DEVELOPMENT OF TRANSACTIVATOR SYSTEM FOR RICE AND ITS APPLICATION FOR STUDIES OF FLORAL DEVELOPMENT

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Chapter 7

LOSS-OF-FUNCTION PHENOTYPES OF TAFET LINES

7.1 INTRODUCTION

Although the purpose for development of the GAL4/VP16 facilitated enhancer trap population was not to induce gene disruption, lines with mutant phenotypes were obtained in our experiment, presumably through random insertion of the T-DNA constructs in the rice genome.

Loss of Function (LoF) mutations have been a major component of functional genomic approaches and several different technologies using insertional mutagenesis have been applied with different levels of success in *Arabidopsis* (Burns et al., 1994; Pereira, 2000) and in rice (Izawa, 1997; Enoki et al., 1999; Greco et al., 2001). It is clear that functional gene redundancy 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 from japonica rice were in a single cluster on the distal end of the long arm of rice chromosome 1 and two of those genes (RBBI2-3) had been identified having similar functions to protect rice from the fungal pathogen *Pyricularia oryzae*. Many genes cloned from mutants belong to the same gene families, for example the *AGAMOUS* and other *MADS-box* genes, and yet display strong phenotypes (Bouche and Bouchez, 2001). Disruption of these genes is not likely to lead to an easily recognisable phenotype (Burns et al., 1994; Springer, 2000; Bouche and Bouchez, 2001). The enhancer trap is one system which is based upon gene expression, instead of induced mutagenesis, to overcome the ameliorating effects of gene redundancy (Sundaresan et al., 1995a; Springer, 2000).

The method used to deliver insertional sequence mutagenesis may also affect the efficiency of gaining mutations (Alonso and Stepanova, 2003). Mutants can also be induced from routine tissue culture; Palmer et al. (2000) and Peschke et al. (1987) provided conclusive molecular evidence that transposable elements activation is responsible for tissue culture-induced mutations in maize.

Hirochika *et al.* (1996) found that the transposable element, retrotransposon *Tos*17, was highly activated during tissue culture in rice. Retrotransposons are class I elements that are functionally and structurally different from the DNA transposon element (class II elements), such as *Ac*, *Ds*, *Spm*, etc. A mechanism of retrotransposon activation is based on a replicative process, which leads to proliferation of these elements in the genome, as the "master copy" of the retrotransposon is not excised from its genome. This propagation is achieved through an RNA intermediate (Girard and Freeling, 1999). Retrotransposons usually induce a stable mutation, since they do not have excision capability. Retrotransposons carry long terminal direct repeats (LTRs) at both ends and an internal domain encoding *Gag* and *Pol* polyproteins. Enhancer-promoter sequences are present on the LTRs and regulate the synthesis of both the RNA template for reverse transcription and mRNA required for protein synthesis (Girard and Freeling, 1999). This unique feature of *Tos*17 suggested that it could be used for transposon-tagging and reverse genetic studies in rice.

The reverse genetic studies in rice are important, as a large number of cloned genes with unknown function have been identified by large scale sequencing of cDNA (Sasaki et al., 1994, Sasaki and Yamamoto, 1997), including a large set of full length cDNAs (Kikuchi et al., 2003). In order to identify the insertion sites of transposable elements or T-DNAs in the gene of interest, a transposable element segment or T-DNA sequence and a mutagenised gene sequence were used in PCR reactions using DNA

from a large population of mutants as a template (Lee et al., 2003b; Kolesnik et al., 2004).

This chapter is a report of the mutant phenotypes observed in the TAFET lines produced in this thesis and attempts to identify at the molecular level the floral morphological changes observed in the pSKC66.1-8e line.

7.2 MATERIALS AND METHODS

7.2.1 MATERIALS

As described in chapter 3, I produced 1,000 TAFET lines. Of these, eight plants from each of 270 lines were observed for any phenotypic change in the T_1 generation. Seeds of the pSKC66.1-8e mutant line were grown and plants of this mutant were observed in T_1, T_2 and T_3 generations.

7.2.2 METHODS

7.2.2.1 Observation of TAFET lines

All plants of the T_1 generation of TAFET lines were observed for any morphological changes and/or phenotypic changes in vegetative and floral parts. Flowers of pSKC66.1-8e mutant line were observed using a Leica Wild M8 microscope or a Leitz Diaplan microscope with bright-or dark-field optics. Images were acquired with a Nikon CoolPix Digital photo camera. Samples were also observed using a Scanning Electron Microscope, Hitachi 4500 (Vesk et al., 1994).

7.2.2.2 Histological analysis

Histochemical detection of GUS (β-glucuronidase) was performed using fresh floral parts of the mutant line as previously described (Jefferson et al., 1987). Samples were viewed using a Leica Wild M8 microscope or a Leitz Diaplan microscope with bright- or dark-field optics.

7.2.2.3 Southern Blot Hybridisation

Molecular analysis of the mutant lines was carried out using a Southern blot hybridisation method on T_0, T_2 and T_3 plants. Two probes, the GAL4/VP16 and *Tos*17 fragments, were hybridised to nylon filters with genomic DNA digested with *EcoR* I. The procedure used was as previously described (Sambrook et al., 1989)

7.3 RESULTS

7.3.1 Phenotype and reporter gene expression of mutants in T_1 generation

Eight plants each of 270 TAFET lines of the T_1 generation were observed for any chlorophyll-deficiency and/or any morphological change phenotypes (for details see Appendix 4.1). These observations resulted in a number of lines with phenotypic changes, mostly in leaf chlorophyll content and floral shape. Chlorophyll-deficient mutations observed ranged from yellow-green (viridis), green with white stripe to completely white (albino) (Figure7.1). The percentage of these phenotypes was 2% (viridis), 3% (white stripes) and 5% (albino). Mutants with an albino phenotype died after two weeks, whereas plants with white stripes or yellow-green colouration survived.

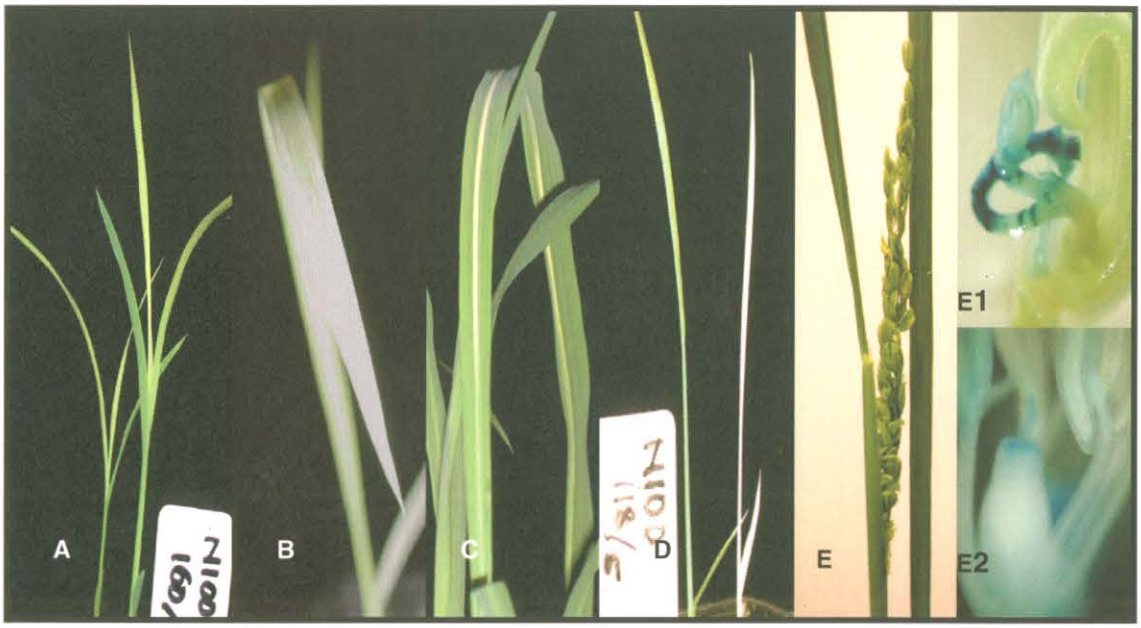

Figure 7.1. Chlorophyl mutation and ectopic phenotypes in spikelets displayed in T1 TAFET lines. A. yellowish green (viridis), **B**. broad white stripe, **C**. narrow white stripe, **D**. albino, **E**. floral (spikelets) morphological changes, **E1**. flower (spikelet, blue colour) within flower (spikelet) and **E2**. abnormal style and stigma (blue colour). Blue colour is GUS reporter gene expression.

