UNTAGGED MUTATION IN RICE GAL4/VP16 TRANSCRIPTIONAL ACTIVATOR FACILITATED-ENHANCER TRAP LINES

Mutasi Tak Berlabel pada Galur Padi GAL4/VP16 Transcriptional Activator Facilitated-Enhancer Trap

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ABSTRACT

An enhancer trap system is an insertional mutagenesis based upon gene expression, instead of gene knock-out, so its insertion in genome is expected not linked to any dramatic changes in plant phenotypes. Gene knock-out, leading to loss-of-function (LoF) mutation, is a dominant approach for rice functional genomic studies. The objective of this study was to find out whether Transcriptional Activator-Facilitated Enhancer Trap (TAFET) T-DNA insertion inducing mutant phenotypes in rice TAFET population. Materials used in this experiment were T_1 generation of 270 rice TAFET lines. Eight plants of each were grown in the greenhouse and observed for any mutant phenotypes. Phenotypic, histochemical, Southern blot analyses were carried out to define a mutant of pSKC66.1-8e. Result showed that about 10% of the 270 lines produced chlorophyll-deficient leaves, ranged from yellowish green (viridis), white stripe green (zebra-like stripe) to completely white (albino). Albino plants died after two weeks, whilst white stripe or viridis mutants became normal in the next generation (T_2). Another mutant was pSKC66.1-8e line which had floral dramatic phenotype change with various spikelet shapes and number of organs, and had a single twisted culm. The flower of mutant also had *gus* gene expression. Plants with wild type did not express *gus* gene and had six or more straight culms. Molecular, histochemical and phenotypic analyses of this particular line for three generations indicated that mutant phenotype was not due to the T-DNA insertion. Since there was approved that *Tos17* is activated during tissue culture and induced mutant phenotype, this line might relate to *Tos17* insertion, but it needs further investigation to gain such conclusion.

[**Keywords**: Rice, mutation, TAFET, loss-of-function mutation, *Tos17* transposition, Southern blot]

ABSTRAK

Sistem perangkap penambah merupakan mutagenesis sisipan berdasarkan ekspresi gen, bukan gen knock-out*, sehingga penyisipan dalam genom diharapkan tidak terkait dengan perubahan dramatis pada fenotipe tanaman. Gen* knock-out*, yang menyebabkan mutasi kehilangan fungsi, merupakan pendekatan yang dominan dalam studi genomik fungsional pada padi. Penelitian ini bertujuan untuk mengetahui penyisipan Trans-criptional Activator-Facilitated Enhancer Trap (TAFET) T-DNA menginduksi fenotipe mutan dalam populasi TAFET padi. Bahan yang digunakan dalam penelitian ini adalah generasi T1 dari 270 galur TAFET padi. Delapan tanaman masing-masing ditanam di rumah kaca dan diamati fenotipenya. Analisis fenotipe, histokimia, dan Southern blot dilakukan untuk menentukan mutan pSKC66.1-8e. Hasil penelitian menunjukkan sekitar 10% dari 270 galur padi menghasilkan daun yang kekurangan klorofil, berkisar dari hijau kekuningan (viridis), hijau bergaris putih (seperti strip zebra) sampai putih (albino). Tanaman albino mati setelah dua minggu, sementara tanaman bergaris putih atau mutan viridis menjadi normal pada generasi berikutnya (T2). Mutan yang lain adalah galur pSKC66.1-8e yang memiliki perubahan fenotipe yang dramatis pada bunga denga bentuk spikelet dan jumlah organ yang bervariasi, serta memiliki batang tunggal yang bengkok. Bunga mutan juga mengekspresikan gen* gus. *Tanaman dengan tipe liar tidak mengekspresikan gen* gus *dan memiliki enam atau lebih batang lurus. Analisis molekuler, histokimia, dan fenotipe pada galur ini untuk tiga generasi menunjukkan bahwa fenotipe mutan bukan karena penyisipan T-DNA. Dikarenakan telah terbukti bahwa* Tos17 *diaktifkan selama kultur jaringan dan menginduksi fenotipe mutan, lini mutan ini kemungkinan berkaitan dengan* Tos17*, tetapi diperlukan penelitian lebih lanjut untuk mendapatkan kesimpulan ini.*

[***Kata kunci:*** *Padi, mutasi, TAFET T-DNA, mutasi kerugian fungsi, transposisi Tos17, Southern blot*]

INTRODUCTION

Rice is the most important staple food in Asia, and together with wheat and maize, are dominant crops in the world. Rice genome size is small (around 430 Mb) compared to other cereals, such as maize (2,400 Mb), barley (4,900 Mb) and wheat (16,000 Mb) (Bennetzen, 2002). This small genome size and highly developed research tools make rice a prominent model system for cereal genomics and a model for mono-cotyledonous plants.

