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REVIEW

C.N. Stewart Jr

The utility of green fluorescent protein in transgenic plants

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Abstract The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has proven to be a powerful tool in plant genetic transformation studies. This paper reviews the history and the progression of the expression of GFP variants in transgenic plants. The distinguishing features of the most useful GFPs, such as those including the S65T chromophore mutation and those with dual excitation peaks, are discussed. The review also focuses on the utility of GFP as a visual selectable marker in aiding the plant transformation process; GFP has been more important in monocot transformation compared with dicot transformation. Finally, the potential utility of new fluorescent proteins is speculated upon.

Keywords Fluorescent proteins · GFP · Gene expression · Marker genes · Plant transformation

Introduction

The jellyfish green fluorescent protein (GFP) has become a very effective marker for use in plant genetic transformation research. For the first time in plant biology, researchers had at their disposal a universal, in vivo, and real-time transgenic visible marker in GFP. Several review papers have been written about the uses of GFP in plant biology (Haseloff and Amos 1995; Leffel et al. 1997; Haseloff and Siemering1998), however the present review focuses on the expression of different *Aequorea victoria* GFP variants in transgenic plants with the specific purpose of assessing their utility in plant transformation research. The historical development of GFP variants will be reviewed. In addition, the trends towards mutational variant development of *Aequorea* GFPs will be extrapolated to speculate on the usefulness of recently cloned non-*Aequorea* GFP genes in plant transformation.

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Early variants of green fluorescent protein

The utility of GFP in plant transformation and expression was first demonstrated in plant cells, not intact transgenic plant tissues. Niedz et al. (1995) were the first to show that wild-type *Aequorea* GFP could be visualized in plant cells – in this case sweet orange (*Citrus sinensis*) protoplasts. There were two other published studies using wild-type GFP. Hu and Cheng (1995) demonstrated that GFP could be synthesized in corn protoplasts. However, they failed to observe GFP in transformed *Arabidopsis thaliana* or tobacco cells, presumably the result of low expression of the wild-type gene. Using a stronger promoter, another group was able to visualize wild-type GFP in corn and arabidopsis cells (Sheen et al. 1995). 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 difference 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) versus incandescent lamp with excitation filters (Hu and Cheng 1995). These studies show that successful GFP detection is highly dependent on the strength and source of the excitation sources. Nonetheless, the experience with a low expression of wild-type 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 wild-type *Aequorea* GFP that caused a 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 wild-type spectral characteristics: maximal excitation at 395 nm and maximal emis-

sion at 509 nm. mGFP4 was successfully expressed in soybean suspension cultured cells (Plautz et al. 1996), arabidopis (Haseloff et al. 1997), tobacco (Stewart 1996) and other plants. 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 (Stewart 1996; Leffel et al. 1997; Harper et al. 1999). A similar synthetic human codon-optimized GFP with a wild-type chromophore was created by Haas et al. (1996) that also eliminated the cryptic intron. Since humans and corn have very similar codon usage, the gene proved to be well expressed in plants. When it was expressed in plants it yielded 20 times more fluorescence than the wild-type gene (Chiu et al. 1996).

Common GFP variants

The various GFP mutants that are most commonly used for plant transformation experiments are shown in Table 1.

Haseloff's group also introduced mutations that conferred increased GFP heat stability and altered spectral qualities. The V163A and S175G mutations proved to provide better folding and hence green fluorescence at 37°C (Siemering et al. 1996). Coupled with the I167T mutation (Heim et al. 1994), the variant, mGFP5, has dual excitation peaks at 395 nm and 475 nm and an emission peak at 509 nm (Siemering et al. 1996).

The variants mentioned above thus far retained a wild-type chromophore of SYG (peptides 65–67) and, therefore, an unaltered emission spectrum from wild-type GFP. The most important chromophore alteration to plant biology has been the S65T change that created a single blue excitation peak (489 nm optimum) and red-shifted the excitation optimum to 511 nm (still green) and also the less often-used S65C mutation (Heim et al. 1995). When in an essentially mGFP4 background (few codons changed, cryptic intron removed), the S65T and S65C mutations increased detection limits by up to 19-fold (Reichel et al. 1996). Chiu et al. (1996) demonstrated that the S65T mutation provided a fluorescence gain of up to 100-fold in plant cells after human codon optimization was performed. The synthetic S65T gene with the cryptic intron removed was called sGFP (S65T) (Haas et al. 1996). Harper et al. (1999) demonstrated in the field that the *sGFP-S65T* gene was expressed up to 0.2% and had strong fluorescence characteristics. Not all mutants have yielded increased fluorescence. A sub-optimal change is the Y66H, which makes a blue fluorescent protein (BFP) that does not fluoresce well in plants (Reichel et al. 1996).