Dramatic changes in plant and floral morphology were observed in one of eight plants of pSKC66.1-8e (Fig. 7.2 and 7.3). Various spikelet morphology changes were observed, such as 1) a spikelet within a spikelet (Fig. 7.3, A, B, H); 2) a spikelet with a bud-like shape (Fig. 7.3, I), 3) spikelets with leaves-like shape of palea and lemma (Fig. 7.3, D and F); 4) spikelet with abnormal carpel and a lesser numbers of anther than 6 (Fig. 7.3, C); 5) spikelets with abnormal style and stigma (Fig. 7.3,J-L) and 6) spikelet with abnormal pedicle (Fig. 7.3, G). Interestingly, the changed floral tissues showed blue GUS staining, while the wild type flowers showed GUS staining only in the anthers and pollen. In addition, the mutant had only one culm instead of an average number of five or more culms, and the culm was slightly twisted (Fig. 7.2)

Figure 7.2. Phenotypes of mutant (B) and wild type (A) of pSKC66.1-8e line.

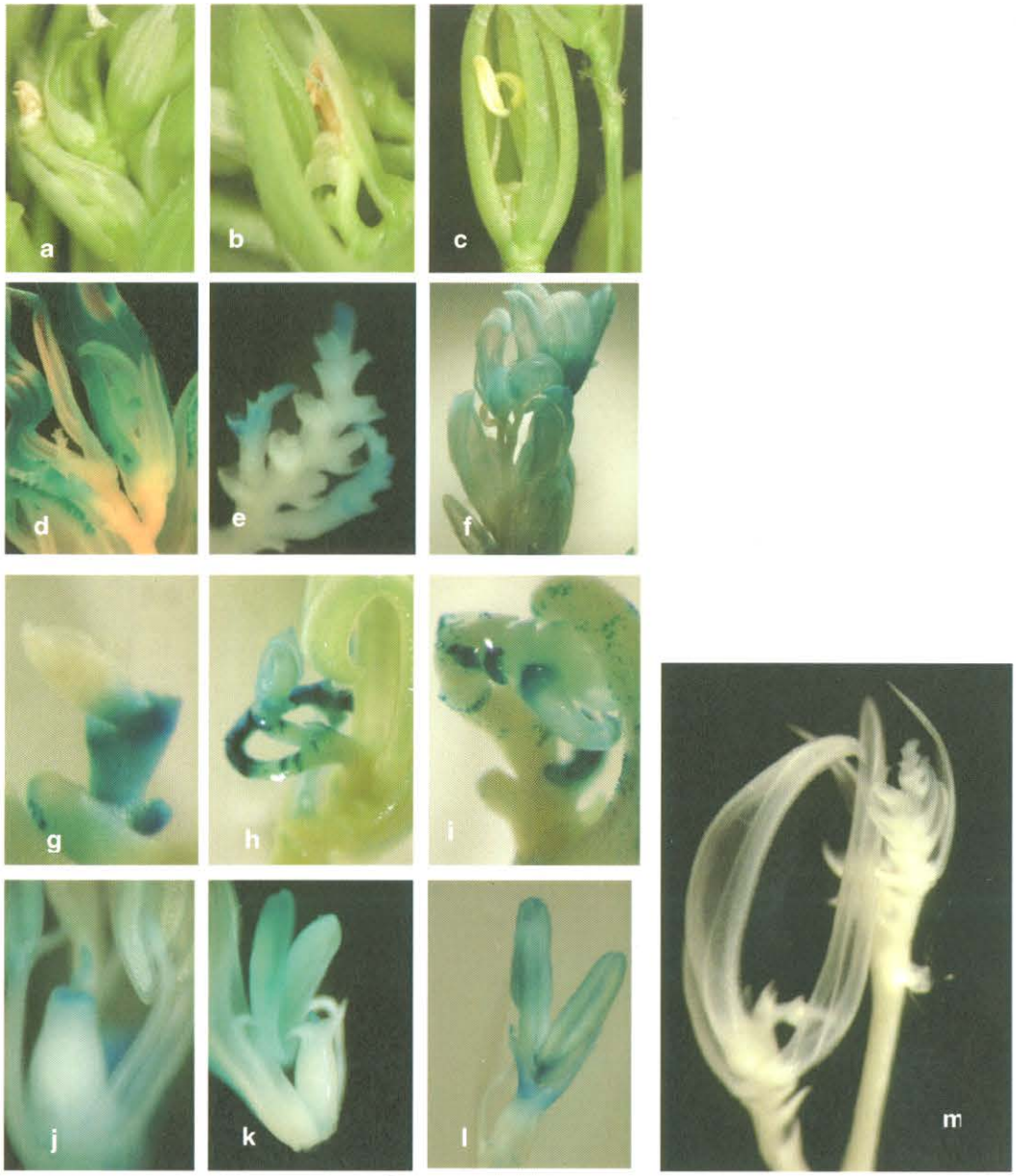

Figure 7.3. Ectopic expressions of pSKC66.1-8e line and its reporter gene expression in various flower organs. a-c: range of flower morphological changes, **a & b.** no sexual organs, **c:** have two anthers but no female organ, **d-f**: leafy- or bud-like structure, **g-i**: spikelet-like structure inside spikelet, **j**: no style and stigma, but small tissues in the middle-top of ovule (carpel), **k**: style-like structure and small tissues in the middle-style, **l**: style and not fully developed stigma (philia) and **m**: spikelet-like structure inside spikelet without GUS reporter gene expression.

Moreover, flower pSKC66.1-8e was observed using Scanning Electron Microscope (SEM). This mutant plant was abnormal in anther, style and stigma tissues development (Figure7.4).

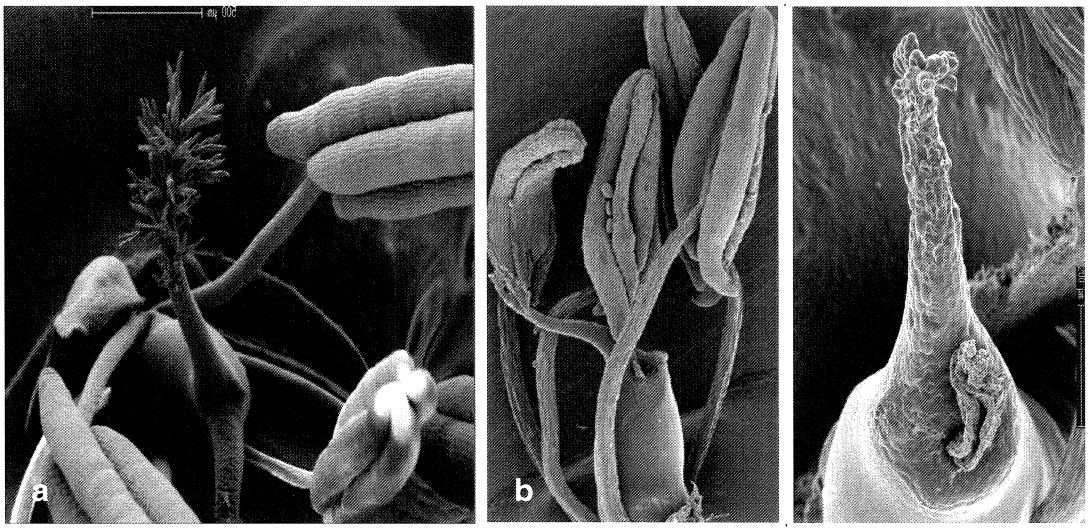

Figure 7.4 Scanning electron microscopy of anther and stigma of the pSKC66.1-8e line. a. fully developed anther, style and stigma of wild type; **b**. not fully developed anther and stigma, **c**. Style and stigma were not developed at all, were observed in mutant flowers.

