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Development of GAL4/VP16 Facilitated-enhancer Trap System for Rice Crop Improvement

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ABSTRAK

Pengembangan Sistem Perangkap Enhancer yang Difasilitasi oleh Aktivator Transkripsi GAL4/VP16 untuk Perbaikan Tanaman Padi. *Sri Koerniati dan Andrzej Kilian*. Peningkatan produksi padi untuk memenuhi kebutuhan nasional dilakukan melalui perbaikan tanaman, termasuk pencarian dan isolasi gen-gen baru melalui mutasi. Aplikasi berbagai sekuen mutagen, elemen loncat atau T-DNA, didukung dengan teknik PCR merupakan perdekatan yang lebih baik dibandingkan dengan metode klasik. Perangkap *enhancer* adalah sistem yang dikembangkan untuk mengatasi masalah rendahnya tingkat perolehan mutan, akibat banyak gen berfungsi sama atau satu gen berfungsi pada beberapa tingkat perkembangan tanaman. Sistem ini mampu menampilkan ekspresi pada jaringan tertentu (*spatial*) dan atau pada waktu tertentu (*temporal*) pada tanaman hemizigot (*hemizygous plants*). Penelitian ini bertujuan untuk (1) mengembangkan *vector cassette* perangkap *enhancer* dengan komponen utama aktivator transkripsi GAL4/VP16 dan dua gen reporter (*gus* dan *gus*Plus), dan (2) memperoleh informasi tentang ekspresinya pada padi. Sepuluh *vector* diperoleh dan ditransformasikan ke kalus padi Nipponbare dan Millin dengan *Agrobacterium tumefaciens*. Kajian vektor melalui ekspresi gen reporter diamati pada kalus 3 hari setelah *co-cultivation* dan jaringan vegetatif dari 745 lini penangkap *enhancer*. Sembilan puluh lima persen nomor memiliki ekspresi dan persentase lebih tinggi daripada yang telah dilaporkan sebelumnya. Lini dengan vektor GAL4/VP16 delesi tidak memiliki ekspresi pada kalus dan jaringan vegetatif, walaupun hasil *Southern Blot* menunjukkan tanaman ini memiliki dua T-DNA. Tiga puluh dua persen lini *gus*Plus memiliki ekspresi yang kuat, sedangkan 30% berekspresi lemah dibandingkan dengan masing-masing 12% dan 47% untuk lini *gus*. Lini *gus*Plus juga tersebar pada lebih banyak pola ekspresi. Jumlah insersi pada lini perangkap *enhancer* berkisar antara 1-7 T-DNA dan 49% di antaranya memiliki satu T-DNA. *gus*Plus merupakan gen reporter yang lebih sensitif daripada *gus* dan GAL4/VP16 terbukti berfungsi.

Kata kunci: Sistem perangkap *enhancer*, gen reporter *gus* dan *gus*Plus, padi.

INTRODUCTION

Rice is one of the world's most important crops used as a staple food for more than one-half of the world population. It is a nutritious grain crop which contains carbohydrate, proteins, lipids, and minerals, etc. Rice is used for food in various forms. Rice is planted on about 150 million hectares annually. More than 91% of world production comes from Asia, 5% from the Americas, 3% from Africa, and another 1% from Europe and Oceania. Rice demand in year 2030 is projected to be approximately 533 million tones of milled rice (FAO 2002). To meet this goal, rice varieties with high yielding potentials, durable resistances to pests and tolerances to a biotic stresses are needed. Addressing these problems requires a coordinated approach using the best available research tools at molecular level. This include use of transgenic, isolation, and transfer of novel genes that confer tolerances or resistances to particular biotic and abiotic stresses, and the use of molecular markers in the breeding program to enhance genetic improvements.

A forward genetic approach has been applied to increase the rate of finding new rice genes. In common, rice mutants are generated using either chemical or physical mutagens, such as ethyl methanesulphonate (EMS) (Inukai *et al.* 2000, Goel *et al.* 2001) and gamma-ray irradiation (Teraishi *et al.* 1999, Biswass *et al.* 2003). Unconventionally, mutants can be generated through the application of insertion sequence mutagenesis, such as Ds or Ac transposable elements and T-DNA of *Agrobacterium tumefaciens*-mediated transformation (Izawa 1997, Jeon *et al.* 2000). The use of insertion sequence mutagenesis in rice was boosted by the efficient transformation method developed by Hiei *et al.* (1997). The technique offers advantages over the conventional mutagenesis in that it facilitates the tagging of the target gene both molecularly and genetically, as reporter and/or selectable marker genes are carried along by the insert (Martienssen 1998). A Thermal Asymmetric Interlaced (TAIL)-PCR was developed to recover the DNA flanking the insert (Liu and Whittier 1995) and this may lead to the isolation of the wild type gene sequences (Ortega *et al.* 2002). The integration of insertion sequences into protein coding regions of the nuclear genome may inactivate or alter the expression of plant genes resulting in recessive or *Loss-of-Function* (LoF) mutations. Not all genes, however, can be uncovered

