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TECHNICAL ADVANCE

Spatial control of transgene expression in rice (*Oryza sativa* L.) using the GAL4 enhancer trapping system

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Summary

We used enhancer trapping with the GAL4 transcriptional activator from yeast to obtain spatial control of transgene expression in all organs of the model monocotyledonous species rice (*Oryza sativa* L. cv. Nipponbare). Our T-DNA enhancer trapping cassette consisted of two principle components: (1) the minimal promoter-equipped *gal4* gene placed adjacent to the right border, and (2) the green fluorescent protein gene (*gfp*) fused to the upstream activation sequence element (UAS) to which GAL4 binds and activates expression, so that *gfp* expression corresponds to *gal4* expression. *Agrobacterium*-mediated integration of the cassette into the rice genome often brings the *gal4* gene under transcriptional control of local genomic enhancers and promoters, resulting in *gal4*/*gfp* expression patterns ranging in specificity from single-cell types to constitutive expression. We produced more than 13 000 enhancer trap lines with this cassette and screened T_0 adult plants (1982 lines), T_1 seed (2684 lines) and T_1 seedlings (2667 lines) for *gfp* expression. Approximately 30% of the lines produced GFP, and we identified lines with *gfp* expression in specific cell types of all major organs of the rice plant. Subsequently, using the GUS reporter gene (*uidA*), we demonstrated that UAS:*geneX* constructs can be transactivated in specific cell types where *gal4* and *gfp* are expressed, thus providing an excellent system for the manipulation of gene expression and physiological function in specific cell types of rice.

Keywords: tissue-specific promoters, GAL4-VP16, ectopic gene expression.

Introduction

Rice feeds more people than any other crop (Cantrell and Reeves, 2002; Yu *et al.*, 2002), yet an estimated 50% increase in production must occur over the next 25–30 years in order to accommodate the world's rapidly growing population (Tilman *et al.*, 2002). Currently, the maximum yield of rice in the tropics is 10 tons ha^{-1}, while under irrigated field conditions only 5 tons ha^{-1} is typically achieved. Significant proportions of this 'yield gap' are attributed to biotic and abiotic stresses such as disease (9%), insect pests (27%), salinity and water stress (Khush, 2001).

Plants generally respond to stress in a cell type-specific manner. For example, pathogen recognition and resistance is sensed and initiated in specific cells; water use efficiency is mainly controlled by stomata (Schroeder *et al.*, 2001); and salinity tolerance largely depends on excluding Na^+ from the shoot by minimizing influx into root cortical cells while maximizing influx into xylem parenchyma cells (Tester and Davenport, 2003). To minimize the yield gap in rice, as well as in other crops, there is clearly a need to understand cell type-specific processes involved in plant responses to biotic and abiotic stresses. In particular, spatial control of transgene expression allowing the manipulation of gene expression in a wide variety of specific cell types is necessary for the modification of stress-related functions.

Currently, most efforts to spatially regulate transgene expression in plants make use of promoters with activity specific to particular tissues, cell types and/or developmental stages. Successful examples of this include the pollen-specific Bp4 and microspore-specific NTM19 promoters from rapeseed and tobacco, respectively, which have been used to arrest pollen development in transgenic tobacco through directed expression of barnase (Custers *et al.*,

1997). A recent study of 15 promoters isolated from genes expressed in the rice seed (Qu and Takaiwa, 2004) demonstrated that many of these sequences direct GUS reporter gene expression in specific cell types of the seed, such as endosperm. Moreover, an elegant combination of tissue-specific promoters and the *alc* gene-expression system has allowed control of transgene expression both spatially and temporally in vegetative apices and inflorescences of Arabidopsis (Deveaux *et al.*, 2003). While providing stable expression of transgenes in particular tissue types, this approach remains limited by the general paucity of tissue-specific promoters that have been identified within species.

Attempts to use tissue-specific promoters from other plant species, even those closely related, may result in changes or loss of expression specificity due to the absence of *trans*-acting factors in recipient species (or presence of novel factors) that interact with *cis*-acting elements in the promoter region to achieve specificity. In a recent study, seed-specific promoters from *Phaseolus vulgaris* and *Vicia faba* that were engineered into species such as tobacco, pea and linseed directed reporter gene expression in non-seed tissues such as pollen (Zakharov *et al.*, 2004). The *cis*-acting elements in promoters appear to be evolving rapidly and a recent comparison of closely related cereal genomes such as rice, sorghum and maize revealed little conservation among conserved noncoding sequence elements in the promoters of orthologous gene pairs (Guo and Moose, 2003).

The GAL4 enhancer trapping system overcomes the problems posed by the paucity of tissue-specific promoters by using native enhancers within a host genome to drive expression of the GAL4 transcriptional activator from yeast (Giniger *et al.*, 1985). GAL4 can then be used to drive any gene of interest by placing that gene downstream of the upstream activation sequence element (UAS), to which GAL4 binds and activates expression. The GAL4 system was first deployed in *Drosophila melanogaster* (Brand and Perrimon, 1993; Phelps and Brand, 1998), where today there are more than 5000 characterized enhancer trap lines, as well as more than 3000 UAS:*geneX* lines, available for study. More importantly, recent use of the GAL4 system with inducible promoters, such as tetracycline, has made it possible to combine the spatial properties of GAL4 with the temporal characteristics of inducible promoters (McGuire *et al.*, 2004).

In order to identify interesting patterns of GAL4, most enhancer trapping cassettes include reporter genes such as *gfp* or *gus* placed downstream of the UAS sequence. We used a T-DNA-based enhancer trapping cassette harbouring a codon-modified *gal4-vp16* fusion gene engineered for high expression in plants (hereafter referred to as *gal4*) placed near to the right border and a UAS:*gfp* reporter construct towards the centre of the cassette to develop our enhancer trap population (Haseloff, 1999). Binding of GAL4 to the UAS element activates expression of the *gfp* reporter gene and, thus, patterns of GFP expression accurately represent activity of the GAL4 transcriptional activator.

The true power of the GAL4 system lies in its ability to spatially transactivate any gene of interest, a feature that has been exploited extensively in *Drosophila* and described more recently in Arabidopsis (Bougourd *et al.*, 2000; Kiegle *et al.*, 2000a; Phelps and Brand, 1998), by introducing genes fused to the UAS element into particular enhancer trap lines displaying cell type-specific patterns of GAL4. While two GAL4 enhancer-trapping populations of rice have recently been described (Wu *et al.*, 2003; Yang *et al.*, 2004), neither study has reported use of those lines to spatially control transgene expression. Through use of the vital GFP reporter gene, we have been able to quickly screen living plants to identify lines with cell-specific patterns of GAL4, and then utilize those lines to spatially control transgene expression.