7.3.2 Genetic analysis

In order to establish the genetic basis of the pSKC66.1-8e mutant phenotypes, 63 T_1 seeds were planted. Floral phenotype changes were observed in 10 of 63 T_1 plants (Table7.1) and the mutants showed GUS staining in the floral tissues. Another 10 of 63 plants died during growth. By assuming that the 10 dead plants and 10 plants with floral mutant phenotypes were in the same class (group), plants in T_1 appeared to segregate in an approximate 3:1 ratio, between plants with normal phenotype (wild-type phenotype) and plants with mutant phenotype. Those 53 of 63 T_1 plants were also observed for GUS gene expression in the floral tissues. Plants showed an approximate a 3:1 segregation ratio between those displaying GUS expression

(36 of 53) and those displaying no expression (17 of 53) (Table 7.1). Gus reporter gene expression was expressed either in the anther wall and pollen of wild-type plants or in the floral tissues showing morphology changes in the T_1 generation. These results suggested a link between mutant phenotypes in this line and TAFET T-DNA insertion. This prompted the analysis of the T_2 generation.

Nine T_2 families were planted and at least 20 plants were observed for each family (Table 7.1). A varying number of plants with mutant phenotypes were observed within each family. As a result, plants within a family did not segregate in a 3:1 ratio (wild type:mutant). In theory, about one quarter of plants should have a mutant phenotype if the mutation is recessive. Interestingly, the GUS staining segregation pattern was consistently shown by plants of families in the T_2 and T_3 generations, except for plants of family 39-54 number 4 (39-59/4) which showed no GUS gene expression (Table 7.1) (Figure7.3, F).

Southern blot analysis was carried out on T_2 and T_3 plants to identify and confirm if T-DNA insertion is linked to the mutant phenotype. The GAL4/VP16 gene fragment was used as a probe. Southern blot analysis of pSKC66.1-8e plant DNA in the T_0 generation showed that four DNA fragments (3.6kb, 3.0kb, 1.6kb and 1.3kb in size) containing TAFET T-DNA insertions (Figure 7.6, A, lane 15). The first two, 3.6kb and 3.0kb, were "fixed"in all plants, while the 1.6kb and 1.3kb fragments co-segregated among plants within a family. Therefore, plants within families had either 4 fragments or 2 fragments. In addition, there were families which had no T-DNA (Fig.7.6, A) (Table 7.1). Interestingly, there were plants which had a different number of T-DNA insertions, showing mutant phenotypes (Fig. 7.6 A, lane 14 and 15). In contrast, there were plants which had a similar number of T-DNA insertions showing different phenotypes, both wild type and mutant. For example, plant 6 (lane 6) showed mutant phenotypes and plant 7 (lane 7) showed wild-type phenotype (Fig. 7.7 A, lane 6 and 7).

	No-/Family	Σ plants	GUS staining			died	Mutant	one culm	normal	T-DNA		
			yes	No	% with GUS					0	2	4
T_0	pSKC66.1-8e		1									1
T_1	39	63	36	17	68	10	10*	0	43	na	na	na
T_2	39-7A	21	16	5	76.2	0	1*(2)	6	14	3	5	0
	39-7B	21	16	5	76.2	3	0	6	12	0	4	0
	39-10	20	13	3	81.3	1	0	6	13	0	0	2
	39-13	24	na	na	na	4	1	2	18	0	1	1
	39-15	24	na	na	na	2	0	5	17	0	1	1
	39-23	20	na	na	na	2	0	3	15	0	2	0
	39-24	32	na	na	na	2	0	7	23	0	6	0
	39-54	20	na	na	na	3	1	5	11	1	5	3
	39-4	23	17	6	73.9	0	2*(2)	7	14	0	3	0
T_3	39-4/8, n	24	17	7	70.8	1	1*(2)	9	14	1	5	0
	39-4/9, n	29	18	6	75.0	3	1*	13	12	0	5	0
	39-63/3, s	24	10	5	66.7	1	1*(2)	4	18	0	2	0
	39-63/6, n	24	18	6	75.0	0	1*	5	18	0	3	0
	39-63/24, n	24	18	6	75.0	0	0	11	18	1	10	0
	39-54/4, M	24	0	20	0	4	1**(0)	4	15	13	0	0
	39-54/9, n	24	17	6	73.9	1	0	7	16	3	0	10
	39-54/20, n	24	na	4	na	0	1**(0)	6	20	10	0	0
	39-54/23, r.	20	10	5	66.7	5	0	6	9	11	0	1

Table 7.1 Segregation of pSKC66.1-8e mutant line and their T-DNA copy numbers. *, mutant plants with staining; **, mutant plants without staining; ( ), T-DNA insertion within mutant plants; na, plants were not analysed.

These results strongly suggested that the mutant phenotypes were not linked to the T-DNA insertion.

Further Southern analysis was carried out on several families in the T_3 generation. Analysis gave confirmation that the mutant phenotypes were most likely not linked to the T-DNA insertion, as two plants of family 39-54 number 4 (39-59/4) and family 39-54 number 20 (39-59/20) with a floral phenotype change (mutant) had no T-DNA insertion (Tabel 7.1). In addition, these flowers did not show GUS staining (Figure7.3, F).

7.3.3 Testing the *Tos*17 status in T2 and T3 generations of line pSKC66.1-8e

We hybridised the same membranes previously hybridised with the GAL4/VP16 probe, with a *Tos*17 fragment as a probe after stripping. Southern blot analysis showed that plant genomic DNA(s) that did not hybridise with the GAL4/VP16 probes (1, 2, 3, 5, 6, 7, 8, 9, 10, and 16) (Fig. 7.6, A), showed eight DNA fragments that appear to contain *Tos*17 retrotransposon transposition (Figure 7.6 B). Another membrane blot also showed that *Tos*17 probe hybridised the plant DNA(s) blots that did not hybridise with GAL4/VP16 probe and also producing eight DNA fragments containing *Tos*17 transposition (Fig. 7.7, B).

Analysis showed a 3kb DNA fragment was only observed in plants with mutant phenotypes. This may suggest that the mutant phenotype was linked to the transposition of the *Tos*17 retrotransposon in the rice genome. However, as the mutant plant contained eight DNA fragments hybridised to the *Tos*17 probe, cloning of the DNA sequence tagged by this *Tos*17 transposition was not pursued.

