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

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Sri Koerniati

Center for the Application of Molecular Biology
to International Agriculture (CAMBIA)
Canberra, ACT, Australia

And

Research School of Biological Sciences
The Australian National University
Canberra, ACT, Australia

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Chapter 4

STABILITY OF TAFET LINES EXPRESSION PATTERNS IN T_1 AND T_2 GENERATIONS

4.1 INTRODUCTION

The stability of transgenic expression patterns is a critical parameter for the further use of the transcriptional activator-facilitated enhancer trap (TAFET) system and of TAFET lines for rice functional genomic investigations.

It has been shown in Chapter 3 that varying levels of specificity in expression patterns in vegetative and floral tissue in rice, were obtained when transactivator constructs of the reporter gene were also displayed in TAFET lines (see Chapter 3, Section 3.3.3). Variation of expression was also reported in *Drosophila* and *Arabidopsis* when the GAL4 enhancer trap system was applied (Brand and Perrimon, 1993; Castelli-Gair et al., 1994; Aoyama et al., 1995; Phelps and Brand, 1998; Kiegle et al., 2000).

In general, variation in the intensity and patterns of transgene expression are attributed to several factors, such as differences with respect to chromosome location (position effect), transgene copy number, and transgene construct fidelity (Hobbs et al., 1990; van Leeuwen et al., 2001). Chromosome location affects transgene expression primarily through the integration of the transgene either into euchromatin which is the transcriptionally active region, or into heterochromatin which is an inactive region. Epigenetic silencing was also described as having an effect on transgene expression (Wolffe and Matzke, 1999).

The quantity and quality (for example a transgene truncation) of inserts have been reported as influencing gene expression and this is also determined by the method of delivery of transgenes. With *Agrobacterium tumefaciens* mediated T-DNA transformation, molecules are sited in between two imperfect direct repeats of 24bp, the right and left borders of the Ti (tumour induced) plasmid of *Agrobacterium*. These borders are the only *cis*-acting elements required for T-DNA transfer and can mediate transfer into the plant nucleus of any DNA sequence located between them (Zambryski et al., 1989). A T-DNA insertion occurs usually at the site of DNA damage, the activated area containing DNA repair enzymes necessary for the process of T-DNA integration. T-DNA integration leads to a different amount of duplication in either the host or the T-DNA. T-DNA inserts randomly in genomes (Ambros, 1986; Chyi, 1986; Wallroth, 1986), but it has been described that T-DNA tends to insert into the gene space; i.e. in the regions of the genome that are transcriptionally active, in tobacco (Herman et al., 1990; Fobert et al., 1991) as well as in rice and *Arabidopsis* (Barakat et al., 2000).

In relation to construct infidelity, the type of transgene construct is considered to influence the copy number and also expression. A direct selection for a T-DNA integration into actively transcribed plant genes leads to an amplification of a promoter-less reporter gene, a truncated or an aberrant T-DNA. Five to twenty copies of promoter-less reporter T-DNAs were found inserted into the plant genome compared to only 1.5 copies for promoter-driven reporter T-DNAs (Koncz et al., 1989, Koncz, 1992). Multiple T-DNA insertions often occur in a single plant, including multiple copies per locus and in multiple loci (Lindsey et al., 1993). This multiplicity is a potential problem when delivering the enhancer trap system because multiple insertions may complicate interpretation of expression patterns (Springer, 2000).

Analysis of the GAL4/VP16 transcriptional activator facilitated enhancer trap (TAFET) constructs and expression patterns induced in the various plant tissues described in Chapter 3 clearly showed that gene expression was due to the activation of the GAL4/VP16 transcriptional activator. It also showed that GUSPlus constructs induced a higher number of lines with staining, strong intensity of expressions, more complexity of patterns, as well as with a greater spread of pattern distribution than GUS constructs (see Chapter 3, Fig. 3.19). Statistical analysis showed significant levels of the influence of each construct to intensity and complexity of patterns. Two copies per line was the average number of TAFET T-DNA insertions and most of the TAFET lines contained a single copy T-DNA insertion (about 49%).

The aim of the experiments reported in this chapter was to characterise the stability and inheritance of the phenotypic patterns of the TAFET lines from the T_0 generation (reported in Chapter 3) in T_1 and T_2 generations. Another objective was to analyse the genetic segregation of pattern traits among progeny plants, and relate this segregation data to the number of T-DNA insertions.

4.2 MATERIALS AND METHODS

4.2.1 PLANTING T_1 and T_2 TAFET LINES

Eight plants of each of 270 lines of the T_1 generation were planted. A total of 2160 T_1 plants were grown and sampled for observation. The seeds from these plants were harvested. Based upon the observed results, 24 seeds from each of 3 families (72 seeds) of each of 40 lines were subsequently planted for the next step of experiment as the T_2 generation. A total of 2880 (24x3x40) plants were grown in this generation.

4.2.2 OBSERVATION AND HISTOLOGICAL ANALYSIS

Observations were conducted to define expression patterns of the reporter gene in rice plant tissues. Histochemical detection of β-glucuronidase genes (GUS and GUSPlus) expression was performed using fresh leaves and flowers of T_1, and only fresh flowers of T_2, as previously described by Jefferson et al. (1987). Leaf samples were prepared by cutting leaves using a hole punch and placing tissues in 96-well plates. Flowers (spikelets) were analysed at three stages of flower development, young (early booting stage), medium (full booting) and mature flowers (just before flower dehiscence). Samples were viewed using a Leica Wild M8 microscope or a Leitz Diaplan microscope with bright- and dark-field optics. Images were acquired with a Nikon CoolPix Digital photo camera.

4.2.3 MOLECULAR ANALYSIS

Molecular analysis was carried out for several families of the T_2 generation to find out whether T-DNA(s) in the rice plant genome related to gene expression patterns in TAFET lines. Southern blot hybridisation of membranes with plant DNA(s) were conducted as previously described (Sambrook et al., 1989) and a GAL4/VP16 fragment was used as a probe.

4.3 RESULTS

4.3.1 INHERITANCE AND CONSISTENCY OF GENE EXPRESSION IN T_1 AND T_2 GENERATIONS

4.3.1.1 Pattern inheritance in T_1 and T_2 generations

Eight plants of each of 270 TAFET T_1 lines were grown and analysed for gene expression patterns. Apart from expression patterns obtained from employing TAFET

constructs in the rice genome, about 10% of the lines displayed *loss-of-function* (LoF) phenotypes which were chlorophyll mutations; ranging from yellow-green to white stripe to completely white phenotypes (albino). In addition, one of the GUS-TAFET lines, pSKC66.1-8e had flower and/or spikelet morphological changes. These materials were observed and are discussed in Chapter 7.

Based upon GUS and GUSPlus histological analysis, most plants of each T_1 line showed segregation of expression patterns. When observational data in leaf and flower were combined, about 92.8% of lines displayed an approximate 3:1 segregation ratio. The data were derived from 219 of the 270 T_1 lines tested, using 8 plants for each. Of the 92.8% segregating lines, about 76.0% had expression in leaf and flower, 14.4% had expression only in the flower and another 3.3% had expression only in the leaf. In addition, about 1.5% of lines showed no segregation (presumably homozygous) and about 5.7% of lines showed no expression.

A total of 40 lines were chosen for segregation analysis in the T_2 generation. From each line 3 families were chosen at random. Twenty-four plants of each family were planted for analysis of expression patterns and segregation analysis. Flowers from 106 families were taken for histological analysis of GUS reporter gene expression (Appendix 4.1).

Segregation patterns were analysed using a χ^2 (chi-square) test to determine if the numbers of plants with and without expression of reporter gene were in agreement with expected Mendelian segregation. About 59.5% of families had a 3:1 segregation ratio between plants with expression versus plants without expression. 32% did not segregate at all, 3.8% showed a 1:1 segregation ratio and 4.7% did not show any gene expression (Appendix 4.1). Surprisingly, one fourth of 24 plants that were suspected to have no gene expression, displayed very weak to moderately weak blue GUS staining

in their pollen with at least some, if not all pollen with staining. This type of expression was considered to be a background level of glucuronidase-like activity, as about 35.0% of segregating families showed this type of staining.

4.3.1.2 Consistency of patterns between T_0, T_1 and T_2 generations

An expression pattern was categorised as "consistent" when a pattern shown in the T_1 plant was similar to the pattern shown in the T_0 plant. There were three classes of phenotypes that fitted this criterion of consistency. The first class, where a pattern shown in T_1 was exactly the same as that shown in T_0; the second class, where a pattern was maintained in T_1, but had an additional expression in another tissue that was not observed in T_0; and the third class, where a pattern was displayed in T_1 that was expressed in a lesser number of tissues (partly disappeared) than that of in T_0.

Analysis of observational data based upon these classes showed that 91.7% of patterns in T_1 were consistent to those in T_0. In more detail, it was 32.4% of patterns that fell into the first class of consistency. About 52.4% of patterns fitted into the second class. These second class patterns divided into two groups: 27.6% had additional expression either in palea, lemma and other tissues, and another 24.8% had additional expression in the lodicule, filament, and stigma or style. The third class of consistency contained 6.9% of patterns, and these patterns were mostly a weak expression on stigma or style in the T_0 plants (Appendix 4.1). Data was only derived from 145 of the 270 T_1 lines planted, because some of lines did not have observational data on the T_0 generation. In comparing T_2 and T_1 patterns, 97.0% of the patterns were found to be consistent. Some of these consistent patterns are shown in Figure 4.1.

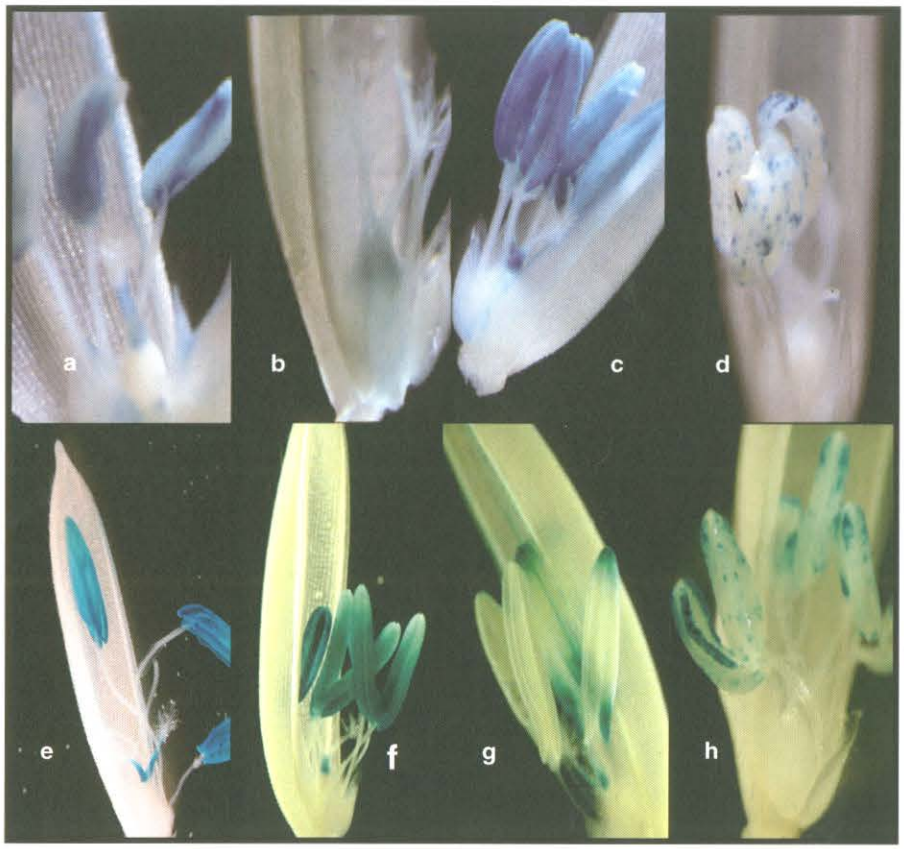

Figure 4.1 Some patterns displayed in T_0 compared to patterns in T_2 plants. **a-d**: T_0 plants, **e-h**: T_2 plants, **a** & **e**: pSMRJ18-506, **b** & **f**: pSMRJ18-3a, **c** & **g**: pSMRJ18-5a, **d** & **h**: pSMRJ18R (1/2)-712.