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Functional studies on rice genome have been intensified in the recent years, since rice structural genomics was finally concluded. Gene knockout, leading to loss of function (LoF) mutations has been a dominant approach to study functions of rice genes with several different technologies using insertional mutagenesis applied with different levels of successes (Burns *et al*. 1994). Chemical (Inukai *et al*. 2000; Goel *et al*. 2001) and physical mutagens (Inukai *et al*. 2000; Biswass *et al*. 2003), such as EMS and gama-ray, respectively, have been commonly used to generate rice mutant populations. T-DNA insertion and Ac/Ds transposon systems were developed for rice functional genomics with the aim of whole genome saturation with the DNA tags (Izawa *et al*. 1997; Enoki *et al*. 1999; Jeon *et al*. 2000).

It is also known that mutants can also be induced by tissue culture technique. Evidences about genetic instability during tissue culture were reported. Peschke *et al*. (1987) and Palmer *et al*. (2000) provided conclusive molecular evidences that transposable element activation has responsible for tissue culture-induced mutation in soybean and maize, respectively. Hirochika *et al*. (1996) successfully found active endogenous retrotransposons in rice and the most active one was called *Tos17*. This retrotransposon can be activated by tissue culture resulted in an increase in its copy number by 5-30. These increased *Tos17* copies shown were inserted throughout the genome (Yamazaki *et al*. 2001).

It is clear, however, that gene redundancy phenomenon can limit the efficiency of technologies applied for inducing mutations. For example, Qu *et al*. (2003) reported that seven *Bowman-Birk inhibitor* (*BBI*) genes that encode serine protease inhibitors in Japonica rice were in a single cluster on the distal end of the long arm of rice chromosome 1 and two of these genes (*RBBI-2* and *RBBI-3*) had been identified having similar functions to protect rice from the fungal pathogen *Pyricularia oryzae*. Many genes were cloned from mutants belong to the same family gene, for example the *AGAMOUS* and other *MADS-box* genes, and yet display strong phenotypes (Bouche and Bouchez 2001). A disruption of genes is not likely to lead to an easily recognizable phenotype (Burns *et al*. 1994; Springer 2000; Bouche and Bouchez 2001). The method used to delivery an insertional sequence mutagenesis may also affect the efficiency of gaining mutations (Alonso and Stepanova 2003).

An enhancer trap is one system based upon a gene expression, instead of inducing mutagenesis to overcome the ameliorating effects of genes redundancy (Sundaresan *et al*. 1995; Springer 2000). One enhancer trap system developed in rice was the GAL4/VP16 Transcriptional Activator-Facilitated Enhancer Trap (TAFET) (Koerniati and Kilian 2009). Although the purpose of the TAFET system development did not for inducing gene disruption, some of lines showed mutant phenotypes (Koerniati and Kilian 2009). Mutants are presumably due to random insertions of the T-DNA constructs in the rice genome, but those need confirmation.

This paper is about mutants produced in the rice enhancer trap (TAFET) population and details observation on one particular mutant of pSK66.1-8e which had dramatic changes on flower and culm phenotypes. Morphological, histological and molecular analyses to elucidate such phenotypes of this mutant line were conducted in three generations. Efforts were also to define its genetic stability of such expression.

MATERIALS AND METHODS

Rice enhancer trap using the properties of GAL4::VP16 Transciptional Activator (TAFET) was generated at Centre for Application of Molecular Biology for International Agriculture (CAMBIA), Canberra, Australia by Koerniati in 1999-2000 (Koerniati and Kilian 2009). Rice enhancer trap population was developed in Millin rice variety (Japonica rice variety grown in Australia). Eight plants each of 270 T_1 generation plants of TAFET lines were planted and observed for any chlorophyll-deficiency and/or morphological phenotype change in vegetative and or in generative (floral) parts. Except for a mutant line of pSKC66.1-8e, 63 T_1 plants were observed for phenotypes. Then this particular line was investigated in T_2 and T_3 generation. Twenty to 32 T_2 plants each of nine families were investigated for segregation of wild and mutant phenotypes. Twenty to 30 T_3 plants each of nine families were investigated for confirmation.

Morphological Analysis

Flowers of the pSKC66.1-8e mutant line were investigated thoroughly using a Leica Wild M8 and Leitz Diaplan microscopes for bright-or dark-field optics. Images were acquired with a Nikon Cool-Pix Digital photo camera.