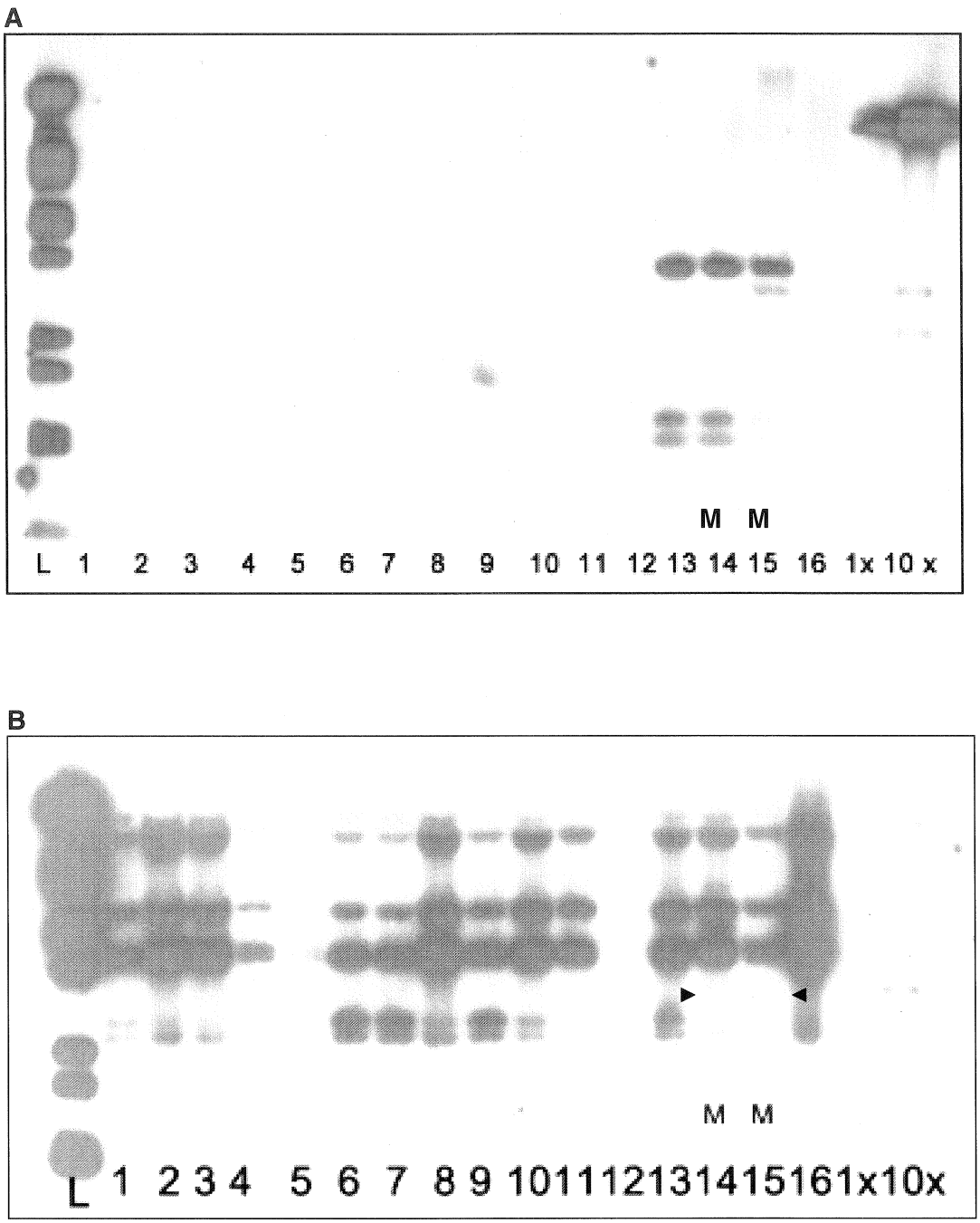

Figure 7.5 Southern blots of plants with mutant and normal phenotypes hybridised with (A) GAL4/VP16 fragment and (B) *TOS17* fragment probes. 1-13 and 16: plants with wild type phenotype, **14-15**: plants with mutant phenotypes. **Arrow**: 3.0kb DNA fragment containing *Tos17* assumed to be linked to the 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 plasmi, **M** plant with flower changed phenotype.

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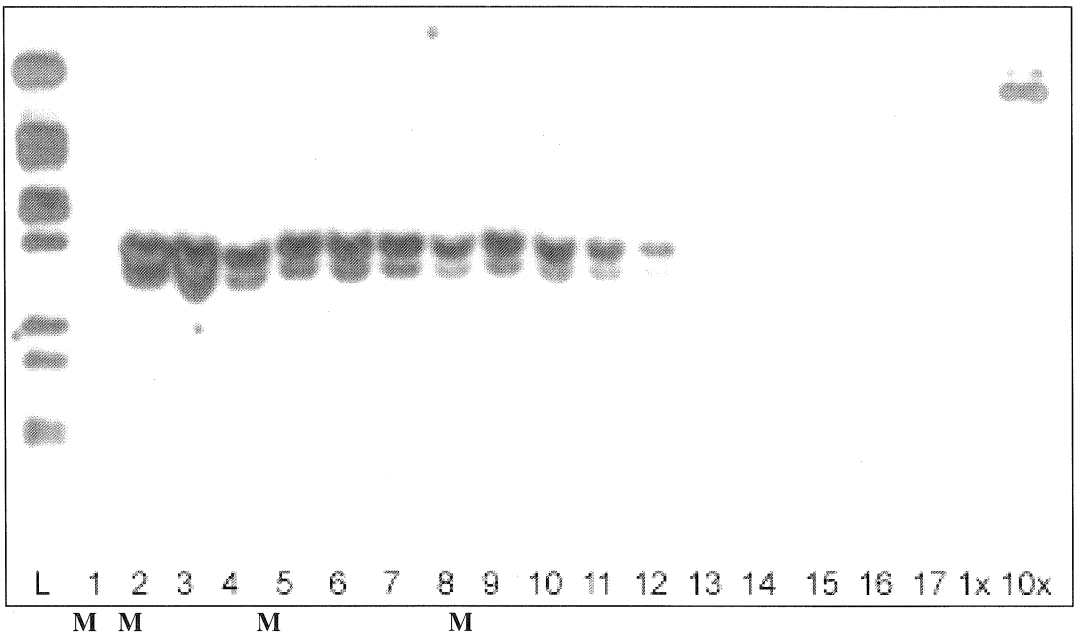
A



B

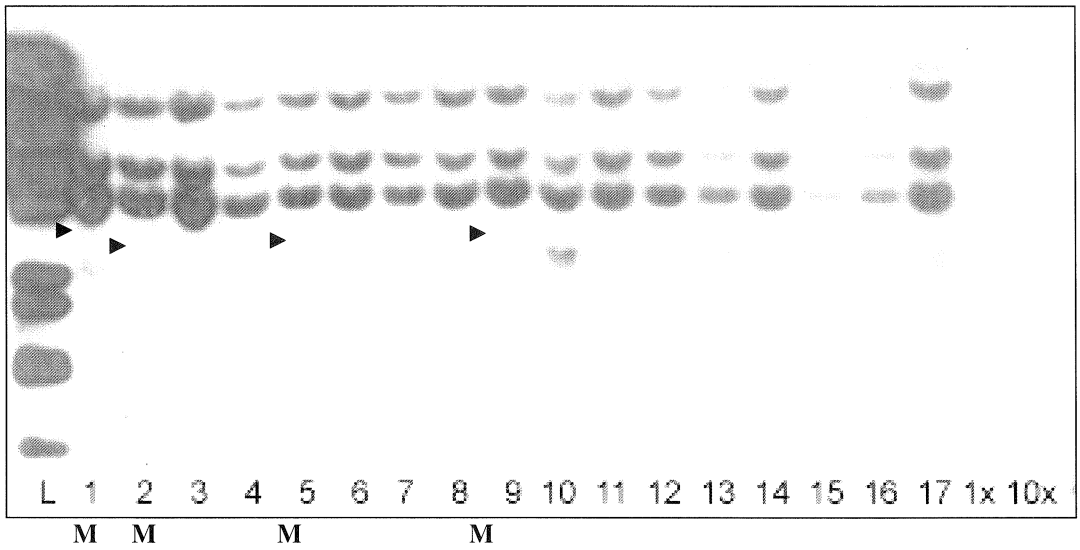


Figure 7.6 Southern of plants with mutant and normal phenotypes hybridised with (A) GAL4/VP16 fragment and (B) *TOS17* fragment probes. 3, 4, 6-8, 10-17: plants with wild type phenotype, **1, 2, 5 and 9**: plants with mutant phenotypes. **arrow**: 3.0kb DNA fragment containing *Tos17* assumed to be linked to the 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.

7.4 DISCUSSION

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 (Maes et al., 1999). 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; Springer, 2000). Indeed, about two thirds of *Drosophila*'s genes (equal to 8000 genes) are predicted to show no obvious LoF phenotypes because of gene redundancy (Miklos and Rubin, 1996). Added to this is the fact that mutations are not always linked to T-DNA and this could be as high as about 60% in *Arabidopsis* and about 50% in rice (McElver et al., 2001).

In my experiments, about 10% of TAFET lines showed various types of chlorophyll-deficient mutations, and such mutations were often produced from tissue culture application (Palmer et al., 2000). Such chlorophyll-deficient phenotypes were also obtained 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 have a T-DNA insertion in the chlorina (*OsCHLH*) gene that is highly homologous to *XANTHA-F* in barley and *CHLH* in *Arabidopsis*. Two other mutants with chlorophyll-deficient phenotypes had a *Tos*-17 insertion in the *OsCHLH* gene (Jung et al., 2003).