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by insertion mutagenesis (Burns *et al.* 1994). The first reason is due to the presence of genes functional redundancy where one or more other loci can substitute for the same function, thus preventing the elucidation of gene function by the LoF approach (Campisi *et al.* 1999, Springer 2000). The second reason that genes knock-out may fail to uncover genes is that many genes function at multiple stages of development. The mutation of such a gene might affect early lethality and could be highly pleiotropic. Both of these effects can mask the role of the genes in specific pathways. In addition, the frequency of the lethal mutant recovery is low (Miklos and Rubin 1996).

One of alternative approaches developed to deal with the gene redundancy problem, and that of is able to reveal spatial (cell or tissue-specific) and/or temporal (developmental stage) gene expression patterns are gene trapping, promoter trapping and enhancer trapping (Topping and Lindsey 1995, Campisi *et al.* 1999, Jeong *et al.* 2002). Each type these approaches have its advantages and disadvantages over the other type. Gene trap is used for expression a construct need to be inserted into the transcriptional unit (transcribed region) and only if the orientation is correct (Springer 2000). One or more splice acceptor sequences preceding a reporter gene allow expression if insertions are in the intron site (Springer 2000). Apart from transcriptional fusions, this trap can also create translational fusions, which may provide information about protein localisation. However, gene trap insertions are more likely to lead to gene disruption. From 2000 *Arabidopsis* gene trap lines screened, 32% of gene trap insertions exhibited expression in seedlings and 10% exhibited expression in floral and reproductive tissues (Gu *et al.* 1998). In rice, maximum *gus* tagging efficiency would be about 25% (Jeon *et al.* 2000). A promoter trap construct contains a reporter gene, either fused to a minimal promoter or without minimal promoter at the T-DNA left border (Topping *et al.* 1991, Topping *et al.* 1994). Similar to the gene trap, promoter trap expression can only be obtained when a construct is inserted into a transcribed region (Topping *et al.* 1991, Topping *et al.* 1994), producing of translational fusion and gene disruption. The system was first developed in tobacco, *Arabidopsis* and potato. Similar percentages of lines with expression in leaf and/or root (73% and 78%), were displayed by transgenic transformed with either construct described (Topping *et al.* 1991).

An enhancer trap construct typically contains a minimal promoter fused to a reporter gene, which is activated by chromosomal enhancer elements, resulting in expression of the reporter gene. In producing a gene expression, the enhancer trap does not suffer from constraints mentioned in the two previous trap systems. A high frequency of reporter gene expression is demonstrated and frequently resembled the expression patterns of endogenous trapped genes (Wilson *et al.* 1990, Sundaresan *et al.* 1995). Due to the transcription is affected by position, expression levels of transgenes at different chromosomal locations may vary greatly. Some advantages of the system, enhancer trap lines will almost always be viable, even when the enhancer trap T-DNA inserts in the middle of an essential gene, because the enhancer trap element is dominant. Patterns can be detected when insertions are in a hemizygous state (Campisi *et al.* 1999). The use of enhancer traps in gene expression studies exploits the fact that enhancers are essential for gene transcription in eukaryotic cells (Bonifer 2000, Martin 2001). Such traps have been used in *Drosophila* (Bellen 1989, Bellen 1999) and in *Arabidopsis* (Sundaresan *et al.* 1995) and a high efficiency of trapping, from 25% to 59%, was displayed in rice (Wu *et al.* 2003).

The first development of an enhancer trap was applied in *Escherichia coli*, using a bacteriophage Mu carrying a promoter-less *lacZ* gene. Insertion of the construct into a gene under constitutive control produced constitutive *lacZ* gene expression, whereas the insertion into a regulated gene led to *lacZ* induction only under specific conditions (temporal) (Casadaban and Cohen 1979). Ten years later, the system was applied using a translation fusion between a *P*-transposase gene and a promoter-less *Escherichia coli* β-galactosidase (*LacZ*) gene was in *Drosophila melanogaster* (Bellen 1989). The enhancer could only be activeted if the transposon integrates in the genome close to a genomic enhancer (O'Kane and Gehring 1987) and incapable of inducing gene regulation in a specific biological pathway since the transposon tends to insert non-randomly (Bellen 1999). Later *Ds* or *Ac*-based enhancer trap constructs were designed to have the shortest 5' end and the core sequence of the CaMV 35S promoter. Expressions of the reporter genes were produced by insertions near the chromosomal enhancer sequence in plants (Sundaresan *et al.* 1995). This system has been applied in *Arabidopsis* and rice (Fedoroff and Smith 1993, Klimyuk *et al.* 1995, Sundaresan *et al.* 1995, Chin *et al*. 1999), resulting in about 48% transpositions associated with reporter gene expression patterns in various organs (Sundaresan *et al.* 1995), and the patterns were stably inherited in the subsequent generations (Klimyuk *et al.* 1995).