We first describe the development of more than 13 000 rice enhancer trap lines transformed with the GAL4-GFP cassette, followed by screening for GFP expression in various tissues of the rice plant at several stages of development. Epifluorescence microscopy and confocal imaging are presented to show that cell-specific expression of the GAL4 transcriptional activator has been achieved. We then demonstrate with the GUS reporter gene (*uidA*) that this library of enhancer trap lines can be used to transactivate genes of interest in a large number of specific cell types in the rice seed, root, leaf and flower.

Results

Construction of the enhancer trap cassette and generation of transgenic lines

The GAL4-based enhancer trap cassette pC4956:ET15 (Figure 1a) contained the *gal4-vp16* gene immediately downstream of a –45CaMV 35S minimal promoter, placed near to the right border. The *gfp* reporter gene (targeted to the endoplasmic reticulum) was placed downstream of a –90CaMV 35S minimal promoter TATA and five tandem UAS sequences to which the GAL4 protein binds and activates transcription. Towards the left border, the hygromycin resistance gene, *hpt*, was placed downstream of the subterranean clover mosaic virus (S4) promoter. Two introns, rice actin 1 and castor bean catalase 1, were placed upstream and within the coding sequence of the *hpt* gene, respectively, to prevent *Agrobacterium tumefaciens* utilization of the resistance gene and thus increase efficiency of bacterial decontamination and selection during tissue culture (Wang *et al.*, 1997).

Screening of 11 654 hygromycin-resistant calli transformed with this cassette showed that 23% displayed *gfp* expression levels ranging from bright to weak. This result demonstrated that the enhancer trap cassette functioned properly in rice tissues, was capable of generating *gfp*

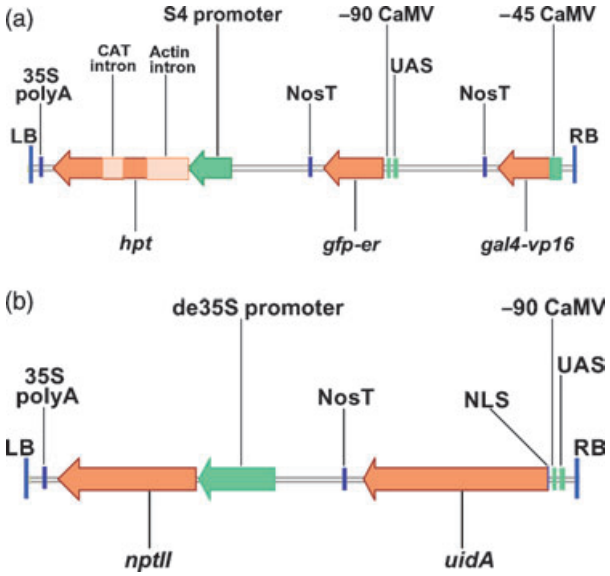

Figure 1. Structures of the two T-DNAs incorporated into rice lines.
(a) The GAL4-based enhancer trap pC-4956:ET15. From the right border (RB): –45CaMV, –45CaMV 35S minimal promoter; *gal4-vp16*, *gal4-vp16* transcriptional activator fusion gene; NosT, bacterial nopaline synthase terminator sequence; UAS, five tandem repeats of the upstream activation sequence element GAL4 binding site; –90CaMV, –90CaMV 35S minimal promoter TATA; *gfp-er*, green fluorescent protein (*mgfp5*) targeted to the endoplasmic reticulum; S4, subterranean clover mosaic virus promoter; actin intron, rice actin intron 1; CAT intron, castor bean catalase intron 1; *hpt*, hygromycin phosphotransferase gene; 35SpolyA, CaMV 35S 3′UTR polyA signal; LB, left border.
(b) The transactivation cassette pC-2300:UAS:*uidA*. Abbreviations the same as in (a), except: NLS, nuclear localization sequence; *uidA*, GUS reporter gene; de35S promoter, duplicated enhancer CaMV35S promoter; *nptII*, neomycin phosphotransferase gene.

expression at varying degrees of intensity, and, in the absence of external activation, was not capable of driving expression. Over three large-scale *A. tumefaciens*-mediated transformations, we regenerated 13 881 primary transformants carrying this enhancer trap cassette.

In planta *patterns of* gfp *expression*

Bright GFP fluorescence, localized to the endoplasmic reticulum (Haseloff *et al.*, 1997), was detected in a wide range of cell types of all major organs of the rice plant (Table 1 and Table S1; Figures 2–4) at an overall expression frequency of 32% for 1052 lines that were analysed at the T_0 adult, T_1 seed and seedling stages. This frequency is remarkably similar to the 30% reported for *Arabidopsis thaliana* T_1 lines transformed with a similar *gal4*/*gfp* cassette (http://enhancertraps.bio.upenn.edu), demonstrating that the GAL4 system achieves similar rates of enhancer trapping in both of these model plant species. This may be due to a bias for T-DNA integration into gene-rich regions and very low integration frequency into repetitive DNA (Sallaud *et al.*, 2004).

Table 1 Frequency of *gfp* expression observed in enhancer trap lines of rice

Tissue	*gfp* expression (%)
Flower (T_0)	**9.9**
Stamen	2.9
Carpel	0.3
Lodicule	4.5
Rachilla	0.4
Lemma/palea	0.3
Sterile lemma	0.2
Pedicel	0.4
Trichomes	0.7
Guard cells	0.4
Vascular	4.4
Seed (T_1)	**10.4**
Embryo	4.2
Scutellum	5.5
Aleurone or Endosperm	0.8
Coleorhiza	1.8
Shoot (vegetative)	**9.5**
Leaf blade (T_0)	**3.5**
Trichomes	0.2
Guard cells	0.3
Mesophyll	0.1
Vascular bundles	3.4
Leaf sheath (T_0)	**6.8**
Trichomes	0.1
Guard cells	0.1
Mesophyll	0.8
Vascular bundles	6.7
Collar	**8.2**
Seedling (T_1)	**29.3**
Shoot	18.1
Root	25.4
Digesting endosperm	13.8

Overall frequencies for particular organs are presented in bold and do not represent the sum of individual percentages due to *gfp* expression in multiple tissues for some enhancer trap lines. The leaf blade and leaf sheath were examined as separate organs, and thus data for each are included. The numbers are based on examination of 1982 T_0 plants (for expression of *gfp* in flowers and leaves), 2684 T_1 seeds (five seeds for each line) and 2667 T_1 seedlings (five seedlings for each line).