In contrast, 5.6% of patterns showed inconsistency in T_1 and these were mostly lines which were transformed with the TAFET-GUS constructs. In addition, the remaining 2.7% of lines showed a silenced expression, for example, pSKC66.1-2a.1 (NI00-33), pSKC66.1-8g (NI00-40) and pSKC66.1-8f (NI00-41) (Appendix 4.1).

In this T_2, T_1 and T_0 comparative analysis, one pattern (line pSMRJ18-21) was exactly the same, with only a different intensity of expression between the T_2 and the T_0 plants. The T_2 flowers showed a weaker expression of the GUSPlus reporter gene in the base part of style than T_0 flowers (Figure 4-2).

In the T_1 and T_0 comparative analysis of TAFET lines, it was shown that only 28.3% of patterns in T_1 lines were without expression in the anther wall and/or in the pollen, compared to 34.0% of patterns in T_0 lines without expression in the anthers. Patterns of reporter gene expression in the anther wall were either in the whole anther wall (58.6%), in the cells (10.3%) or in the connective tissues of the anther (2.8%) and only 5.8% of the lines in T_0 had a pollen-specific expression. Surprisingly, about 35.0% of families which showed a 3:1 segregation ratio, displayed very weak to moderately weak expression (blue staining) in pollen in one quarter of their plants. Since one quarter of plants were expected not to have T-DNA insertion, this weak staining was considered to be background activity, rather than a GUS or GUSPlus expression, while remaining plants showed expression in other tissues.

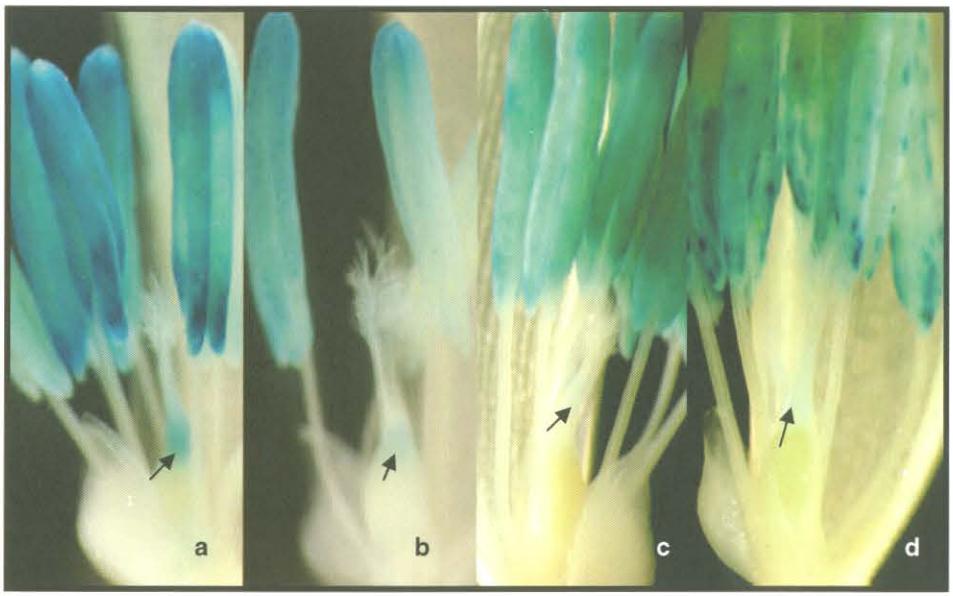

Figure 4.2 Expression pattern of the pSMRJ18-21 line displayed in T_1 and T_2 generations. **a**: T_1 flower with strong GUSPlus expression in anther wall and the base part of style (arrow); **b-d**:T_2 flowers with less intensity of expression in the base part of style.

4.3.2 MOLECULAR ANALYSIS

Molecular analysis to identify T-DNA segregation was carried out only on some lines. Southern blot analysis was employed to define whether T-DNA segregation had occurred. T-DNA segregation that linked to reporter gene expression was observed in floral tissues of pSMRJ18R (1/2)-9a line. After plant DNA blots were hybridised with a DIG-labelled GAL4/VP16 fragment probe, analysis showed that there were three DNA fragments containing T-DNA insertions. One DNA fragment of 5kb was fixed, whereas DNA fragments of 3kb and 1kb were co-segregated among plants (Fig.4.3). Nine plants (1, 2, 4, 5, 7, 9, 13, 14, and 15) contained these two fragments, while another 6 plants (3, 6, 8, 10, 11 and 12) did not (Fig.4.3). This segregation was likely linked to the segregation of reporter gene expression patterns in the floral tissues of pSMRJ18R (1/2)-9a plants (Table 4.1)

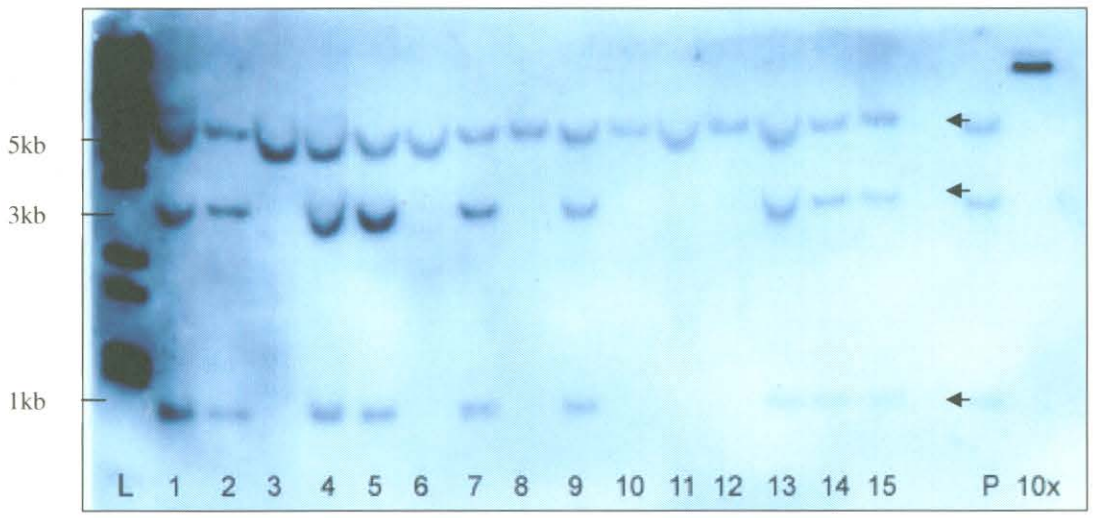

Figure 4.3. T-DNA segregation within T_2 plants (1-15) of the pSMRJ18R (1/2)-9a TAFET line. **arrow**: 5kb (band 1)fragment; 3kb (band 2) and 1kb (band 3) fragments that co-segregated within T_2 (**1-15**) (refer to Table 4.1) and T_0 plants of pSMRJ18R(1/2)-9a (**P**), **10x**: plasmid pSMRJ18R; **L**: λ-DNA digested with *BstE* II.

Plant No.	GUSPlus Expression Tissues	Plant No	GUSPlus Expression Tissues
1	an, po, stig, sty	9	an, po, stig, sty
2	an, po, stig, sty	10	an, po,
3	an, po	11	an, po,
4	an, po, stig, sty	12	an, po,
5	an, po, stig, sty	13	an, po, stig
6	po	14	an, po, stig, sty
7	an, po, stig, sty	15	an, po, stig, sty
8	an, po		

Table 4.1 Pattern segregation among T_2 plants (1-15) of pSMRJ18R (1/2)-9a family number 6 (NI01-12/6). an: anther wall; po: pollen; stg: stigma; sty: style.

Another Southern blot analysis was carried out using plants of the pSMRJ17R-301 line, family 7 and 8. DNA(s) blots were again hybridised with a DIG-labelled GAL4/VP16 probe. A membrane contained plants DNA blots of family 8 showed 4 bands (7.2kb, 5.6kb, 3.6kb and 2.5kb) which contained T-DNA insertions (Fig. 4.4). Three of the bands (7.2kb, 3.6kb and 2.5kb) were fixed, while the 5.6kb fragment was segregated among plants within the family. However, plants of the pSMRJ17R-301 line did not display segregation in their pattern of expression (Table 4.2). These results could suggest that a T-DNA represented by the 5.6kb fragment did not trap any enhancer, as the existence of this T-DNA did not contribute to an expression pattern, and that the three fixed T-DNA insertions had trapped enhancers, inducing the pattern observed (Table4.2).

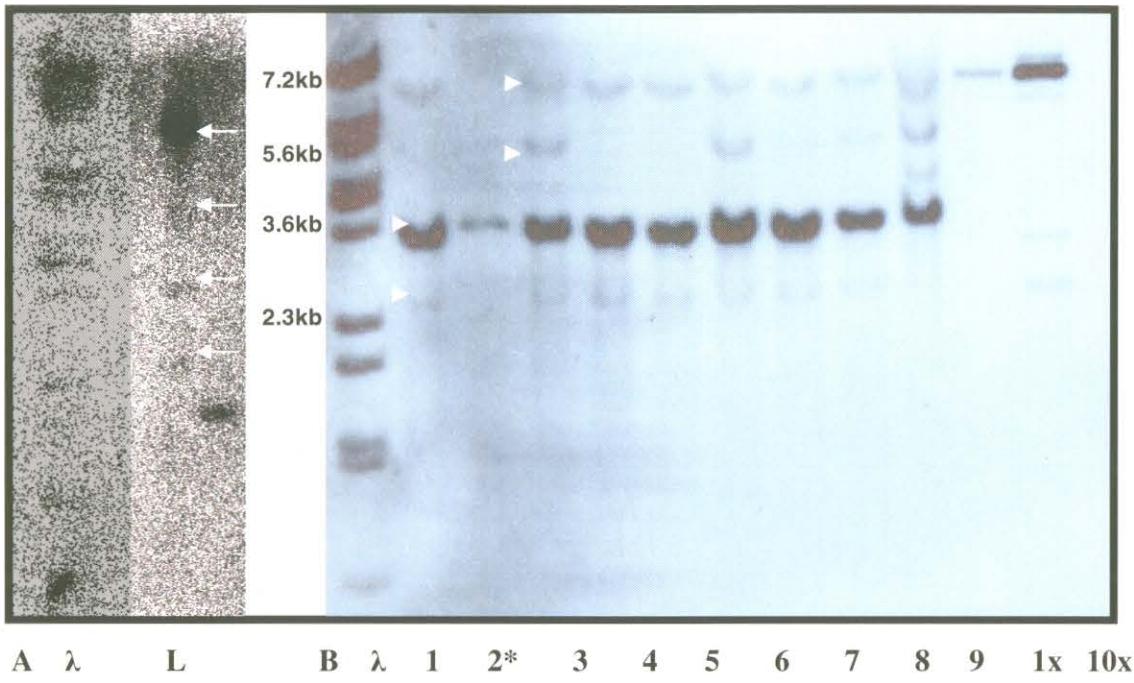

Figure 4.4. T-DNA segregation in a TAFET line, pSMRJ17R-301. A: Southern blot of pSMRJ17R-301 (**L**) in T_0, **B**: Southern blot of T_2 plants of pSMRJ17R-301 (**1-9**). **Arrow**: 7kb (band 1), 5.6kb (band 2), 3.6kb (band 3), and 2.5kb (band 4). **λ**: λ-DNA digested with *BstE* II, **1x**, **10x**: pSMRJ17R. *: less amount of DNA.

Plant No.	GUSPlus Expression		Plant No.	GUSPlus Expression	
	sites	intensity		sites	intensity
1	aw, bsty	s	5	aw, po, bsty	s
2	aw, po	m	6	po, bsty	s
3	aw, po, bsty	s	7	aw, bsty	s
4	aw, po, bsty	s	8	aw, po, bsty	s
			9	na	

Table 4.2 Pattern displayed among plants in pSMRJ17R-301 family. aw: Anther wall; po: pollen; bsty: base of style; s: strong; and m: medium; na: plant was not analysed.