A T-DNA-based enhancer trap construct containing a minimal CaMV 35S promoter that fused into the *gus* reporter gene and located next to the right T-DNA border was developed recently. Rice and *Arabidopsis* enhancer trap lines generated with this construct exhibited tissue specific expression patterns (Campisi *et al.* 1999, Jeon *et al.* 2000). The more recent T-DNA enhancer trap construct utilized a transcriptional activator GAL4. The GAL4 that functions through the recognition of the upstream activating sequence of the GAL gene (UAS_{GAL4}) by DNA binding domain, allowing transcription of any gene linked to the UAS (Fischer *et al.* 1988). Therefore, this system offers an advantage over enhancer trap systems described above, as it allows subsequent use of GAL4 lines as "effectors" or pattern lines to direct the expression of any gene in a spatially and temporally regulated fashion by introducing a second construct in which the gene of interest is placed downstream of the UAS_G as a "receptor" or target (Brand and Perrimon 1993, Castelli-Gair *et al.* 1994). Expression of a toxin gene, such as DTA gene was used to kill or inactivate specific cells under investigation (Brand and Dormand 1995).

This paper reports result of research carried out at CAMBIA, Canberra, Australia, in 1999-2001, with objectives to develop a new enhancer trap system using the GAL4/VP16 transcriptional activator and to test its expression in rice. Our analyses to evaluate two different reporter genes β-glucuronidases, *gus* and *gus*Plus, used for visualizing the activity of the GAL4/VP16-facilitated enhancer trap system and the ability of GAL4/VP16 to reveal expression are mainly discussed, as both are critical components of the system. A behavior of T-DNA en-hancer trap insertion in the rice genome (TAFET lines) was presented.

MATERIALS AND METHODS

Design and Construction of Transcriptional Activator-facilitated Enhancer Trap Binary Vectors

A *p*TG113 vector backbone (source of Castor bean C*atalase*-1 gene intron and the *gus* gene) and the *p*DAMSNS-Luc L containing 6 repeats of the Upstream Activating sequence (UAS_{GAL4}) of the GAL gene, GGAAGACTCTCCTCCG, were digested with *Hind*III and *Nco*I. The fragments were purified from an agarose gel using the Qiagen gel extraction kit and DNA were subsequently ligated using T_4 DNA ligase enzymes (NEB) and transformed into DH5-α competent-cell. After shaking for 30 minutes in 37°C shaker, aliquots of cells were plated on solid medium contained 100 mg ampicilin/L and grown in a 37°C incubator overnight. 10 individual colonies were cultured in LB liquid medium containing ampicilin[100] overnight. Plasmid DNA was isolated using a CTAB method (Del Sal *et al.* 1989) and identified by digestion with *Hind*III/*Nco*I and *Spe*I. These steps produced intermediate plasmid *p*SKC2.1. Plasmid *p*FX-B61.1 (source for the GAL4/VP16 and NPTII gene driven by 35S) and *p*SKC2.1 were digested with *Hind*III and ligated, producing *p*SKC59.1 (containing 6 x UAS-*gus* proximal, 1.6 kb to 35S-NPTII, and GAL4/VP16) and *p*SKC59.2 (containing 6 x UAS-*gus* distal, 7 kb from 35S-NPTII and GAL4/VP16). Plasmid *p*FX-B75.1-1 (containing the GAL4/VP16 with *Cat-1* gene intron) was treated similarly as *p*FX-B61.1, produced *p*SKC66.1 (6 x UAS-*gus* cassette proximal, 1.6 kb to 35S-NPTII and GAL4/VP16-*Cat-1* intron) and *p*SKD76.2 (6 x UAS-*gus* cassette distal, 7 kb from 35S-NPTII, and GAL4/VP16-*Cat-1* intron).

Plasmids *p*SKC2.1 and *p*TANH114 (containing a *gus*Plus gene) were digested with *Nco*I and *Afl*II, produced intermediate plasmid *p*SKE15.11 containing 6 x UAS-catalase intron-*gus*Plus. Subsequently, *p*SKE15.11 and *p*FX-B61-1 were digested with *Hind*III, treated with SAP-dephosphorylated (Boehringer Mannheim protocol) and ligated, producing *p*SMRJ18 (6 x UAS-*gus*Plus cassette proximal, 1.6 kb to 35S-NPTII) and *p*SMRJ18R (6 x UAS-*gus*Plus cassette distance, 7 kb from 35S-NPTII). Similar procedure was applied to clone *p*SKE15.11 into *p*FX-B75.1-1, resulting of *p*SMRJ17 and (containing 6 x UAS *gus*Plus proximate, 1.6 kb to the 35S-NPTII and GAL4/VP16-*Cat*-1 intron) and *p*SMRJ17R (6 x UAS *gus*Plus distance, 7 kb from the 35S-NPTII and GAL4/VP16-*Cat*-intron).