The GFP expression frequencies for particular organs (leaf, flower, seed) consistently averaged 10% while young seedlings displayed a much higher frequency of 29.3% (Table 1). This difference is due partly to the fact that the seedling screen comprised three different organs (root, digesting seed, shoot), but also suggests that young seedlings possess a more active gene expression profile than adult plants. In mature T_0 plants in the greenhouse, similar frequencies of expression were observed in reproductive and vegetative organs (10%), with by far the highest frequency of expression observed in the collar region connecting the sheath and blade tissues (occurring in 8.2% of all plants screened, Figure 2g). The collar possesses an intercalary meristem involved in blade growth, and the presence of these rapidly dividing, undifferentiated cells

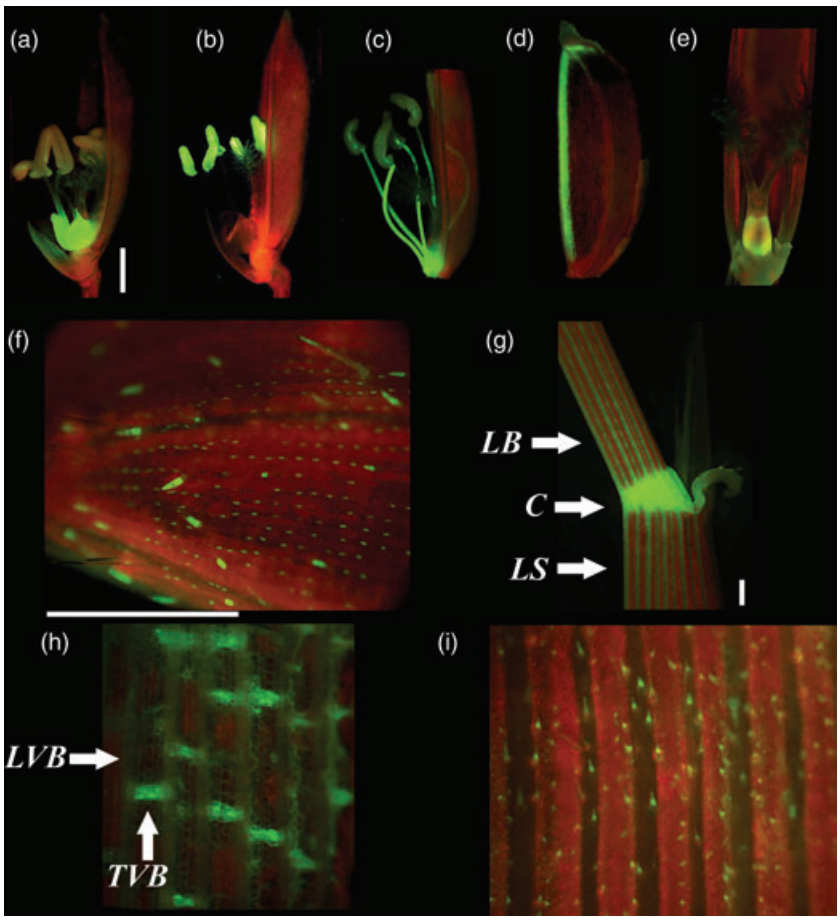

Figure 2. Enhancer trap lines (T_0) expressing *gal4* and *gfp* in specific tissues and cell types of the rice flower (a–f), leaf and sheath (g–i), visualized using epifluorescence microscopy. Fluorescence patterns: (a), lodicules; (b), pollen; (c), anther filaments; (d), vascular bundle of lemma; (e), ovary; (f), trichomes and guard cells on lemma surface; (g), collar (*C*), or lamina joint, and surrounding longitudinal vascular bundles of the leaf blade (*LB*) and leaf sheath (*LS*); (h), sheath vascular network with expression specific to the transverse vascular bundles (*TVB*) but not the longitudinal vascular bundles (*LBV*); (i), trichomes on leaf blade epidermis. Green colour due to fluorescence of GFP; red due to autofluorescence of chlorophyll. Scale bar in (a) =1 mm for upper floral images; scale bar in (f) =1 mm for images (f), (h) and (i).

may explain the high frequency of *gfp* expression observed in this organ. Expression of *gfp* associated with vascular bundles of shoots was also observed frequently (Figure 2g,h).

The predominant cell types expressing *gfp* in rice flowers were lodicules and vascular cells in the lemma and palea (4.5 and 4.4% of all plants examined; Figure 2a,d). Other striking patterns included lines with expression restricted to guard cells and trichomes of the palea (Figure 2f) and the ovary of the carpel (Figure 2e). A high frequency of expression was detected in the stamen (2.9%) with the vast majority of those lines expressing *gfp* in the pollen and/or anther loculi (Figure 2b) and only 5% with expression specific to the filament (Figure 2c).

Germinating seeds 16 h after imbibition rarely expressed *gfp* in coleorhiza and aleurone/endosperm layers (0.8%, Figure 3b), while most expression was observed in the scutellum (5.5%, Figure 3c) and embryo (4.2%, Figure 3a). As seedlings grew, expression was often clearly seen in the hydrolysing endosperm (Figure 3h; Table 1).

In 1-week-old seedlings the root showed a higher frequency of *gfp* expression than the shoot (25% versus 18% respectively), resulting from the fact that root-specific patterns were more commonly observed than shoot-specific patterns. Patterns were detected in many different organs and cell types of seedlings and ranged in specificity from single cell types such as guard cells (Figure 3j) or root hairs to constitutive plant expression (0.5% of patterns). Root patterns of varying specificity with regard to cell type (epidermal, stellar, endodermal, etc.) and root type (crown versus lateral) were also commonly observed (Figure 3d–g). In leaves *gfp* expression was detected in many different tissues such as mesophyll cells, trichomes, longitudinal vascular bundles (Figure 3i) and the basal meristem.