In contrast, family 7 displayed no reporter gene expression in the floral tissues and they did not show T-DNA in their Southern blot when it was hybridised with a DIG-labelled GAL4/VP16 probe (data is not presented).

Molecular analysis also showed that plants of deletion lines in the T_2 generation had T-DNA insertions, and those plants displayed no GUS reporter gene expression at all, either in vegetative and generative parts (Fig. 4.5).

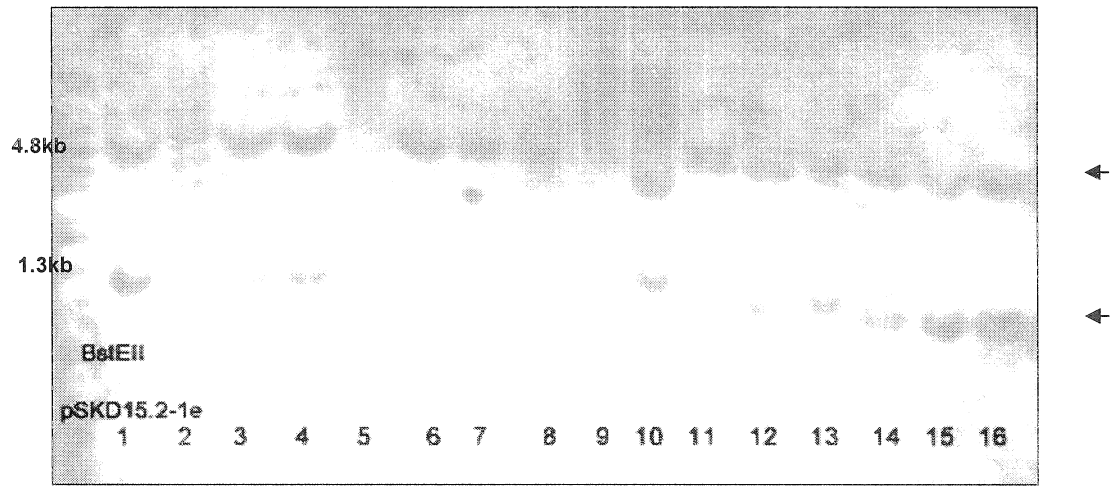

Figure 4.5 Southern hybridisation of T_2 plants of pSKD15.2-1e deletion line. DNA of 16 plants of pSKD15.2-1e family number 6 digested with *EcoR* I restriction enzyme and hybridised with DIG-labelled GAL4/VP16 fragment probe. **Arrow**: two T-DNA insertions, about 1.3kb and 4.8kb were displayed by plants. Plant DNA of sample number 5 and 9 were not properly digested. **1-16**: plants were tested; **BstEII**: λ ladder were digested with *BstE* II restriction enzyme; **arrow**: T-DNA(s) insertion (about 1.3kb and 4.8kb in size).

4.3.2 MOLECULAR ANALYSIS

Molecular analysis to identify T-DNA segregation was carried out only on some lines. Southern blot analysis was employed to define whether T-DNA segregation had occurred. T-DNA segregation that linked to reporter gene expression was observed in floral tissues of pSMRJ18R (1/2)-9a line. After plant DNA blots were hybridised with a DIG-labelled GAL4/VP16 fragment probe, analysis showed that there were three DNA fragments containing T-DNA insertions. One DNA fragment of 5kb was fixed, whereas DNA fragments of 3kb and 1kb were co-segregated among plants (Fig.4.3). Nine plants (1, 2, 4, 5, 7, 9, 13, 14, and 15) contained these two fragments, while another 6 plants (3, 6, 8, 10, 11 and 12) did not (Fig.4.3). This segregation was likely linked to the segregation of reporter gene expression patterns in the floral tissues of pSMRJ18R (1/2)-9a plants (Table 4.1)

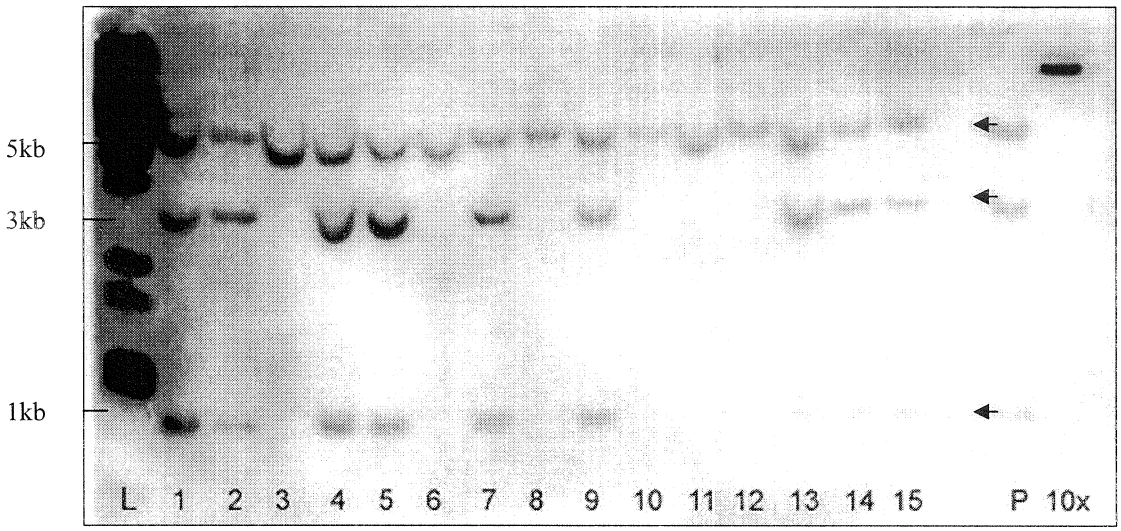

Figure 4.3. T-DNA segregation within T_2 plants (1-15) of the pSMRJ18R (1/2)-9a TAFET line. **arrow**: 5kb (band 1)fragment; 3kb (band 2) and 1kb (band 3) fragments that co-segregated within T_2 (**1-15**) (refer to Table 4.1) and T_0 plants of pSMRJ18R(1/2)-9a (**P**), **10x**: plasmid pSMRJ18R; **L**: λ-DNA digested with *BstE* II.

Chapter 5

VALIDATING THE GAL4/VP16 TAFET SYSTEM THROUGH SEXUAL CROSSING

5.1 INTRODUCTION

It has been described that the yeast transcriptional activator GAL4 functions via recognition of a DNA binding domain to the GAL upstream activating sequence (UAS_{GAL4}) (Fischer et al., 1988). The GAL4 activates genes adjacent to the UAS_{GAL4} (Fields, 1989) and was also able to direct cell- or tissue-specific gene expression patterns in *Drosophila* (Brand and Perrimon, 1993).

Activity of the GAL4 transcriptional activator can be visualized in a bipartite system by the expression of the reporter gene fused downstream of the UAS_G (Brand and Perrimon, 1993). This system offers an advantage over other enhancer trap systems developed for plants system (Sundaresan et al., 1995a) as it allows subsequent use of GAL4 lines as "effectors" or pattern lines to direct 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 sequence as a "receptor" or a target in *Drosophila* (Castelli-Gair et al., 1994; Brand and Dormand, 1995). Random insertion of the GAL4 construct into the *Drosophila* genome affects GAL4-driven expression from a diverse array of genomic enhancers. Subsequent to this, an introduced gene containing GAL4 binding sites (UAS_{GAL4}) within its promoter would be expressed at sites where the GAL4 was expressed. This mechanism might be able to target the expression of a gene to particular cells where it is not normally active, resulting in *Gain-of-Function* (GoF) phenotypes as demonstrated in *Drosophila*

melanogaster (Brand and Perrimon, 1993). For example, the crossing between lines with the GAL4 directed transcription *even-skipped* gene and UAS lines resulted in mis-expression of the *even-skipped* gene in the naked cuticle cells and produced denticle secreting cells in *Drosophila* (Brand and Perrimon, 1993). The UAS line is considered as a target line, commonly silent, unless it has been crossed with pattern lines (GAL4 lines).

From my experiments described in Chapter 3, a wide variety of tissue- or cell-specific patterns of expression in rice plants were obtained when transactivator (the transcriptional activator GAL4/VP16-facilitated enhancer trap) GUS and GUSPlus constructs were applied to the rice genome. Moreover, the GAL4/VP16-deleted constructs, containing a 108bp deletion of the GAL4 binding domain, produced calli and lines without reporter gene expression. The interpretation of these results is that expression was due to transactivator activities.

Whether the transactivator GAL4/VP16 constructs could function in rice in the same way as the GAL4-UAS_{GAL4} in *Drosophila*, needed to be validated. In order to validate this TAFET system, sexual crosses were conducted between TAFET lines which had GUSPlus reporter gene expressions in the flower tissues (patterns) and UAS_{GAL4}-EGFP lines which had no EGFP (Enhancer Green Fluorescent Protein) gene expression (targets). UAS_{GAL4}-EGFP lines used in this experiment containing T-DNA insertions which had a 108bp deletion in the GAL4 DNA-binding domain and $6xUAS_{GAL4}$ fused to the EGFP reporter gene. Those were observed expressing no EGFP in their tissues. A scenario in which the transactivator GAL4/VP16 activates a gene linked to UAS_{GAL4} is presented in Figure 5-1. If the transactivator GAL4/VP16 functioned as expected, F_1 plants would be expected to have both GUSPlus and EGFP reporter gene expression in similar patterns and in a tissue-specific manner, directed by the rice endogenous enhancer driving GAL4 GAL4/VP16 transcription.

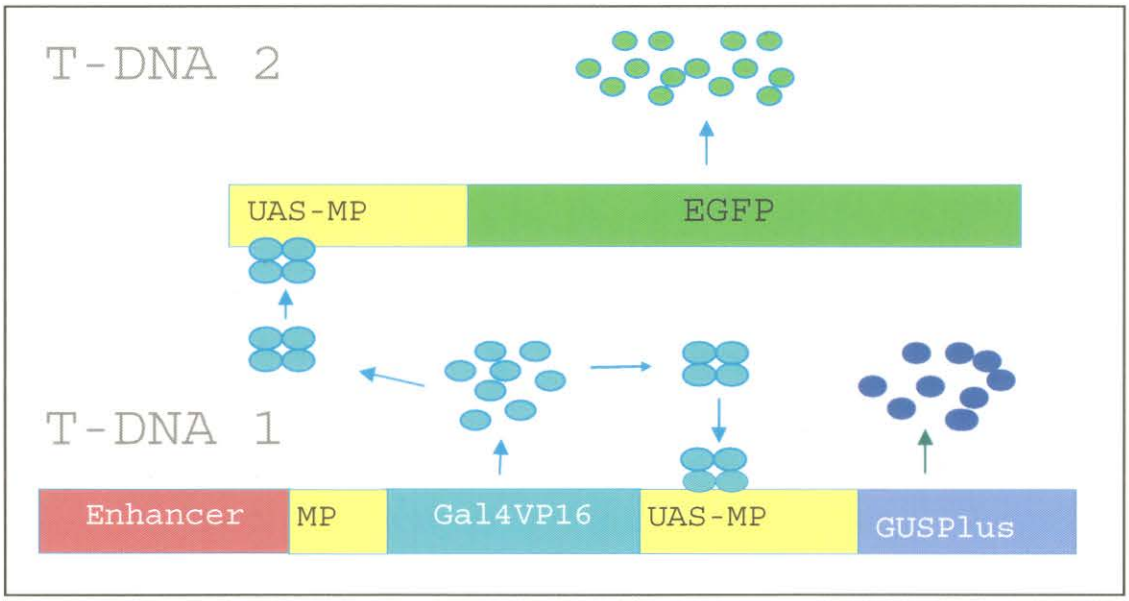

Figure 5.1 The Activation test by sexual crossing between transactivator GUSplus lines (patterns) and UAS-EGFP lines (targets). T-DNA1: T-DNA in TAFET lines; **T-DNA2**: T-DNA in target lines; **Enhancer**: rice endogenous enhancer; **MP**: minimal promoter of the CaMV35 promoter; **light blue dots**: transactivator proteins; **dark blue dots**: GUSPlus; **Green-Fluorescent dots**: EGFP

Such experiments have mostly been conducted in *Drosophila* (Brand and Dormand, 1995; Rorth, 1996; Phelps and Brand, 1998) with plant work still limited to *Arabidopsis* (Haseloff, 2002). Validation of the system in rice has not been previously attempted.

