus pyralis*) luciferase catalyzes the ATP-dependent oxidative decarboxylation of luciferin. After the reaction occurs the luciferase is inactive until the oxyluciferin is released from the enzyme complex. This is a slow process and the LUC half life is very short; thus, it is believed that LUC activity more accurately reflects transcriptional activity than some other reporter genes that are more stable and accumulate over time. Luciferase is often used with other marker genes as an internal control and is also used as a visual marker of transformation for the manual selection of transgenic material undergoing selection (Ow *et al.* 1986; Miki and McHugh 2004; Tian *et al*. 2006). The bacterial enzyme β-galactosidase, which is coded by the *E. coli lacZ* gene, has been a useful marker gene in many cell systems because it can be easily assayed and can form N-terminal translational fusions with other proteins. This enzyme also uses the synthetic substrate *O*-nitro-phenyl-β-D-galacto pyranoside (ONPG) and tissues that express the enzyme will stain with 5-bromo-4-chloro-3-indoyl-β-D-galactosylpyranoside (X-Gal). Oxalate oxidase activity has a narrow range of expression in cereals and appears to be absent in dicots. The wheat gene coding for OxO can function as a conditional reporter gene for monocot and dicot species. The assay depends on the relatively inexpensive substrates, oxalic acid and 4-chloro-1-naphthol and permits rapid histochemical localization of enzyme activity. Quantitative measurements of OxO enzyme activity can also be performed (Miki and McHugh 2004). The maize *R*, *C1*, *P1* and *B* transcription factor genes regulate the anthocyanin biosynthetic pathways in specific plant tissues. Ectopic expression of *R* or *B* initiated the non-selective accumulation of anthocyanins in plant cells raising the potential use of the transcription factors as non-conditional reporter genes that do not require the application of external substrates or destructive assays. Although the *R*, *C1* and *B* transcription factor genes showed promise as visible markers for optimizing transformation methods, expression of the genes was toxic to transformed cells and expression was subject to environmental stimuli. The system has therefore not been extensively adopted as a marker gene system (Miki and McHugh 2004; see Teixeira da Silva 2006 for a few reviews).

Much of the success of GFP as enabling technology in transgenic plants hinges on the success of seeing GFP in plants. In laboratory work most researchers use epi-fluorescence microscopes fitted with mercury lamps (approx. 100 W) with blue filters (e.g., 470/40 nm) equipped with 515 nm long-pass emission filters. Of course, without the emission filters, one only sees blue reflectance. In using such arrangements several researchers have reported background fluorescence that interferes with observing GFP (Hass *et al.* 1996; Elliot *et al*. 1999; Jeoung *et al.* 2002; Hraska *et al.* 2005). Altering the filter choices, such as choosing emission filters of a narrower band width, or alternative emission filters helps. Empirical optimization by plant species and tissue types may need to be performed when using blue light-excited GFPs. If one desires to visualize whole plants or organs, then a microscope is not the best tool. The fluorescence excitation spectrum of GFP exhibits peaks at wavelengths of 395 and 475 nm, with the 395 nm peak predominating (Haseloff 1999). The variant, mGFP5, has dual excitation peaks at 395 and 475 nm and an emission peak at 509 nm. This is a useful property for simple detection of the protein using a longwavelength 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 photonics of a microscope system; Lightools (Encinitas, Calif) produces a blue light source with the proper cutoff or band-pass 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-150 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 (Snape 1998; Sunilkumar *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), DNA integration patterns and their efficiency (Snape 1998). GFP expression has been successfully used as an efficient tool for the evaluation and subsequent modification of various parameters and procedures associated with particle bombardment transformation, such as selection of the appropriate tissue to be bombarded (Huber *et al*. 2002; Tee *et al*. 2003), modification of gene gun settings (Richards *et al*. 2001), optimization of bombardment parameters (Jordan 2000) 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 (Kamate *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 for transformation. Furthermore, GFP can be easily detectable at all stages of development during transformation, but it can sometimes be confused with a false auto-fluorescence of wounded tissues (Molinier *et al*. 2000; Kim *et al.* 2004). Other problems are that, in some cases, transformed tissue could possess such strong auto-fluorescence that green fluorescence could not be easily distinguished (Knapp *et al.* 2001; Hraska and Rakousky 2005). Maize cell walls were observed to autofluoresce, probably due to their lignin content (van der Geest and Petolino 1998). This was much less of an issue with *Arabidopsis* transformed with a similar construct. Although toluidine blue staining largely circumvented this problem, a modified GFP with different fluorescence properties would be more desirable (van der Geest and Petolino 1998). The autofluorescence could perhaps be avoided by using a different excitation wavelength. Heim *et al*. (1994) found that by substituting certain amino acids in the fluorophore of the protein, mutated versions of GFP with altered excitation and emission spectra could be generated. Indeed, brightgreen fluorescence was observed upon excitation with 490-nm light in transgenic maize with a GFP construct modified such that the serine at position 65 was replaced with a threonine or a cysteine (van der Geest and Petolino 1998). GFP fluorescence signal is usually visible within a few hours of co-cultivation, and also decreases within few days (Jeoung *et al*. 2002). A few studies also reported that the level of GFP fluorescence differs depending on the target genotype and tissue, *gfp* variant and the promoter used (Cho *et al*. 2003; Hraska *et al*. 2006). It was also found that GFP fluorescence is usually visible in new young tissues and organs but that it declines to give a weak signal in older ones. This might be due to the presence of increasing content of chlorophyll, which possess strong red autofluorescence or other fluorescing compounds (Kamate *et al*. 2000; Zhou *et al*. 2004). This also leads to the conclusion that the expression of *gfp* might be influenced by the positional effect of inserted transgenes or by co-suppression due to a higher transgene copy number (Tamura *et al*. 2003). Another cause of GFP fluorescence quenching in older leaves may be the change in cytoplasmic density of cells. Other possible reasons for the poor expression are developmental or cell-specific expression of the 35S promoter, dilution of GFP content in dividing and growing cells or gene silencing (Voinnet and Baulcombe 1997; Ponappa *et al*. 1999; Zhou *et al*. 2004). GFP can be visualized in mature plant tissues macroscopically in real time. Therefore, for the first time, gene expression can be simultaneously assessed in all plant tissues. In tobacco grown in both the greenhouse and field, GFP expression patterning is essentially the same as GUS when both genes are under the control of the 35S promoter (Harper and Stewart 2000). Very similar results have been obtained for canola (Halfhill *et al.* 2001). Young leaves, roots, and vascular tissue had particularly high expression. Similar results were obtained with artificial light-grown *Arabidopsis* and *Lotus japonica* when a GFP (EGFP or sGFP-S65T) GUS fusion was expressed under the control of the 35S promoter (Quaedvlieg *et al.* 1998).

GFP has rapidly become a standard reporter in many biological systems (Abelson and Simon 1999), although it can be toxic under high light conditions in plants that express high levels of the protein (Haseloff *et al*. 1997). GFP has been proved extremely useful as a reporter of plant gene expression in living cells, and has several advantages over luciferase, the other commonly used *in vivo* reporter. Luciferase activity is readily visualized in living plants and can be detected with great sensitivity; however, uneven penetration of luciferin substrate through plant tissues sometimes makes it difficult to interpret spatial patterns of luciferase activity, and most detection systems produce only relatively low resolution images. But GFP does not require an externally applied substrate and is easily visualized at higher resolution even at the subcellular level (Mercuri *et al.* 2001; Halfhill *et al*. 2007). GFP expression in *A. thaliana* increased after altering the codon usage (mGFP) in the region that is incorrectly spliced, 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 widely used 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 vacuum infiltration and transformants were obtained with about 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 white 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 (*npt*II) 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-conditional reporter is the direct visualization of GFP in living tissues in real time without invasive procedures 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 would be extremely helpful to have a 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 in intact whole-root tissues of *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 *pgfp* 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. However, 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 virus X* and *Tobacco 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 in the environment, and it is desirable to track 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 maternal 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 reversion 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 homoplasmic 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, transplastomic plants were produced using a GFP-antibiotic resistance marker fusion gene (Pang *et al*. 1996; Khan and Maliga 1999). This approach helped the researchers visualize recovered chimeric plants and also plastid segregation within plants. GFP was synthesized at very high levels.

