

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

A thesis submitted for the degree of Doctor of Philosophy
of the Australian National University

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



March 2004

Chapter 1

GENERAL INTRODUCTION

1.1 RICE AS AN IMPORTANT AGRICULTURAL CROP

Rice is a monocotyledonous angiosperm. The genus, to which it belongs, *Oryza*, contains more than 20 species, only two of which are referred to as cultivated rice: *Oryza sativa*, (Watanabe, 1997) cultivated in South-east Asian countries and Japan, and *Oryza glaberrima* cultivated in West Africa. Rice was originally cultivated in tropical Asia, the oldest record dating 5000 years BC, but then extended also to temperate regions (Watanabe, 1997).

Rice is the most important staple food in Asia. More than 90% of the world's rice is grown and consumed in Asia, where 60% of the world's population lives. Rice accounts for between 35-60% of the caloric intake of three billion Asians (Guyer et al., 1998). Over 150 million hectares of rice are planted annually, covering about 10% of the world's arable land. In 1999/2000, this amounted to some 600 million tonnes of rice seed, equal to 386 million tonnes of milled rice. With the world population estimated to increase from 6.2 billion in the year 2000 to about 8.2 billion in the year 2030, the global rice demand will rise to about 765 million tonnes, or 533 million tonnes of milled rice (FAO, 2002). For almost three decades since the Green Revolution, the rice yield growth rate was approximately 2.5% per year. During the 1990s, however, this has decreased to only 1.1% (Riveros and Figures, 2000).

Attempts to overcome the rice yield limitation by improving yield, resistance to pests and diseases, and adaptability to diverse growing conditions, have consisted of breeding programmes and the development of hybrid rice varieties. Hybrid rice has

been developed in China since 1974 and now is planted in almost 40% of Chinese rice fields (Fujimaki and Matsuba, 1997; Sasaki, 1997.; IRRI, 1999).

1.2 RICE AS A MODEL SYSTEM FOR MONOCOTYLEDONOUS PLANTS

Apart from its economic significance, rice has become an important plant for genetic and genomic studies. Rice is diploid with 24 chromosomes which can be distinguished individually using cytogenetic techniques (Fukui and Lijima, 1991). The rice genome is small (about 430 Mb) compared to other cereal crops such as maize (2,400 Mb), barley (4,900 Mb) and wheat (16,000 Mb) and contains an estimated 32,000 to 62,000 genes (Bennetzen, 2002; Sasaki and Sedoroff, 2003). This small genome size has contributed to rice becoming the prominent model system for cereal genomics as well as a model for monocotyledonous plants.

Rice researchers have developed important tools for genetic analysis, for example developing high density molecular genetic maps for rice (Harushima et al., 1998) and efficient genetic transformation techniques (Hiei et al., 1997). Comparative genetic maps within the grass family indicate the existence of conserved gene content and gene order (synteny) among grass genomes (Ahn et al., 1993; Devos and Gale, 1997; Devos et al., 2000). Drafts of the rice genome sequence for two rice subspecies have been released by the Beijing Genomics Institute (BGI) (Yu et al., 2002) and the Syngenta's Torrey Mesa Research Institute (TMRI) (Goff et al., 2002). An ongoing effort by International Rice Genome Sequencing Project (IRGSP) to compile a complete high-quality draft of the rice genome sequence promises to deliver a very useful tool for science and rice breeding (Sakata et al., 2002; Sasaki and Sedoroff, 2003).

1.3 RICE GENOMICS

The term genome is more than 75 years old and refers to an organism's complete set of genes and chromosomes. The term "genomics" was coined more recently by Thomas Roderick (1986) to describe the scientific discipline of mapping, sequencing and analyzing genomes. Genomics is now undergoing an expansion from mapping to an emphasis on genome functions and now consists of structural and functional genomics (Hieter and Boguski, 1997).

1.3.1 RICE GENETIC MAPPING

Genetic maps of the rice genome have been developed using molecular markers, for example RFLP (Kishimoto et al., 1993) and Simple Sequence Repeats (SSRs) (Temnykh et al., 2000; Wu et al., 2002). About 70% of the RFLP-based maps were developed using rice cDNAs as probes and, of those RFLP markers, about 30% had significant sequence homology to sequences of known genes (Kishimoto et al., 1993).