This chapter is a report of crossing experiments that were conducted in rice between pattern lines and target lines, with analysis of the patterns produced in F_1 and F_2 plants, and the molecular analysis of T-DNA(s) linked to reporter gene expression.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

T_1 plants of several TAFET lines (patterns) were used in sexual crosses with T_0 plants of TAFET EGFP lines (targets) with a deleted version of the transactivator. Deleted transactivator EGFP lines contained a 108bp deletion in the GAL4 DNA-binding domain and $6xUAS_{GAL4}$ fused to EGFP reporter gene, and these lines did not express EGFP. The EGFP gene used was the mGFR5ER-modified gene, developed by Xiqin (2003).

5.2.2 METHODS

5.2.2.1 SEXUAL CROSSING

Crossings were conducted between the TAFET lines that expressed GUSPlus in the flower (pattern lines) and the UAS_EGFP lines that contained UAS fragments fused to EGFP reporter gene (target lines). F_1 seeds were grown and flowers were taken for histological analysis of β-glucuronidase reporter gene expression. Sexual crossings were performed just before the panicle emerged from the booting sheath. Emasculation was done one day before crossing by cutting one third off the top part of paleas and lemmas (spikelets) and then placing the panicles into warm water (45^0C) for about 5 minutes. Through this treatment the male organ (pollen) in all spikelets on the panicle was killed, whereas the female organ remained alive. After emasculation the panicles were covered immediately with paper bags, ready to cross the next day (Qixin Fu, personal communication). Pollination was carried out when several spikelets on the cut panicle opened, and pollen from the male parent flower was sprinkled on to the cut spikelets of the female parent. The fertilised panicles were kept bagged for 10 days after crossing and F_1 seeds were harvested 40 to 45 days after pollination.

5.2.2.2 HISTOLOGICAL AND GENETIC ANALYSIS

Flowers of F_1 were taken for analysis. Flowers were first examined under a microscope to observe patterns of EGFP expression. Subsequently, those flowers were put into a GUS staining solution (Jefferson et al., 1987). After overnight incubation, flowers were taken out of the GUS solution and put into 70% ethanol for 2 or 3 days. 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. EGFP expression from fresh tissues was analysed with a Leica MZFLIII using a Leica GFP3 filter, which was set with 480/40-nm excitation and images were acquired with a Nikon N-2000 photo camera.

5.2.2.3 MOLECULAR ANALYSIS

Molecular analysis of the F_1 plants to determine the presence of T-DNAs from each parent was performed. DNA was extracted from fresh leaf tissues ground in liquid nitrogen using the CTAB method as previously described by (Del Sal et al., 1989). Electrophoresis and Southern blot hybridisation of DNA were performed as previously described (Sambrook et al., 1989). DNA was digested with *EcoR* I restriction enzyme and radioactively-labelled GUSPlus and EGFP probes were used for hybridisation. The Southern blot membrane was first hybridised with the EGFP fragment as a probe (obtained from a PCR reaction, using primers for EGFP) (Table 5.1), and the same membrane was also hybridised with a GUSPlus fragment as a probe, after stripping. To define whether reporter gene expression patterns were correlated with the presence of T-DNAs in the genome, fragments of the reporter genes were amplified using PCR (Sambrook et al., 1989). In addition to these two genes, a *hpt*II gene was also included in PCR and three sets of primers were used for amplification of relevant gene fragments (Table 5.1).

Oligos	Sequence (5' – 3')
HYG R	GATGCCTCCGCTCGAAGTAGCG
HYG F	GCATCTCCCGCCGTGCACAG
EGFP R	TTCTGCTGGTAGTGGTCGGC
EGFP F	ATGGTGAGCAAGGGCGAGGA
GUSPlus R	GTTGGCGATGCTCCACATCA
GUSPlus F	AGCGAGCAATGTGATGGATTTC

Table 5.1 Oligos used in PCR to amplify fragments of Hygromycin (HYG), EGFP and GUSPlus genes in F_1 plants.

5.3 RESULTS

5.3.1 SEXUAL CROSSING

In this crossing experiment several different TAFET lines with GUSPlus reporter gene expression in floral tissues (pattern lines) (Table 5.2) were chosen and crossed with different UAS::*EGFP* lines (target lines) (Table 5.3).

TAFET lines (pattern lines)			GUSPlus Expressions	
	T_1 tested no. / plant no.	Lines no.	Patterns	Levels
1	NI00-117/4	pSMRJ17R-10a	tip or whole palea, lemma, trichome, anther wall, stigma, style	strong
2	NI00-280/3	pSMRJ17R-37d	pollen and stigma	weak
3	NI00-90/5	pSMRJ18(50)-505-1	anther wall cells	medium
4	NI00-110/3	pSMRJ18R-133b	pollen	medium
5	NI00-122/1	pSMRJ18-21	anther wall and pollen	weak
6	NI00-121/1	pSMRJ18-20	anther, pollen and base of style	strong
7	NI00-161/8	pSMRJ18R-7g	anther wall cells	strong
8	NI00-173/5	pSMRJ18R(50)-132a	midrib or whole, anther, pollen	medium
9	NI00-110/3-13	pSMRJ18R-133b	pollen	medium
10	NI00-169/3	pSMRJ18R(1/2)-6d	trichome, midrib, anther wall	strong
11	NI00-141/1	pSMRJ18R(50)-23b	midrib or whole, anther and pollen	medium
12	NI00-159/3	pSMRJ18R-47a	palea, lemma, lodicule, anther	Medium

Table 5.2 TAFET lines (T_1 plants) used in crossing experiments and their expression patterns in the floral tissues.

Crossing number	Female parents	Male parents
FU01-1	pFXH13.3-5	NI00-117/4
FU01-2	pFXH13.3-8	NI00-280
FU01-3	pFXH13.3-3	NI00-90/5
FU01-4	pFXH13.3-206	NI00-110/3
FU01-5	pFXH13.3-244	NI00-122/1
FU01-6	pFXH13.3-265	NI00-121/1
FU01-8	PFXH13.3-123	NI00-161/2
FU01-9	pFXH13.3-176	NI00-173/5
FU01-11	pFXH13.3-12	NI00-110/3-13
FU01-12	pFXH13.3-130	NI00-169
FU01-13	pFXH13.3-140	NI00-141/1
FU01-14	pFXG74.1-246	NI00-159/3

Table 5.3 Crossing numbers, parental plants (male and female) were used in experimental crosses.

5.3.2 PATTERN OF GENE EXPRESSION IN F_1 PLANTS

Fresh material was observed for EGFP gene expression, and histological analysis of F_1 flowers was carried out for GUS or GUSPlus gene expression. Most F_1 plants showed expression of both reporter genes, GUSPlus and EGFP, with similar expression patterns (Table 5.4). Patterns of EGFP expression were similar to those previously seen in the GUSPlus reporter gene TAFET lines (male parents) (Table 5.2).

Crossing no.	Reporter genes		Patterns	plants with expression out of total plants	
	GUSPlus	EGFP			%
FU01-3	Y, s	Y, s	trichome, Anther, base of pedicle	4/18	22.2
FU01-4	Y, s	Y, s	anther wall	5/8	62.5
FU01-5	Y, s	Y, s	midrib of palea & lemma, anther, style	6/13	46.2
FU01-6	Y, s	Y, s	anther wall and style	7/17	41.2
FU01-8	Y, s	Y, m	anther wall and pollen	3/9	33.3
FU01-11	Y, s	Y, s	anther	7/21	33.3
FU01-12	Y, s	Y, s	Tip or midrib palea & lemma, anther	8/20	40.0
FU01-13	Y, m	Y, m	Tip, midrib, anther	3/5	60.0
FU01-14	Y, s	Y, s	Palea, lemma, lodicule and anther	13/26	50.0

Table 5.4 Patterns displayed by F_1 plants visualised as EGFP and GUSPlus reporter genes expressions in floral tissues. Y: yes; s: strong; m: medium.

Consistent patterns of EGFP and GUSPlus staining were displayed by most F1 plants, especially those with strong levels of expression. Consistency in expression was shown, for example by F_1 plants from crossing number 14 (FU01-14), which had strong expression in both anther and lodicule; by F_1 plants of FU01-12 which had expressions in the tips of palea, lemma, anther, base of spikelet and pedicle and trichome; and by F_1 plants of FU01-13 which had expressions in the tips of palea and lemma, base of spikelets, and anthers (Fig. 5.2). These results were similar to those described in Chapter 4, where strong expression patterns of TAFET lines were consistently displayed in subsequent generations of selfing.

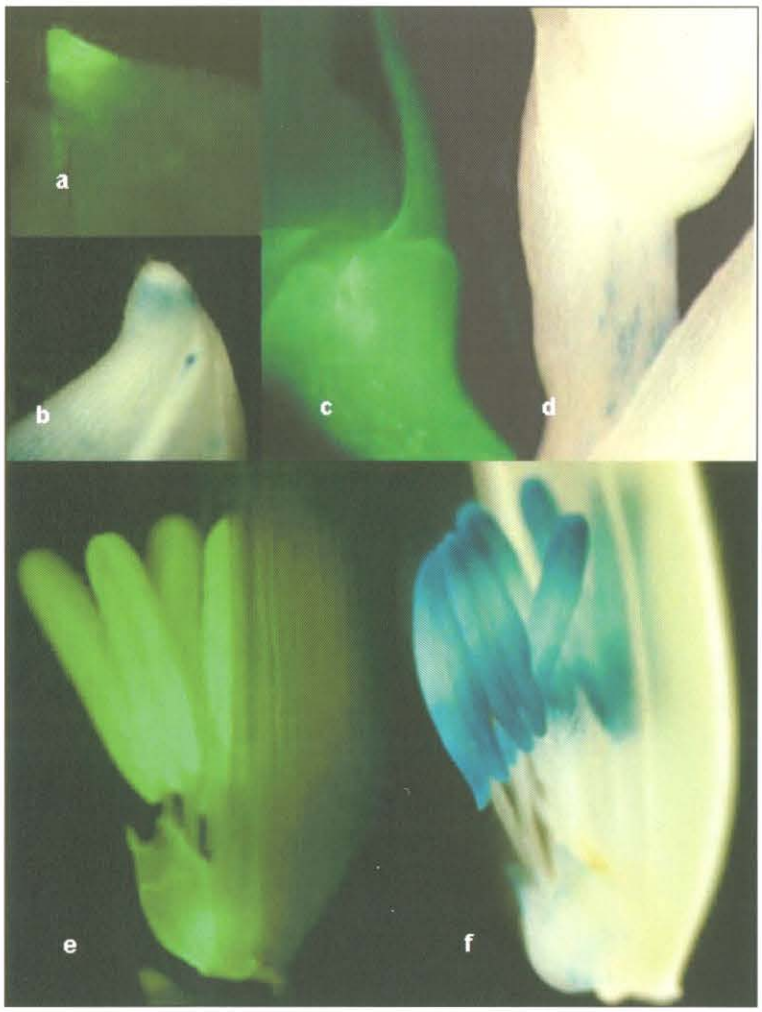

Figure 5.2 Activation of EGFP gene by the GAL4/VP16 transcriptional activator through sexual crossing. **a,c & e**: EGFP expression, **b, d & f**: GUSPlus expression, **a+b**: line FU01-12/ 23, **c+d**: lines FU01-13/2 and **e+f**: line FU01-14/3.