Very recently, Hraska *et al*. (2008) defined the fluorescence patterns of GFP in the leaves of transgenic tobacco plants, using a simple method of image analyses. Various variables in the fluorescence were identified based on the leaf tissue type selected for the investigation. Based on these results, it was evident that the GFP manifestation differs in various leaf tissues and in leaves of different physiological ages, although the exact physiological reasons are still debatable. This fact strengthens the necessity to perform comparative studies of GFP fluorescence/promoter activity using the same methodology for all plants and tissues of comparable physiological age or developmental stage. Moreover, the influence of each individual plant within cloned genotypes was revealed. It is evident that the origin/position of investigated leaf tissue could affect the interpretation of data obtained based on the detection of GFP fluorescence. 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 study should be provided with a wider numbers of individuals, clones or populations (Hraska *et al*. 2008). This study also showed that the origin of leaf tissue in tobacco plants selected for the GFP quantification is crucial and that the fluctuations in the fluorescence intensity should be taken into account when comparing the GFP fluorescence patterns of different plants. Moreover, the degree of fluorescence variability seemed to be individually affected in tobacco plants (Hraska *et al.* 2008). Regardless of the exact cause, some reports indicate that the expression and/or the detection of marker genes differ in various developmental stages and tissues of plants (Halfhill *et al.* 2003). Therefore, it is evident that the selection of proper tissue for marker gene quantification is a crucial point for accurate study of GFP fluorescence and can affect the final interpretation of obtained data and their reliability (Hraska *et al.* 2008).

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 often in monocots, while mGFP5-ER and wt chromophore GFPs have been more frequently used in dicots. Ponappa *et al.* (1999) found a greater number of transient GFP spots of smRS-GFP than of smGFP-ER that is targeted to the ER; spots of the latter were greater than those of the former. Generally if GFP accumulates and can be visualized, then the choice of GFP variants is not likely to be critical. Stewart (2001) reported the use of mGFP5-ER for most applications because of its dual wavelength excitation, which gives flexibility in excitation. For field work, the combination of the heat-stability mutations and ER targeting may allow it to be better expressed and fluorescent in the field under hot, summer conditions (Harper *et al.* 1999). The best GFP today will not be the best GFP in the near future as new GFPs are being discovered and mutagenized. The technology advances will lead to brighter GFPs in the future.

EXPRESSION OF GFP IN PLANTS: CASE STUDIES

Model plants: Tobacco, *Arabidopsis* and carrot

Hraska *et al.* (2008) reported the study of GFP fluorescence intensity using the T_1 generation of transgenic tobacco (*Nicotiana tabacum* L. cvs. '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 particular genotype and environment. Their study also showed that the origin of leaf tissue selected for the GFP quantification is crucial, and that the fluctuations in the fluorescence intensity should be taken into account when comparing the GFP fluorescence patterns of different plants. Moreover, the degree of fluorescence variability seems to be individually affected in transgenic tobacco expressing the *m-gfp5*-ER gene.

GFP expression also coupled with some limitations, and obstacles. First, the loss or the quenching, of the fluorescence signal in older tissues, especially leaves, usually occurs (Hraska *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-

Table 1 Examples of genetic transformation experiments using various variants of *gfp* gene.

Plant species	Transformed tissue	References
Abies fraseri, *A. nordmanniana*	Mature zygotic embryos	Tang and Newton 2005
Acanthopanax sciadophylloides	Mature zygotic embryos and leaf explants	Taniguchi *et al.* 2004
Allium cepa	Immature embryos	Eady *et al*. 2000
A. porrum	Immature embryos	Eady *et al*. 2005
A. sativum	Immature embryos	Eady *et al*. 2005
Antirrhinum majus	Hypocotyls	Cui *et al.* 2003
Arabidopsis thaliana	Roots	Haseloff *et al*. 1997; Moseyko and Feldman 2001; Darnowski and Vodkin 2002
Artemisia annua	Leaf explants	Han *et al*. 2005
Astragalus sinicus	Cotyledons	Cho and Widholm 2002
Avena sativa	Shoot meristem	Cho *et al*. 2003
Banana (Musa species)	Embryogenic tissue	Yang *et al.* 2003
Brassica campestris	Cotyledons	Malyshenko *et al*. 2003
B. napus (canola)	Calli	Richards *et al.* 2003
B. rapa	Cotyledons	Wahlroos *et al*. 2003
Cajanus cajan	Mature embryo axis	Mohan and Krishnamurthy 2003
Carica papaya	Embryogenic calli	Zhu *et al.* 2004
Castanea dentata	Somatic embryos	Polin *et al.* 2006
Chamaecyparis obtuse	Zygotic and leaf explants	Taniguchi *et al.* 2004, 2005
Citrus aurantium	Stems and epicotyls	Ghorbel *et al.* 1999
Conyza canadensis	Seedling explants	Halfhill *et al*. 2007
Cryptomeria japonica	Zygotic explants and leaf explants	Taniguchi *et al.* 2004
	Embryogenic tissue	Taniguchi *et al.* 2008
Cucumis melo	Cotyledons	Galperin *et al*. 2003
Daucus carota	Root discs	Baranski *et al.* 2006
Dendrobium spp.	Tips of influorescence, various types of calli	Tee *et al*. 2003
Festuca arundinacea	Embryogenic calli	Wang and Ge 2005
Glycine max	Embryogenic tissue	Ponappa *et al*. 1999; Nishizawa *et al.* 2006
Helianthus annuus	Shoot apices	Weber *et al.* 2003
Hordeum vulgare	Immature embryos	Cho *et al.* 2002
	Microspore culture	Carlson *et al*. 2001
	Embryogenic calli	Ahlandsberg *et al*. 1999
Ipomoea batatas	Protoplasts and somatic embryos	Lawton *et al.* 2000
Juglans regia	Embryos	Escobar *et al.* 2000
Lactuca sativa	Coleoptiles, calli, leaf explants	Elliot *et al*. 1999
Larix kaempferi	Zygotic explants	Taniguchi *et al.* 2004
Malus domestica	Leaf explants	Maximova *et al*. 1998
Medicago truncatula	Cotyledons, embryo axis	Zhou *et al.* 2004
	Floral organs	Kamate *et al*. 2000
Nicotiana tabacum	Zygote	Li and Yang 2000
	Leaf discs	Molinier *et al.* 2000
	Embryogenic tissue	Yang *et al.* 2003
	Leaf discs	Chen *et al*. 2005
	Coleoptiles, calli, leaf explants	Elliot *et al*. 1999
Oryza sativa	Immature embryos	Vain *et al*. 1998
	Embryogenic callus	Sallaud *et al*. 2003
	Leaves	Lu *et al*. 2008
Picea glauca, *Pinus mariana*, *P. strobus*	Embryogenic tissue	Tian *et al*. 1997, 1999
Pinus densiflora	Mature zygotic embryos	Taniguchi *et al.* 2004
P. thunbergii	Mature zygotic embryos	Taniguchi *et al.* 2004
P. virginiana	Mature zygotic embryos	Tang and Newton 2005
Prunus armeniaca	Leaves	Petri *et al.* 2004
P. persica	Embryo sections	Perez-Clemente *et al.* 2004
P. salicina	Hypocotyl	Urtubia *et al.* 2008
Punica granatum	Zygotic embryos and leaf tissues	Terakami *et al.* 2007
Pyrus communis	Leaf explants	Yancheva *et al*. 2006
Rhododendron spp.	Leaves	Knapp *et al.* 2001
Rosa hybrida	Embryogenic calli	Kim *et al.* 2004
Saccharum spp.	Coleoptiles, calli, leaf explants	Elliot *et al*. 1999
Sorghum spp.	Embryogenic calli	Jeoung *et al*. 2002
Spinacia oleracea	Cotyledons	Zhang and Zeevaart 1999
Theobroma cacao	Cotyledons	Maximova *et al.* 1998, 2003
Triticum aestivum	Embryos	Jordan 2000
	Immature embryos	Huber *et al*. 2002
Verbena × hybrida	Shoots	Tamura *et al*. 2003
Vigna angularis	Epicotyls	Yamada *et al*. 2001
Vitis vinifera	Somatic embryos	Li *et al*. 2001
	Leaf tissues	Hraska *et al.* 2008; Zottini *et al.* 2008
V. rotundifolia	Leaf tissues	Dhekney *et al.* 2008
Zea mays	Embryogenic calli	van der Geest and Petolino 1998
	Coleoptiles, calli, leaf explants	Elliot *et al*. 1999