Beside those constructs, double digestions at *Eco*47 and *Stu*II restriction sites deleted 108 bp of the GAL4/VP16 activating domain of *p*SKC59.1 and the *p*SKC66.1, resulting *p*SKD15.1, and *p*SKD15 vectors, respectively. These constructs were designed as negative controls of the GAL4/VP16 transcriptional activator system.

Generating Rice TAFET Lines

All binary vector plasmids were introduced into *Agrobacterium tumefaciens* (strain EHA-105) by electroporation. Three to four weeks embryogenic calli derived from rice scutellum was transformed using *Agrobacterium tumefaciens* containing binary vectors (Hiei *et al.* 1994). Independent rice transgenic rice lines were regenerated on 100 mg/L hygromycin containing medium. The regenerated lines were grown in the green house that was set at 28°C during the day and 20°C at night. The light/dark cycle in the greenhouse was set for 14/10 hours.

Gus and *Gus*Plus Assays and Reporter Gene Expression Analysis of TAFET Lines

Histochemical detection of *gus* (β-glucuronidase) and *gus*Plus was performed using fresh plant organs from vegetative and generative parts of the first generation of TAFET lines (T_0), as described by Jefferson *et al.* (1987). The vegetative parts were collected at the plantlet stage, just before transfer to the green house for histochemical analysis of *gus* and *gus*Plus. Samples were viewed using a Leica Wild M8 microscope or a Leitz Diaplan microscope with bright-field optics. Images were acquired with a Nikon CoolPix Digital photo camera. The images were acquired with a Nikon N-2000 photo camera.

To determine the effect of the reporter genes and the transcriptional activator GAL4/VP16 on expression patterns in the vegetative tissues of T_0 generation of TAFET lines tabulation, grouping and analysis were carried out.

Molecular Analysis of TAFET Lines

Plants DNA were extracted from fresh leaf tissue ground in liquid nitrogen using a CTAB method as previously described (Del Sal *et al.* 1989). This DNA was then digested with *Eco*RI restriction enzyme. Electrophoresis and Southern blot hybridization of DNA were performed as previously described (Sambrook *et al.* 1989), using the GAL4/VP16 radioactive-labeled probe.

RESULT AND DISCUSSION

In eukaryotic promoters, a TATA-box (which is a component of a gene promoter about 100 base pairs), operates in close proximity to the transcription initiation site (Martin 2001), whereas an enhancers which links in *cis* to a promoter, acts from several hundred base pairs away from the transcription start site (Bohmann *et al.* 1987, Martin 2001). Enhancers are binding sites for transcriptional activators (regulatory proteins). The binding of an enhancer to a UAS produces much closer distances between regulatory proteins and the UAS in the TATA box (Guarente 1988). These characteristics were exploited in development of a new enhancer trap system.

Design and Construction of Transcriptional Activator-facilitated Enhancer Trap Binary Vectors

The *gus* reporter gene that is a widely used β-glucuronidase from *Escherichia coli* (Jefferson *et al.* 1987) and a newly developed β-glucuronidase *gus*Plus from *Staphylococcus* sp. (Nguyen 2002) were tested, beside a transactivator GAL4/VP16. In order to maximize the trapping ability of the GAL4/VP16 enhancer trap system, a minimal promoter (mp) of the transcriptional activator cassettes was always positioned at the right (5') border of the T-DNA. The F_1 replication origin, a coding sequence of *bla* and a replication origin of the *p*MB1 mutant of *p*Blue-Script backbone (total 3.2 kb) are sequences providing a separation between the UAS-reporter gene and transactivator in TAFET plasmids. Transactivator plasmids were constructed to position UAS-β-glucuronidase reporters were relatively close (1.6 kb) to or distant (7 kb) from the CaMV35S promoter driving gene resistance to hygromycin (*hpt*II), for example in the plasmid of *p*SKC59.1 (Figure 1) and in the plasmid *p*SKD76-1 (Figure 2), respectively. The nomenclature and description of the binary vectors produced in our research are shown in Table 1. All constructs also contained the mgfp5-ER reporter gene, but this reportter gene was proved to have very low sensitivity and as observed only in the context of transactivation tests to ensure lack of Green-Fluorescence background in TAFET lines used for crossing.