5.3.3 GENETIC ANALYSIS

Observations of the expression patterns of each plant, within each family, were carried out to find out whether they segregated following a Mendelian fashion. Bacause of the limited number of plants within families, and the unequal numbers of plants produced in individual F_1 crosses, statistical analysis was not performed.

In three F_1 families, the percentages of plants with both genes (GUSplus and EGFP) expressed were between 22 and 33%, consistent with a 3:1 segregation ratio which would be expected if hemizygous T_1 TAFET plants were crossed with hemizygous T_0 EGFP plants. This was shown, for example in crossing numbers FU01-3 and FU01-4. These families showed approximately one quarter of plants displaying both EGFP and GUSPlus reporter gene expression, and similar expression patterns (Table 5-3).

The remaining six families showed expression of both genes in 40 to >60% of plants. This type of segregation is consistent with an assumption that homozygous T_1 TAFET plants were crossed with hemizygous T_0 EGFP. Such segregation was observed for example in crossings number FU01-5 and FU01-14 with 50% of plants within the families displaying both reporter genes (Table 5-3).

Expressions of both reporter genes were confirmed using Southern blot analysis. Because of limited time, blotting membranes containing plant DNA(s) of analysed F_1 plants were hybridised only with DIG-labelled EGFP fragment probes. Most of the female parents used in the crossing experiments had been identified in the previous generation (T_0). Southern blot analysis showed that some F_1 plants contained UAS::*EGFP* T-DNA insertions and some did not (Figure 5-3).

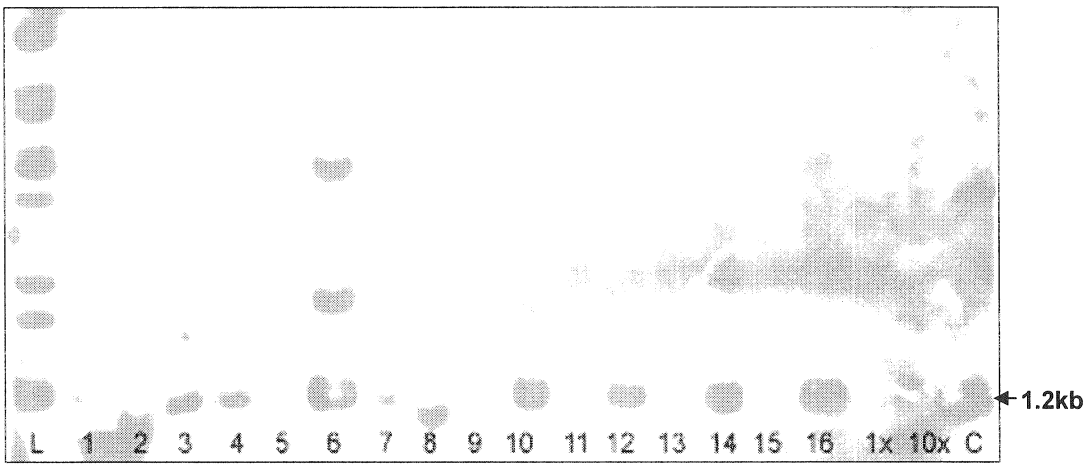

Figure5-3. DNA Southern Blot of F1 plants (TAFET line x UAS::EGFP line) hybridized with DIG labeled of EGFP fragment probe. **L**: λ-DNA digested with BstEII, **1**: 6-12, **2**: 6-13, **3**: 6-20, **4**: 6-24, **5**: 9-1, **6**: 9-21, **7**: 11-23, **8**: 11-28, **9**: 11:31, **10**: 11-34, **11**: 11-37, **12**: 11-39, **13**: 12-17, **14**: 12-23, **15**: 12-28, **16**: 14-10, **1x and 10x:** pSMRJ18R (GAL4/VP16_UAS-GUSPlus), and **C**: pFXH13.3 (UAS-EGFP T-DNA).

	Plant no.															
Expression	1	2	3	4	5	6	7*	8	9*	10	11	12	13	14	15*	16
GUSPlus	y	y	y	y	y	y	n	y	y	y	0	y	y	y	y	y
EGFP	y	y	y	y	n	y	y	y	y	y	na	y	n	y	y	y

Table 5.4 Expression of reporter genes in the floral tissues of F_1 plants. y: plant showed expression, **n**: plant showed no expression, **na**: plant was not analysed, **plant number with** *: plant had a contradictive result.

When reporter gene expression data (Table 5.4) were compared with molecular data, results showed that most of the F_1 plants that displayed both EGFP expression and GUSPlus expression also contained the UAS::*EGFP* T-DNA insertion (1, 2, 3, 4, 6, 8, 10, 12, 14 and 16) (Table 5.4 and Fig. 5.3). Moreover, some F_1 had no UAS::*EGFP* T-DNA (5, 9, 11 and 13) and showed no EGFP expression as well (Table 5.4).

Three F_1 plants numbers 7, 9 and 15 showed inconsistent results. Plant number 7 which had the UAS::*EGFP* T-DNA insertion showed no EGFP expression and only very weak expression of GUSPlus in the anther. It may be presumed that this plant did not contain the GAL4/VP16 T-DNA, since the expression of GUSPlus was very weak and the expression was only in the anther. The results described in Chapter 4 indicated a very weak background of glucuronidase-like expression observed in the anther. Plant number 9 showed EGFP expression in the anther, but contained no T-DNA insertion. This inconsistent observation could be due to autofluorescence that is commonly observed in tissues containing chlorophyll, such as anthers (Haseloff et al., 1997). Plant number 15 showed expression of both reporter genes in the palea, lemma, lodicule, filament and anther, but Southern blot analysis indicated that it contained no T-DNA insertion. Progenies of these three F_1 plants need further investigation before conclusions can be made.

5.4 DISCUSSION

The ability of the GAL4/UAS system to induce targeted gene expression occurs through the ability of the GAL4 transcriptional activator to activate transcription of any gene fused to the UAS_{GAL4}. It occurs through the recognition of the UAS_{GAL4} region by the GAL4 DNA binding domain, and has been demonstrated in *Drosophila*, mammalian cells and plants (Fischer et al., 1988; Kakidani and Ptashne, 1988).

The GAL4- UAS_{GAL4} system has also been found to be efficient for trapping enhancers in *Drosophila* and *Arabidopsis*, producing temporal and spatial expression patterns of reporter genes (Brand and Dormand, 1995; Haseloff, 2002). It was also able to direct expression of a target gene in certain tissues (spatial) under investigation, or of a random endogenous gene (Brand and Perrimon, 1993; Brand and Dormand, 1995; Rorth, 1996). Rorth (1996) reported that about 4% of target inserts gave dominant phenotypes (*Gain-of-Function* phenotypes) when the GAL4- UAS_{GAL4} system was applied to activate the developing eye in *Drosophila*.

Our crossing experiments between TAFET GUSPlus lines and UAS::*EGFP* lines showed that the transactivator GAL4/VP16 constructs were able to activate the *EGFP* gene fused to the UAS_{GAL4}, producing F_1 plants showing expression of both EGFP and GUSPlus reporter genes in similar patterns (Figure 5.2). F_1 plants showed EGFP over-expression in similar tissues (spatial) where the GAL4/VP16 was active (visualised using GUSPlus reporter gene).

Southern blot hybridisation analysis confirmed that F_1 plants showing both EGFP and GUSPlus reporter gene expression contained both GAL4/VP16_UAS-GUSPlus (previously identified in T_0) and UAS-EGFP T-DNA(s) (Fig. 5.4), whereas plants displaying only GUSPlus or EGFP expression may have correlated with the presence of the relevant single T-DNA insert. Detection of EGFP may have involved a scoring error because of some autofluorescence produced by anther tissues. Green autofluorescence in plant tissues containing chlorophyll has been previously reported and may have been difficult to distinguish it from the GFP (Haseloff et al., 1997; Jach et al., 2001).

Contradictory results between expression and the presence of T-DNA were shown for plants number 7, 9 and 15. Plant number 7, which had no EGFP expression

and only very weak GUSPlus expression in the anther, did contain the UAS::*EGFP* T-DNA. The plant presumably did not have any GAL4/VP16 T-DNA, as the GUSPlus expression in the anther was very weak. A weak background glucuronidase-like expression had been observed in the anther of plants from segregated families that were not expected to have expression. In the case of plant number 9, which was observed to have EGFP expression in the anther, but contained no T-DNA insertion, this could be due to autofluorescence, as described above (Haseloff et al., 1997). In relation to plant number 15 which was observed to have both reporter gene expressions in the palea, lemma, lodicule, filament and anther, but it did not have any T-DNA insertion, conclusions cannot be drawn without further investigation of the progeny of these F_1 plants.

From these experiments, we can conclude that the GAL4/VP16 TAFET system was able to activate reporter gene expression through a mechanism in which the GAL4 DNA binding domain recognised the UAS, and the VP16 activating domain transcribed a gene linked to the UAS. The implications of the GAL4/VP16 system to activate any gene linked to the UAS is that the system may be used to direct the expression of any gene under study (a target) in specific-tissue types, generating ectopic expression, and possibly producing *Gain-of-Function* (GoF) phenotypes. Haseloff (2002) has described attempts to select enhancer-trap GAL4/VP16 lines to activate cloned genes (*HASTY, EARLY TRICHOME, SQUINT*) involved in regulating the developmental phase of shoot in *Arabidopsis*.

Various attempts to use the GAL4-UAS to direct a gene fused to the UAS_{GAL4} have been conducted in *Drosophila* (Castelli-Gair et al., 1994; Brand and Dormand, 1995; Gustafson and Boulianne, 1996; Ito et al., 1997; Phelps and Brand, 1998). Results obtained from these experiments showed the ability of GAL4 to activate cloned genes (mostly reporter genes) fused to the UAS_{GAL4}. The purpose of these studies was

mainly to express cloned genes in the site where the GAL4 transcriptional activator was active, producing GoF phenotypes. By this approach the functionalities of cloned genes were identified (Castelli-Gair et al., 1994; Brand and Dormand, 1995; Gustafson and Boulianne, 1996; Ito et al., 1997; Phelps and Brand, 1998).

5.5 CONCLUSION

From these experiments, two conclusions can be drawn as follows.

- First, the GAL4/VP16 TAFET system was able to activate reporter gene expression through a mechanism in which the GAL4 DNA binding domain recognised and bound to the UAS, and the VP16 activating domain transcribed a gene linked to the UAS.

- Second, a regulatory protein was bound to an enhancer and that subsequently the enhancer bound to the minimal promoter upstream of the GAL4/VP16, and its activity was visualised by reporter gene expression in the particular tissues where the regulatory protein was active.

Chapter 6

TESTING THE CAPABILITY OF THE GAL4/VP16 TAFET SYSTEM TO INDUCE PHENOTYPIC CHANGES

6.1 INTRODUCTION

The identity of a cell is determined by its characteristic profile of gene expression. The ability of an introduced transactivator to direct gene expression in cells in which a particular gene is not normally (commonly) active, known as ectopic gene expression, is thus a powerful way to investigate the role of a particular gene in defining cellular identity.

A common approach for analysing of the function of genes is through generating *Loss-of-Function* (LoF) phenotypes. This approach relies on a single mutational event and only detects genes which, when mutated, resulted in obvious mutant phenotypes.

Ectopic expression phenotypes, however, can be equally informative as they can be useful for identifying a gene with multiple functions (pleiotropic). For example, the LoF phenotype of *eyeless* in *Drosophila* is the partial or complete absence of the compound eyes, whereas ectopic expression of *eyeless* is sufficient to generate extra eye structures that are not only morphologicaly normal but also electrically active on illumination (Phelps and Brand, 1998). Ectopic expression can also provide unique functional information in cases where there is no LoF phenotype because of genetic redundancy (Perrimon, 1998); (Phelps and Brand, 1998). The existence of the phenomenon of gene redundancy has been clearly shown. For example, a severe segmentation phenotype was obtained only when both the sloopy paired (*slp*) loci (*slp1*

and slp2) encoding proteins containing a forkhead domain were deleted in *Drosophila* (Cadigan et al., 1994). It was reported that about two thirds of *Drosophila*'s genes (equal to 8000 genes) are predicted to show no obvious LoF phenotypes (Miklos and Rubin, 1996).