orescence 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 strong enough to conclude the dominant role of the chlorophyll only. Therefore, it seems that the GFP fluorescence intensity may be affected by synergic incidence/action 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 (Halfhill *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.* 2003). Although 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 or using mixture of two *A. tumefaciens* strains were compared, and showed that the double T-DNA vector method could produce marker-free transgenic tobacco (*N. tabacum*) plants more efficiently (**Table 1**). A dual marker double T-DNA vector was then constructed by assembling the green fluorescent protein (GFP) gene *mgfp5* and the *npt*II gene into the same T-DNA driven under the control of CaMV 35S promoter. The frequency of cotransformants produced by this vector was 56.3%. Coexpression of *mgfp5* and *npt*II was found in 28 out of 29 T1 lines, and segregation of the reporter GUS gene, *gusA*, from *mgfp5* to *npt*II was found in 12 out of 29 T1 lines. Therefore, GFP could be used as a vital marker to improve the transformation efficiency and to easily monitor the segregation of marker genes, thus facilitating screening of marker-free progeny. This work has succeeded in constructing a dual marker (*mgfp5-nptII*) double T-DNA vector system and demonstrating that GFP can be a vital marker to improve the efficiency of tobacco transformation and monitor the segregation of the marker gene *nptII*, thus facilitating screening of marker-free progeny. Since GFP has been used successfully as a vital marker in many transgenic plant species (Maximova *et al.* 1998; Escobar *et al.* 2000; Lawton *et al.* 2000; Li and Yang 2000; Zhou *et al.* 2004), it can be concluded that this dual marker double T-DNA vector approach might be widely applicable for use in producing marker-free transgenic plants for many crop species. In addition, GFP might also be combined with other high efficiency co-transformation systems or site-specific recombination systems to easily screen marker-free transgenic plants (Chen *et al.* 2005).

Expression of GFP linked to an actin binding domain is a commonly used method for live cell imaging of the actin cytoskeleton. One of these chimeric proteins is GFP-mTalin (GFP fused to the actin binding domain of mouse talin) (Ketelaar *et al.* 2004). Although it has been demonstrated that GFP-mTalin colocalizes with the actin cytoskeleton, its effect on actin dynamics and cell expansion has not been studied in detail. Therefore, Ketelaar *et al.* (2004) created *A. thaliana* plants harbouring alcohol-inducible GFP-mTalin constructs to assess the effect of GFP-mTalin expression *in vivo* (**Table 1**). They have also focused on the growing root hair as this is a model cell for studying cell expansion and root hair tip growth that requires a highly dynamic and polar actin cytoskeleton. Their results showed that alcohol-inducible expression of GFP-mTalin in root hairs causes severe defects in actin organization, resulting in either the termination of growth, cell death, and/or changes in cell shape. Fluorescence recovery after photobleaching experiments demonstrates that the interaction of GFP-mTalin and actin filaments is highly dynamic. To assess how GFP-mTalin affects actin dynamics we performed cosedimentation assays of GFP-mTalin with actin on its own or in the presence of the actin modulating protein, actin depolymerizing factor. They also indicated that the GFP-mTalin does not affect actin polymerization but that it does inhibit the actin depolymerizing activity of actin depolymerizing factor. These observations demonstrate that GFP-mTalin can affect cell expansion, actin organization, and the interaction of actin binding proteins with actin.

Darnowski and Vodkin (2002) reported the use of a derivative of GFP to directly label the plant vacuole in live, unfixed tissues of stably transformed transgenic plants. They used the developmentally regulated soybean seed lectin promoter and the 32 amino acids of the soybean lectin amino terminal signal sequence to create an in-frame fusion polypeptide with GFP (pLGFP5). This construct was transferred into *A. thaliana* by vacuum infiltration, and the transformed lines were characterized by DNA blotting and immunoblotting to detect the presence and expression of the GFP gene. GFP fluorescence was detected in the protein storage vacuoles of developing *Arabidopsis* embryos with the objective of labeling storage vacuoles in developing seeds when imaged by fluorescence microscopy. Very little signal was detected in any other compartments including the cell wall. Thus, despite the absence of vacuolar sorting signals in GFP and other foreign proteins fused to the lectin sequence, the 32-amino-acid lectin signal sequence has general utility to direct foreign proteins to the protein storage vacuoles in seeds. The plant vacuole serves as a storage site for many products, including the storage proteins of the seed that supply energy during germination of the seedling. In general, lines that were transformed continued to express the GFP-lectin gene in a consistent pattern through several generations. LGFP5 fluorescence could be seen both in both epidermal cells and in subepidermal cells, though most of the cells shown were epidermal cells. GFP expression in both the GFP5ER and the LGFP5 lines was sometimes patchy, with strong expression in some cells and weak or no expression in others. In a very few cells found in only several of the more than 80 embryos examined, a small number of cell walls showed a yellowish-green autofluorescence that was difficult to distinguish from GFP fluorescence. Such fluorescence was aberrant with respect to the overall pattern of LGFP5 expression and could have been phenolic autofluorescence due to cellular damage. Otherwise, all of the LGFP5-expressing cells examined showed a pattern of subcellular localization. GFP signal occurred in the large vacuolar body that filled most of the cell volume of those

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 genomics scale by random fusion to cDNA libraries.

Moseyko and Feldman (2001) first reported the use of GFP as a pH reporter in plants. Proton fluxes and pH regulation play important roles in plant cellular activity and therefore, it would be extremely helpful to have a plant gene reporter system for rapid, non-invasive visualization of intracellular pH changes. In order to develop such a system, they constructed three vectors for transient and stable transformation of plant cells with a pH-sensitive derivative of GFP. Using these vectors, transgenic *A. thaliana* (Columbia and Wassilevskija ecotypes), and tobacco (*N. benthamiana*) plants were produced (**Table 1**). Here the application of pH-sensitive GFP technology in plants is described and, for the first time, the visualization of pH gradients between different developmental compartments in intact whole-root tissues of *A. thaliana* was reported. The utility of pH-sensitive GFP in revealing rapid, environmentally induced changes in cytoplasmic pH in roots was also demonstrated. Two binary plasmid vectors, pHGFP-35S and pHGFP-SP, were constructed for stable plant transformation with the *phGFP* gene under the control of the CaMV 35S and the chimeric *(ocs)3mas* promoters, respectively. On average, the plants transformed with the *phGFP* gene under the control of the chimeric *(ocs)3mas* promoter show brighter fluorescence in comparison with plants transformed with this gene under the control of the CaMV35S promoter. As a rule, fluorescence was readily detectable in roots and hypocotyls, and to a lesser extent in other tissues such as leaves and stems. These authors demonstrated that phGFP: (a) was expressed at a high level, sufficient for ratio imaging in *A. thaliana* and tobacco plants; (b) was suitable for cytoplasmic pH measurements in plants; (c) showed an additive effect of aluminium ions on cytoplasmic acidification at low extracellular pH; (d) could be applied for non-invasive monitoring of pH dynamics in individual plant cells as well as in whole plant tissues; and (e) showed for the first time the existence of significant pH gradients between different developmental regions in roots of *A. thaliana* (**Table 1**).