Confocal laser scanning microscopy (CLSM) enabled a more detailed examination of *gfp* expression patterns in the roots of enhancer trap lines selected by epifluorescence microscopy to be cell type specific. CLSM verified that, indeed, cell-specific expression of *gfp* had been achieved and identified lines with expression in specific cell types of the root such as the root cap (Figure 4a), epidermis (Figure 4b,c,f) as well as internal cell types such as the endodermis (Figure 4e), pericyle (Figure 4g) and protoxylem (Figure 4d). In addition to these cell type-specific expression patterns, lines with *gfp* expression determined by cell position rather than anatomically discernible cell type were also identified. Examples include a line where only root epidermal cells within two to three cells contact with

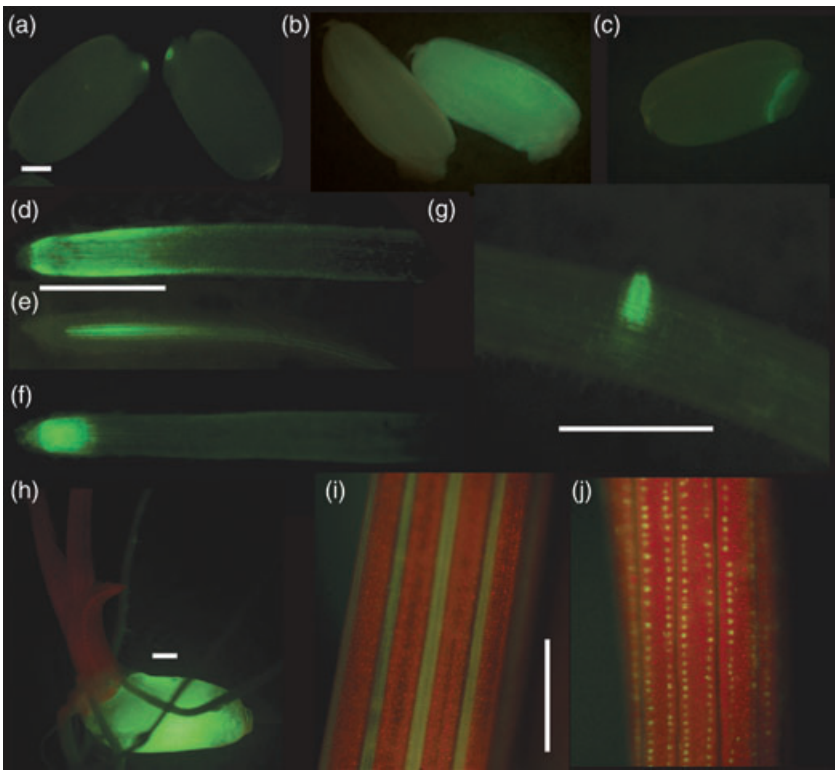

Figure 3. Enhancer trap lines (T_1) expressing *gal4* and *gfp* in specific tissues and cell types of the rice seed (a–c), root/endosperm (d–h) and shoot (i, j), visualized using epifluorescence microscopy. Fluorescence patterns: (a), embryo; (b), aleurone/endosperm; (c), scutellum; (d), crown root epidermis; (e), crown root stele; (f), crown root primary meristems (protoderm, ground, procambium and apical); (g), lateral root endodermis (emerging from crown root); (h), endosperm; (i), vascular bundle-associated leaf cells; (j), stomata. Green colour due to fluorescence of GFP; red due to autofluorescence of chlorophyll. Scale bar in (a) =1 mm for upper seed images; scale bar in (d) =1 mm for crown root images; scale bar in (i) = 1 mm for shoot images.

emerging lateral roots are marked with GFP (Figure 4h), and another where root epidermal cells undergoing cell division specifically produce GFP (Figure 4b). These results indicate that the rice enhancer trap lines possess patterns of GAL4/GFP that are specific not only to discrete cell types but also to cells dependent on their maturity and location.

Stability of gfp *expression in the enhancer trap lines*

To determine the stability of GFP patterns observed in our enhancer trap population across generations, we selected 30 lines displaying various patterns of *gfp* throughout the rice plant and examined expression in five to 10 plants of the T_2 generation. In all but one case (where expression was lost), we observed stable, consistent expression in seedlings and adult plants that corresponded to the patterns detected in the original T_0 and T_1 screens, indicating a transgene silencing frequency of 3% across two sexual cycles. More than half of the lines (55%) segregated for *gfp* expression in the T_2 generation while the remainder produced only GFP-positive progeny. In addition, the *gus* transactivation experiments (described in subsequent section) involved the use of T_2 seed from selected enhancer trap lines to produce callus for a second round of transformation. Second-round transformants were regenerated from those experiments that have continued to express GFP in the original specific pattern until at least the T_1 generation (present time), indicating that the enhancer trap patterns in those lines are robust and have not experienced silencing after a total of three generations with an intervening *in vitro* callus phase.

Spatial control of transgene expression through transactivation of uidA

The pC-2300:UAS:*uidA* transactivation cassette (Figure 1b) contained the *uidA* gene placed downstream of a –90CaMV 35S minimal promoter TATA and five, tandem UAS sequences. As with *gfp* in the pC4956:ET15 cassette (Figure 1a), transcription of the *uidA* gene could only be initiated by binding of GAL4 to the UAS sequences. No GUS activity was detected in regenerating calli and the leaves and roots of 136 plants produced by *Agrobacterium*-mediated transformation of wild-type rice (cv. Nipponbare) with this cassette, demonstrating that, in the absence of activation by GAL4, the cassette was incapable of driving *uidA* expression.