The activation tagging system was developed for ectopic expression of genes as an approach to deal with genetic redundancy. Multiple copies of 35S enhancers, instead of the promoter itself, have been used in activation tagging constructs (Hayashi et al., 1992; Weigel et al., 2000). Application of this system results in mis or over-expressing of endogenous genes in *Arabidopsis* (Huang et al., 2001). Among transgenic plants generated using this system a number of mutant phenotypes behaved genetically in a dominant fashion suggesting that at least some of them resulted from *Gain-of-Function* (GoF) mutations (Nakazawa et al., 2003). The activation tagging system has mostly been applied in *Arabidopsis* (Weigel et al., 2000; Huang et al., 2001; Nakazawa et al., 2003).

This chapter represents an important part of developing another approach aimed at generating GoF mutation in rice. This approach, called TransGenomics, uses a similar strategy to that employed by Rorth (1996) in *Drosophila melanogaster*. Rorth mated a population of flies with defined patterns of Gal4 expression in embryo (as determined via analysis of a reporter gene under the control of UAS_{Gal4} promoter) with a population of flies containing UAS_{Gal4} promoter on the P-element, which is highly active in the *Drosophila* germline (Rorth, 1998). The application of the GAL4-UAS system induced 4% dominant mutations (Rorth et al., 1998).

To explore the potential to produce *Gain-of Function* (GoF) phenotypes, two populations of plants needed to be developed; firstly, pattern lines containing the GAL4/VP16 transactivator, which we refer to as Transcriptional Activator Facilitated

Enhancer Trap (TAFET) lines and secondly, target lines containing either a cloned gene under the control of the UAS promoter (specific targets) or containing random insertions of the UAS_{GAL4} minimal promoter in the rice genome (random targets). The experiments aimed at mating plants from both populations to achieve either targeted expression of specific genes (when using specific targets) or, when using random targets, to deploy a transactivation-based mutagenesis process.

In my experiments, the maize *AGAMOUS*-related gene, the *ZAG1* gene, was chosen to create a specific target. This gene was cloned through low stringency hybridisation from a maize female inflorescences cDNA library using the *AGAMOUS* (*AG*) cDNA from *Arabidopsis* (Schmidt et al., 1993). It encodes a putative polypeptide of 286 amino acids, having 61% homology with the *AGAMOUS* (AG) protein of *Arabidopsis*. *ZAG1* RNA accumulates in the early stamen and carpel primordial stages, but it then diminishes during stamen development (Schmidt et al., 1993). A mutant zag_1–mum_1 obtained from transposon Mutator (*Mu*) application showed some flower changes, such as development of a variable number of extra silks which were partly sterile (Mena et al., 1996). It was concluded that the absence of *ZAG1* resulted in a change in the fate of the cells that are destined to become carpel primordial. The *OsMAD3* gene, which is highly homologous to members of the AGAMOUS family, was cloned and studied in rice (Kang, 1998). *Loss-of-function* (LoF) mutation of this gene alternates the third and forth whorls; filaments were changed into thick and fleshy bodies, similar to lodicules, whereas carpels were changed to several abnormal flowers. These results suggested that the *OsMAD3* gene belongs to the class C gene family of floral organ identity determination (Kang, 1998).

A second specific target chosen was the Diphtheria toxin-*A* gene (DT-*A*). This gene has been previously used to determine a particular cell's fate during development, utilising cell ablation caused by expressing the Diphtheria toxin-*A* gene

(DT-*A*) construct molecule in a cell-specific manner (Day et al., 1995). The DT toxin contains two functional domains: chain A which carries the active site for ADP-ribosylation of the elongation factor 2 (*EF2*), inhibits all protein synthesis, and chain B is needed for the binding of the toxin to cells and for the entry of chain A into the cytosolic compartment (Greenfield et al., 1983). Chain A cannot be transported across the plasma membrane without chain B, and remains in the cells where it is expressed. Using this characteristic, DT-*A* has been used for ablation of specific cells or tissues (Day et al., 1995; Tsugeki and Fedoroff, 1999) and/or specific cells during a certain stage of development (van de Geest et al., 1995). It was reported that DT-*A* driven by tissue specific promoter resulted in the ablation of cells where the promoter was active (Day et al., 1995).

In this chapter, I report on the construction of plasmids containing either the UAS_{GAL4} or the CaMV35S promoters driving the maize *AGAMOUS*-related gene, the *ZAG1* and generating UAS::*ZAG1* and 35S::*ZAG1* populations through co-transformation of those plasmids into rice calli. Results from crosses between TAFET lines and UAS::DT-*A* lines are also reported.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

In this experiment, a plasmid containing *ZAG1* gene was provided by Martin Yanofsky (Schmidt et al., 1993). The TAFET lines used for crossing either with UAS::*ZAG1* or with UAS::DT-*A* lines were T_2 and F_1 (from previous experiment, see Chapter 5 Table 5.3). The DT-*A* lines were provided by CAMBIA.

6.2.2 METHODS

6.2.2.1 Constructing binary vectors containing *ZAG1* gene (*Zea mays AGAMOUS*-like)

The cloning steps to produce plasmids containing either the UAS_{GAL4} or the CaMV 35S promoter driving the maize *AGAMOUS*-related gene (*ZAG1*) were as follows. A plasmid pCAMBIA0390 containing the CaMV 35S promoter (a backbone) was digested *with Pml* I, while pBS-SK-*ZAG1* containing the *ZAG1* gene (in pBlueScript backbone) was digested with *EcoR* I and *Xho* I. Two fragments: pCAMBIA0390 and the *ZAG1*, were purified after electrophoresis using a gel extraction method from Qiagen. Protruding ends of restricted fragments were eliminated by a filling reaction using T4 DNA polymerase for 20 minutes at 12°C and the DNA was purified again. DNA fragments were dephosphorylated in a 25 µL reaction containing 2 units of Shrimp Alkaline Phosphatase (SAP, Boehringer Mannheim) and 1x dephosphorylation buffer (10x buffer contains 0.5 M Tris-HCl and 50 mM Mg_2Cl, pH 8.5) at 37°C for 1 hour. The reaction was terminated by heat inactivation at 65°C for 20 minutes. Both fragments were ligated using T4-DNA ligase overnight at 16°C. 2 µl of ligation product were subsequently transformed into *E. coli* competent cells (DH5-α) using electroporation at 1.8 Volt, 500 µL of SOC solution (refer to p23, **2.1.1**) was added and then incubated with shaking for 30 minutes at 37°C. Cells were cultured in solid agar medium containing kanamycin^{100} and incubated overnight at 37°C. Colonies were picked and cultured in liquid media with Kanamycin^{100} and incubated at 37°C with shaking overnight. DNA was purified using the alkaline lysis method (Sambrook et al., 1989) and the correct plasmids were identified through digestion using *BamH* I restriction enzyme. The plasmid containing the *ZAG1* gene was designated as pSKG46.22. The final step was to clone the UAS minimal promoter upstream of the *ZAG1* gene. A fragment of $6xUAS_{GAL4}$ was excised from pSKB72.1 through a double digestion reaction using *Sma* I and *Nco* I restriction enzymes. Plasmid pSKG46.22 was digested

with *Sma* I. The protruding ends of the *Nco I* site were eliminated using a filling reaction with T4 DNA polymerase for 20 minutes at 12°C, DNA purified again and then ligated. The same procedure was carried out for transformation and bacterial culture as mentioned above. The plasmid containing the UAS $_{GAL4}$*_ZAG1* gene, designated pSKG53.1 (Fig. 6.1), was verified through digestion using *Aat* I and *Sac* I restriction enzymes.

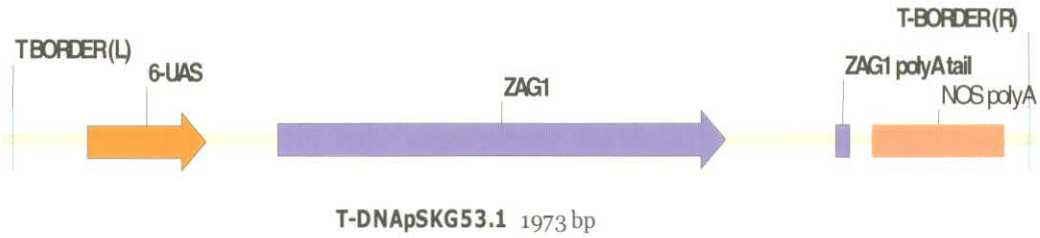

Figure 6.1 The plasmid containing the UAS$_{GAL4}$ driving *ZAG1*.

Another cloning experiment was undertaken to produce a plasmid containing the CaMV 35S promoter driving the *ZAG1* gene. The first step was to amplify the *ZAG1* gene with new restriction sites (*Nco* I and *Bgl* II sites) using the Polymerase Chain Reaction (PCR). The primers designed for the reaction were ZAG1-B and ZAG1-T (Table 6.1). This PCR product was cloned into TOPO vector strain 10F (Progen) using the heat shock method. Transformed cells were grown onto solid agar medium containing ampicilin[100], IPTG [40] and X-GAL [40]. Media plates were incubated overnight at 37°C. A white colony containing the plasmid, with appropriate size confirmed by PCR, was identified and named pSKH8.7. Sequencing to verify that the PCR process did not introduce mutation in to *ZAG1* gene was carried out (Appendix 6.1). The second step was to digest pCAMBIA0390 (backbone) and pTNT C-98.2 (source for the CaMV 35S promoter) with *Hind* III and *Bgl* II and to purify the pCAMBIA0390 fragment and the 35S promoter fragment using a gel extraction method. DNAs from these two fragments were ligated using T4 DNA ligase and then transformed to *E. coli* competent cells

(DH5-α). Cells were cultured on solid agar medium containing kanamycin^{100} and incubated for 24 hours at 37°C. Colonies were picked and cultured in the liquid media with Kanamycin^{100}, overnight at 37°C with shaking. This cloning step produced pSKG92.10. The final step was to clone the *ZAG1* fragment from pSKH8.7 into pSKG92.10 by digesting both plasmids with *Nco* I and *Bgl* II restriction enzymes, gel purifying and ligating the *ZAG1* fragment and pSKG92.10 fragment to produce pSKH26.5 plasmid containing the CaMV35S promoter driving the *ZAG1* gene (Figure 6.2).

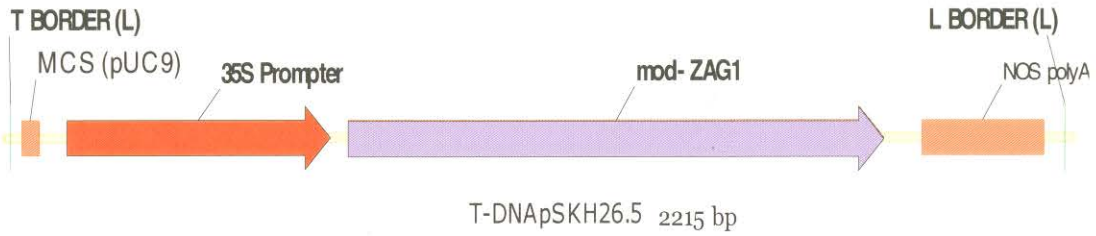

Figure 6.2 The plasmid containing the CaMV35 promoter.driving *ZAG1* gene.

6.2.2.2 Co-transformation and generating pSKG53.1 (UAS::*ZAG1*) and pSKH26.5 (35S::*ZAG1*) lines.

Both pSKG53.1 and pSKH26.5 plasmids were transformed into *Agrobacterium tumefaciens* strain EHA-105 competent cells by electroporation (Biorad). A plasmid containing a *hpt*II gene driven by the CaMV 35S promoter, pFX-B114-1, was used in co-transformation experiments to introduce both plasmids into the rice genome, as described in Chapter 2.