Using tobacco (*N. tabacum* cv. 'Samsun NN') as a model system, Molinier *et al*. (2000) demonstrated that GFP can be used as a visual selection marker for transformed tissues. Based on differences in the intensity of GFP fluorescence, homozygous and hemizygous states could be easily visualized in seeds and seedlings of the T_1 generation. These results were confirmed by genetic analysis in tobacco (Molinier *et al.* 2000), in which *A. tumefaciens* strain LBA 4404 was transformed by electroporation with plasmid pHB2892 (Molinier *et al.* 2000). This plasmid contains the S-GFP gene, optimized for human codon usage under the control of the double CaMV 35S promoter. The study by Molinier *et al*. (2000) indicated that although GFP is naturally absent from wt tobacco plants, autofluorescence with similar spectral characteristics does occur in wounded tissues. For this reason, unambiguous distinction was difficult between leaf discs treated with *Agrobacteria* carrying pHB2892, a plasmid encoding GFP, and those treated with a control construct, immediately after co-culture (Rouwendal *et al*. 1997). However, 2 weeks after transformation, the characteristic green fluorescence was easily detectable in the calli developing on transformed leaf discs. Although weak yellowish or greenish fluorescence occurred in control calli, the identification of GFP-expressing calli was unambiguous. In contrast, the red chlorophyll autofluorescence limited the detection level for GFP fluorescence in shoots regenerating from GFP-positive calli when equipment with long-pass cut-off filters was used. The use of appropriate bandpass filters alleviates this problem in tobacco. Under 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. In contrast to the results of Hasseloff *et al.* (1997) who observed a reduced regeneration frequency from 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 root system of wt tobacco seedlings (**Table 1**). This is in contrast to the roots of *Arabidopsis* where no such artifactual fluorescence was detected (Hasseloff *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 without specialized equipment allowing quantification of the fluorescence levels. Nevertheless, the ratio of fluorescing 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 T_1 plants belonging to the high-fluorescence class were uniform and 100% of the analyzed seedlings showed a high level of fluorescence. Such T_1 and T_2 tobacco plants thus appear to be homozygous for the T-DNA. Similarly, the progeny of the T_1 plants belonging to the non-fluorescing class were uniformly lacking the fluorescence characteristic of GFP. The non-fluorescing class therefore, corresponds to wt segregants. T_1 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 hemizygous plants. Thus, simple observation 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

screen.

Haseloff and Amos (1995) reported that GFP accumulated in the cytoplasm and nuclei of *Arabidopsis* leaf cells but was omitted from the nucleolus, vacuoles, components of the endomembrane system, and small organelles. Later, cytoplasmic streaming and the movement of organelles in *gfp*-expressing cells of *Arabidopsis* were observed (Haseloff *et al.* 1997). The option exists to remove a small number of fluorescent cells for examination of cellular infrastructure under higher magnification. However, for routine transformation studies, the accumulation of GFP in plant nuclei acts as a quick and significant indicator of plant transformation (Elliot *et al.* 1999). 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 sugarcane plants (Elliot *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 cell clusters 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 (pEmuKN), 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 of the GFP gene was obtained in electroporated 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, 8 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 discs 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).

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 (GAPA1::YFP) (YFP=yellow fluorescent protein) and mitochondria (GFP) (Zottini *et al.* 2008). By combining different genotypes and physiological conditions, they have developed a protocol for efficient transformation of selected grapevine cultivars. Among the four cultivars analyzed 'Sugraone' and 'Aleatico' exhibited high levels of transient transformation. Transient expression occurred in the majority of grapevine cells within the infiltrated tissue several days after agroinfiltration and, in a few cases; it later spread to a larger portion of the leaf. Three different *A. tumefaciens* strains viz. LBA4404, GV3101 and AGL1 to the cloning vectors pBI121, pAVA554, and pGreen 0029 having 35S as the promoter with different virulence levels were used for agroinfiltration assays on grapevine plants. Confocal microscope analyses demonstrated that these subcellular compartments could be easily visualized in grapevine leaf cells. In addition, from leaves of cv. 'Sugraone', agroinfiltrated with ER-targeted GFP-construct, stable transformed cells were obtained that show the opportunity to convert a transiently transformed leaf tissue into a stably transformed cell line in grapevine. By comparing the fluorescent intensities of the regions expressing the GFP, an increase of the fluorescent signal from 6 to 12 days was observed in grapevine cells. The three *Agrobacterium* strains have given similar results in grapevine. The availability of different fluorescent proteins, with characteristic excitation and emission spectra, allow the simultaneous visualization of two or more different fluorophores in the same transformed cells of grapevine.

A GFP/neomycin phosphotransferase II (*gfp*/*nptII*) fusion gene that allowed for simultaneous selection of transgenic cells based on GFP fluorescence and kanamycin resistance was used to optimize parameters influencing genetic transformation in *Vitis rotundifolia* (Dhekney *et al.* 2008). Transgenic plants exhibited uniform GFP expression in cells of all plant tissues and organs including leaves, stems, roots, influorescence and the embryo and endosperm of developing berries. They used *A. tumefaciens* strain EHA105 with CsVMV promoter during the transformation of *V. rotundifolia*. No visual difference in GFP expression was observed among transgenic plants with varying transgene copy number in *V. rotundifolia*.

Activity of three constitutive promoters and enhanced derivatives in transgenic grape (*V. vinifera* L. cv. 'Thompson Seedless') was characterized using a bifunctional fusion marker containing the enhanced GFP (EGFP) and *npt*II genes (Li *et al.* 2001). Relative differences in transient GFP expression and stable transformation efficiencies were used to compare promoter activity. Expression patterns in transformed somatic embryos revealed that the ACT2 promoter from *A. thaliana*, previously shown to be a strong constitutive promoter in *A. thaliana* and other species, failed to promote strong expression in grape (Li *et al.* 2001). In contrast,