A high resolution rice genetic-linkage map has been constructed using EST clones as RFLP probes. Two thousand two hundred and seventy five markers, distributed in 1,450 loci were obtained from Nipponbare callus, root, and shoot libraries and from 186 F₂ plants of a single cross between the japonica rice subspecies, variety Nipponbare and the indica subspecies, variety Kasalath. Of these 1,450 loci, about 600 showed significant similarities to known genes, including single-copy genes and gene families (Harushima et al., 1998).

Two highly saturated molecular-linkage maps, localizing the numerous genes and the quantitative trait loci (QTLs), have been generated (McCouch and Doerge,

1995). In addition, a RLFP-based map linked to a drought resistance-related trait was constructed from the drought-resistant varieties Azucena and Bala (Price et al., 2002). Another QTL map for the AI tolerance-related traits has been developed using molecular markers obtained from 171 F₆ recombinant inbred lines (RILs), derived from crosses between *Oryza sativa* (IR64) which is the AI susceptible parent, and *Oryza rufipogon* which is the AI tolerant parent (Nguyen et al., 2003).

Another application of rice molecular maps is their use in comparative genetics to identify conserved synteny between rice chromosomes and those of other species. For example, RFLP comparative genetic maps showed that rice, wheat and maize were nearly identical in overall gene content and gene order (Ahn et al., 1993; Ahn and Tanksley, 1993). Examination of gene distribution among rice, barley and maize, using a different set of probes, produced similar results (Barakat et al., 1997). The greatest advantage of establishing synteny is that grasses with a smaller genome can be used to “walk” the chromosomes of larger genome cereals, using the smallest possible number of steps (Bennetzen and Freeling, 1993). This map-based technique was used in an attempt to clone the barley stem rust resistance gene *Rpg1* using nine rice probes (Kilian et al., 1995). A high degree of micro-synteny was shown in the telomeric region of barley chromosome 1P (6.5 cM) and the most terminal 2.7 cM of rice chromosome 6 and colinearity established for barley chromosome 7 and rice chromosome 3 (Kilian et al., 1997).

A comparative genetic analysis using a QTL map for the AI tolerance-related trait showed that QTLs for the root relative length (RRL) which mapped on chromosomes 1 and 9 appeared to be consistent among different rice populations. A major QTL for the RRL was found on chromosome 3 of rice and the linkage to some molecular markers possibly conserved across cereal species (Nguyen et al., 2003).

1.3.2 RICE PHYSICAL MAPPING

Physical maps of the rice genome have been constructed based upon Expressed Sequence Tags (ESTs), Sequence-Tagged Connectors (STCs), bacterial artificial chromosome (BAC), yeast artificial chromosomes (YAC), P1-derived artificial chromosomes (PAC), or shotgun sequence analysis. About 68,000 rice ESTs are available in public databases and another 120,000 are kept in private databases (Tarchini et al., 2000). Of these, however, only about 25% show significant homology to known genes, and the function of most of the genes is undetermined (Jeon et al., 2000b).

Rice YAC-based physical maps with six-fold genome coverage have been developed by the Tsukuba Rice Genome Projects (RGP). Recently, new YAC-based maps were constructed using about 1,450 genetically mapped ESTs (Saji et al., 2001). Another YAC-based map was also constructed using 3'-untranslated regions (UTRs) (Matsumoto, 2001). Another 4,300 rice YAC clones were mapped based upon PCR, using primers designed from about 6,700 ESTs, derived from 19 cDNA libraries (Wu et al., 2002). Two BAC-based rice maps were generated from *Oryza sativa* subspecies Japonica var. Nipponbare using *Hind* III and *EcoR* I as cloning restriction enzymes by the Clemson University Genomic Institute (CUGI) (Presting et al., 2001). Other BAC libraries were constructed from several rice varieties: IR64, Lemont, Teqing, Azucena, Muinghui 63 and Guang Lu A4 (Kurata et al., 1997).

Moreover, P1-derived artificial chromosome-based (PAC) rice maps were constructed using PCR markers from cDNA sequences (EST markers) and STS markers by RGP research program. About 4,500 EST and 970 STS markers distributed throughout the genome and were used to anchor PAC clones (Matsumoto, 2001).