Generating Rice TAFET Lines

Almost 1,000 independent TAFET lines were produced from the transformation of 8 TAFET constructs mediated by *Agrobacterium tumefaciens* (Hiei *et al.* 1994). Three hundred and twenty lines were produced using the TAFET *gus* constructs, while 663 lines were produced using the TAFET *gus*Plus constructs. These lines were developed in two Japonica rice varieties Nipponbare and Millin. Among these, only three independent lines were produced using the *p*SKD15.2

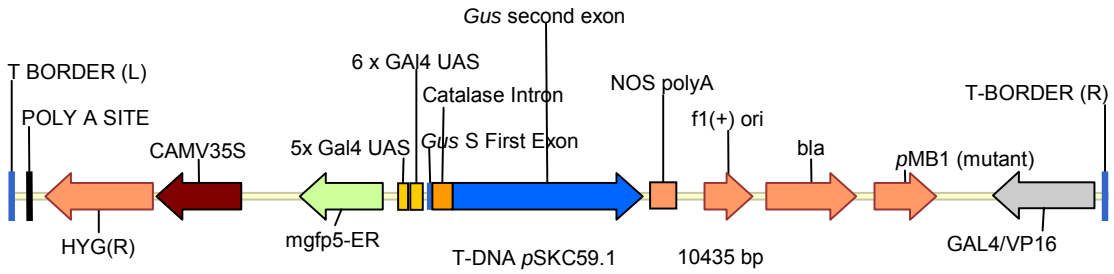

Figure 1. Plasmid *p*SKC59.1 containing 35S_HPTII-5UAS_mGFP5ER-6UAS_*gus*-*p*BS-GAL4/VP16.

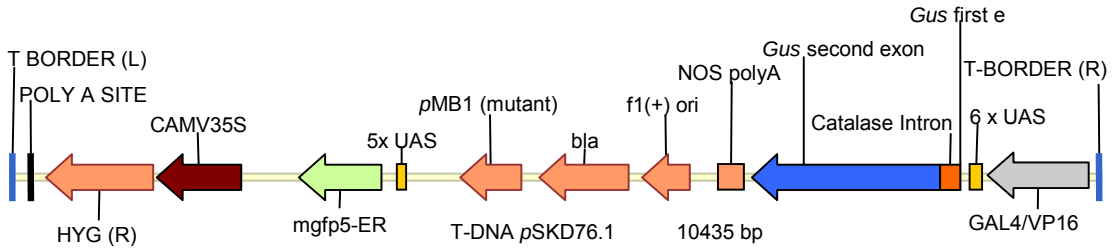

Figure 2. Plasmid *p*SKD76.1 containing 35S_HPTII-5UAS_mGFP5ER-*p*BS-6UAS_*gus*-GAL4/VP16.

Table 1. TAFET constructs containing *gus* and *gus*Plus reporter genes.

Binary vector description	*Gus*	*Gus*Plus
35S_HPTII-5UAS_mGFP5ER-6UAS_reporter gene-pBS_GAL4/VP16	*p*SKC59.1	*p*SMRJ18
35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_reporter gene_GAL4/VP16	*p*SKD76.1	*p*SMRJ18R
35S_HPTII-5UAS_mGFP5ER-6UAS_reporter gene-*p*BS-intron_GAL4/VP16	*p*SKC66.1	*p*SMRJ17
35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_reporter gene-intron_GAL4/VP16	*p*SKD76.2	*p*SMRJ17R
35S_HPTII-5UAS_mGFP5ER-6UAS_reporter gene-*p*BS_deletion GAL4/VP16	*p*SKD15.1	
35S_HPTII-5UAS_mGFP5ER-6UAS_reporter gene-*p*BS-intron_deletion GAL4/VP16	*p*SKD15.2	

35S = the CaMV35 promoter, *hpt*II = *hpt*II Hygromycin resistance gene, mGFP5ER = modified Green Fluorescent Protein, 5UAS, 6UAS = upstream activation sequence of the GAL4, *p*BS = plasmid Blue-Script, intron = the castor bean *catalase* intron, GAL4/VP16 = transcriptional activator fusion.

construct, one of negative control of TAFET constructs, and 64 independent lines were that of with *p*CAMBIA1201, as positive control.

Gus and *Gus*Plus Reporter Genes Expression Analysis of TAFET Line

An effort to evaluate the principal component of the system, which is the reporter gene β-glucuronidases *gus* and *gus*Plus and the transactivator, was first carried out in rice calli. The information obtained will be useful to define whether the system is working or not working and also to define sensitivity of the reporter gene itself, since this is critical in the enhancer trap system. The GAL4/VP16 deletion constructs, *p*SKD15.1 and *p*SKD15.2 were developed for that purpose. Observation was performed on calli 3 days after co-cultivation with *Agrobacterium*. Results showed that the average percentage of calli exhibiting blue foci of *gus* expression of four *gus* TAFET constructs ranged from 14.4 to 54.3%, while that of with four *gus*Plus constructs ranged from 42 to 71.5% (Figure 3).