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 enhanced double CaMV 35S promoter was highly 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 by the ACT2 promoter precluded the recovery of any transgenic grape SEs under selection conditions. The cause for lack of expression activity with the ACT2 promoter in grape SE cells remains unknown, although the ACT2 promoter may be subjected to developmental and tissue-specific regulatory control in grape SE cells. This has been shown to be the case in tobacco (*N. tabacum* cv. 'Dynes') in which high levels of promoter activity were mainly associated with vegetative tissues. That is, regulatory *cis*-elements within the *A. thaliana* ACT2 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 tissues, this promoter produced relatively low levels of expression in roots of transgenic grape plants through all developmental stages. 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 mesophyll cells 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 *as*1 motif and the synergistic interactions between these elements in the CsVMV promoter, as in the case of the CaMV 35S promoter (Li *et al.* 2001). However, the number and the relative position of both *as*1 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 *npt*II 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 the maximum transformation rate (transformation rate = number of GFP-positive explants/number of explants × 100) with GFP expression in the transgenic shoots of pomegranate. Transient GFP activity was efficiently observed in adventitious shoots, particularly in the shoots inoculated with EHA105. Transient GFP activity was observed to a slight extent in calli (60-day-old cultures) but gradually disappeared at the time of selection. Transient GFP activity was not detected in leaf segments and 30-day-old cultures of 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 transformants at each stage of plant development and in the T_1 generation of pomegranate. 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/ 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 (T_1 generation) were obtained from four self-pollinated T_0 plants, and the GFP assay was performed for the investigation of transgene segregation. However, Terakami *et al.* (2007) showed GFP fluorescence in T_1 plantlets. The inheritance of *gfp* gene indicated that T_0 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 *npt*II genes. High expression levels of GFP were detected in transgenic cells as early as 7 days after transformation. GFP marked callii and transformed plants were observed after 14 and 24 days, respectively. Fluorescence microscopy screening of transformed plant material, under the selection of kanamycin, increased the transformation frequency 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, strong GUS staining was detected in the whole plant. Following transformation with pPZP, the *npt*II 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 microprojectile bombardment of 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 (mgfp50), 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* gene was sufficiently bright and distinct under UV light that it could easily be used for the isolation of transformed segments of the callus. The brightness of fluorescence was maintained at full intensity during the subculture, growth, and development of the cultures until the differentiation of the small shoots regenerated from the cultures. The regenerated shoots showed fluorescence, but the intensity was reduced. They did not mentioned the reason for this reduction but suggest that it might appear to be diminished by one or more factors such as differential GFP expression, dilution of GFP during cell expansion, and interference from chlorophyll autofluorescence. Note that the cell cultures did not exhibit any autonomous fluorescence so that it was not necessary to filter out background fluorescence. This was not the case after plants had regenerated. The regenerated green plants exhibited the typical red autofluorescence of chlorophyll in both the non-transformed control plants and in the *gfp*-transformed plants. In the plants transformed with *gfp*, the red autofluorescence 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-*npt*II-Noster cassette as a selectable marker and the CaMV35Spro-*sgfp*-CaMV35Ster 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 and, after selection, transgenic shoots were regenerated. Shoots that survived exhibited high-level of sGFP expression mainly visible in the young leaves of the apex. *In vivo* monitoring of GFP expression permitted an early, rapid and easy discrimination of both transgenic and escape buds. After elimination of escapes, transgenic shoots were rooted *in vitro* and the recovered plantlets were screened using PCR amplification. Southern analysis confirmed stable genomic integration of the *sgfp* transgene. The high levels of GFP expression were also maintained in the second generation of transgenic peach plants. GFP expression was stable during the development of the transgenic plants and green fluorescence was easily detected in young tissues shoot apices, young leaves and root tips, but also in the different flower tissues, like petals and carpel. The stability of GFP expression was maintained in the transgenic peach plants after 2 years in the greenhouse not only in the vegetative tissues but also in the flowers and in the resulting fruits and seeds. The high levels of GFP expression were also maintained in the second generation of plants originated after germination of the transgenic seeds, indicating the stability of transgenes. *In vivo* fluorescence could be an easily scorable marker for the ecological monitoring of transgene dispersion through the pollen and to investigate transgene persistance and stability in woody perennial plants which are clonally propagated and grown over long periods of time in the field. The fluorescence intensity varied among the different parts of the transgenic plants. In old tissues, lower metabolic activity and chlorophyll accumulation partially masked the green fluorescence provided by GFP and differences in fluorescence intensity could be observed associated with different GFP expression levels in apical leaves from independent transgenic lines. It has been suggested that GFP can be used as a reporter of gene expression for the early non-destructive identification and selection of transgenic buds expressing the highest levels of protein. In some rare cases, transgenic peach shoots showed sectorial expression of GFP. These chimeric plantlets were derived in origin from a mixture of transgenic and non-transgenic cells. Transgenic chimeras can be identified at very early stages of development and this fact could be very useful to isolate transgenic sectors and to produce fully transgenic plants. *In vivo* monitoring of GFP expression permitted a rapid and easy discrimination of transgenic and escape shoots. Following this approach non-transformed buds would be periodically eliminated to avoid competition with their transgenic counterparts in order to favour the proper development of shoots derived only from the transgenic events, thus increasing the frequency of GFP-positive shoots regenerating on a medium without kanamycin selection. This fact opens the possibility to rescue the transgenic cell clusters and to regenerate transgenic plants without using selectable marker genes conferring antibiotic or herbicide resistance.

Ornamental and medicinal plants

Five different DNA plasmids carrying a synthetic *gfp* gene driven by different promoters, CaMV 35S, HBT, and Ubi1 were tested for the genetic transformation of *Dendrobium* Sonia 17 (Tee *et al.* 2003). 35S-*sgfp*-TYG-nos (p35S) with the CaMV 35S promoter showed the highest GFP transient expression rate, while the HBT and Ubi1 promoters showed a relatively lower expression rate in the entire target tissues of *Dendrobium* Sonia 17 tested. The highest number of GFP-expressing cells was observed on day 2 post-bombardment, and the number declined gradually over the course of the next 2 weeks. The non-destructive assay feature of the GFP system is valuable for application in an orchid transformation system, as orchid callus tissue is well known for its slow growth rate and low regeneration rate (Tee *et al.* 2003).

Verbena (*Verbena* × *hybrida*), an important floricultural species, was successfully regenerated from stem segments, and a transformation system was developed using cvs. 'Temari Scarlet', 'Temari Sakura', 'Tapien Rose' and 'TP-P2' (Tamura *et al.* 2003). *A. tumefaciens* strain AGL0 with binary vector pSPB1044 harboring the sGFP gene was infected into stem segments. Transformation efficiency was improved by evaluating and manipulating the age of the plant material, the concentration of kanamycin in the medium during selection, and the length of the culture period in the dark. After 2–3 months of culture on the selection medium, GFP-positive shoots were obtained in all four of the cultivars tested (Tamura *et al.* 2003). These shoots were successfully acclimated and set flowers within 2–3 months in a greenhouse. GFP was expressed in all of the organs including the floral parts. Stable genomic transformation was confirmed by Southern blot analysis. No morphological differences were observed between the transformed plants and their host plants. The lines with a single copy had stronger GFP expression than those with multiple copies. According to Tamura *et al.* (2003), the level of gene expression might

have been influenced by the positional effect of the transgene or by co-suppression due to the integration of multicopies. 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 all parts of flowers of two lines of *Medicago truncatula* 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 Enod12A* promoter-*uidA* reporter gene fusion and plasmid pLP35*gfp* containing a CaMV35S promoter-*gfp* reporter gene fusion derived from pMon30049 were transformed into *A. tumefaciens* EHA 105. In both cases the binary vector was derived from pLP100 and contained a chimeric *nos* promoter-*nptII-nos poly*A 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. Desite *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 plants 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 rearrangment of the transgenes have taken place in the *M. truncatula* 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* strains LBA4404 and GV2260 harboring the binary vector pBIGFP121, which contains the *npt*II gene as a selectable marker and the *gfp* gene as a visual marker linked to the CaMV 35S promoter. Putative 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 a morphologically normal phenotype and the transgene was shown to be inherited in a Mendelian manner. Cui *et al*. (2003) successfully improved previous methods that had existed for *Agrobacterium*-mediated transformation of *A. majus*. Transformants generated using this method showed stable expression of the GFP reporter gene at each stage from inoculation to plant development, and the transgene was integrated into the genome of *A. majus*, as confirmed by fluorescence microscopy and molecular analyses. Furthermore, the transformation frequency, based on the numbers of transformed plants obtained after 5 months of culture, reached 8–9% of the inoculated explants. These results suggest that the improved protocol will be useful for studying the roles of identified genes in *A. majus* plants.