Several genes involved in rice flower development 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. Transgenics 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., 2003a). Ectopic expression of *OsMAD3* in rice plant caused homeotic transformation of lodicule to stamens (Kyozuka and Shimamoto, 2002). The homeotic mutation spw1 converted stamens and lodicules into carpels and palea-like organs, respectively. Two spw1 alleles, *spw1*-1 and *spw1*-2, show 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).

The pSKC66.1-8e mutant line had dramatic floral tissue changes, such as spikelets with leaf-like organs of palea and lemma (Fig. 7.3, D and F), a spikelet within a spikelet (Fig. 7.3, A, B, H), a spikelet with bud-like organs (Fig. 7.3, I), and spikelet with a lesser numbers of anthers and abnormal carpel (Fig. 7.3, C, J-L). With reference to the results above, ectopic phenotypes of the pSKC66.1-8e mutant might be related to a disruption of a MADs-box family gene.

In addition, this mutant phenotype was one example of the phenomenon that a mutation is not always linked to a T-DNA insertion. Although an approximate 3:1

segregation between plants with wild type and plants with mutant phenotype was observed in the T_1 generation (Fig 7.2, Fig 7.3 and Fig 7.4). However, observation in the T_2 generation showed that mutant phenotypes did not segregate among plants in a 3:1 Mendelian fashion as expected (Table 7.1). Indeed, plants of the pSKD66.1-8e families were segregated in 3:1 segregation ratio between plants with GUS gene expression and that of without GUS expression in the T_2 generation.

Southern blot analysis showed that the presence of DNA fragments which hybridised with the GAL4/VP16 fragment as a probe varied among families in T_2 and T_3 generations. These suggested that there was no link between the mutant phenotype and the T-DNA insertion and it was reconfirmed by Southern blot in the T_3 generation, where some of mutant plants did not have any T-DNA insertion (Table 7.1).

Further attempts were made to determine whether the mutant phenotype was due to *Tos*17 transposition, as *Tos*17 transposition has been known to be related to a tissue culture-induced mutation (Hirochika et al., 1996; Jung et al., 2003). Results showed that plant DNA blots with mutant and normal phenotypes had different numbers of DNA fragments when the membranes were hybridised with the *Tos*17 fragment as a probe. Eight fragments containing *Tos*17 transposition were produced by plants with mutant phenotypes, whereas plants with normal phenotype had only 7 DNA fragments (Fig. 7.6B). The 3.0kb DNA fragment containing the *Tos*17 transposition could be responsible for the mutant phenotype. Very few plants with mutant phenotype were observed in T_2 and T_3 families. The most likely explanation of this segregation distortion is lethality of *Tos*17 insertion when in double dose and sub-lethality in a single dose. The high level of sterility and seedling death observed in these families is sufficient for such an assumption.

Several mutants induced by *Tos*17 transposition have been identified. 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 are 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).

7.5 CONCLUSION

There are three conclusions that can be drawn from the experiments.

- First, the mutant phenotypes with dramatic changes in plant and floral morphology of pSKC66.1-8e were not related to TAFET T-DNA insertion; instead they might be linked to the transposition of the *Tos*17 retrotransposon in to a 3kb *EcoR I* fragment of rice DNA.

- Second, the *Tos*17 retrotransposon could be used to identify mutant phenotypes in transgenic rice, as previously described by Hirochika (2001).

- Third, the low frequency of mutations may suggest that *Loss-of-Function* (LoF) phenotypes are not easily induced using insertional sequence mutagenesis, due to a gene redundancy phenomenon, and there is a need to develop other mutational systems for *Gain-of-Function* (GoF) for which the Transcriptional Activator-Facilitated Enhancer Trap (TAFET) system was originally intended.

Chapter 8

CONCLUSION AND FUTURE WORK

8.1 RESULTS IN PERSPECTIVE

In this thesis five major areas have been studied, as follow:

1. The development and testing of GAL4/VP16 transactivator-facilitated enhancer trap (TAFET) constructs in rice.
2. The stability and inheritance of patterns induced by TAFET constructs.
3. Validation of the GAL4/VP16 system to see whether it works in the same way as the mechanism of the GAL4 system in *Drosophila* (Brand and Dormand, 1995) and *Arabidopsis* (Haseloff, 2002).
4. Validation of the GAL4/VP16 system to see whether it is able to direct expression of a gene fused to the UAS_{GAL4}, inducing a phenotypic change (ectopic expression) and producing of a *Gain of Function* (GoF) phenotype.
5. Identification of a mutant line observed among TAFET lines and testing whether the phenotypes were linked to T-DNA insertion.

Transactivator constructs developed and tested in the experiments had the following components: two β-glucuronidase reporter genes cassettes (GUS and GUSPlus); two different transactivator cassettes (with and without the *catalase*-1 intron upstream of the GAL4/VP16) and two different relative distances between the UAS_{GAL4} and the CaMV 35S promoter driving a hygromycin gene (1.6 kb and 7 kb).

The results presented in this thesis showed the GAL4/VP16 transcriptional activator-facilitated enhancer trap (TAFET) system was able to reveal varying levels of

tissue specificity (spatial) expression patterns in the roots, leaves or flowers of rice. . About 95% of the TAFET lines showed gene expression in the roots, leaves or floral tissues, which was higher than previously reported by Wu et al. (2003) (about 70%) using a similar type of construct. A higher percentage of expression may be due to the constructs used to generate the transgenics. In contrast, the deletion lines have T-DNA insertions, but do not display any gene expression. Results showed that the percentage of lines with expression in the floral tissues was 55%, which was higher than previously reported by Campisi et al. (31%) (Campisi et al., 1999), but comparable to that previously reported by Sundaresan et al. (Sundaresan et al., 1995b). About 17.3% of lines showed tissue-specific expression in the flower, higher than previously found in rice (1.9%) (Jeon et al., 2000a). The percentage of lines showing expression in the ovule was only about 0.5% and it was also reported as the least frequent expression in rice flowers by Wu et al (2003). These patterns may become invaluable sources for the study of rice floral tissue development, as previously described (Kiegle et al., 2000; Geisler et al., 2002).

Indeed, one construct used by Wu et al. (2003) to generate rice enhancer trap line was containing similar elements as the eight constructs used in generation of TAFET lines presented in this thesis, since it was part of the Rice Transgenomic project of CAMBIA. However, as we mentioned about transactivator constructs were developed and tested, the eight different construct induced different levels of intensity and also complexity of expression patterns. In our analysis, it is indicated that the use of different reporter genes and intron upstream GAL4/VP16 TAFET in combinations had some effect on the patterns produced. It is also apparent that 35S enhancer used affected the expression patterns in a number of lines. However, patterns are likely representing primarily the effect of endogenous enhancer and of complex interaction between rice genomic enhancer and those from the T-DNA. Beside that, our analysis also showed that different sensitivity of reporter gene affected patterns of gene

expression. GUSPlus constructs produced more lines with staining, strong expression and with complexity of patterns, produced more patterns and a greater spread of pattern distribution than GUS constructs. It supports efforts devoted for developing a better and more sensitive of reporter gene (Jefferson et al., 1987; Haseloff et al., 1997)

The work also proved that patterns produced were due to the GAL4/VP16 activation of reporter gene fused to the UAS_{GAL4} in rice, as previously reported in plant cells (Schwechheimer et al., 1998) and *Arabidopsis* (Kiegle et al., 2000).

As patterns of the transactivator-based enhancer trap lines were mimicking endogenous rice enhancers, enhancer trap lines are equally valuable materials for plant development studies in rice, as those previously reported in *Arabidopsis* (Kiegle et al., 2000; Haseloff, 2002) and *Drosophila* (Brand and Perrimon, 1993; Rorth, 1998).