The average percentage of calli expressed *gus* gene of *p*CAMBIA1201 was about 60.6% (a positive control). In contrast, the calli transformed using *p*SKD15.1 and *p*SKD15.2 displayed no blue foci of the *gus* gene (Figure 3). Transformations using these two deletion constructs were replicated three times and observations were carried out 3, 7, and 14 days after co-cultivation. Lines of *p*SKD15.2 did not exhibit *gus* expression in their vegetative tissues. These results were interpreted as a clear indication that the expression was due to the GAL4/VP16 activity. A deletion of 108 bp in the binding domain of the GAL4 disrupted the binding of GAL4 to the UAS_{GAL4} and stopped the trans-cription of the reporter gene fused to the UAS_{GAL4}, and this was in compromise with that of previously reported (Sullivan *et al.* 1998).

Efficiency of the system to induce the gene expression was continuously evaluated, in 745 T_0 TAFET lines. Only about 5% of 745 lines did not have any reporter gene expression in their vegetative tissues. Of expressing reporter gene in rice vegetative tissues, about 34%, 36 and 25% of lines displayed weak (scored as 1), medium (2) and strong (3) expression in vegetative tissues (Figure 4). When three levels of expression were separated between the *gus* and *gus*Plus TAFET lines, it was indicated that the number of *gus*Plus lines with weak expression were lower (30%) than those of the *gus* lines (47%). In contrast, the *gus*Plus lines with strong expression were higher (32%) than those of the *gus* lines (12%) (Figure 4).

Analysis of the gene expression on the vegetative tissues was attempted by comparing percentages of total unique patterns divided by total lines with expression to a percentage of total patterns divided by total lines for each construct. For TAFET *gus* lines, the percentage of total unique patterns divided by total lines with the expression is higher than that of the percentage of total patterns divided by the total lines analysis. In contrast, the percentages of these two calculations for each *gus*Plus constructs were similar but lower than those of the *gus* constructs (Figure 5). These results were due to the fact that the *gus*Plus

constructs produced more lines with expression than that of the *gus* constructs, confirming the observation in the callus stage. These may indicate that *gus*Plus is a more sensitive reporter gene than *gus*.

A diversity of patterns produced by TAFET constructs in the vegetative rice tissues was also analyses. Each expression pattern was classified into a score like, which was defined as "a" for the root tissue, "b" for the shoot tissue, and "c" for the leaf tissue and all the possible combinations of these tissues. In theory, there are 7 possible patterns occurred in the TAFET lines. Data on the most abundant patterns produced by each constructs are shown in Table 2 and Figure 6.

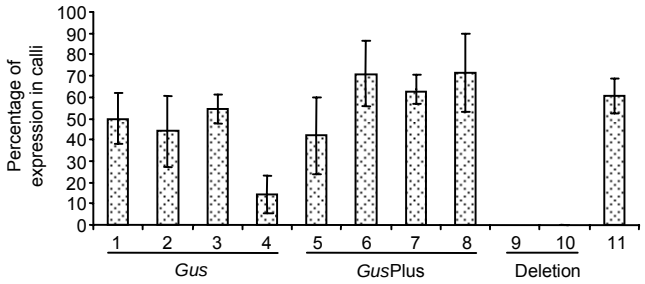

Figure 3. Expression of enhancer trap constructs in rice calli stage. 1-8 = transactivator constructs, 9-10 = GAL4/VP16 deletion constructs (refer to Table1), 11 = *p*CAMBIA 1201 (a positive control).

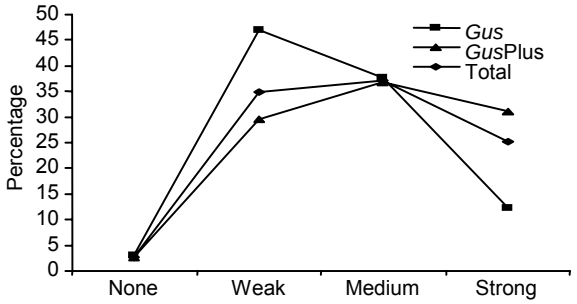

Figure 4. Percentages of TAFET lines with different intensities of reporter gene expressions. Note: The intensities of the expression were observed among 745 *gus* and *gus*Plus lines.