Histochemical Analysis of *Gus* Gene

A histological detection of *gus* (β-glucuronidase) gene on plant tissues using buffer containing X-glucuronidase was applied to flowers of pSKC66.1-8e mutant line as described by Jefferson *et al.* (1987). Samples were viewed using a Leica Wild M8 microscope and a Leitz Diaplan microscope for bright- and dark-field optics.

Molecular (Southern Blot) Analysis

Molecular analysis using Southern blot hybridization was carried out to analyze the pSKC66.1-8e mutant line in three generations (T_1, T_2 and T_3). DNAs were prepared using CTAB method (Sambrook *et al.* 1989) and followed by digestion with *Eco*RI restriction enzyme. DNAs were then separated in 0.7% of 20 cm x 20 cm agarose gels in 1x TAE running buffer at 0.5V cm^{-1}. DNA were then transferred from the gel onto a positively charged nylon membrane (Boehringer, Manheim) by an alkaline transfer method using 0.4N NaOH and 0.6N NaCl buffer for overnight (Sambrook *et al.* 1989). Following the transfer process, the membrane was washed briefly in 2x SSC buffer to remove excess salt. The DNA was fixed on the membrane by baking the membrane at 80°C for about 2 hours. A baked nylon filter was then pre-hybridized in 50 ml pre-warmed (65°C) pre-hybridization solution containing 1x HSB and 1x Denhardt's solution for about 5 hours at 65°C (in a plastic container) in an oven. Hybridization was then carried out in 20 ml hybridization solution containing 1x HSB, 1x Denhardt's solution and 10 mg denatured herring sperm DNA and a radioactive labeled Gal4/Vp16 probe for about 18-24 hours at 65°C in the plastic container in the oven. Following the hybridization, a membrane was washed and hybridization signal was detected using either Biorad GS-250 molecular imager or X-ray film.

RESULTS AND DISCUSSION

Phenotypes and *Gus* Gene Expression Analysis of Mutants in T_1 Generation

Eight T_1 generation plants each of 270 TAFET lines were observed for any chlorophyll deficiency and/or any morphological change phenotypes. Of 270 lines observed, there were lines having chlorophyll-deficient leaves and floral morphological change phenotypes. Chlorophyll deficient mutations ranged from yellowish green (viridis) (Fig. 1a), white stripe green (zebra - like stripe) (Fig. 1b and 1c) to completely white (albino) (Fig. 1d). Percentages for each mutant category were 2%, 3% and 5%, respectively. Albino mutant (Fig. 1d) died after been grown for about two weeks in the greenhouse, whilst mutants with white stripes or viridis survived and some of those became normal in the next generation.

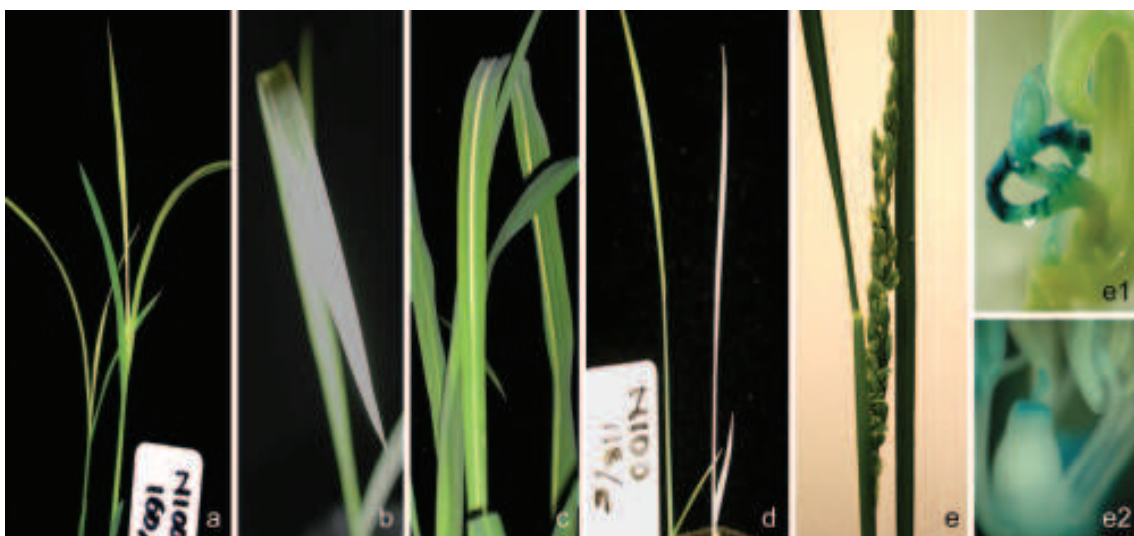Another mutant was the one with dramatic change in the plant and flower and only observed in one of