GAL4 enhancer trap lines displaying interesting and specific patterns of GFP were transformed with the UAS:*uidA* plasmid, and plants were regenerated after nine weeks of tissue culture (Table S2). We performed a preliminary experiment using an enhancer trap line with bright GFP fluorescence in regenerating calli to determine if GAL4 was capable of transactivating a secondary UAS:transgene at an unlinked locus (Figure 5). The majority of regenerating calli (80%) from the line were brightly fluorescent (Figure 5b), while the remaining 20% experienced varying degrees of silencing of the GAL4 enhancer trap cassette, displaying

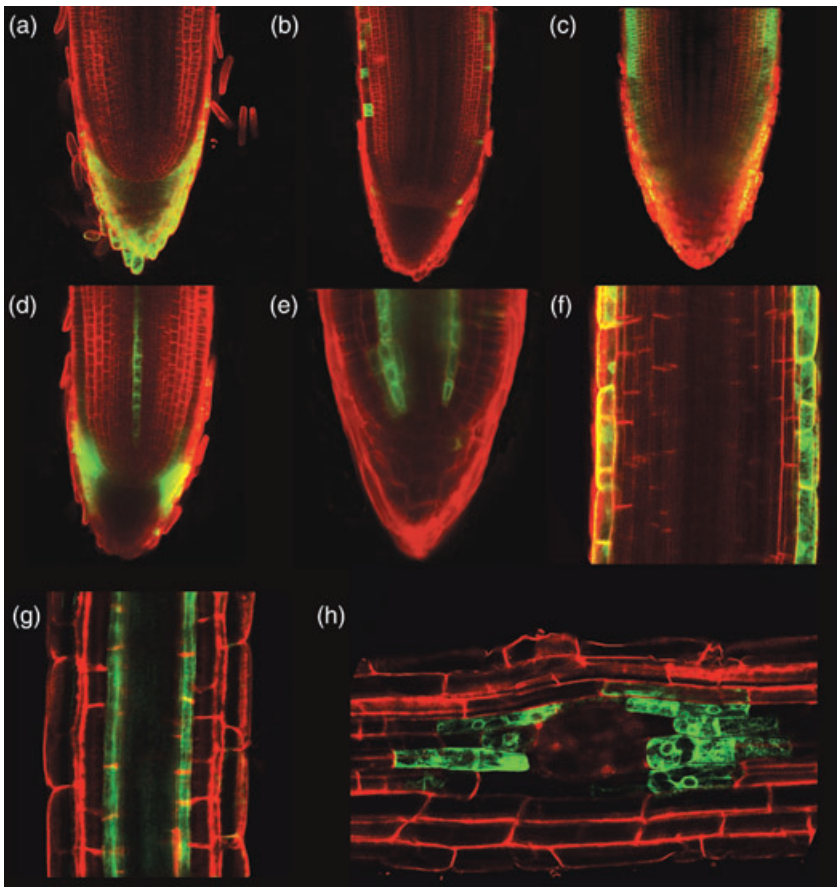

Figure 4. Identification of cell types responsible for enhancer trap patterns in the rice root tip (a–e) and mature root (f–h), using confocal laser scanning microscopy. Fluorescence patterns: (a), root cap; (b), dividing epidermal cells; (c), continuous throughout epidermis; (d), protoderm, cortex and root cap initials near cell division zone, as well as protoxylem in elongation zone; (e), endodermis; (f), epidermis; (g), pericycle; (h), crown root epidermal cells within two to three cells contact with an emerging lateral root. The lateral is emerging towards the reader, the cells in the focal plane are epidermal cells. Green colour due to fluorescence of GFP; red due to fluorescence of propidium iodide.

medium to no fluorescence of the GFP marker (Figure 5e,h). GUS staining of bright- (Figure 5a–c), medium- (Figure 5d–f) and non-fluorescent calli (Figure 5g,h) revealed that GFP fluorescence levels were positively correlated with GUS staining intensity, verifying that the GAL4 transcriptional activator was indeed transactivating a secondary gene placed downstream of UAS, in this case *uidA*, at a level of transcriptional control similar to that observed for the GFP marker.

Additional assays were conducted with the regenerated plants at seed, seedling and adult stages of development to determine if GAL4 could transactivate the UAS:*uidA* cassette in specific cell types of the rice plant (Figure 6). Figure 6(a) documents the GFP pattern of an enhancer trap line showing root epidermal expression, most notable for increased expression in the elongation zone, as well as scattered expression on the root cap. GUS histochemical staining of roots from this enhancer trap line revealed the identical pattern, not only in terms of pattern type (epidermal) but also with respect to intensity, as evidenced by enhanced GUS staining in the elongation zone (Figure 6b). Transactivation of UAS:*uidA* in more localized regions of the root was also demonstrated. Figure 6(c) documents GFP expression specific to epidermal cells of the root cap, with the corresponding GUS stain in Figure 6(d) revealing the same pattern.

The aleurone layer is a clearly defined cell type in the rice seed that surrounds the endosperm tissue. Cross-sectioning of seed from enhancer trap lines showing bright overall GFP expression 16 h post-imbibition (Figure 3b), enabled the identification of lines with expression specific to the aleurone layer (Figure 6e). The UAS:*uidA* cassette, when introduced into such lines, produced GUS patterns specific to the aleurone layer (Figure 6f) and provided clear visualization of these characteristically small, cubical cells. Transactivation of *uidA* was also documented in a variety of cell types in the rice leaf, such as the large hydathodes involved in guttation that are found at the margins of blade tips (Figure 6g,h).

Figure 6(i) documents GFP fluorescence of an enhancer trap line with several cell type-specific patterns in the rice flower: lodicules, loculi tissue of the anther, and floral trichomes on the lemma and palea. Figure 6(j) shows flowers of the same line after GUS staining and reveals the identical expression profile for GUS (note that lodicule expression is clearly specific to upper parts of the organ with both the GFP and GUS reporter genes). Closer examination of GFP fluorescence and GUS staining in floral trichomes shows that reporter gene expression is specific to this cell type and not present in the surrounding epidermal and mesophyll cells of the lemma/palea (Figure 6k,l). Figure 6(m,n) examines in