An effort to sequence the rice genome was undertaken at the Beijing Genomics Institute (BGI) and the Syngenta's Torrey Mesa Research Institute (TMRI). These two institutions independently produced rice sequence data using the whole genome shotgun approach. The BGI provided the sequences for Indica rice varieties 93-11 and a PA64s, the most widely cultivated subspecies in China and most of the rest of Asia (Yu et al., 2002). The TMRI provided sequences of the Japonica rice subspecies, variety Nipponbare (Goff et al., 2002). Sequences are publicly available at the BGI and TMRI web sites (<http://bgi.genomic.org.cn/rice> and <http://www.tmri.org>). Extensive efforts to sequence the rice genome with high accuracy have also been carried out by the International Rice Genome Sequence Project (IRGSP). The IRGSP has accumulated more than 137 Mb of the Japonica rice subspecies genome sequence and made this available to the public in 2001 (Sakata et al., 2002). The complete sequence of chromosome 1, the longest chromosome in the rice genome, was reported and the whole genome sequence has been published (Leach et al., 2002).

In addition to genomic data, about 28 thousand full-length cDNA clones from ssp. *Japonica* (cv. Nipponbare) are available to the public at the Knowledge-based Oryza Molecular Encyclopedia web-site (KOME, <http://cdna01.dna.affrc.go.jp/cDNA>) (Kikuchi et al., 2003). These are necessary to identify exon-intron boundaries and gene-coding regions within rice genomic sequences. Mapping of cDNA clones and comparison of genome sequences indicate the correct structure of the genes in rice and this may also be used to understand gene structure in other *Poaceae* species.

1.3.3 RICE GENOME ANALYSIS

As more of the rice genome sequence and mapping of markers becomes available, it becomes critical to identify the functions of thousands of new rice genes. The reverse genetics approach attempts to do this by comparing sequence similarity among plant genes using rice EST markers.

The first rice gene cloned using a map-based cloning strategy was a *Xanthomonas campestris* cv. *Oryzae* resistance gene, *Xa21* (Song et al., 1995) and *Xa1* (Yoshimura et al., 1998). A location of the blast resistance locus *P1-2t* on a map that was constructed from 22 BAC clones covered the whole *P1-2t* region. It provides a molecular marker to aid in selection of new blast resistance material in rice (Fu et al., 2000).

In relation to rice ESTs development which are available in public databases (Tarchini et al., 2000), nine of 109 rice ESTs were mapped into three regions on chromosomes 6 and 11 that contain genetically defined resistance genes. Two of them which code for a receptor-like kinase and a putative membrane channel protein respectively, were mapped to the *Pi2* locus, and were induced by rice blast infection as early as 4 hours after inoculation (Wang et al., 2001). Efforts to identify rice genes associated with drought stress responses were carried out by analysing 1,540 high-quality Expressed Sequence Tags (ESTs) constructed from drought-stressed seedlings of Indica rice. About 120 of 320 novel ESTs were localized to BAC clones and about 120 ESTs were identified with putative functions (Babu et al., 2002).

The use of molecular probes from one species to clone a gene from other species has also been attempted in rice. The *OsMADS1* gene was isolated from a rice cDNA clone using probes from *Arabidopsis*. The *OsMADS1* encodes a MADS-domain-containing protein and its amino acid sequence shows 56.2% identity to *AGL2* and 44.4% identity to *Arabidopsis AP1* gene (Chung et al., 1994). Another approach to clone rice genes has been to screen the genomic library with DNA fragments obtained from PCR using degenerate primers. For example, other rice MADS box genes, such as *OsMADS2* and *OsMADS4*, were cloned using the *OsMADS1* fragment as a probe for cDNA library screening (Chung et al., 1995). In addition, two rice gibberellin (GA) 3 beta-hydroxylase genes, *OsGA3ox1* and *OsGA3ox2*, have been identified using the

conserved sequence of the target gene from other species (Itoh et al., 2001). Molecular and linkage analysis maps the OsGA3ox1 gene to the distal end of the short arm of chromosome 5; the OsGA3ox2 gene maps to the distal end of the short arm of chromosome 1. The association of the OsGA3ox2 gene with the d18 locus is confirmed by sequencing and complementation analysis of three d18 alleles. Although both genes showed transient expression, the OsGA3ox1 gene was highly expressed in the unopened flower, whereas the OsGA3ox2 gene was expressed in elongating leaves.

1.4 RICE GENES DISCOVERY USING INSERTIONAL SEQUENCE MUTAGENESIS

In order to increase the rate of finding new rice genes, a forward genetics approach has also been applied in rice. In general, rice mutants can be generated using either chemical or physical mutagens such as ethyl methane-sulphonate (EMS) (Inukai et al., 2000; Goel et al., 2001) or Gamma ray irradiation (the classical approach) (Teraishi et al., 1999; Biswass et al., 2003), or by applying insertional sequence mutagenesis such as transposable elements and T-DNA of *Agrobacterium tumefaciens*-mediated transformation (Izawa, 1997; Jeon et al., 2000b).