Fig. 1. Mutant phenotypes of T1 generation of rice TAFET lines. a = yellowish green (viridis), b = whitish green, c = green white stripe green, d = albino, e = morphological change spikelets, e1 = spikelet within spikelet with blue colour of GUS and e2 = abnormal style and stigma with blue colour of GUS.

eight T_1 plants of pSKC66.1-8e (Fig.1e, e1 and e2). Various phenotypes in the individual rice flower (spikelet) shape were performed by the mutant (Fig. 2). Histochemical analysis for *gus* gene expression showed that blue of GUS staining was expressed in the floral tissues such as in palea, lemma and stigma of the mutant. In contrast, the flower of the wild type did not produce blue of GUS staining. These may indicate that floral change phenotypes link to T-DNA. Added to that the mutant had only a single culm (shoot) that was slightly twisted (Fig. 3 right), in contrast, the wild type had six to eight straight culms (Fig. 3 left).

Due to those evidences, another 55 T_1 seeds of pSKC66.1-8e line were sown and seedlings were grown and evaluated for their phenotypes. Results showed that 43 plants exhibited wild type, 10 were mutant phenotypes, and another 10 died. Of 53 plants alive, analysis of *gus* gene expression showed that

about 3:1 segregation ratio occurred between plants without *gus* (36 of 53 plants) and those of with gus (17 of 53 plants) expression.

Phenotypes and *Gus* Gene Expression Analysis of pSKC66.1-8e in T_2 and T_3 Generations

Following that indication, T_2 plants of pSKC66.1-8e were analysed. Twenty to 32 T_2 plants each of six families were planted. In theory, about a quarter of plants should have a mutant phenotype if the mutation is related to a recessive gene. Chi-square test result showed a ratio of 3:1 between wild type and mutant type plants was performed only by one of six families; the other five had either 1:1 or 2:1 ratio instead (Table 1). Interestingly, a 3:1 pattern of segregation between plants with and without *gus*
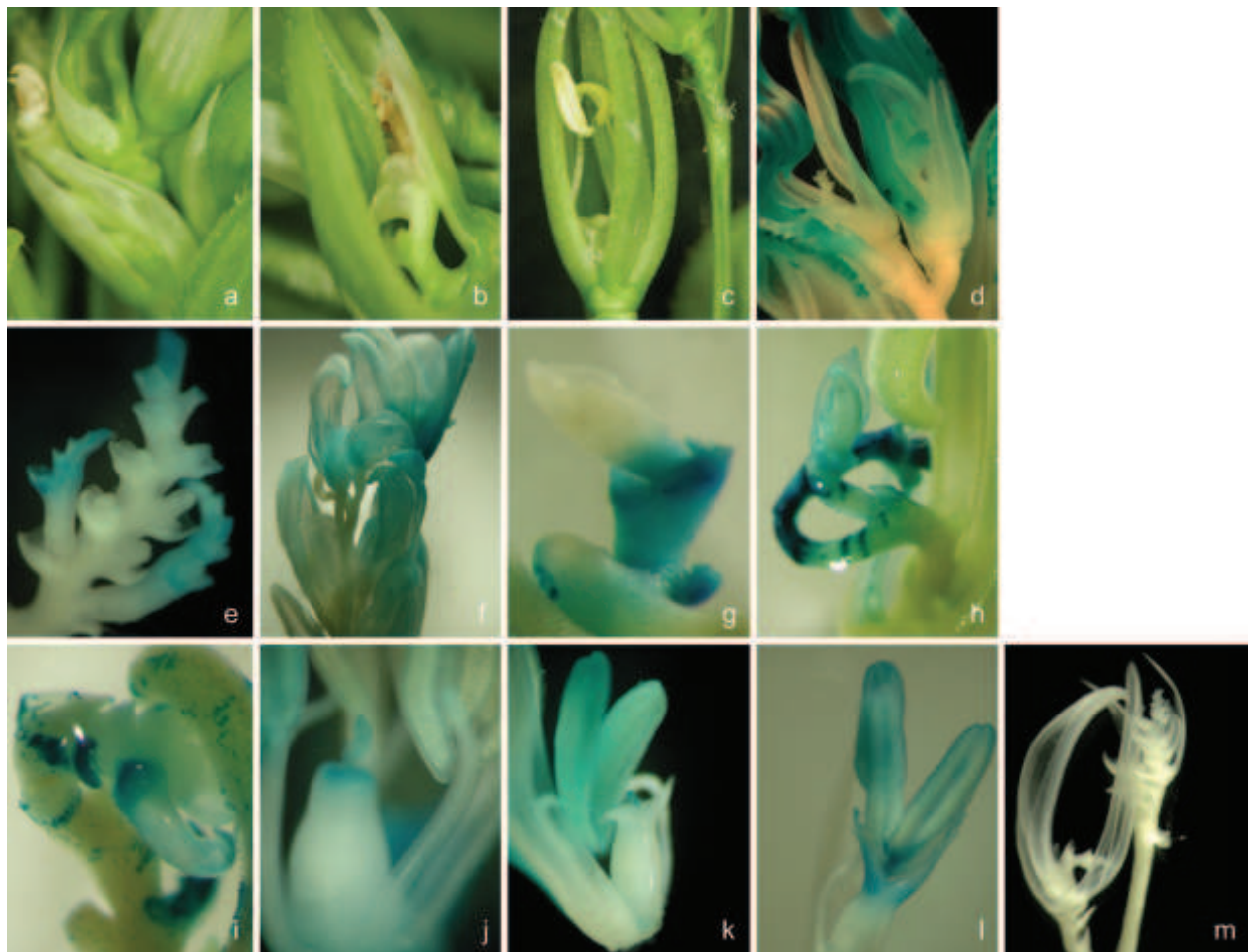