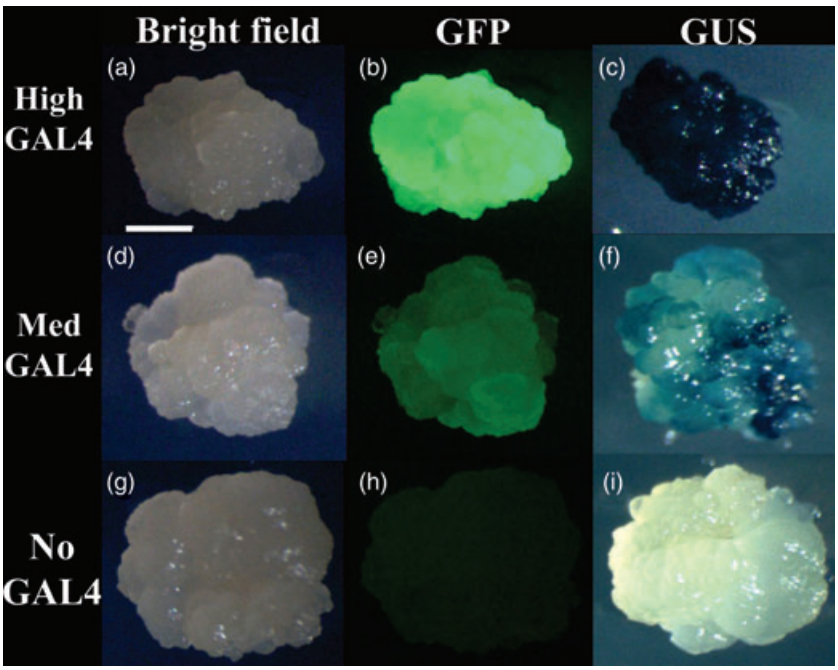

Figure 5. Intensity of GFP fluorescence correlates with GUS staining in calli transformed with both the GAL4 enhancer trap (Figure 1a) and the UAS:*uidA* transactivation cassette (Figure 1b), indicating that GAL4 is transactivating both reporter genes at equivalent levels of transcriptional control. A GAL4 enhancer trap line with brightly fluorescent calli was transformed with the UAS:*uidA* cassette. Roughly 80% of the regenerating calli were brightly fluorescent, while the remaining 20% ranged from medium to no fluorescence due to tissue culture-induced silencing of the GAL4 enhancer trap. Light and UV images were captured of live calli, GUS images of the same calli were captured after 2-h staining in X-Gluc at 37°C. (a–c), a high GAL4-expressing callus; (d–f), a medium GAL4-expressing callus; (g–i), a non-GAL4-expressing callus.

more detail the expression profile of reporter genes in the anther; GFP fluorescence indicates that expression is specific to the loculi ends where pollen dehiscence occurs (Figure 6m), and GUS staining verifies this observation by enabling visualization of the pollen grains which are free of GUS (Figure 6n). These results demonstrate that spatial control of transgene expression using the GAL4 system is possible in all organs of the rice plant.

Discussion

In addition to being a major food crop, rice has become the main molecular genetic model for cereal and monocot plants (Gale and Devos, 1998). Possessing a relatively small genome (430 Mb) with significant synteny to other cereals (Bennetzen and Freeling, 1998), rice was the first crop to have draft sequences of its genome published (Goff *et al.*, 2002; Yu *et al.*, 2002), and rapid progress with high quality sequences is being made (Feng *et al.*, 2002; Sasaki *et al.*, 2002; The Rice Chromosome 10 Sequencing Consortium, 2003). High-throughput *Agrobacterium*-mediated transformation techniques, producing low T-DNA copy numbers, are now routine (Hiei *et al.*, 1994; Sallaud *et al.*, 2003) and large collections of mutant lines have been generated (Chin *et al.*, 1999; Hirochika *et al.*, 2004; Jeon *et al.*, 2000; Jeong *et al.*, 2002; Miyao *et al.*, 2003; Sallaud *et al.*, 2004; Wu *et al.*, 2003). The abundance of available genetic resources, which continue to grow rapidly, has made rice the species of choice for many genetic and physiological studies of cereals.

A T-DNA-based GAL4 enhancer trapping cassette using the GFP reporter gene has been engineered for utilization in Arabidopsis (Haseloff, 1999; Haseloff and Hodge, 1997; Haseloff *et al.*, 1997) and roughly 250 lines with distinct and stable root expression patterns (http://www.plantsci.cam.ac.uk/Haseloff/Home.html) as well as 3500 lines showing a wide variety of expression patterns (http://enhancertraps.bio.upenn.edu) are now publicly available. The 13 000 enhancer trap lines described here demonstrate that the GAL4-GFP system functions equally well in rice, achieving a similar level of enhancer trapping efficiency as in Arabidopsis, and represent a significant step towards establishing GAL4 resources in the model monocot, rice. Furthermore, by introducing a UAS:*uidA* construct into selected enhancer trap lines, we have clearly demonstrated that this rice collection can be used to control transgene expression in specific cell types of the rice root, seed, leaf and flower. Many of the cell types in which we demonstrated transactivation of *uidA*, such as the seed aleurone layer or leaf hydathode cells, are involved in specific physiological processes in plants and the ability to activate transgene expression exclusively in those cell types should allow novel investigations into their function.

Wu *et al.* (2003) recently described the development of more than 30 000 rice enhancer trap lines employing a T-DNA-based GAL4 system with *GUSPlus* as the reporter gene. Surprisingly, the frequency of *GUSplus* expression in calli was 84%, while expression in plant organs ranged from 25% in stamens/pistils to 68.5% in leaves at seeding stage. Only 30% of lines showed no *GUSplus* expression. Little has been reported regarding reporter gene expression frequencies in the collection developed by Yang *et al.* (2004), although 24% of lines expressed *gus* in roots. These frequencies of expression are far higher than those reported for Arabidopsis, or for our own work in rice, and perhaps are