Vegetables, cereals, tuber and oil-yielding crops

Cho and Widholm (2002) reported an efficient protocol for the transformation of the legume *Astragalus sinicus* (Chinese milk vetch), cotyledon segments were infected with *A. tumefaciens* strain EHA105 harbouring the binary vector pBIN*m-gfp5*-ERwhich carries the *gfp5* gene encoding green fluorescent protein and the kanamycin (Km) resistance gene *nptII*. Putative transformed shoots were selected and transformation was monitored by observation of GFP expression under a dissecting fluorescence Microscope with appropriate filters. Plants were regenerated from seven independent transgenic events and five plants set seed. GFP expression segregated in the T_1 seedlings of the two lines tested in a 3:1 ratio. In addition to the GFP expression of the transgenic plants, the transgenic nature of individual plants of *A. sinicus* was confirmed by Southern and Western blot analyses. They also indicated that attempts to count cells expressing GFP 5 days after cocultivation were unsuccessful as the variation within treatments and between cotyledon segments was high as many cotyledon explants showed no fluorescence, while some showed dozens of fluorescing cells. However, four weeks after transformation, the characteristic green fluorescence was easily detectable in the adventitious buds and shoots and callus developing on cotyledon segments transformed by *A. tumefaciens* strain EHA105 containing pBIN*m-gfp5*-ER. The CaMV 35S promoter-GFP fusion contained in the pBIN*mgfp5*- ER binary vector showed constitutive GFP expression in all tissues of the transgenic plants on a gross level. The tissues of the presumed transformed plantlets of *A. sinicus* were bright green while the leaves of the untransformed plantlets were reddish purple due to chlorophyll autofluorescence when illuminated by a black light fluorescent lamp. GFP fluorescence in dark-green leaves of mature plants of *A. sinicus* was usually masked by the chlorophyll red autofluorescence so that leaves were pink to green, but the untransformed leaves were red. GFP-positive tissue was easier to identify in young plantlets than in the older, more developed parts of regenerating shoots of *A. sinicus*. This could be due to chlorophyll autofluorescence or to the higher cytoplasmic density in young tissues compared to the more vacuolated older tissue of *A. sinicus.* Other explanations could include lower expression and GFP turnover (Yang *et al.* 2003; Ketelaar *et al.* 2004). Cho and Widholm (2002) also observed no signs of GFP toxicity or reduced regenerability or phenotypic effects with the green-fluorescent *A. sinicus* plants in the greenhouse, compared with untransformed control plants.

Stable transformation and regeneration was developed for a grain legume, azuki bean (*Vigna angularis* Willd. Ohwi & Ohashi) for the expression of *gfp* (Yamada *et al.* 2001). Two constructs containing the *npt*II gene and either the GUS gene or the modified GFP [sGFP(S65T)] gene were introduced independently *via A. tumefaciens*-mediated transformation. Surviving plants were transferred to soil and grown in a greenhouse to produce viable seeds (Yamada *et al.* 2001). *A. tumefaciens* strains LBA4404, AGL1 and EHA105 were used with two plant vectors, pIG121 and a GFP expression vector (pSG65T). Binary vector pIG121 has an intron-containing GUS gene driven by the CaMV 35S promoter and an *npt*II gene, conferring kanamycin tolerance, under regulatory control of the *nos* promoter (Yamada *et al.* 2001). 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* under the control of CaMV 35S promoter has been 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 other 3 had multiple copies. Transgenic leek (*Allium porrum*) and garlic (*Allium sativum*) plants have been recovered by the selective culturing of immature leek 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 *npt*II antibiotic selectable marker gene, was used (Eady *et al.* 2005).

Huber *et al.* (2002) reported transformation for the expression of *gfp* using immature embryos 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 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, this was now due to stable transformation. In leaves of rooted *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 proved to be difficult or impossible, because minor 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 T_0-plants when *gfp* was controlled by the 35S promoter. Young (10 to 15 days after anthesis) transgenic grains obtained by selfing T_0-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 T_1 embryos and plants was possible. The same was true with T_2 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 followed by culturing these embryos on PPT-containing callus medium. Only *gfp*-expressing calli which had developed on this medium were transferred to PPT-containing 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 *sGFPS65T* 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 *sgfpS65T* as a marker gene in an experiment comparing bombardment parameters allowed the rapid identification of variables 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 T_0 plants were grown to maturity, and in some cases immature seeds were collected 20 days after anthesis. Southern blots confirmed transformation in the T_0 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 expression from maternal endosperm tissue (Jordan 2000). Segregation data on the T_1 progeny of four plants showed that for three of the four T_0 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. Applying antibiotic selection can increase the number of transgenics 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 and facilitated the identification of transformed cell clusters. 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 production of greater numbers of transformed plants in a 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 produced bright-green fluorescence 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 (T_0) and their progeny (T_1). 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 leaves of seedlings grown in the dark. The limited strength of the CaMV 35S promoter in rice might be responsible for the low apparent expression of GFP in leaf tissue (Vain *et al.* 1998). The use of highly constitutive promoters and introns lead to GFP expression in all plant tissues of transgenic tobacco (Chen *et al.* 2005), papaya (Zhu *et al.* 2004), *Medicago truncatula* (Kamate *et al*. 2000), *Allium cepa* (Eady *et al.* 2000) and *O. sativa* (Sallaud *et al.* 2003). The pBINmGFP4 construct used in the study of rice transformation was primarily designed for gene-expression studies in dicotyledonous species and it is reasonable to expect that GFP expression may be improved using stronger promoter sequences (Vain *et al.* 1998). GFP fluorescence was observed in T_1 embryos and seedlings of rice (Vain *et al.* 1998). Segregation studies at the expression level, using GFP fluorescence followed by histochemical GUS staining, showed both Mendelian and non-Mendelian inheritance of GFP and GUS expression in the progeny of rice. Most deviations from the expected 3:1 ratio were due to transformed seedlings expressing GUS but not detectable levels of GFP (observed using a simple hand-held ultraviolet lamp for GFP excitation).