Fig. 2. Morphological variations of the pSKC66.1-8e mutant rice line spikelets; a,b,h = spikelet within a spikelet, l,m = A spikelet with a bud-like shape, d, f = Spikelets with leaves-like shape of palea and lemma, c = Spikelet with abnormal carpel and less number of anther than 6, j,j,l = Spikelets with abnormal style and stigma, g = Spikelet with abnormal pedicle.

gene expression which had blue staining, was consistently shown by those six families.

Following that result, T_3 plants of nine families of pSKC66.1-8e (fourth generation) were analysed. Result showed that a 3:1 ratio between plants with wild type and mutant type was displayed only by a third of families tested. Once again, a 3:1 ratio between plants with and without *gus* gene expression was performed by almost all families, except family 39-54 number 4 (39-59/4) that had mutant phenotypes, but did not express *gus* gene (Table 1) (Fig. 2 m). This evidence may indicate that mutant phenotype is not related to T-DNA enhancer trap, and might be related to something else, for example *Tos17*. It, however, needs a further investigation.

Molecular Analysis by Southern Blot

Molecular analysis (Southern blot) was carried out to define whether the mutant phenotype is linked or is not linked to the T-DNA insertion. Southern blot of T_0 (first generation) plant of a pSKC66.1-8e line indicated that it had four copies of T-DNA insertion in position of 3.6 kb (strong), 3.0 kb, 1.6 kb and 1.3 kb (faint).

Southern blot of T_2 (third generation) plants of the pSKC66.1-8e line with Gal4/Vp16 probe showed that the first two T-DNA insertions (positioned at 3.6 and 3.0 kb) were "fixed" in all plants, while that of in the position of 1.6 kb and 1.3 kb were co-segregated among plants within a family, shown in Figure 4, lane 13-15, and in Figure 5, line 1-12. Those resulted in a segregation between plants of some families either had 2 or 4 copies of T-DNA. Added to that, plants of some families had no T-DNA insertion, as shown in Figure 4, lane 1-12 and Figure 5, lane 13-17. Southern blot also showed that plants with mutant phenotypes

Fig. 3. The pSKC66.1-8e mutant plants: a wild type (left), a mutan type (right).

Table 1. Segregation ratio among T2 and T3 rice plants of pSKC66.1-8e tested families and their T-DNA copy numbers.

Generation	Family no.	GUS staining			Die	Flower change	One culm	Wild	Ratio of wild: mutant	T-DNA copy number		
		Yes	No	Ratio						0	2	4
T_0	pSKC66.1-8e	1										1
T_1	39	47	16	3:1	10	10[1]	0	43	2:1	na	na	na
T_2	39-7A	16	4	3:1	0	1[1] (2)	6	14	2:1	3	5	0
	39-7B	16	5	3:1	3	0	6	12	2:1	0	4	0
	39-10	13	3	3:1	1	0	6	13	2:1	0	0	2
	39-13	na	na	na	4	1	2	18	3:1	0	1	1
	39-54	na	na	na	3	1	5	11	1:1	1	5	3
	39-4	17	6	3:1	0	2[1] (2)	7	14	2:1	0	3	0
T_3	39-4/8, n	17	7	3:1	1	1[1] (2)	9	14	1:1	1	5	0
	39-4/9, n	18	6	3:1	3	1[1]	13	12	1:1	0	5	0
	39-63/3, s	10	5	2:1	1	1[1] (2)	4	18	3:1	0	2	0
	39-63/6, n	18	6	3:1	0	1[1]	5	18	3:1	0	3	0
	39-54/4, M	0	24	0:1	4	1[2] (0)	4	11	1:1	13	0	0
	39-54/9, n	17	6	3:1	1	0	7	16	2:1	3	0	10
	39-54/20, n	na	na	na	0	1[2] (0)	6	20	3:1	10	0	0
	39-54/23, n	10	5	2:1	5	0	6	8	1:1	11	0	1