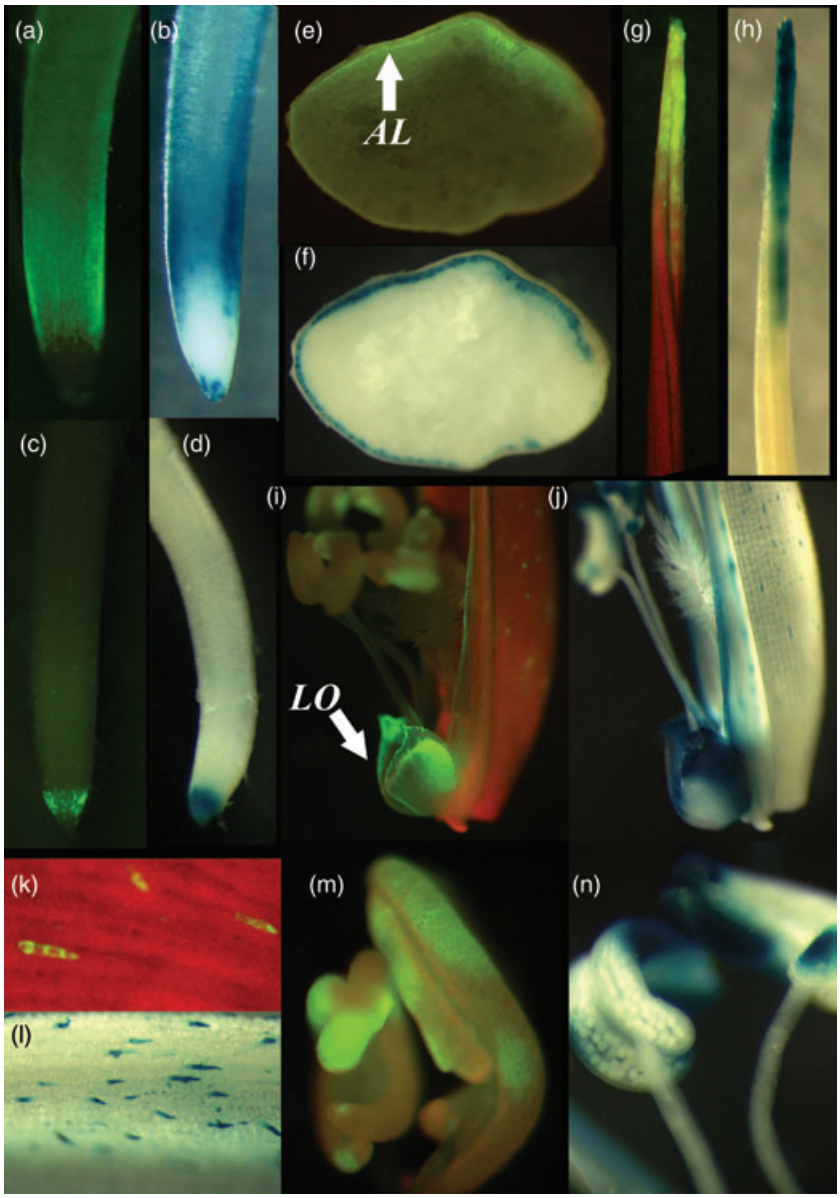

Figure 6. GAL4 enhancer trap lines transformed with the UAS:*uidA* cassette demonstrate *in planta* transactivation of *gfp* and *gus* in specific cell types of the rice root (a–d), seed (e, f), leaf (g, h) and flower (i–n).
(a, b) GFP image (left) and GUS staining pattern (right) of an enhancer trap line with root epidermal expression, most pronounced in the elongation zone with scattered expression also observed on the root cap.
(c, d) GFP image (left) and GUS staining pattern (right) of an enhancer trap line with root expression specific to epidermal cells of the root cap.
(e, f) GFP image (top) and GUS staining pattern (bottom) of an enhancer trap line with seed expression 16 h post-imbibition specific to cells of the aleurone layer (*AL*) surrounding the endosperm tissue.
(g, h) GFP image (left) and GUS staining pattern (right) of an enhancer trap line with leaf expression specific to large hydathode cells found along the margins of blade tips.
(i, j) GFP image (left) and GUS staining pattern (right) of an enhancer trap line showing floral expression in lodicules (*LO*), anthers and trichomes.
(k, l) Detailed images of GFP activity (top) and GUS staining (bottom) in the floral trichomes.
(m, n) Detailed images of GFP activity (left) and GUS staining (right) in the anthers reveal that expression is specific to the loculi, not pollen grains, at zones of pollen dehiscence.

inflated due to cryptic enhancer effects in the trapping cassette or staining/fixation artefacts. Alternatively, *GUS-Plus* may function as a more sensitive reporter gene than *gfp* at low levels of activity; although we have detected no differences between *gfp* and *gus* activity in our transactivation studies (Figures 5 and 6).

The fact that our enhancer trap collection is based on the GFP reporter gene offers several major advantages over GUS in its use as a traditional enhancer trapping tool and, more crucially, for the spatial control of transgene expression. The vital GFP reporter gene permits rapid, non-destructive screening of live plants, free from staining or fixation artefacts. This is particularly beneficial for the rice plant with a relatively long generation time and, when the lines were used for transactivation studies, allowed us to verify that target cell types were indeed producing GAL4 before commencing with further studies. Because reporter gene expression can be followed over time, simply by repeated viewing of the same specimen, lines with patterns specific to particular developmental stages have been identified and additional screens for GFP up- or down-regulation after exposure to stress, such as high salinity, are currently being performed.

Furthermore, confocal microscopy can be used for high-resolution imaging of GFP in specific cell types (Figure 4) as well as time-lapse imaging, where dynamic events in whole living cells or files of cells can be monitored for hours without photobleaching of the GFP (see http://www.plantsci.cam.ac.uk/Haseloff/downloads/downloadFrameset.html). Finally, individual cells marked with GFP can be identified and isolated for single cell studies, such as patch clamping

(Kiegle *et al.*, 2000b) and fluorescence-activated cell sorting (Makridou *et al.*, 2003; Sheen *et al.*, 1995). The ability to study the effects of transgene activation in a specific cell type that has developed in a normal physiological context (i.e. within the plant) is likely to provide novel insights relevant to *in planta* functions beyond those possible with conventional transgenic techniques.

The GAL4 enhancer trap lines presented here allow spatially controlled transgene expression in a wide variety of cell types throughout the rice plant and, as such, provide a powerful tool for probing cell type-specific processes important for crop productivity. We envisage that these lines will be used primarily for the transactivation of genes in specific cells marked by GFP and for cell-specific manipulation of native gene expression using techniques such as RNAi silencing. In addition, they could be used for identifying transcriptionally distinct cell types, visualizing cell position and fate during development, cloning of cell- and tissue-specific regulatory elements, and activation of random genes in specific cell types for gain-of-function screens.

Experimental procedures

Construction of the enhancer trap cassette

The *gal4*-based enhancer trap cassette pET15 (pBIN mGAL4-VP16 UAS_{GAL4}::mGFP5ER-enhancer trap) carrying the *mgfp5-er* reporter gene (Haseloff *et al.*, 1997) was the source of our enhancer trap. The T-DNA region from the deleted 35S promoter upstream of the *gal4-vp16* gene to the Nos terminator 3′ to *gfp* was PCR-amplified from pET15 with primers containing *Kpn*I restriction sites (5′-GATCGGTACCAACACTGGATCTTCGCAAGACC-3′; 5′-GCGCGGTACCCGCTTAGACAACTTAATAACACATTG-3′) and subsequently ligated into a unique *Kpn*I site near the right border of the binary plasmid pC-4956 (A. Betzner and W. Tucker, ANU, Canberra, Australia, unpublished data). The insertion of the T-DNA region into pC-4956 resulted in the pC-4956:ET15 plasmid (Figure 1a).