Sallaud *et al.* (2003) investigated the potential of an improved *A. tumefaciens*-mediated transformation procedure of japonica rice (*O. sativa*) for the expression of *gfp*. Using a T-DNA construct bearing the *hpt*, *gfp* and *gus*A genes, each individually driven by a CaMV 35S promoter, they established a highly efficient seed-embryo callus transformation procedure that results both in a high frequency (75–95%) of co-cultured calli yielding resistant cell lines and the generation of multiple (10 to more than 20) resistant cell lines per co-cultured callus. Efficiencies ranged from four to 10 independent transformants per cocultivated callus in various japonica cultivars. Sallaud *et al.* (2003) also further analysed the T-DNA integration patterns within a population of more than 200 transgenic plants. In the three cultivars studied, 30–40% of the T_0 plants were found to have integrated a single T-DNA copy. Analyses of segregation for hygromycin resistance in T_1 progenies showed that 30–50% of the lines harbouring multiple TDNA insertions exhibited hpt gene silencing, whereas only 10% of lines harbouring a single T-DNA insertion was prone to silencing (Voinnet and Baulcombe 1997). Most of the lines silenced for *hpt* also exhibited apparent silencing of the *gus* and *gfp* genes borne by the T-DNA. Moreover, Sallaud *et al.* (2003) found that plants with multiple T-DNA copies were more affected by gene silencing than plants with a unique TDNA. Tandem structures such as inverted repeats have shown to be responsible for gene silencing by a mechanism known as post transcriptional gene silencing (PTGS), also termed RNAi. Establishing the correlation between T-DNA integration pattern and transgene expression in a significant number of lines has not been previously reported in rice. T-DNA inverted repeats are most likely responsible for the gene silencing which occurred in transgenic lines (Sallaud *et al.* 2003). Forward genetic screening to identify tissue-specific or induced gene expression is also a classical approach. If silencing occurred in plants with multiple insertions of the T-DNA bearing a gene detector, which may account for more than half of the rice T-DNA population, a large proportion 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 microspore culture was biolistically transformed with two synthetic forms of GFP, *sgfp* and *pgfp*. Thirty-seven fluorescing multicellular structures were isolated using epifluorescent microscopy. Sixteen structures developed shoots, but only five regenerated into green plants. Three events had been co-bombarded 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 method to chemical selection of transgenic plants from barley microspore 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. Proliferating shoot meristematic cultures 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 *npt*II. Cultures were selected with bialaphos, hygromycin B and geneticin (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 T_0 plants and their progeny in 13 out of 23 independent transgenic lines. Stable GFP expression was observed in T_2 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 their progeny of some transgenic lines of *A. sativa*. Transient expression of the *sgfp*(S65T) gene driven by the rice actin promoter (pAct1IsGFP-1) resulted in large numbers of GFP-expressing foci in the bombarded tissues of *A. sativa*. Different tissues formed from stably transformed shoot meristematic cultures. T_0 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 transgene silencing or physical loss of the transgene 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 (*N. 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 discerning 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-m*gfp*5-ER. However, in this study, pBin.35S-m*gfp*5-ER did not confer increased green fluorescence in *Agrobacterium*. The reasons for this were unclear, but low-level *gfp* expression in *Agrobacterium* may be masked by its own low fluorescence. Detection of strong green GFP fluorescence in lettuce or 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. With microprojectile bombardment, where genes are frequently introduced via separate plasmids, application of GFP as a secondary marker requires a high frequency of cotransformation and coexpression of both genes (Elliot *et al.* 1999). These authors reported that the frequency of coexpression was very high, above 96%, as judged by the proportion of green-fluorescent calli that formed under stringent selection and was consistent with the integration of multiple copies of reporter and antibiotic resistance genes.

gfp-expressing cells from four different plant species (sugarcane, maize, lettuce, and tobacco) were readily distinguished, following either *Agrobacterium*-mediated or particle bombardment-mediated transformation (Elliot *et al.* 1999). 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*. Sugarcane leaf tissue and maize coleoptiles were bombarded with pGEM.Ubi1-s*gfp*S65T to test for detection of GFP fluorescence using a dissecting fluorescence microscope. The leaf exhibits substantial red chlorophyll autofluorescence upon illumination with blue light (490 nm). Green-fluorescent cells were also easily visualized in dark-grown maize coleoptiles after bombardment (Elliot *et al.* 1999). Elevated green fluorescence in the nuclei of the fluorescent cells was consistent with the accumulation of GFP in other species, in the cytoplasm and at higher levels in plant nuclei (e.g. Haseloff and Amos 1995). Expression of Ubi1-s*gfp*S65T was also examined in cells isolated from a sugarcane suspension culture. Green fluorescence was clearly visible in a percentage of cells at 48 h after bombardment. No green fluorescence was observed in control bombardments of calli or suspension cells using tungsten only or tungsten coated with pEmuKN. However the study of Elliot *et al*. (1999) indicated that pBin.35S-m*gfp*5-ER did not confer increased green fluorescence in *Agrobacterium*. The reasons for this were unclear, but low-level *gfp* expression in *Agrobacterium* may be masked by its own low fluorescence (Zhang *et al.* 1999; Eady *et al.* 2005). Detection of strong green GFP fluorescence in lettuce or tobacco was not impeded by endogenous fluorescence of *Agrobacterium* cells. Also, GFP fluorescence was always confined to plant cells with no leakage, whereas diffusion of the blue staining product produced during histochemical GUS staining is often observed. 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 *ubi*-1 promoter and intron region, and transformed into maize (van der Geest and Petolino 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 samples revealed bright-green fluorescence, presumably caused by GFP accumulation, in the cells. Non-transformed callus emitted a faint bluish glow that was much weaker than the green fluorescence seen with the transformed callus samples. The low level of background fluorescence observed in extracts of non-transformed callus was probably due to cellular components producing this bluish glow. Thin sections of leaves of various ages and young roots of both transformed and non-transformed plants displayed bright fluorescence in their cell walls, making it difficult to observe GFP fluorescence. Young leaves appeared to autofluoresce less than older leaves, probably because of their lower lignin content. Staining sections with toluidine blue significantly quenched the cell wall fluorescence. This allowed the authors to detect GFP in transgenic tissue while non-transformed, control samples did not fluoresce following staining. GFP expression from the *ubi*-1/GFP construct was apparent in mesophyll, epidermis, and vascular cells of leaves and in cortex, endodermis, and pith cells of young roots. These observations were generally consistent with those made using the GUS reporter gene driven by the maize *ubi*-1 promoter region, which has been shown to drive strong, constitutive transgene expression. However, in contrast to the results with GUS, not all cells within a given tissue displayed GFP expression. Although fluorescence was consis-

tently 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 cells that remained intact within a given section 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 plants regenerated from transgenic cultures.

Nishizawa *et al.* (2006) evaluated the red fluorescent protein *DsRed2* (a mutant form of *DsRed* from *Discosoma* sp.) for its suitability as a visual marker in combination of soybean (*Glycine max*). Transient and stable expression of *DsRed2* in somatic embryos of soybean was readily detected by fluorescence microscopy, allowing easy confirmation of gene introduction. *DsRed2* is a modified form of *DsRed* from *Discosoma* sp. whose maximum excitation and emission wavelengths are 563 and 582 nm, respectively-as visual marker in combination with antibiotic selection for the production of transgenic soybean by particle bombardment mediated transformation. They obtained several fertile transgenic lines, including homozygous lines that grew and produced seeds in an apparently normal manner. The red fluorescence of *DsRed2* 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 *DsRed2* 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 *DsRed2*, 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 *DsRed2* 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-based transformation 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 efficient and rapid visual selection of primary 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/pDM96.0501. Uninoculated SEs and cultures of *A. tumefaciens* EHA101/ pDM96.0501 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 (Escobar *et al.* 2000). If such an 'embryogenically 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_1 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 specipcity, 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 effective scorable marker in walnut SE culture and does not interfere with subsequent walnut development in 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 AGL cells comprising a binary vector *pBinm-gfp5-ER* with a selectable *npt*II 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 (T_0 generation) were obtained. Expression of GFP in T_1 generation 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, some of these 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) and *Arabidopsis* plants (Niwa *et al.* 1999). Additionally antibiotic selection-based screening steps may be avoided (Niwa *et al.* 1999) or the low level of antibiotic selection can be executed by using GFP-based selection (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). Another use of GFP in whole plants is to use it 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).

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 macerozyme R-10 had a negative effect. The 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 after the termination of coculture by determining the 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 at a significantly higher frequency than any sonication treatment.

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 planta* 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, suggesting that the fluorescence-based method 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-cm^2 disk (approximately 10 mg of tissue), while the protein extracts included 200 mg of leaf 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 roll 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 data do 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 transgene condition (Richards *et al.* 2003). The homozygotes 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 *npt*II 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) examined two types of *gfp* genes: the *mgfp4* described earlier and a modified GFP construct, *mgfp5ER*, 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 *mgfp5ER* vector. 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 endoplastic 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 *mgfp4* construct had a different pattern of accumulation of GFP compared with the *mgfp5ER* 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 (Molinier *et al.* 2000; Zhu *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 was mostly detected in vascular tissues from primary transformants. Because poplar transformation was performed with the *mgfp5ER* 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 cell lines expressing *gfp*, embryonal masses of four 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 *Arabidopsis* 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).