[1] : Mutant plants with staining; [2]: Mutant plants without staining;

( ): number of T-DNA insertion, na: no analysis

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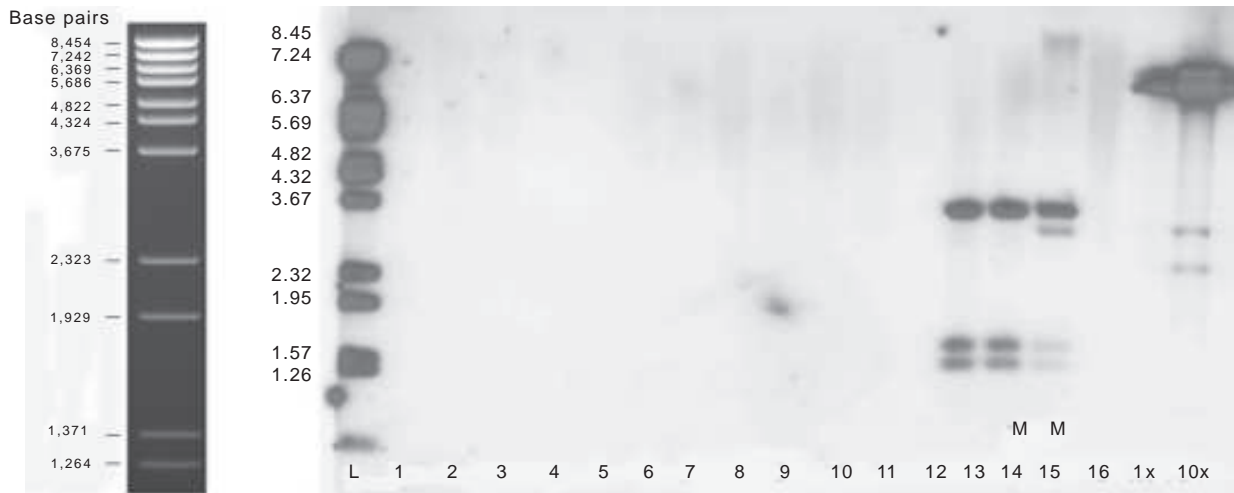*Indones. J. Agric. Sci. Vol. 14 No. 1 April 2013: 27-35*

Fig. 4. Southern blots of rice plants with mutant and normal phenotypes hybridised with GAL4/VP16 fragment as a probe. 1-13 and 16 = plants with wild type phenotype; 14-15 = plants with mutant phenotypes. L = λ-DNA digested with *BstE* II restriction enzyme; 1 39-54/4-2, 2 39-54/4-3, 3 39-54/4-4, 4 39-54/4-6, 5 39-54/4-7, 6 39-54/4-8, 7 39-54/4-9, 8 39-54/4-13, 9 39-54/4-14, 10 39-54/4-15, 11 39-54/4-16, 12 39-54/3, 13 39-54/1, 14 39-54/2, 15 39-63/6-14, 16 39-54/4-9, 1x 10x: pSKC66.1 = plasmid; M = plant with flower changed phenotypes.
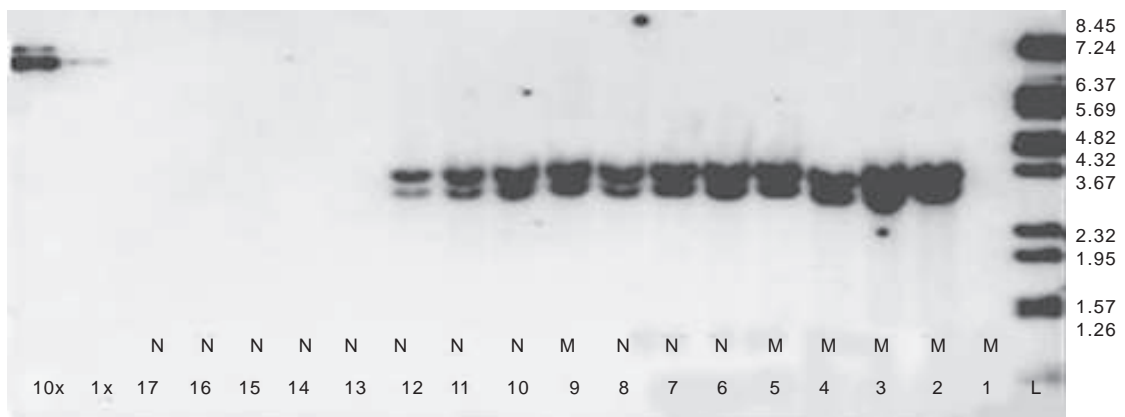