Several other important modifications have been made to improve GFP expression in plants. Pang et al. (1996) produced a synthetic (*pgfp*) S65T and S65C variants, with versions with and without the substitution of a potato ST-LS1 intron 2 in place of the cryptic intron. The effect of adding the intron boosted the fluorescence of the synthetic S65T/S65C versions 150-fold compared with that of the wild-type GFP.

Other GFP variants have been expressed in plants. The commercially available EGFP (Clontech) has the S65T as well as the F64L and Y145F mutations and is human codon-optimized (Yang et al. 1996). Gene shuffling was used to produce a mutant that has greater solubility and fluorescence (Crameri et al. 1996). The mut3GFP has the V164A (V163A) mutation that putatively improves folding at higher temperatures (Siemering et al. 1996) as well as the F100S (F99S) and M154T (m153T) mutations. Davis and Vierstra (1998) further modified mGFP4 to include the mut3 mutations and called it smGFP (sm = soluble, modified). In addition, they added the S65T mutation, in yet another variant, Y66H. They called these smRS-GFP (RS = red-shifted) and smBFP, respectively. Surprisingly, when expressed in arabidopsis, there was no difference found in fluorescence between smGFP and smRS-GFP, but both were improvements over mGFP4. smBFP, like all BFPs described to date, had little fluorescence. smGFP, like mut3, showed primarily UV excitation (with a minor blue peak) and smRS-GFP had an excitation maximum of 495 nm and emission maximum at 507 nm.

Table 1 The various GFP mutants that are most commonly used for plant transformation experiments. The mutations listed are amino acid substitutions in standard format (for example: S65T = serine to threonine at the 65th amino acid)

Mutant (source)	Excitation/emittance (nm)	Mutations[a]
Wild-type *Aequorea victoria*	395[b], 475/507	None
mGFP4 (Haseloff)	395[b], 475/509	CI, SDM
mGFP5 (Haseloff)	395, 475/509	CI, V163A, S167T, S175G, SDM
SGFP S65T (Chiu)	489/511	CI, S65T, synth
PGFP (S65T) (Pang)	489/511	CI, S65T, synth, intron added
EGFP(Clontech, Yang)	488/507	CI F64L, S65T, Y145F, synth
smGFP (Davis and Vierstra)	395[b], 475/509	CI F99S, M153T, V163A, SDM
smRS-GFP (Davis and Vierstra)	490/510	CI S65T, F99S, M153T, V163A, SDM

[a] *CI* The cryptic intron in the wild-type gene has been altered; *SDM* site-directed mutagenesis has been performed; *synth* the gene is codon-optimized (humanized)

[b] Major excitation peak where more than one excitation peak is present

Future fluorescent proteins

GFPs from other marine organisms have recently been cloned (Matz et al. 1999; Szent-Gyorgyi et al. 2000). Matz et al. (1999) have cloned FP genes from non-bioluminescent Anthozoan species that fluoresce green, yellow, and orange. Szent-Gyorgyi et al. (2000) have cloned and optimized genes from *Renilla* and *Ptilosarcus* species that have very narrow stoke shifts and that fluoresce green. While many of these, in their wild-type form, are not as bright as current *Aequorea* mutants, it is reasonable to expect that plant-optimized green, yellow, and orange fluorescent proteins will work better in plant applications than those currently available. In fact, a priori, *Renilla reniformis* GFP has spectral qualities that should make it brighter in heterologous systems (Ward 1998). In particular, orange-red fluorescent proteins, such as DsRed (from reef corals *Discosoma* species), may have interesting applications in plant research because they provide a second excitation (558 nm) and emission (583 nm) spectra that are much different from those of the much-used GFPs. So far, there are no published data available on the over-expression in transgenic plants of DsRed or any of the Anthozoa-derived fluorescent proteins or, in fact, of any of the fluorescent proteins mentioned in this paragraph.