The use of insertional sequence mutagenesis in rice was boosted by the efficient transformation method developed for rice by Hiei (1997). Since this is a recently developed technique, the outcomes are still largely anticipated (Izawa, 1997; Jeon et al., 2000b; Jeong et al., 2002). The technique offers advantages over chemical and physical mutagenesis in that it facilitates the tagging of the target gene both molecularly and genetically, as reporter and/or selectable marker genes are carried along by the insert (Martienssen, 1998). A polymerase chain reaction (PCR) can be used to recover the DNA flanking the insert and this may lead to the isolation of the

wild type gene sequences (Ortega et al., 2002). Insertional sequences are currently also being used for other purposes such as gene-trapping or gene-activation (Springer, 2000).

1.4.1 TRANSPOSABLE ELEMENTS

Transposable elements commonly used for insertional sequence mutagenesis in many plants, including rice, were *Dissociation* (*Ds*) and *Activator* (*Ac*), which are endogenous in maize (Izawa, 1997; Chin, 1999; Enoki et al., 1999). These transposable elements are well studied and were first recognised by Barbara McClintock (Fedoroff, 2000).

The *Activator* (*Ac*) is an autonomous element carrying a single, 2,421bp open reading frame (ORF) encoding the single, 807–amino acid protein *Ac* transposase (*AcTPase*) which catalyses the process of *Ac* transposition by interacting with specific *cis*-acting sequences near the termini of the element (Kunze and Starlinger, 1989). In contrast, the *Dissociation* (*Ds*) is a non-autonomous element that lacks the ability to encode TPase, but retains the *cis*-acting sequences necessary for transposition. The *Ds* can therefore be trans-activated by the presence of *Ac* within the same cell, as shown in *Arabidopsis* and rice (Hehl, 1989; Fedoroff and Smith, 1993; Izawa, 1997). The *Ac* displays very high levels of activity in maize, tobacco and tomato, but reduced activity in *Arabidopsis* (Hehl, 1994; Jarvis et al., 1997). In two generations of tomato, one copy of the *Ac* insertion increased to 15 copies (Yoder, 1990). Recent studies in *Arabidopsis* showed a high level of *Ac* transposition when it was adjacent to the cauliflower mosaic virus (CaMV) 35S promoter.

Almost 80% of *Ds* elements were excised from the original insertion sites when *Ac* cDNA driven by CaMV 35S promoter was applied in rice (Chin et al., 1999). It has

been shown that *Ac/Ds* elements transpose preferentially to linked sites. *Ac* tends to transpose to protein-coding regions in rice and is therefore considered a valuable asset for generating mutants (Enoki et al., 1999). Because of this characteristic, transposable elements can be highly efficient for regional mutagenesis but are not efficient for global mutagenesis (Bancroft and Dean, 1993; Fedoroff and Smith, 1993; Jones et al., 1994; Walbot, 2000). The tomato *Cf-9 Cladosporium fulvum* resistance gene was isolated after *Ac* re-inserted into a new location close to the original insertion site (Jones et al., 1994). A rice cytochrome P450 (CYP86) was isolated from the application of *Ac* (Enoki et al., 1999).

Another transposable element which has proved useful as a mutagenesis tool in rice is the endogenous retrotransposon *Tos17* (Hirochika et al., 1996; Hirochika, 1997; 2001). This class I element is highly activate during tissue culture but inactive in generated plants. Mutants induced by *Tos17* insertion are relatively stable (Hirochika, 1997). In comparison to other transposable elements, the copy number of *Tos17* is quite low. In addition, this retrotransposon shows preferential insertion to genic rather than intergenic regions (Miyao et al., 2003).

1.4.2 T-DNA of *Agrobacterium tumefaciens*-mediated transformation

T-DNA insertional sequence mutagenesis exploits the property of the tumor inducing (Ti) plasmid of *Agrobacterium tumefaciens* to transfer any DNA delimited by two 25-bp direct repeats at its right and left borders to plant cells (Zambryski, 1992).