In my experiments, the observation was focused on reporter gene expression in the floral tissues, and it resulted in 17.3% of TAFET lines showing tissue-specific expression patterns in floral parts. Overall, 2.1% had expression only in a single tissue, 10.5% had expression in two tissues, and 4.8%, had expression in three tissues. These GAL4/VP16 enhancer trap lines could be useful for floral tissue-specific development studies, using a similar approach as described above. For example, lines that had expression in the ovule (0.5%) of the pattern lines developed could be crossed with UAS lines (a target gene or a random target), and any gene lying next to the UAS would be activated in the ovule, producing lines with phenotypic changes in the ovule tissues. Screening of such materials might lead to identification of genes involved in ovule development. Ovule development is an important subject as it is related to the apomixis phenomenon that is a subject of very active research (Koltunow, 1993; Moore et al., 1997; Chaudhury et al., 1998; Grossniklaus and Schneitz, 1998; Grossniklaus et al., 2001; Koltunow and Grossniklaus, 2003). Most studies in ovule development have

8.2 SOME REMAINING ISSUES

Attempts to generate an obvious phenotypic change in a rice tissue under investigation through ectopic expression of maize *AGAMOUS*-like *ZAG1* gene, using the GAL4/VP16 system, have not been successful. Other genes should be tested using this system to evaluate its performance.

As varying levels of specificity of expression patterns were observed in TAFET lines, and these could aid the identification of regulatory proteins involved in cells or tissue development in rice. Screening of those lines under various conditions would be very useful. Field screening would be particularly valuable, as the performance of transactivator in normal growing conditions for rice is essential for potential practical applications of the system. Unfortunately field screening of transgenic material in Australia is very difficult to carry out because of current regulations.

8.3 CONCLUSIONS

Experimental results presented in this thesis allow the conclusion as follows.

- The GAL4/VP16 transcriptional activator-facilitated enhancer trap (TAFET) system is functional. Gene expression patterns are revealed by a reporter gene, both in ubiquitous and cells- or tissue-specific manner, mimicking endogenous enhancers.

- These patterns were inherited in a Mendelian fashion and stably expressed over three generations in rice. The implication of these observations is that selection of GAL4/VP16 enhancer-trap lines can be carried out in the T_0 generation (hemizygous state).

- The GAL4/VP16 TAFET system was able to direct expression of a "target" gene fused to the UAS_{GAL4}, in a cell or tissue of rice, in which the GAL4/VP16 was active (reflection of the site where an enhancer and regulatory proteins were active). This ablility has a potential use for inducing *Gain-of-Function* (GoF) mutations. The GAL4/VP16 TAFET system is therefore a prospective system and a novel tool for rice gene discovery and rice functional genomics investigations.

- The TAFET-GUSPlus constructs induced more complex patterns and greater diversity of patterns than GUS constructs in rice enhancer trap lines. It was confirmed that GUSPlus is a more sensitive reporter gene than GUS (Nguyen, 2002).

8.4 FUTURE WORK

Crossing between GAL4/VP16 lines and target lines containing genes other than *ZAG1* to produce F1 plants with phenotypic changes needs to be conducted. This will confirm wether the GAL4/VP16 system is able to direct expression of a target gene (gene under study or random insertion) fused to UAS in the cell- or tissue-specific manner, hopefully producing novel *Gain-of-Function* (GoF) phenotypes.

Appendix 4.1 TAFET lines were investigated for patterns of expression in three generations and χ^2 test in T_2 generation.

No.	Constructs	Lines	G	T-DNA	No	F	C1	C 2	E1	E2	Expression patterns	chi-squared	P<0.05 (3.84)	status
			T_0	2							anther wall, strong			
1	pSKC76.1	56f	T_1								anther wall, strong			
			T_2		1	2	16	6	16.5	5.5	anther wall, strong:pollen, weak	0.06	accept hypothesis	heterozygote
					2	3	23	0	17.25	5.75	anther wall, strong:pollen, weak	7.67	reject hypothesis	homozygote
					3	6	15	8	17.25	5.75	anther wall, strong:pollen, weak	1.17	accept hypothesis	heterozygote
				2							anther wall cells			
2	pSMRJ18(0)	31a	T_0								anther wall cells			
			T_1		4	2	17	7	18	6	anther wall cells	0.22	accept hypothesis	heterozygote
			T_2		5	3	24	0	18	6	anther wall cells	8.00	reject hypothesis	homozygote
					6	6	21	1	17.25	5.75	anther wall cells	4.74	reject hypothesis	homozygote
			T_0	1							Midrib palea&lemma, anther wall, (stigma and style, weak)			
3	pSMRJ18R	3a	T_1								midrib palea & lemma, anther wall			
			T_2		7	1	14	0	10.5	3.5	midrib palea & lemma, anther wall (stigma and style, weak)	4.67	reject hypothesis	homozygote
					8	7	0	8	6	2	pollen	24	reject hypothesis	Background
					9	8	9	2	9.75	3.25	anther wall	0.55	accept hypothesis	heterozygote
			T_0								Not observed			
4	pSMRJ18 (50)	506	T_1								anther wall cells			
			T_2		10	5	24	0	18	6	anther wall cells	8.00	reject hypothesis	homozygote
					11	6	24	0	18	6	anther wall cells	8.00	reject hypothesis	homozygote
					12	8	24	0	18	6	anther wall cells	8.00	reject hypothesis	homozygote
			T_0								pollen			
5	pSMRJ18	103c	T_1								pollen			
			T_2		13	2	16	7	17.25	5.75	pollen	0..36	accept hypothesis	heterozygote
					14	4	13	7	15	5	pollen	1.07	accept hypothesis	heterozygote
					15	5	15	5	15	5	pollen	0	accept hypothesis	heterozygote
6	pSKC66.1	8e	T_0	5							Anther wall, pollen			
			T_1								anther wall, pollen (5/8) and 3/8 morphological changes *			

No.	Constructs	Lines	G	T-DNA	No	F	C1	C 2	E1	E2	Expression patterns	chi-squared	P<0.05 (3.84)	status
			T_2		16	54	14	3	12.75	4.25	anther wall, pollen:pollen	0.49	accept hypothesis	heterozygote
					17	52	15	16	23.25	7.75	anther wall, pollen:pollen	11.71	reject hypothesis	uncertain
					18	55	10	9	14.25	4.75	anther wall, pollen:pollen	5.07	reject hypothesis	uncertain
7	pSMRJ18	3a	T_0								Palea &lemma, pollen, base of style			
			T_1								base of style, middle part of carpel			
			T_2		19	1	13	8	16.5	5.5	base of style, anther wall: anther wall	1.88	accept hypothesis	heterozygote
					20	2	17	6	17.25	5.75	anther wall:pollen	0.01	accept hypothesis	heterozygote
					21	3	16	8	18	6	base of style, anther wall: anther wall	0.89	accept hypothesis	heterozygote
8	pSMRJ17R	301	T_0	4							anther, base of style			
			T_1								anther, base of style, medium-strong			
			T_2		22	5	17	6	17.25	5.75	base of style, strong:pollen, style weak	0.01	accept hypothesis	heterozygote
					23	7	12	7	14.25	4.75	base of style:pollen	1.42	accept hypothesis	heterozygote
					24	8	22	1	17.25	5.75	base of style, strong:pollen	5.23	reject hypothesis	homozygote
9	pSMRJ18(50)	22a	T_0								Filament, anther, pollen			
			T_1								Anther wall, (stigma, style, ovule, weak)			
			T_2		26	4	18	6	18	6	anther wall cells : pollen	0	accept hypothesis	heterozygote
					27	8	0	24	16	8	Pollen	48.00	reject hypothesis	background
					28	7	18	6	18	6	anther wall cells : pollen	0	accept hypothesis	heterozygote
10	pSMRJ18R -1/2	9a	T_0	3							tip of palea, lemma (weak), anther, stigma, style			
			T_1								midrib of palea, lemma, anther, stigma, style			
			T_2		29	1	18	6	18	6	anther, style, stigma: anther	0	accept hypothesis	heterozygote
					30	4	19	5	18	6	anther, style, stigma: anther	0.22	accept hypothesis	heterozygote
					31	6	18	6	18	6	anther, style, stigma: anther	0	accept hypothesis	heterozygote
11	pSMRJ18 (50)	770-1	T_0								Not observed			
			T_1								Lodicule, anther wall, pollen, (base of style)			
			T_2		32	2	16	8	18	6	Lodicule, anther wall, pollen : pollen	0.89	accept hypothesis	heterozygote
					33	4	20	0	15	5	anther wall cells , pollen	6.67	reject hypothesis	homozygote
					34	6	16	4	15	5	anther wall cells, pollen : pollen	2.67	accept hypothesis	heterozygote