Non-*Aequorea*-derived fluorescent proteins will also enable the dual-color labeling of transgenic plants with less chance of post-transcriptional gene silencing (PTGS). PTGS can be induced upon the introduction of transgenes with similar sequences, among other situations. In fact, GFP has been used as a powerful tool to study PTGS. Ruiz et al. (1998) demonstrated that the expression of GFP was silenced in mGFP5 transgenic tobacco plants when potato virus X (PVX) with an mGFP5 tag was infected onto the GFP-transgenic plants.

Which GFP is best in plants?

The performance of GFP variants has seldom been compared in plants other than trivial comparisons (wild-type GFP versus sGFP-S65T). However, some directed experiments have been performed. Ponappa et al. (2000) found a greater number of transient GFP spots of smRS-GFP than of smGFP and sGFP-S65T in soybean somatic embryos, although by 24 h post-bombardment, the number of mGFP5-ER [mGFP5 that is targeted to the endoplasmic reticulum (ER)] spots was greater than those of smRS-GFP. Elliot et al. (1999) reported that SGFP-S65T had brighter fluorescence in sugarcane callus than mGFP5-ER. The S65T mutation variant and derivatives have been used more often in monocots, while mGFP5-ER and wild-type chromophore GFPs have been more frequently used in dicots. There are no easy answers to the question of which is the best GFP. If GFP accumulates and can be visualized, then the choice of GFP variants is not likely to be critical. My laboratory uses 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. Technology advances will lead to brighter GFPs in the future.

Toxicity of GFP

While many researchers are on the GFP bandwagon, for a time the opinion prevailed that GFP was cytotoxic to plant cells. 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 and Amos 1995; Haseloff et al. 1997; Haseloff and Siemering 1998). 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 endoplasmic reticulum (Haseloff et al. 1997). In fact, this targeting does isolate the GFP away for the nucleosome and does seem to enhance expression (Haseloff et al. 1997; Harper et al. 1999). Many researchers have failed to observe this apparent toxicity in plants (Chiu et al. 1996; Pang et al. 1996; Leffel et al. 1997; Quaedvlieg et al. 1998; Ghorbel et al. 1999; Tian et al. 1999; Molinier et al. 2000). To specifically address this issue, Harper et al. (1999) tested plants for yield drag and biomass decreases associated with GFP synthesis and fluorescence in the field for two growing seasons with three GFP variants; no associations were found. 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, the adaptations between animal cells and plants are different. Evidence indicates 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.

GFP as a visual selection agent for nuclear transformation

GFP has been used successfully as a β-glucuronidase (GUS) replacement for assessing the transient expression of transgenes. Because GFP is an in vivo reporter, the dynamics of expression may be followed in time, both for *Agrobacterium*- and gene gun-mediated transformation. For example, sGFP-S65T has been used to optimize the *Agrobacterium*-mediated transformation of wheat (McCormac et al. 1998). In apple, in which endogenous GUS activity is problematic, sGFP-S65T was

helpful in assessing the transient expression of *Agrobacterium*-mediated transformation in four different varieties (Maximova et al. 1998). A variety of GFPs (sGFP-S65T, mGFP4, mGFP5-ER, smGFP, and smRS-GFP) were used in similar gene gun-mediated transformation experiments in soybean (Ponappa et al. 2000). SGFP-S65T was used for similar purposes in tall fescue and red fescue also (Cho et al. 2000). Canola was produced with GFP-aided selection in which the events were tracked over time (Halfhill et al. 2001). In all of the above experiments, GFP was used in conjunction with antibiotic selection.

GFP can partially replace antibiotic selection and be of great use when the organogenesis or conversion segments of transformation procedures are inefficient under antibiotic or herbicide selection. It could be helpful in isolating events during the early stages of transformation experiments. An example of this was the sugarcane transformed with sGFP-S65T (Elliot et al. 1998). Initial callus was cultured under herbicide selection, and green fluorescent sectors were isolated and subcultured in the absence of the herbicide. Elliot and colleagues subsequently showed that similar techniques were effective in tobacco, corn, and lettuce transformation (Elliot et al. 1999). Therefore, GFP might increase the regeneration frequency. One prerequisite for such a technique is the continual fluorescence of transgenic material throughout development. In soybean fluorescence is lost for several weeks between transient expression and stable transformation (Larkin and Finer 2000).