Fig. 5. Southern blots of rice plants with mutant and wild type phenotype hybridised with GAL4/VP16 fragment probe. 6-8, 10-17 = plants with wild type phenotype; 1-5 and 9 = plants with mutant phenotypes; 1 39-4/8-5, 2 39-4/9-10, 3 39-7A/10, 4 39-7A/13, 5 39-7A/23, 6 39-7B/11, 7 39-7B/18, 8 39-4/8-23, 9 39-4/8-25, 10 39-4/9-4, 11 39-4/9-13, 12 39-4/9-14, 13 39-54/20-16, 14 39-54/20-13, 15 39-54/20-12, 16 39-54/20-11, 17 39-54/20-1, 1x 10x pSKC66.1 = plasmid, M = plant with flower changed phenotype.

had different numbers of T-DNA insertion, either 3 or 4 copies, as shown by Figure 4, lane 14 and 15. On the other hand, plants that had similar number of T-DNA showed different phenotypes, either a wild or a mutant type (Fig. 5, lane 2-12). Contrary result, however, was shown by a plant with mutant phenotype, but did not have T-DNA insertion (Fig. 5, lane 1). Then these results strongly indicated that the mutant phenotypes did not relate to the T-DNA enhancer trap insertion.

A further analyses using Southern blot on six families of T_3 plant generation (fourth generation) gave another confirmation that the mutant phenotypes were most likely not linked to the T-DNA insertion. Plants of two families (39-59/4 and 39-59/20) had floral with phenotype changes (a mutant type), but did not have T-DNA insertion (Table 1). Their flowers did not have blue GUS staining at all (Fig. 2 m).

Mutant phenotypes have been commonly used for plant functional genomic investigations. Ethyl

methane sulphonate (EMS) and gamma- or X-ray are conventional methods for mutagenesis. These have a higher efficiency in inducing mutant phenotypes than from the less conventional method of insertional mutagenesis (Koornneef *et al.* 1982). Insertional mutagenesis, however, is a preferred approach for functional genomics, as it may generally tag genes (inducing mutant phenotypes). DNA sequences adjacent to the insertion may be cloned using PCR and TAIL-PCR (Maes *et al.* 1999; Liu *et al.* 1995). However, it has been shown that not all genes can be uncovered through application of insertional mutagenesis (Burns *et al.* 1994; Campisi *et al.* 1999; Spinger 2000). Indeed, about two thirds of *Drosophila*'s genes (equal to 8,000 genes) are predicted to show no obvious loss-of-function phenotypes because of gene redundancy (Miklos and Rubin 1996). Mutations are also not always linked to T-DNA and this could be as about 60% in *Arabidopsis* and about 50% in rice (McElver *et al.* 2001).

In this experiment, about 10% of rice TAFET lines showed various types of chlorophyll-deficient mutations. Such mutations were previously reported often induced by tissue culture application (Palmer *et al.* 2000). Such chlorophyll-deficient phenotypes were also reported from previous work using a T-DNA insertional sequences in rice, in about similar percentage (Jeon and An 2001; Jung *et al.* 2003). One mutant line was identified having a T-DNA insertion in the *chlorina* (*OsCHLH*) gene that is highly homologous to *XANTHA-F* gene in barley and *CHLH* gene in *Arabidopsis*. Two other mutants with chlorophyll-deficient phenotypes had a *Tos17* insertion in the *OsCHLH* gene (Jung *et al.* 2003).

Relating to rice flower development, several genes involved have been identified, those were *OsMADS1*, *OsMADS16*, *SUPERWOMAN1* (*SPW1*), *DROOPING LEAF* (*DL*) (Nagasawa *et al.* 2003) and *OsMADS3*. A mutation of the *OsMADS1* altered spikelets morphology to elongated leafy palea and lemma, two pairs of leafy palea-like and lemma-like lodicules, decrease in a stamen number and an increase in the number of carpels. The gain-of-function of *OsMADS1* transgenic lines presented the transformation of outer glume to lemma-/palea-like organs and no changes in length lemma and palea, but the loss-of-function of *OsMADS1* transgenic lines displayed the overdeveloped lemma and palea (Prasad *et al.* 2005). Moreover, it was indicated that *OsMADS1* up-regulated the transcript level of *APETALA3* homologue *OsMADS16*, using real-time PCR analysis on *gain* and loss-of-function of *OsMADS1* transgenic lines. Transgenic