6.2.2.3 Identification of lines with T-DNA insertions

Identification of UAS::*ZAG1* lines and 35S:: *ZAG1* containing T-DNA insertion(s) was carried out using PCR. Two sets of primers were used to amplify both the *ZAG1* gene and the *hpt* II gene (a hygromycin resistance gene) (Table 6.1). Plants containing both elements (UAS::*ZAG1* and 35S driving *hpt* II gene) were used as crossing materials.

Oligonucleotides	Sequence (5' – 3')
HYG R	GATGCCTCCGCTCGAAGTAGCG
HYG F	GCATCTCCCGCCGTGCACAG
cZAG1T	TCGCGCTCATCGTCTTCTCC
cZAG1B	AAACCGTGCTGCTGATACTCACTT
ZAG1-T	GATCCATGGTGCACATCCGAGAAG *Nco* I
ZAG1-B	ACATAGATCTCCCCCTCGAGTTT *Bgl* II

Table 6.1 Oligonucleotides used in PCR to amplify the *hpt* II gene (hygromycin resistance gene) and *ZAG1* gene.

6.2.2.4 Crossing

Two crossing experiments were carried out: 1) crosses between TAFET lines and UAS::*ZAG1* lines and 2) crosses between TAFET lines and UAS::DT-*A* lines. Sexual crossings were performed just before spikelets emerged from the booting sheath. Emasculation and crossing procedures were carried out, as previously described in Chapter 5 Section 5.2.2.1.

6.2.2.5 Southern blot analysis of F_1 plants

Southern blot hybridisation was carried out to define whether reporter gene expression coincided with the presence of T-DNA in selected F_1 plants. Plant DNAs were extracted from fresh leaf tissues ground in liquid nitrogen using a CTAB method as previously described (Del Sal et al., 1989). DNA was digested with *EcoR* I restriction enzyme. Electrophoresis and Southern blot hybridisation of DNA were performed as previously described (Sambrook et al., 1989) using DIG-labelled *ZAG1* probes. A *ZAG1* gene fragment was amplified from pSKG53.1 using cZAG1-B and cZAG1-T primers, and a DT-*A* fragment was amplified from pSMRJ54.3 using M13-F and M13-R TOPO-TA cloning primers (Invitrogen).

6.3 RESULTS

A plasmid containing the *ZAG1* gene driven by the CaMV35S promoter (pSKH26.5) was constructed (Fig. 6.2) and transformed into the rice genome to evaluate the phenotypic changes resulting from over-expression of *ZAG1*. Also constructed was a plasmid containing the *ZAG1* gene (a target gene) driven by the UAS_{GAL4} (pSKG53.1) (Fig. 6.1). A 35S::*ZAG1* population was developed as a control for the *ZAG1* transactivation experiment (Fig. 6.2). UAS::*ZAG1* transgenic lines were generated and crossed to TAFET lines to produce F_1 plants. These plants were evaluated for any phenotypic changes in the rice floral tissues.

6.3.1 Identifying T-DNA insertion of pSKH26.5 and pSKG53.1 lines using PCR

A hundred and twenty two pSKH26.5 lines (containing 35S::*ZAG1*) and 100 pSKG53.1 lines were generated from co-transformation. PCR amplification of *hpt* II and ZAG1 genes identified 51 pSKG53.1 lines (51%) containing pSKG53.1 and pFX-

B114-1 T-DNAs and 59 pSKH26.5 lines (48%) containing pSKH26.5 and pFX-B114-1 T-DNAs insertion.

6.3.3 Observation 35S::ZAG1 and UAS::ZAG1 T_0 populations

Of the 59 pSKH26.5 lines containing the 35S::*ZAG1* T-DNA, only half were assessed for any floral change. Most of the lines had no obvious floral change and only five displayed abnormalities (20.8%) in some spikelets (Table 6.2). For example, line pSKH26.5-5d did not have any lodicule or had lodicules that converted into a filament-like organ, and some of the spikelets did not have stigmas. Line pSKH 26.5-3b had several ovules, and not fully developed stigmas, whereas pSKH26-93 had two ovules with four stigmas.

Interestingly, when seed setting was observed, there were differences in the percentage of lines with a high level of sterility between the population with and without 35S::*ZAG1* T-DNA insertion. In this experiment, plants were classified as "high" sterility when more than 50% of spikelets were empty (no seed), whereas "low" sterility plants had less than 10% of spikelets empty. 22 of 41 pSKH26.5 lines (about 56.7%) with 35S::*ZAG1* T-DNA insertion displayed high sterility, whereas this phenotype was only shown by 15 of 41 pSKH26.5 lines (about 36.6%) without 35S::*ZAG1* T-DNA. Moreover, the opposite result was shown for low level of sterility, in which 10 of 41 pSKH26.5 (24.4%) with T-DNA showed low sterility, whereas 16 of 41 pSKH26.5 lines (39%) without T-DNA showed such a level. In addition, 8 of those 22 pSKH26.5 lines containing 35S::*ZAG1* T-DNA with high sterilities as described above, displayed some floral abnormality phenotypes, whereas lines with low or medium sterilities, displayed normal floral phenotypes (Table 6.2).

No	with T-DNA	Sterility level	without T-DNA	Sterility level
1	93*	high	1f	medium
2	62a	high	4b	medium
3	14b	medium	4d	low
4	37b	high	1a	low
5	71*	high	5L	high
6	182	high	6a	low
7	8f**	high	6c	low
8	69f	low	7b	high
9	64a	medium	9i	high
10	172**	medium	14a	high
11	24a**	low	16a	high
12	16b	high	18a	low
13	9*	high	19c	low
14	3a	medium	19d	medium
15	60b	low	19a	low
16	8c	high	20d	high
17	43d**	low	22a	medium
18	25b	high	22b	low
19	21b*	high	23a	medium
20	4e	medium	23c	high
21	4f	medium	7	medium
22	92**	low	29a	low
23	23b	high	29h	high
24	25d	low	19b	medium
25	12a*	high	30d	medium
26	174*	high	43c	low
27	186	high	44c**	low
28	31e	medium	49b**	medium
29	11a**	medium	50a	medium
30	72	medium	61b	high
31	38c	high	62b**	low
32	48d**	low	66b	high
33	26	low	32a	low
34	3b*	high	69e	low
35	5d*	high	70b	high
36	14b	medium	100c*	high
37	8b	high	100f	high
38	63a	high	36a	low
39	69k	low	33d	low
40	29f	high	91	high
41	42a	high	92	high

Table 6.2 Sterility level of pSKH26.1 lines with and without 35S::*ZAG1* T-DNA insertion. *: lines with floral abnormalities, **: lines with normal floral phenotypes. Categories of sterility refer to the text.

The higher percentage of 35S::*ZAG1* lines displaying poor seed set may indicate that over-expression of *ZAG1* gene had some effect on reproductive organs. Of the 37 pSKG53.1 lines observed for floral phenotypes, only four had apparent changes (10.8%). Those were pSKG53.1-48b and pSKG53-43c that had stamens changed to ovules, therefore producing multiple ovules. Line pSKG53.1-16b had attached filaments producing a lodicule-like organ and had extra palea and lemma, and pSKG53-296 had undeveloped anthers. However, these phenotypes only occurred in some spikelets.

6.3.4 Analysing F_1 plants phenotypes

Crossing was carried out between the GAL4/VP16 pattern lines and the UAS::*ZAG1* and between the GAL4/VP16 pattern lines and the UAS::*DT-A* line (Table 6.3). The UAS::*ZAG1* lines used as materials for crossing were identified containing T-DNA insertions using PCR, while TAFET and TAFETxUAS::EGFP lines were identified for both T-DNA and reporter gene expressions (Table 6.3).

NI02	Female parent	Male parent	ΣF_1	Phenotypic observations
	UAS::*ZAG1*	**TAFETxUAS::*EGFP***		
12	pSKG53-18b	FU01-14/3 [c]	11	3 died
13	pSKG53-18b	FU01-6/20 [c]	2	1 died
14	pSKG53-47b	FU01-5/7 [c]	5	1 died
15	pSKG53-47b	FU01-6/20 [c]	13	2 yellow
16	pSKG53-55	FU01-9/21 [c]	8	2 died
17	pSKG53-50b	FU01-14/10 [c]	4	3 yellow and died
18	pSKG53-18b	FU01-226	0	
19	pSKG53-47b	FU01-136	1	
	TAFET	**UAS::DT-A**		
2	NI01-14/4-Ga	FU01-298b	9	2 died
3	NI01-12/6-3a	FU01-297 b	18	2 died
4	NI01-12/6-1a	FU01- 297 b	8	1 died
5	NI01-12/6a	FU01-297 b	12	1 died
8	NI01-12/6-2a	FU01-298 b	18	5 stunted, yellow; 4 died
10	NI01-24/5-8a	FU01-297 b	6	2 stunted, yellow

Table 6.3 Description of F_1 progeny resulting from crossing experiments between UAS::*ZAG1* and TAFET lines and between UAS:DT-*A* and TAFET lines. [a]. code given for TAFET lines in the experiment year 2001; [b]. code given for UAS::DT-*A* line in the experiment year 2001; [c]. F_1 plants (GUSPlus TAFET line x UAS::EGFP line), except for FU01-136 is a TAFET-EGFP line.

In general, the number of F_1 plants produced from crossing experiments was very low (Table 6.3). Four lines of pSKG53.1 (UAS::*ZAG1*) were used for crosses with TAFET lines (GAL4/VP16) (Table 6.3). Most of the F_1 plants from these crossings did not show any apparent phenotypic change in their flowers. For example, F_1 plants of crossing between a pSKG53.1-18b line and a TAFET line (FU01-14/3) had normal floral phenotypes. Only a small number of spikelets showed anthers that changed to ovule-like shape, producing multiple ovules.

Three TAFET lines were crossed with two UAS::DT-*A* lines (Table 6.3). F_1 plants displayed various degrees of severity of phenotypic changes including stunted plants, yellow leaf colour, or lethality (Fig. 6.3). A limited number of seed settings due to a high percentage of sterile spikelets were also observed in these F_1 plants. For example most of F1 plants of crossing # 8 (NI01-12/6-2 x FU01-298) showed high to completely sterile or died in the seedling stage (Table 6.4).

Figure 6.3 A plant with stunt phenotype (B) compared to a normal plant (A) in F2 NI02-8 (NI01 12/6-2 x UAS::DT-*A*) family.

F1 NI02/8	Plant Phenotypes	Sterility level	∑panicle	∑Seeds	GUS Expression patterns	T-DNA number	
						UAS-DTA	GAL4/VP16
1	ok	High	na	na	Anther	1	3
2	ok	high	4	3	na	0	3
3	ok	high	na	na	Anther	0	1
4	ok	high	na	na	Anther wall	0	3
5	died	-	-	0	na	0	3
6	yellow, stunt and died	-	-	0	Anther wall, pollen	0	1
7	died	-	-	0	Anther wall	1	3
8	ok	high	na	2	na	0	1
9	ok	high	na	3	na	1	3
10	Yellow, stunt	high	1	3	na	0	1
11	ok	low	4	39	Anther wall, pollen	0	1
12	Yellow, stunt	complete	1	0	na	1	1
13	ok	complete	na	0	na	0	3
14	ok	complete	na	0	Anther wall, midrib	1	3
15	ok	high	na	17	Anther wall	1	3
16	Yellow, stunt	high	2	1	na	-*	-*
17*	died	-	-	-	-	-	-
18*	died	-	-	-	-	-	-

Tabel 6.4 The phenotypes and T-DNA insertions of F1 plants of the NI02/8 cross. ok, normal, **na,** not analysed, -, no data due to plant died, -*, not hybridised, **plant no with** *, plants died at very early stage of development.

6.3.5 Molecular analysis of F_1 Plants

Molecular analysis was carried out on the F_1 plants to identify whether plants contained T-DNA from both parents. F_1 plants from cross number 12, a crossing between pSKG53.1-18b and FU01 14/3 (refer to Table 6.3) had two bands, about 6.7kb and 3.0 kb in size. These two fragments were segregated among eight of 11 F_1 plants, when their DNA blots were hybridised with a *ZAG1* fragment as a probe (Table 6.5) (Blot not shown). Only eight of 11 F_1 plants can be analysed, because the other three plants were died (refer to Table 6.3). Both parents had been identified previously and proven to contain T-DNA insertions. Unfortunately, this membrane was not hybridised with GAL4/VP16 probe and consequently this result can not be used to show that phenotypes are linked to the activation of *ZAG1* gene by the GAL4/VP16 transactivator. However, as described before, there were no obvious morphological changes in the floral tissues.