GFP is beginning to be used for in vivo promoter analysis. Nehlin et al. (2000) fused several promoters to sGPP-S65T and bombarded the complex into *Brassica napus* microspores to aid in determining which promoter might be optimal for the development of a microspore transformation method. Most importantly, GFP can enable the unique sorting of GFP transgenic microspores using flow cytometry (Nehlin et al. 2000). GFP will be important in the development of cell-based (rather than tissue-based) transformation methods. Kamaté et al. (2000) demonstrated that GFP could be an effective marker in the transformation of *Medicago truncatula* floral organs

One of potential beneficial uses of GFP is as a complete substitute for non-visual selectable markers. One potential problem is that transgenic tissue could be outcompeted by non-transgenic tissue. This overgrowth was reported to have occurred with sugarcane (Elliot et al. 1999). For GFP to be effective alone as a visible selectable marker, GFP fluorescent tissues should grow preferentially and homogeneously (Fig. 1). Therefore, it is desirable to isolate and subculture small pieces of GFP transgenic tissue (e.g., callus). Paradoxically, if small pieces of GFP-fluorescing tissue are isolated (or any small bits of tissue), if may be impossible to regenerate the tissues (Elliot et al. 1999). Waiting until the pieces are larger could result in losing GFP-transgenic tissue among the non-transgenic tissue. However, barley was transformed with GFP using visible selection only

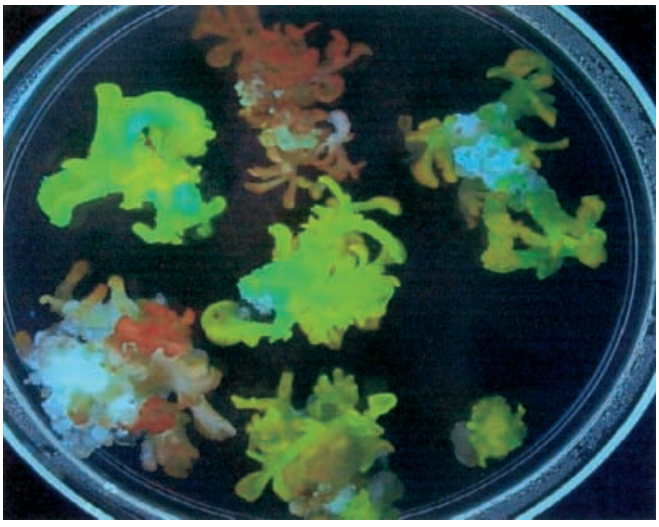

Fig. 1 Transgenic tobacco shoots and callus under selection with GFP (mGFP5-ER) under the control of a 35S promoter. Notice the variation of fluorescence among events and escapes (*red*). GFP is visualized under UV (365 nm) illumination with no emission filter

(Ahlandsberg et al. 1999); green fluorescing callus pieces were selected and plants regenerated. Likewise, gene gun-mediated transformation of rice using a embryogenic callus method was performed using mGFP4 as the sole selectable marker (Vain et al. 2000). This rice study was the first to directly compare antibiotic selection and GFP selection. Vain et al. (2000) reported that while there was no difference in transformation frequency, GFP did increase the efficiency of rice transformation by decreasing the amount of tissue that needed to be handled by a factor of 4 and the time involved by a factor of 2. Kaeppler et al. (2000) showed that sGFP-S65T can be used as a visual selectable marker in oat. The same variant has been used to help produce transgenic wheat (Jordon 2000). One potential benefit is that only high-expressing events are selected (Halfhill et al. 2001).

A GFP selection system seems to hold the most promise for tissue culture/transformation systems that are inefficient, for recalcitrant genotypes, and for plant species for which no system exists. For example, Ghorbel et al. (1999) used sGFP-S65T for the transformation of three citrus genotypes. Prior to using GFP, there were difficulties with escapes and chimeric citrus plants. GFP was an effective tool in screening tissues and plants at various stages of recovery (Ghorbel et al. 1999). One problem often associated with transformation experiments is that of escapes, which result from leaky antibiotic selection procedures. In conjunction with antibiotic selection GFP has been shown to decrease the numbers of escapes for a number of forest tree species (Tian et al. 1999). Ghorbel et al. (1999) also performed experiments directly comparing kanamycin selection and GFP-only selection. They found the transformation frequency to be the same, but curiously there were fewer GFP-positive shoots per experiment using GFP selection. GFP has helped to track *Agrobacterium* to aid in optimizing the sonicated-assist-

ed *Agrobacterium*-mediated transformation (SAAT) procedure (Finer and Finer 2000). sGFP S65T was used recently to produce the first transgenic switchgrass (*Panicum virgatum*) (Richards et al. 2001). With the availability (but not yet commercially available) of GFP plant transformation vectors, GFP will be the first gene transformed into many "new" plant species for which no experience exists in plant transformation.