No.	Constructs	Lines	G	T-DNA	No	F	C1	C 2	E1	E2	Expression patterns	chi-squared	P<0.05 (3.84)	status
12	pSMRJ18R(50)	881-1	T_0								Vascular bundle of palea/lemma, anther, pollen			
			T_1								Vascular bundle, whole, tip palea/lemma, lodicule vessel, anther wall, (base of style)			
			T_2		35	2	16	0	12	4	Midrib, vascular bundle palea/lemma, anther wall, pollen	5.33	reject hypothesis	homozygote
					36	3	16	0	12	4	Midrib, vascular bundle palea/lemma, anther wall, pollen	5.33	reject hypothesis	homozygote
13	pSMRJ18	5a	T_0								not observed			
			T_1								Midrib,lodicule ,anther, base of style, stgma			
			T_2		37	4	19	5	18	6	Midrib, lodicule , anther, (stigma, some), base of style: pollen	0.22	accept hypothesis	heterozygote
					38	6	18	6	18	6	Midrib, lodicule, anther, stigma, base of style: pollen	0	accept hypothesis	heterozygote
					39	7	17	7	18	6	Midrib, lodicule, anther, base of style: pollen	0.22	accept hypothesis	heterozygote
14	pSMRJ17R	204	T_0								not observed			
			T_1								Anther, base of style			
			T_2		40	4	14	9	17.25	5.75	Anther, base of style : pollen	2.45	accept hypothesis	heterozygote
					41	5	14	5	14.25	4.75	Anther , base of style , (lodicule, palea, some) : pollen	0.01	accept hypothesis	heterozygote
					42	8	11	7	14.25	4.75	Anther, base of style : pollen	1.81	accept hypothesis	heterozygote
15	pSMRJ18	100	T_0								lodicule, anther			
			T_1								lodicule, anther			
			T_2		43	2	18	6	18	6	lodicule, anther : pollen	0	accept hypothesis	heterozygote
					44	5	17	4	15.75	5.75	lodicule, anther : pollen	0.63	accept hypothesis	heterozygote
					45	8	20	4	18	6	lodicule, anther : pollen	0.89	accept hypothesis	heterozygote
16	pSMR J17	806	T_0								not observed			
			T_1								lodicule			
			T_2		46	5	19	5	18	6	pollen	0.22	accept hypothesis	heterozygote
					47	6	11	9	15	5	lodicule, anther : pollen	4.27	reject hypothesis	
					48	7	18	5	17.25	5.75	lodicule, anther : pollen	0.13	accept hypothesis	heterozygote
17	pSMR J18R	610	T_0								not observed			
			T_1								Trichoma, strong			
			T_2		49	2	8	0	6	2	Trichome, palea/lemma, anther wall	2.67	accept hypothesis	heterozygote
					50	3	11	0	8.25	2.75	Trichome, palea/lemma, anther wall	3.67	accept hypothesis	heterozygote

No.	Constructs	Lines	G	T-DNA	No	F	C1	C 2	E1	E2	Expression patterns	chi-squared	P<0.05 (3.84)	status
17	pSMR J18R	610	T_2		51	8	0	17	12.75	4.25	pollen, very weak	51.00	reject hypothesis	
18	pSMRJ17	101e	T_0								not observed			
			T_1								end of filament, lodicule			
			T_2		52	7	10	8	13.5	4.5	end of filament, base of style : pollen	3.63	accept hypothesis	heterozygote
					53	3	12	6	13.5	4.5	end of filament, lodicule, base of style	0.67	accept hypothesis	heterozygote
					54		17	7	18	6	end of filament, lodicule, base of style	0.22	accept hypothesis	heterozygote
19	pSMRJ17R	10a	T_0								anther			
			T_1								whole, trichoma, anther wall, stigma			
			T_2		55	1	16	8	18	6	whole, trichoma, anther wall, stigma	0.89	accept hypothesis	heterozygote
					56	2	18	4	17.5	5.5	whole, trichoma, anther wall, stigma	0.42	accept hypothesis	heterozygote
					57	3	18	4	17.5	5.5	whole, trichoma, anther wall, stigma	0.423377	accept hypothesis	heterozygote
20	pSKC D59.2	14d	T_0	1							midrib palea/lemma, lodicule, anther			
			T_1								midrib palea/lemma, lodicule, anther			
			T_2		58	3	12	9	15.25	5.75	midrib palea/lemma, lodicule, anther: pollen	2.53	accept hypothesis	heterozygote
					59	4	18	6	18	6	midrib palea/lemma, lodicule, anther: pollen	0	accept hypothesis	heterozygote
					60	5	18	6	18	6	midrib palea/lemma, lodicule, anther: pollen	0	accept hypothesis	heterozygote
21	pSMRJ18R	25g	T_0	4							Palea/lemma, anther			
			T_1								Palea/lemma, lodicule, anther, stigma, style			
			T_2		61	1	21	3	18	6	trichoma+anther:non	2.00	accept hypothesis	heterozygote
					62	6	16	4	15	5	Palea/lemma,trichoma, anther non	0.27	accept hypothesis	heterozygote
22	pSMRJ18 (50)	505-1	T_0	5							anther, stigma			
			T_1								anther wall cells, strong			
			T_2		63	7	15	0	11.25	3.25	anther wall cells	4.5	reject hypothesis	
					64	5	14	4	13.5	4.5	anther wall cells : non	0.07	accept hypothesis	heterozygote
					67	4	10	3	9.75	3.25	anther wall cells : non	0.03	accept hypothesis	heterozygote
23	pSMRJ18	20	T_0								anther, base of style			
			T_1								base of style, anther			

No.	Constructs	Lines	G	T-DNA	No	F	C1	C 2	E1	E2	Expression patterns	chi-squared	P<0.05 (3.84)	status
23	pSMRJ18	20	T_2		68	1	17	3	15	5	base of style, anther	1.07	accept hypothesis	heterozygote
					69	7	15	2	12.75	4.25	base of style, anther	1.59	accept hypothesis	heterozygote
24	pSMRJ18	21	T_0								anther, base of style			
			T_1								base of style			
			T_2		70	2	12	0	9	3	anther wall, strong	4.00	reject hypothesis	homozygote
					71	6	11	3	10.5	3.5	anther wall, strong	0.10	accept hypothesis	heterozygote
					72	7	15	2	12.75	4.25	anther wall, strong	1.59	accept hypothesis	heterozygote
25	pSMRJ18 (50)	23b	T_0								not observed			
			T_1								anther wall			
			T_2		73	3	24	0	18	6	anther wall	8.00	reject hypothesis	homozygote
26	pSMRJ18R(0)	47a	T_0								Whole, vascular bundle or midrib palea/lemma, anther			
			T_1								Palea/lemma, anther			
			T_2		74	1	17	6	17.25	5.75	Whole/vascular bundle/midrib palea/lemma, anther wall:pollen	0.01	accept hypothesis	heterozygote
					75	4	16	6	16.5	5.5	Whole/vascular bundle/midrib palea/lemma, anther wall:pollen	0.06	accept hypothesis	heterozygote
					76	6	18	6	18	6	Whole/vascular bundle/midrib palea/lemma, anther wall:pollen	0	accept hypothesis	heterozygote
27	pSMRJ17R	25a	T_0								not observed			
			T_1								whole palea/lemma,trichome			
			T_2		77	1	20	3	17.25	5.75	whole palea/lemma,trichome : non	1.75	accept hypothesis	heterozygote
					78	2	18	6	18	6	whole palea/lemma,trichome : non	0	accept hypothesis	heterozygote
					79	5	17	5	16.5	5.5	whole palea/lemma,trichome : non	0.06	accept hypothesis	heterozygote
28	pSKC76.1	2	T_0								not observed			
			T_1								stigma, style			
			T_2		80	4	14	10	15	5	stigma, style:pollen	5.07	reject hypothesis	uncertain
					81	6	15	9	18	6	stigma, style:pollen	2.00	accept hypothesis	heterozygote
					82	8	0	21	15.75	5.25	anther wall and or pollen	63.00	reject hypothesis	background
29	pSMRJ17R	37d	T_0											
			T_1								pollen, stigma			