Mature zygotic embryos of recalcitrant Christmas tree species Fraser fir (*Abies fraseri*) and Nordmann fir (*Abies nordmanniana*), and Virginia pine (*Pinus virginiana*) were used as explants for *A. tumefaciens* strain GV3850-mediated transformation using the *gfp* (green fluorescent protein) gene as a reporter (Tang and Newton 2005). These authors reported the transient expression of *gfp* in three Christmas tree species Fraser fir, Nordmann fir, and Virginia pine, and stable *gfp* expression in Virginia pine. The binary expression vector *pBINm-gfp5-ER* contains the *m-gfp5-ER* gene under the control of the CaMV 35S promoter during the transformation of Christmas tree species Fraser and Nordmann firs, and Virginia pine. Visible GFP expression was observed in embryos from all three species tested. GFP expression remained high at the early stages (5-6 days) following cocultivation, but declined to low levels 21 days after co-cultivation of tissues. The most intense tissue green fluorescence and the highest frequency of embryos expressing GFP was observed in cotyledons and hypocotyls of *A. fraseri*, *A. nordmanniana*, and *P. virginiana*. Transient *m-gfp5-ER* expression in Fraser and Nordmann firs, and Virginia pine was detectable as early as 3 days after co-cultivation and reached a peak with more than 70% of the embryos expressing GFP after 6 days. Three weeks after co-cultivation, the number of visible areas expressing GFP had declined considerably. It was also recommended that evaluation of transient GFP expression takes place 5-10 days after cocultivation since 48 h time point used for determination of transient CAT and GUS was not optimal for GFP. Integration of the *m-gfp5-ER* was confirmed by visual observation of callus derived-transformed embryos of Fraser and Nord-

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* Sieb. et Zucc., Hinoki cypress, was developed after co-cultivation of embryogenic tissues with disarmed *A. tumefaciens* strain C58:pMP90, which harbours the *sgfp* (synthetic green fluorescent protein) as visual marker and *npt*II 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 GFP fluorescence, observed under a fluorescence microscope varied from very faint to relatively strong, depending on the transgenic line or part of the transgenic *C. obtusa* plants. The disarmed *A. tumefaciens* strain *C58/pMP90* containing a binary vector *pBin19-sgfp* which codes a gene for *sgfp* and *nptII* are 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* (S65T) was shown to be available as a reporter in *C. obtusa*. However, the transient expression of GFP after particle bombardment was clearer in cotyledonary somatic embryos of *Larix kaempferi* and zygotic 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. obtuse*, 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 appeared that GFP transgenic cells were growing from definite loci. The GFP expressing shoots appeared morphologically normal when visualized under white light and fluoresced 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. Meristematic regions of intact transgenic plants of horseweed hybrids in pots were fluorescent green.

The *A. tumefaciens* strains LBA4404, EHA105 harboring the binary vector pBI GFP were used for transformation of *Artemisia annua* (Han *et al.* 2005). This study also confirmed the integration of GFP in the transgenic plants using PCR and Southern analyses indicating the integration of gene into the plant genome successfully. They also concluded the frequency of transgenic fascicled shoots is approximately 4 to 10%. Because one cluster of fascicled shoots consists of 10–40 shoots in our cases, the transformation efficiency is quite high (Han *et al.* 2005). Bacterium strains and plant genotypes played a very important role in transformation (Han *et al*. 2005; Malabadi and Nataraja 2007c). EHA105 was superior to LBA4404 when the infectious *A. tumefaciens* was EHA105 during the transformation studies of *A. annua* (Han *et al.* 2005). The chromosome in *Agrobacterium* and activating potency of genes in virulence region are important internal factors influencing the infecting ability of *A. tumefaciens*. EHA105 and LBA4404 not only have different chromosome background but also different Vir-helper plasmid with different levels of activating potency. It was likely for these reasons that EHA105 had stronger ability to infect *A. annua* than LBA4404. *A. tumefaciens* has different ability to infect different species of plants and in some cases, even has different ability to infect different genotypes of the same species. Generally speaking, the specificity of genotype is related to the cell physiological conditions, which include 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* are leaves since the frequency of shoot induction of leaves is the highest and since *A. tumefaciens* has the strongest 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 was 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 cotyledonary explants from primary somatic embryos (SEs) and *A. tumefaciens strain* AGL1. Transgenic plants carrying the visible marker gene EGFP, the selectable marker *npt*II (NPTII), the class I chitinase gene from cacao (*chi*), 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 high levels of GFP expression in lines of transgenic plants 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 EGFP expression – a near-perfect 1:1 segregation was observed, indicating 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-

dons. 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 plantlets expressing GFP, indicating stability of the EGFP transgene expression through meiosis, fertilization and into the T_1 progeny. Maximova *et al.* (2003) also observed that despite the high green fluorescence/GFP expression of all transgenics (T_0) selected for multiplication via tertiary embryogenesis, the new embryos that were produced were not always fluorescing. Complete silencing (no green fluorescence) was observed in two lines from a total of eight lines established containing the primary vector and in one line from a total of four lines established with the chitinase vector.

Taro bacilliform virus (TaBV) is a pararetrovirus of the genus *Badnavirus* which infects the monocotyledonous plant, taro (*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 four different 50 deletion fragments (T600, T500, T250 and T100) (Yang *et al.* 2003). In transient assays, only the T1200, T600, T500 fragments were shown to have promoter activity in taro leaf, banana cv. 'Lady finger' (*Musa* species AAB) suspension cells and tobacco (*N. tabacum* cv. 'Dynes') callus (Yang *et al.* 2003). When these three promoters were evaluated in stably transformed, *in vitro*-grown transgenic banana and tobacco plants, all were found to drive nearconstitutive expression of either the GFP protein or GUS reporter gene in the stem (or pseudostem), leaves and roots, with strongest expression observed in the vascular tissue. In transgenic banana leaves, the T600 promoter directed 4-fold greater GUS activity than that of the T1200, T500 and the maize polyubiquitin-1 promoters. In transgenic tobacco leaves, the levels of GUS 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 GFP expression was difficult to assess due to the green fluorescence being obscured by red chlorophyll autofluorescence. Therefore, 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, seven 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 *A. tumefaciens* strain LBA4404 carrying *npt*II, and the reporter gene sm*gfp*, encoding soluble-modified green-fluorescent protein, driven by the CaMV 35S promoter. Southern blot analysis indicated that the sm*gfp* gene was integrated (multiple copies) into the spinach genome while Northern and Western blots showed that the sm*gfp* 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 regeneration, perhaps due to the fluorescent or catalytic properties of the protein. In jellyfish photocytes, where high levels of GFP are well tolerated, 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 (Elliot *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 Viestra 1998). Various modified forms of GFP – targeted to the ER (Haseloff *et al*. 1997) or with increased solubility (Davis and Viestra 1998) – have been constructed, and these 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 *sgfpS65T* 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 genetic transformation. 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; Leffel *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 confusion during genetic transformation (Elliott *et al*. 1999; Zhou *et al*. 2004). 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 in monitoring trafficking and subcellular localization of protein. At the organ level and up, many exciting applications are rapidly emerging. GFP has 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 homozygote 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 transgenesis. GFP protein has been targeted to the endoplasmic reticulum which solves its potential problem of toxicity when localized in the nucleus (Haseloff *et al*. 1997). There are some subtle techniques when using GFP in the selection for the transformation of plants. Tracking transgenic events as early as possible is desirable for the purpose of keeping them segregated. The isolation of high-expressing events is important, but if green fluorescent tissue is excised from the mother explant source when it is too small it may die.

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