Construction of the gene transactivation cassette

The *uidA*-based cassette pBIB mGAL4-VP16 UAS_{GAL4}: *uidA* was the source of our transactivation construct. The T-DNA region from the –90CaMV 35S minimal promoter TATA to the Nos terminator 3′ to *uidA* was restricted as a *Hin*dIII/*Eco*RI fragment and ligated into the corresponding restriction sites of the binary plasmid pC-2300 multiple cloning site. The insertion of the T-DNA region into pCAMBIA 2300 (CAMBIA, Canberra, Australia) resulted in the pC-2300:UAS:*uidA* plasmid (Figure 1b).

Production, growth conditions and maintenance of transgenic plants

Oryza sativa L. cv Nipponbare was used. Embryogenic nodular units arising from scutellum-derived callus were inoculated with supervirulent *A. tumefaciens* strains EHA105 and AGL1 (carrying the pC-4956:ET15 plasmid) and 50 mg l^{-1} hygromycin-resistant shoots were regenerated after nine weeks according to the protocol described by Sallaud *et al.* (2003, 2004). Overall, 3467 inoculated calli yielded 13 881 regenerated plants with an average transformation efficiency of four primary transformants per callus, consistent with that obtained with other T-DNA cassettes (Sallaud *et al.*, 2004). The same transformation procedure was used for transferring the pC-2300:UAS:*uidA* T-DNA to hygromycin-resistant, scutellum-derived calli of selected enhancer trap lines except that geneticin (200 mg l^{-1}) was used for selection. Rooted T_0 plantlets were transferred to the greenhouse in Jiffy peat pots, and moved to soil after 15 days. Seed was harvested from fertile lines, dried for 3 days at 37°C, and placed into cold storage (4°C, 30% humidity).

Screening for GFP in rice tissues

Expression of *gfp* was assessed at four different stages: (1) 5-week old, hygromycin-resistant calli on pre-regeneration medium; (2) unopened flowers just prior to anthesis and a leaf section comprising sheath, collar (lamina joint) and blade tissue of T_0 greenhouse plants; (3) T_1 seed 16 h after imbibition in Petri dishes on moist filter paper; and (4) 1-week-old T_1 seedlings at the second-leaf stage. GFP fluorescence in living tissue was detected using a Leica MZ FLIII fluorescence stereomicroscope (Leica Microscopie Systémes SA, Heerbrugg, Switzerland) and GFP Plus fluorescence filter set [GFP2, 480 nm excitation filter (bandwidth of 40 nm) and 510 nm barrier filter]. Images were collected using a Leica DC 300F digital camera. For CLSM, roots were immersed in propidium iodide (10 μg ml^{-1}) for 5 min to outline cell walls and provide a counter fluor to the GFP. A Leica TCS-SP1 laser scanning confocal microscope equipped with an argon laser was used for imaging at an excitation wavelength of 488 nm. The GFP and propidium iodide emissions were collected in separate channels (500–550 nm and 600–700 nm respectively) and then overlaid to create composite images.

GUS assay

Visualization of *uidA* expression was performed by histochemical GUS-staining according to Jeon *et al.* (2000). Callus tissue was incubated at 37°C for 2 h, while plant tissue was incubated at 37°C for 12 h, and then rinsed through a series of 70% ethanol washes to remove chlorophyll. GUS activity was examined using a Leica MZ FLIII fluorescence stereomicroscope.

The O. sativa *L. GAL4/GFP database*

A database has been constructed which contains all of the images and observations made during the four screens described in this paper – this is being constantly updated as further lines are screened. The FilemakerPro® database, which, at publication, contains descriptions of more than 500 enhancer trap lines that have yielded sufficient seed for distribution, is searchable by expression level, expression pattern, developmental stage, etc. and is being made accessible at http://www.plantsci.cam.ac.uk/Hibberd/Home.html and http://plantscience.acpfg.com.au/project/detail/36. A pdf version of an extract from this database is provided as Figure S1. A similar, searchable database developed under the Oracle Management System can be found at http://genoplante-info.infobiogen.fr/OryzaTagLine.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2339/TPJ2339sm.htm

Figure S1. Extract of the GALt/GFP database.

Table S1 Frequency of developmental- and tissue-specific *gfp* expression patterns in the enhancer trap library. (A) Nearly half (48%) of the 346 GFP-positive lines detected during the screening of 1052 lines at the T_0 mature, T_1 seed and T_1 seedling stages displayed patterns specific to one particular developmental stage, 41% had patterns present at two stages, while 11% displayed GFP patterns at all stages of development. Expression data for individual lines at the callus regeneration stage were not collected (23% overall expressed *gfp*), although this can be determined for selected lines by inducing calli from seed. If callus expression poses a problem for transactivation, UAS transgenes can be introduced via sexual crosses. (B) Among the 280 GFP-positive lines detected in the T_0 mature screen (1982 lines), 63% had patterns specific to either the flower or shoot, while the remainder displayed GFP activity in both. (C) Approximately one-third of the 780 GFP-positive lines detected in the T_1 seedling screen (2667 lines) had patterns specific to the root, shoot or endosperm, 42% had patterns present in two of the tissues, while 26% had GFP activity in all three of the tissues

Table S2 Reporter gene expression in plants produced by super-transformation of two different enhancer trap lines with the UAS:*uidA* cassette. (A) The AOR G08 line had GFP patterns at both the mature and seedling stages and thus data are included for both periods. Two-thirds of mature T_0 lines regenerated from this transformation did not produce GFP, possibly resulting from the segregation of multiple, silent enhancer trap copies in this line. Of the GFP-positive regenerants, 64% produced a similar GUS staining expression profile, while the remainder did not stain for GUS. A higher number of the lines exhibited GFP activity at the seedling stage (45%), and 71% of the GFP-positive regenerants produced a similar GUS staining expression profile. None of the GFP-negative regenerants at both the mature and seedling stages stained positive for GUS. (B) Half of the transformants regenerated from AOU F04 retained the original GFP pattern (54%), and all of the GFP-positive regenerants produced a similar GUS staining expression profile. None of the GFP-negative regenerants stained positive for GUS. These results clearly indicate the importance of regenerating sufficient super-transformants to account for any silent enhancer trap copies segregating in the line and/or faulty integration (truncation, tandem insertion, etc.) of the transactivation cassette

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