Plant No.	3.6kb fragment	6.7kb fragment
1	no	no
2	yes	yes
3	yes	no
4	yes	yes
5	no	no
6	no	yes
7	yes	yes
8	yes	yes

Table 6.5 T-DNA(s) segregation among F_1 plants of cross number 12 (refer to Table 6.3).

In other experiment, the Southern blot membrane of F_1 plant DNA(s) of NI02/8 [crossing between the TAFET lines and UAS::DT-*A* lines (a T-DNA also contained a UAS::GUS)] was hybridised with a 1.5kb UAS-DT-*A* fragment as a probe. For details of crossings see Table 6-3. It showed no hybridisation at all. However, when it was hybridised with a fragment of GUS gene as a probe, a weak band of 8.5 kb in size was

observed on some plants DNAs blots (Fig. 6.4, B and Table 6.4)). In addition, when the blot was hybridised with the DIG-labelled GAL4/VP16 probe, it had either 1 or 3 copies of T-DNA insertion in 5.6kb, 3.6kb and 1kb DNA fragments (Fig. 6.4, A and Table 6.4). Of these 3 fragments, only the 3.6kb and 1 kb fragments segregated among plants, but all F_1 plants had at least one copy of GAL4/VP16 T-DNA. Some F_1 plants contained both UAS::DT-*A* (1 copy) and GAL4/VP16 (either 1 or 3 copies) T-DNAs, for example by NI02/8 number 1, 7, 11 and 16 (refer to Fig. 6.4 A and B; Table 6.4). In contrast, another F_1 plant, for example NI02/8 number 6, 8 and 13, did not contain UAS::DT-*A* T-DNA insertion and only had GAL4/VP16 T-DNA (refer to Fig. 6.4 A and B; Table 6.4).

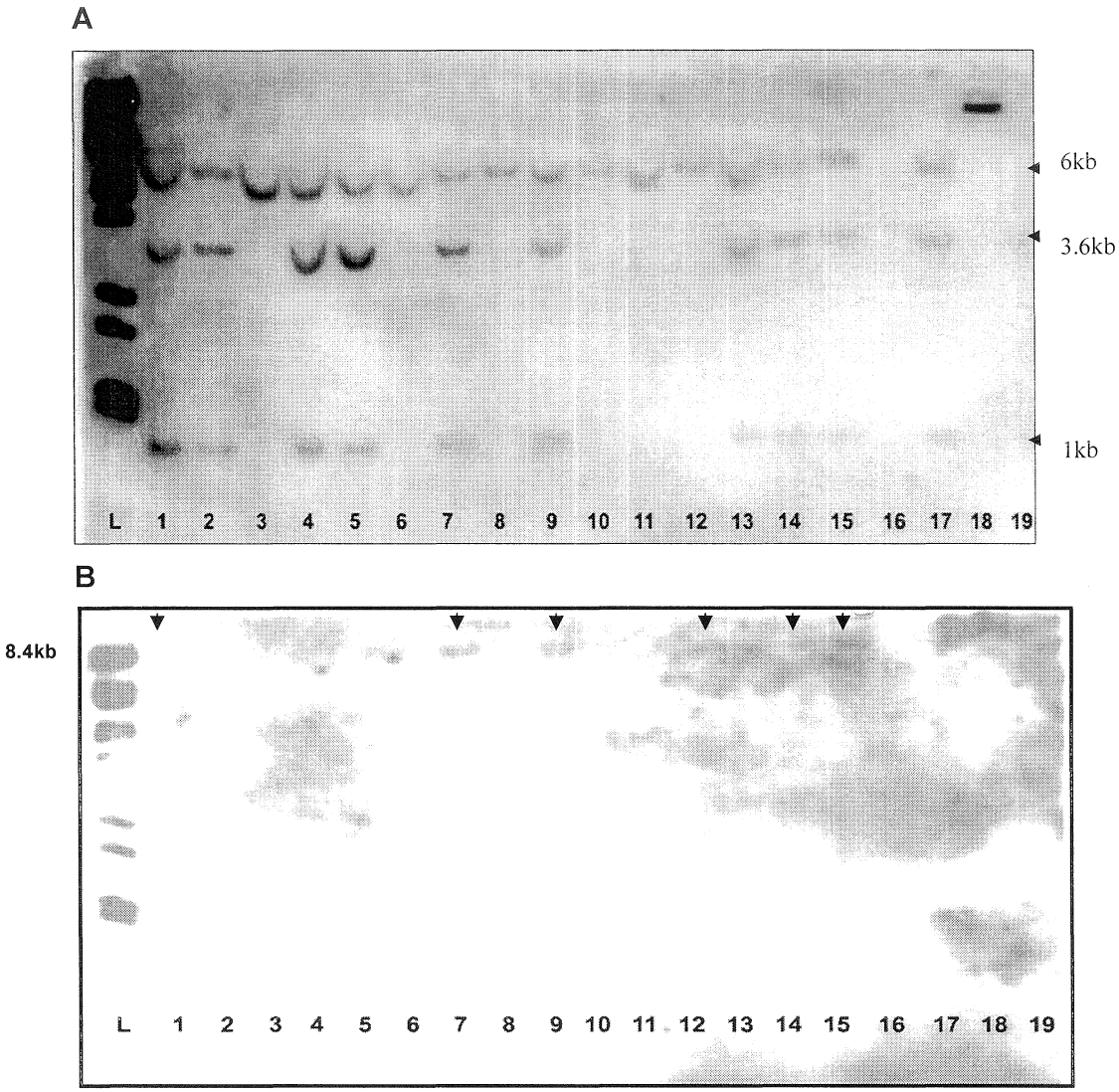

Figure 6.4. Southern Blot analysis F1 plants of NI02/8 [NI01 12/6-3 (pSMRJ18R(1/2)-9a) x FU01-298 (UAS::DTA)]. Plants DNA were digested with *EcoR*I restriction enzyme. **A**, the membrane was hybridised with DIG-labelled GAL4/VP16 fragment as a probe. **L**: λ BstE-II; **1-16**, number of plants (refers to no1-16, Table 6-4); **17**, pSMRJ18R(1/2)-9a (female parent); **18**, 1x pSMRJ18R; **19**: 1x pWSA89-18, and **arrows**: fragments containing T-DNA insertion.

B: the membrane was hybridised with DIG-labelled GUS fragment as a probe; L: λ BstE-II; 1-16: number of plants (refers to no1-16, Table 6-4); 17: pSMRJ18R(1/2)-9a (female parent); 18: 1x pSMRJ18R; 19: 1x pWSA89-18, and arrows: plants with T-DNA insertion.

6.3 DISCUSSION

From the previous experiment, presented in Chapter 5, it was confirmed that the GAL4/VP16 system was able to activate a reporter gene fused to the UAS_{GAL4}. From the crossing experiment between TAFET lines containing GAL4/VP16_UAS-GUSPlus T-DNA and a UAS line containing UAS-EGFP T-DNA, F_1 plants had similar patterns of expression for both GUSPlus and EGFP reporter genes and these patterns were consistently displayed in the F_2 generation.

In this chapter, I reported attempts to generate phenotypic changes through crossing between TAFET lines (containing transactivator GAL4/VP16) as "effectors" and UAS_*ZAG1* lines and UAS_DT-*A* lines, as "target" lines in rice.

Over-expression of the *ZAG1* gene by the CaMV35S promoter (pSKH26.1) showed that it affected the development of reproductive organs in rice, as some of pSKH26.1 lines displayed floral phenotype changes, although the changes were only shown in some spikelets. Moreover, 56.7% of the pSKH26.1 lines with T-DNA insertions displayed high sterility or complete sterility compared to 36.6% of the pSKH26.1 lines without T-DNA displayed similar sterility level (refer to Table 6.2). A higher percentage of sterility phenotypes might be influenced by the over-expression of the *ZAG1* gene, as it is known that the *ZAG1* gene is expressed primarily in a primordial stage of reproductive organ development in maize (Schmidt et al., 1993). A possible reason for these phenotypes (floral change phenotypes shown in some spikelets, leading to high sterility) is co-suppression (Jorgensen et al., 1996). High level of expression of transgenes introduced into the plant can inhibit the expression of the plant's own gene by triggering sequence-specific destruction of similar transcript, instead of producing large quantities of new proteins (Jorgensen et al., 1998). Co-suppression was shown by a single-copy sense construct of a chalone synthase (*Chs*) gene driven by a strong promoter (the 35S promoter) (Que et al., 1997). Plants with

multiple transgene copies are subject to somatic events that result in either a complete loss of the co-suppression stage or a qualitative change in pattern of co-suppression, and it was related to a transgene dosage (Que and Jorgensen, 1998). If the transcriptional silencing only affects the transgenes, then the endogenous expression will not be suppressed and the plant will produce a normal phenotype (Jorgensen et al., 1996). Variation in phenotypes of 35S::*ZAG1* lines might be related to different levels of co-suppression among lines and even spikelets in a single inflorescence.

Another possible reason for this phenotype in F_1 plants is gene silencing. Gene silencing can be homolog-dependent and in our case, the UAS::*ZAG1* sequences might be homologous to an endogenous rice gene (Matzke et al., 1994). The *ZAG1*-homolog gene identified in rice was the *OsMAD3* which was actively expressed in the third and fourth whorls of the rice flower (Kang, 1998).

The crossing experiment between the GAL4/VP16 lines (pattern lines) and UAS::*ZAG1* lines (target lines) produced F_1 plants without any obvious floral phenotypic changes. A possible reason for the lack of floral phenotype change in F_1 plants may be due to the insufficient production of *ZAG1* RNA through transactivation, compared to the 35S promoter that induced an obvious level of the phenotypic changes in the spikelets. While there is little doubt that the GAL4/VP16 is capable of increasing transcription level beyond basal levels there was likely insufficient expression of ZAG transgene. However, some changes were observed in few spikelets (anthers changed to ovule-like shape, producing multiple ovules) suggesting that on those tissues the level of *ZAG1* activation by GAL4/VP16 was sufficiently high.

F1 plants from the crossing experiment between the GAL4/VP16 pattern lines and UAS::*DT-A* lines had severe phenotypic changes and a limited number of seeds (high percentage of sterile spikelets or even complete sterility). A severely affected

phenotype in limited number of seeds has been seen in previous work using the DT-*A* gene in *Arabidopsis* (Day et al., 1995; Nilsson et al., 1998). However in the current work, plants showing severely affected phenotypes with T-DNA and plants showing severely affected phenotypes without the DT-*A* T-DNA are almost equal in number, it is therefore not possible to link the phenotypes with the DT-*A* T-DNA.

6.3 CONCLUSION

From these experiments, three conclusions can be taken, as follows.

- First, attempts to validate the ability of GAL4/VP16 TAFET system to generate a phenotype change in floral tissues did not fully succeed. This might be due to insufficient RNA product produced by the activation of *ZAG1* by the GAL4/VP16 to be able to induced obvious phenotypic changes in the floral tissue. Only limited changes in the floral tissues leading to higher sterility were observed in 35S:: *ZAG1* lines.

- Second, although there was an indication that the GAL4/VP16 activated a DT-*A* target gene fused to the UAS_{GAL4}, producing F_1 plants with severe phenotypes in plant growth and in a limited number of seeds, F_1 plants segregated 1:1 between plants with and without T-DNA and it was not possible to link the phenotypes to T-DNA insertion.

- Third, as the ability of the GAL4/VP16-UAS to activate a target gene is important for rice functional genomics, attempts to confirm the system are needed for future work, by choosing another target gene that might be expressed in sufficient amount to be able to induce an obvious phenotypic change.