containing double-stranded RNA with the *OsMADS16* cDNA fragment were male sterile, and lodicules were converted into palea/lemma-like organs and some stamens into carpels (Xiao *et al.* 2003). The carpels had been replaced by stamen-like organs when *OsMADS16* was ectopically expressed under the control of the Maize Ubiquitin1 promoter (Lee *et al.* 2003). The homeotic mutation *spw1* converted stamens and lodicules into carpels and palea-like organs, respectively. Two *spw1* alleles, *spw1*-1 and *spw1*-2, showed the same floral phenotype and did not affect vegetative development and *SPW1* is a rice *APETALA3* homolog, *OsMADS16* (Nagasawa *et al.* 2003). In contrast, two strong alleles of the *dl* locus, drooping leaf-superman1 (*dl-sup1*) and drooping leaf-superman2 (*dl-sup2*), cause the complete transformation of the gynoecium into stamens (Nagasawa *et al.* 2003). Moreover, the *DL* is regulated negatively by the *SPW1*, as stamens were converted into carpels in *spw1* mutants and carpels were converted into stamens in *dl* mutants (Yamaguchi *et al.* 2004). Ectopic expression of *OsMAD3* in rice plant caused homeotic transformation of lodicule to stamens (Kyozuka and Shimamoto 2002).

The pSKC66.1-8e mutant line had dramatic floral tissue changes, such as spikelets with leaf-like organs of palea and lemma (Fig. 2 d, e and f), a spikelet within a spikelet (Fig. 2 a, b, h), a spikelet with bud-like organs (Fig. 2 e, g, h, i), and spikelet with a lesser numbers of anthers and abnormal carpel (Fig. 2 c, j, k, l). Based on previously reported, those ectopic phenotypes of the pSKC66.1-8e mutant might be related to a disruption of a MADs-box family gene. An approximate 3:1 segregation between plants with wild type and plants with mutant phenotype may indicate the link between mutant phenotypes of the pSKC66.1-8e and T-DNA insertion. However, observation in the T_2 and T_3 generations showed that mutant phenotypes did not segregate among plants in a 3:1 ratio. Nevertheless, plants of T_2 and T_3 generations of the pSKC66.1-8e families segregated in 3:1 between plants with *gus* and that of without *gus* expression, except mutant plants of 39-59/4 and 39-59/20 families which did not express *gus* gene. This mutant phenotype was one example of the phenomenon that a mutation does not always link to a T-DNA insertion.

These results are evidence that the loss-of-function (LoF) phenotypes are not easily induced using insertional sequence mutagenesis, due to a gene redundancy phenomenon. Indeed, development of other mutational systems for *gain-of-function* (GoF) for which the TAFET system was originally intended is a needed.

In relation to pSKC66.1-8e mutant line, efforts to find out whether such mutant phenotype relates to rice endogenous retrotransposon of *Tos17* are needed, since several mutants induced by *Tos17* transposition were identified and the *Tos*17 retrotransposon proven can be used to identify mutant in transgenic rice (Hirochika 2001). For example *Osaba1*, a strong viviparous mutant that displayed low abscisic acid level and almost no further increase in its levels upon drought, and *OsTATC*, a mutant with weak phenotype, exhibited the pale green phenotype and slight increase in abscisic acid levels upon drought (Agrawal *et al*. 2001). Other examples were a chlorophyll-deficient phenotype due to a disruption of the *OSCHLH* gene (Jung *et al*. 2003) and *phyA* mutants which showed insensitivity to far-red light (Takano *et al*. 2001). An attempt to investigate can be followed by sequencing which could be fruitful of gene controls of rice flower development. Since a discovery of gene controlling rice flower development is still limited, this research should be continued in the future.

CONCLUSION

Of 270 lines observed, about 10% were having chlorophyll-deficient leaves, ranging from yellowish green or viridis (2%), white stripe green or zebra stripe (3%) to completely white or albino (5%) mutant phenotypes. Albino mutants were dying after growing for two weeks in the greenhouse, whereas mutants with white stripes or viridis survived and became normal plants in the next generation.

One mutant line (0.4%) had dramatic morphological changes in flower phenotypes. The line with dramatic change in the plant and floral phenotype was having various spikelet shapes and single slightly twisted culm.

Molecular analysis showed that these mutant phenotypes (of pSKC66.1-8e line) were not due to the TAFET T-DNA insertion. Thus this result proved that the TAFET is a system based upon gene expression.

ACKNOWLEDGEMENTS

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