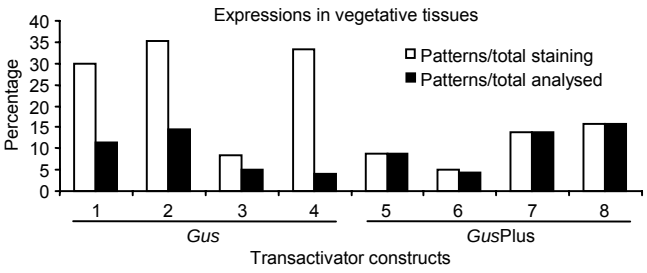

Figure 5. Percentages of a number of patterns/total rice lines with staining and number of patterns/total lines analyses for each transactivator construct in the rice vegetative tissues. For details of constructs refer to Table 1.

Data analysis of the three most abundant patterns of expression in the vegetative tissues indicated a clear difference in the distribution of the three patterns among the constructs, wherein the main difference was between the *gus* and the *gus*Plus constructs (Figure 7). Only one of the three dominant patterns in the vegetative tissues was exhibited by the rice lines with the *gus* reverse_intron construct (Table 1 No. 4 and Figure 7 No. 8). On the other hand, lines with the *gus*Plus reverse_intron construct (Table 1 No. 8 and Figure 7 No. 8) exhibited all dominant patterns (Figure 7). This may indicate that the *gus*Plus is a more sensitive reporter gene than the *gus* gene.

In general, about 95% of the TAFET lines exhibited reporter gene expressions in their vegetative tissues. These results were higher when compared to

Table 2. Three most abundant patterns expressed by each transactivator construct in vegetative tissues. For detail of constructs refer to Table 1.

Unique patterns	*Gus* constructs				*Gus*Plus constructs			
	1	2	3	4	5	6	7	8
1st	b	b	b	b	abc	abc	abc	abc
2nd	ab	abc	abc		ab	bc	ab	b
3rd	bc	bc	ab or c		b	b	b or bc	ab

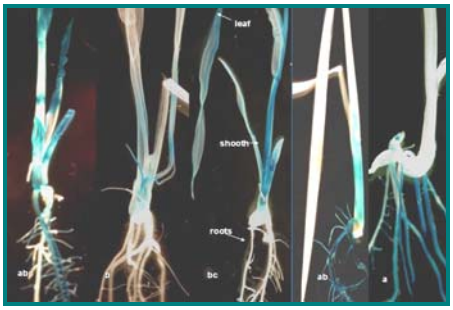

Figure 6. Five most abundant patterns expressed in vegetative tissues of TAFET lines. abc = root, shoot, and leaves; b = shoot; bc = shoot and leaves; ab = root and shoot, a = only in the root.

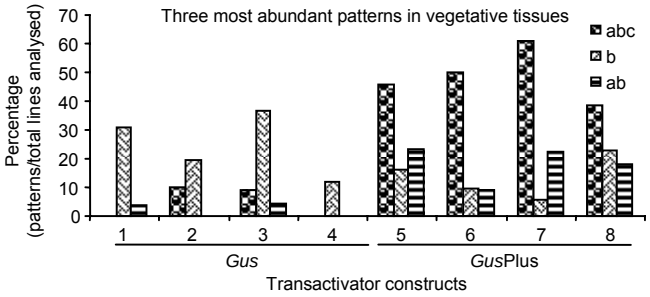

Figure 7. The three most abundant patterns expressed by TAFET lines in vegetative tissues. abc: root, shoot, and leaves b: shoot, ab: root and shoot. For details of the constructs refer to Table 1.

those reported by Klimyuk *et al.* (1995) and Sundaresan *et al.* (1995). This difference might be due to sensitivity of the reporter gene *gus*Plus and the GAL4/VP16 transcriptional activator.

The result, however, was slightly higher than that reported by Wu *et al.* (2003), where only about 70% of the rice transactivator-based enhancer trap lines (Wu *et al.* 2003). It might be due to the use of a similar system by Wu *et al.* (2003), but only one construct which is resemble to the construct number 6, *p*SMRJ18, as shown in Table 1. In our experiments, lines were generated with 8 constructs, four of them containing a *catalase* 1-intron.

Molecular Analysis of TAFET Lines

Insertions of the GAL4/VP16 enhancer trap molecule in the TAFET lines were analyses using the Southern Blot (Sambrook *et al.* 1989). These were conducted in 253 of the T_0 TAFET lines. Based upon patterns of DNA fragments hybridised with a P^{32}-labelled GAL4/VP16 fragment as a probe, it appeared that the TAFET T-DNA was inserted randomly into the rice genome and independent lines were produced from the transformations (Figure 8). Numbers of the T-DNA insertions in the TAFET lines ranged from 1 to 7 copies with an average of two copies per line. About 48.4% of 235 independent lines investigated had a single copy, 22.5% have two copies and less than 30% have three or more copies. This experiment showed