GFP for use in plastid transformation

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 the problem of homoplasmy: the need for every plastid in a putatively transformed cell 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 Pang et al. (1996) S65T GFP and found that GFP did indeed help to confirm that homoplasmic status was achieved. High expression levels (up to 5%) were reported (Siderov et al. 1999). sGFP-S65T was used to produce fertile transgenic rice in which a nuclear transgene was targeted for expression in chloroplasts (Jang et al. 1999). These researchers reported cytoplasmic expression of 0.5% and a surprisingly high 10% expression level for GFP that was targeted for chloroplasts. Their report demonstrated that transplastomic approaches are not needed for high expression in plastids (Jang et al. 1999). In another study, transplastomic plants were produced using a GFP-antibiotic resistance marker fusion gene (Khan and Maliga 1999). This approach helped the researchers visualize recovered chimeric plants and also plastid segregation within plants (Khan and Maliga 1999). GFP was synthesized at very high levels.

GFP for monitoring the expression and presence of transgenes in the field

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).

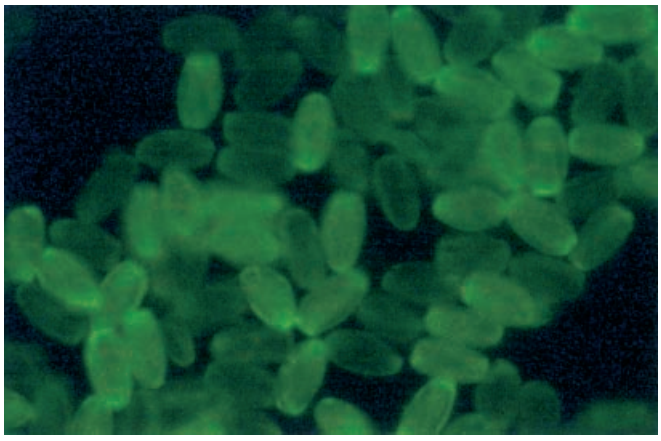

Fig. 2 Pollen grains tagged with GFP using mGFP5-ER fused to a pollen-specific promoter and visualized under a epifluorescence microscope (100×). Transgenic pollen is seen at a 1:1 Mendelian ratio

GFP has proven to be valuable in gene expression analysis. The expression of a linked transgene (*Bt cry1Ac*) could be monitored by GFP (mGFP5-ER) analysis in tobacco in growth chambers (Harper et al. 1999). Unpublished results demonstrate that this system is also effective in the field (C.N. Stewart et al., unpublished data). GFP exclusively allows for the monitoring of transgene movement in agronomic and ecological studies. GFP could also be used to mark seeds, flowers or other organs. For example, pollen could be painted with GFP to study gene flow and pollination behavior (Hudson et al. 2001, Fig. 2).

Finally, GFP has been reported to be valuable in genetic studies as an indicator of zygosity (Niwa et al. 1999; Molinier et al. 2000). For this to occur, GFP must be synthesized on a transgene copy-dose basis. In this case, non-transgenic segregant leaves are red, homozygous leaves are green, and hemizygous leaves appear intermediate between the two. However, these studies have been performed only under controlled conditions and not in the field. Field-grown plants often have more phenolic and blue-green auto-fluorescent compounds that can mask GFP fluorescence (Leffel et al. 1997; Harper et al. 1999).

Photonics and visualization

Much of the success of GFP as an enabling technology in transgenic plants hinges on the success of seeing GFP in plants. For laboratory work most researchers use epifluorescence 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 (Haas et al. 1996; Elliot et al. 1999). Altering the filter choices, such as choosing emission filters of a narrower band width, or alternative emission filters should help. 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. 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 (Little Luma LT 9700). For UV-excited GFPs, my group and others typically use a portable UV lamp (UVP 100 AP, Upland, Calif.) with no emission filter or the lighter Spectroline BIB-150 produced by Spectronics (Westbury, N.Y.). While the 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 eyewear should be used.

There are some subtle techniques when using GFP as a 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. We have been unsuccessful if we isolate fluorescent *Brassica* callus if the tissue piece is much smaller than 0.5 cm. The UV lamp makes it quite easy to weekly screen several plates. It also adds the additional benefit of "lighting-up" contaminants that are otherwise hard to see on petri dishes.

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