No.	Constructs	Lines	G	T-DNA	No	F	C1	C 2	E1	E2	Expression patterns	chi-squared	P<0.05 (3.84)	status
			T_2		83	4	16	7	17.25	5.75	anther wall, stigma, style: pollen	0.36	accept hypothesis	heterozygote
					84	6	24	0	18	6	Palea/lemma, anther, stigma, style	8.00	reject hypothesis	homozygote
					85	8	0	24	18	6	pollen	72.00	reject hypothesis	background
30	pSMRJ17R	78a	T_0								Midrib palea/lemma, stigma, style			
			T_1								Midrib palea/lemma, stigma, style			
			T_2		86	1	14	7	16.5	5.5	Midrib, anther wall, stigma, style	0.79	accept hypothesis	heterozygote
					87	6	16	0	12	4	Midrib, anther wall, stigma, style	5.33	reject hypothesis	homozygote
					88	7	24	0	28	6	Midrib, anther wall, stigma, style	6.57	reject hypothesis	homozygote
31	pSMRJ18R(50)	112d	T_0								not observed			
			T_1								pollen			
			T_2		89	1	17	1	13.5	4.5	pollen : non	3.62	accept hypothesis	heterozygote
					90	5	16	1	12.75	4.25	pollen : non	3.31	accept hypothesis	heterozygote
					91	8	19	1	15	5	pollen : non	4.27	reject hypothesis	homozygote
32	pSMRJ18R(50)	132a	T_0								not observed			
			T_1								whole palea/lemma, anther wall and or pollen			
			T_2		92	4	17	7	18	6	whole palea/lemma, anther wall:pollen	0.22	accept hypothesis	heterozygote
					93	6	24	0	18	6	whole palea/lemma, anther wall	8.00	reject hypothesis	homozygote
					94	5	24	0	18	6	whole palea/lemma, anther wall	8.00	reject hypothesis	homozygote
33	pSMRJ18R(1/2)	6d	T_0								vascular bundle palea/lemma, trichome, anther wall			
			T_1								vascular bundle palea/lemma, trichome, anther wall			
			T_2		95	6	15	9	18	6	vascular bundle,midrib or whole, anther wall:pollen	2.00	accept hypothesis	heterozygote
					96	7	0	24	18	6	anther wall, pollen	72.00	reject hypothesis	background
					97	3	22	2	18	6	vascular bundle,midrib or whole, anther wall:pollen	3.56	accept hypothesis	heterozygote
34	pSMRJ18R	2e	T_0								not observed			
			T_1								vascular bundle palea/lemma, anther			
			T_2		98	1	19	5	18	6	midrib, vascular bundle, anther, stigma:pollen	0.22	accept hypothesis	heterozygote
					99	3	0	11	7.5	2.5	pollen	36.40	reject hypothesis	background
					100	5	17	7	18	6	midrib, vascular bundle, anther,stigma:pollen	0.22	accept hypothesis	heterozygote

No.	Constructs	Lines	G	T-DNA	No	F	C1	C2	E1	E2	Expression patterns	chi-squared	P<0.05 (3.84)	status
35	pSKD15.2	1e	T_0								no expression			
			T_1								no expression			
			T_2	2	101	2	24	0	16	8	no expression	12.00	reject hypothesis	homozygote
					102	3	24	0	16	8	no expression	12.00	reject hypothesis	homozygote
					103	6	24	0	16	8	no expression	12.00	reject hypothesis	homozygote
36	pC1201		T_0								not observed			
	control		T_1								whole flower, strong			
			T_2		104	1	20	4	16	8	whole flower, strong	3.00	accept hypothesis	heterozygote
					105	3	17	7	16	8	whole flower, strong	0.18	accept hypothesis	heterozygote
					106	7	15	5	15	5	whole flower, strong	0	accept hypothesis	heterozygote

G: generation, **F**: family, **C1& C2**: actual number of plants for each class of observation and **E1 & E2**: expectated number of plants of each class.

STATEMENT

This thesis contains no material which has been previously submitted for an academic record at this university or any other university and is the original work of the author, except where acknowledged.

Sri Koerniati

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ABSTRACT

The development of a system for the discovery of rice genes and their function is a high priority. Two different β-glucuronidase reporter gene cassettes (GUS and GUSPlus) and two GAL4/VP16 transactivator cassettes (with and without the *catalase*-1 intron upstream of the GAL4/VP16) as well as their relative position (1.6 kb and 7 kb relative distances between the upstream activating sequence (UAS_{GAL4}) and the CaMV35S promoter driving a hygromycin gene) within T-DNA were tested for their performance in the Transcriptional Activator-Facilitated Enhancer trap (TAFET) constructs to induce cell- or tissue-specific expression patterns in rice. A total of 10 TAFET constructs, including two negative controls, were developed and introduced into the rice genome of varieties Millin and Nipponbare using *Agrobacterium*-mediated transformation and about 1,000 rice TAFET lines were produced and investigated.

The GAL4/VP16 TAFET system was able to reveal varying levels of tissue specific expression patterns in rice in the vegetative and generative tissues, and the expression patterns were proven to be due to the activity of the GAL4/VP16 transcriptional activator.

Most of the TAFET lines examined showed reporter gene expression in many tissues. Patterns shown often in the vascular bundle, pericycle layer of cells in the root suggest that the CaMV35S enhancers affected the expression patterns in numbers of lines. However, the observed patterns are likely to be primarily due to a complex interaction between rice genomic endogenous enhancers and elements within T-DNAs.

Most patterns observed in TAFET lines (91.7%) were inherited by the next generations (T_1 and T_2) and segregated in Mendelian fashion. T-DNA inserts segregated among progeny plants in agreement with expected ratios. About 49% of

lines contained a single T-DNA insertion and the average number of T-DNAs in the whole TAFET population was 2.0.

The crossing experiments between selected GUS-TAFET lines and selected Enhancer Green Fluorescence protein (EGFP) target lines proved that the GAL4/VP16 TAFET system is capable of activating a gene (a reporter gene) adjacent to UAS. The most likely mechanism of activation is through the GAL4 DNA binding domain recognising the UAS_{GAL4}, and the VP16 activating domain promoting transcription of a gene linked to the UAS_{GAL4}. Expression patterns of EGFP reporter gene in F_1 plants were in general consistent with GUSPlus patterns observed in parental GUS-TAFET lines.

Attempts to generate phenotypic changes in floral tissues by induced ectopic expression of the *ZAG1* target gene from maize did not fully succeed, as the changes were only limited to some spikelets. The positive control in these experiments, 35S promoter driven over-expression of *ZAG1*, produced limited phenotypic consequences (increased sterility in some plants), suggesting that the inability of the TAFET system to induce clearly observable changes in floral development were due to poor expression of the *ZAG1* gene.

A number of TAFET lines showed phenotypic changes that segregated in T_1 and T_2 generations. An attempt to clone a gene responsible for a dramatic change in floral development in line pSKC66.1-8e failed due to the lack of a link between T-DNA insertion and mutant phenotypes. *Tos17* retrotransposon mobility during tissue culture was determined to be the most likely cause of floral development change observed in this line.

In general, good performance of the GAL4/VP16-based TAFET system was determined both through observations of TAFET lines in several generations and through sexual crossing with TARGET EGFP lines. The implication of this research is that the system can be used to direct the expression of any gene under study (a target) in specific tissue types to generate ectopic expressions and possibly also *Gain-of-Function* (GoF) phenotypes. The TAFET system therefore can be applied in rice functional genomics studies and could possibly contribute to the generation of a novel germplasm with useful traits for agriculture.

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