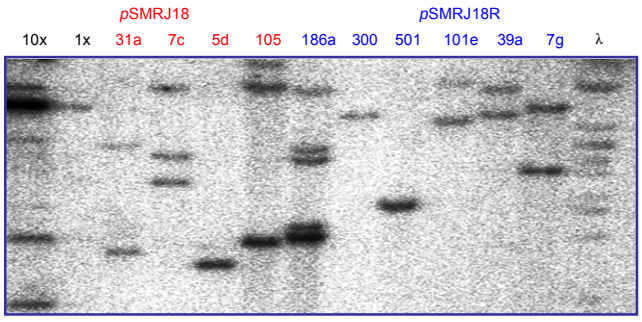

Figure 8. Southern Blot hybridization of *p*SMRJ18 and *p*SMRJ18R lines with the GAL4/VP16 fragment as a probe. Plant DNA(s) was digested with *Eco*RI restriction enzyme and hybridised with ^{32}P-labelled GAL4/VP16 probe. Lanes marked 10X and 1X contained 10 and 1 copy equivalent of *p*SMRJ18 as positive controls. The line numbers are shown on the top. DNA of phage λ was digested with *BstE* II restriction enzyme served as molecular size marker.

Table 3. T-DNA copy number insertion in T_0 generation of 253 TAFET lines.

TAFET lines	Copy number							Total plants	Mean copy number
	1	2	3	4	5	6	7		
Gus	70 (27.77)	30 (11.9)	27 (10.7)	10 (3.96)	6 (2.38)	2 (0.8)	2 (0.8)	147	2.0
*Gus*Plus	52 (20.63)	27 (10.7)	13 (5.16)	9 (3.57)	3 (1.2)	1 (0.4)	1 (0.4)	106	
Total	122 (48.4)	57 (22.6)	40 (15.86)	19 (7.53)	9 (3.58)	3 (1.2)	3 (1.2)	253	

that only 1% of the 253 lines had 7 copies of T-DNA insert in their genomes (Table 3).

The range of the T-DNA insertions numbers the average numbers of the two copies line and the percentage of single copy (Table 3 and 8) in the rice TAFET lines were similar to those reported by Hiei *et al.* (1997) and Wu *et al.* (2003), but the mentioned was slightly higher than those reported by Jeon *et al.* (2000) in rice and by Campisi *et al.* (1999) in *Arabidopsis.* Although plants from the GAL4/VP16 deletion line (*p*SKD15.2-1e) (Table 1 No. 10) had two copies of T-DNA insertions, they did not exhibit *gus* expression 60th in their calli and vegetative tissues. These results provide another evidence that a 108 bp deletion of the GAL4 binding domain. May disrupt the binding of GAL4 to the Upstream Activation Sequence (UAS_{GAL4}). Consequently, it stopped transcription of the reporter gene fused to the UAS_{GAL4}. The GAL4 amino acids 1-147 is the amino terminal portion of the 881 amino acids GAL4 protein, recognizes and binds to the UAS_{GAL4} (Fischer *et al.* 1988). In the absence of an activating domain, fails to activate transcription in yeast and mammalian cells (Ma *et al.* 1988).

A further investigation to define the effect of other components than reporter gene within of enhancer trap constructs on patterns of expression on rice generative tissues needs to be done. The ability of the GAL4/VP16 transcriptional activator to transcribe gene fused to the UAS_{GAL4} needs to be validated. These will support subsequent uses of the GAL4/VP16 lines as "effectors" or pattern lines to direct expression of any gene in spatially and temporally regulated fashions through introducing a second construct in which the gene of interest, such as a gene responsive to drought is placed downstream of the UAS as a "receptor" or target, as previously reported in the GAL4 system (Brand and Perrimon 1993, Castelli-Gair *et al.* 1994).

CONCLUSION AND SUGGESTION

Transactivator-facilitated enhancer trap constructs containing the GAL4/VP16 transcriptional activator and 6 x UAS_{GAL4} were able to reveal patterns of the expression in the rice plant tissues. The expression patterns were due to the activity of the GAL4/VP16 transcriptional activator.

Transactivator *gus*Plus constructs affected patterns of the gene expression more than transactivator *gus*, as they produced more lines with stained, strong expression, more patterns, and a wider spread of pattern distribution than transactivator *gus*. These were due to differences in the reporter gene sensitivity. The *gus*Plus gene was proved to be a more sensitive

reporter gene than the *gus* gene for revealing the gene expression patterns.

A further investigation of reporter gene expression on rice generative tissues and analysis of the effect of additional component fused to GAL4/VP14, such as a*catalase*-1 intron and a validation of the ability of GAL4 to transcribe any gene fused to the UAS_{GAL4} need to be conducted, since the information will support further uses of the TAFET lines for rice crop improvement.

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