The major advantage of T-DNAs over transposable elements is the more random insertion into the genome (Ambros, 1986); (Wallroth, 1986). Although copy numbers are lower than those of most transposable elements, the T-DNA does not transpose after integration, but remains stable in the original insertion site through multiple

generations (Krysan et al., 1999). The lower copy number may more easily facilitate the characterisation of transgenic lines and the isolation of flanking sequences. In addition, it allows the use of specialised T-DNA harbouring trap- or activation-tagged systems (Springer, 2000). A promoterless reporter gene links to the T-DNA border tended to insert more into transcriptional active regions (Koncz et al., 1989). This means a T-DNA has a preferential integration into genomic regions that potentially can be transcribed. T-DNA insertions were found more in “gene space” than expected in rice (Sallaud et al., 2003) and this characteristic is good for functional genomics.

Various T-DNAs have been applied in rice, and mutants with a number of phenotypic changes have been previously described (Jeon et al., 2000b; Jeong et al., 2002; Wu et al., 2003). For example, the *OsCHLH* gene encoding the largest subunit of the rice Mg-chelatase enzyme was identified from T-DNA insertional lines (Jung et al., 2003).

1.5 APPROACHES AND SYSTEMS FOR PLANT FUNCTIONAL GENOMICS

The development of different approach-based systems is pivotal for gene discovery in rice. Recent studies have demonstrated that results obtained from experiments using *Arabidopsis* as the plant model cannot be applied to a highly diverse species such as rice (Kyoizuka et al., 1998; Devos et al., 1999). For example, a *LFY* homolog gene in rice seems to be involved in panicle branch initiation, whereas in *Arabidopsis* the same gene regulates the formation of floral meristems (Kyoizuka et al., 1998). A study of the co-linearity between two small segments of chromosome 1 in *Arabidopsis* and rice using comparative mapping has shown that conservation of gene order is no longer identified (Devos et al., 1999). Each system has advantages and limitations when applied to plant functional genomics.

1.5.1 GENE KNOCK-OUT

As previously mentioned, the most common approach used in plant functional genomic investigations is the generation of mutant phenotypes (gene knock-out), using either transposable elements or T-DNA(s) insertional mutagenesis (Topping and Lindsey, 1995); (Dean, 1991). The integration of insertional sequences into protein coding regions of the nuclear genome may inactivate or alter the expression of plant genes resulting in recessive or *Loss-of-Function* (LoF) mutations.

Not all genes, however, can be uncovered by insertional mutagenesis (Burns et al., 1994). The first reason for this is functional redundancy where one or more other loci can substitute for the same function, thus preventing the elucidation of gene function by the LoF approach (Campisi et al., 1999), (Springer, 2000). A clear example of functional redundancy is the *Abscissic-Acid Insensitive-1* (*ABI-1*) and *ABI-2* loci (Leung et al., 1997). 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 second reason why gene knock-out may fail to uncover genes is that many genes function at multiple stages of development. The mutation of such a gene might affect early lethality and could be highly pleiotropic. Both of these effects can mask the role of the gene in specific pathways. In addition, the frequency of lethal mutant recovery is low (Miklos and Rubin, 1996).

1.5.2 GENE SILENCING

Gene silencing using sense or antisense suppression of selected genes is another approach developed for elucidating gene function (Baulcombe, 1999). Plant gene expression can be suppressed in a sequence-specific manner by infection with virus vectors carrying fragments of host genes.

It has been shown that the mechanism of gene silencing is based on an RNA-mediated defence against viruses (Baulcombe, 1999). Up to 50% of petunia transformants that contained a sense copy of the chalcone synthase (CHS) gene produced floral sectors as a result of post-transcriptional loss of mRNA encoding CHS (Flavell, 1994; Metzlaff et al., 1997). This approach, however, has some disadvantages: it needs several independent transgenic lines generated for every gene, and essential genes cannot be down-regulated in this way, as suppression would lead to a dominant lethal mutant that would not be maintained (Gu et al., 1998).

A further development of the gene silencing system was the design of an inverse/reverse β -glucuronidase (GUS) construct to express RNA with self-complementarity, a hairpin RNA (hpRNA) (Wesley et al., 2001). The RNA-interference (RNAi) system was found to be efficient in inducing silencing of both endogenous genes and transgenes in plants (Wang and Waterhouse, 2000).

1.5.3 INDUCIBLE GENE EXPRESSION

Inducible gene expression systems were developed based upon de-repression, inactivation and activation of transcription of the target gene. These systems use a heat shock promoter (*hs* gene from soybean), chemically inducible promoters such as a tetracycline promoter, dexamethasone, ecdysone, copper, salicylic acid, or a glucocorticoid steroid hormone receptor (Aoyama and Chua, 1997; Gatz et al., 1992). This is considered a useful approach because the methods allow gene expression

experiments to be performed in a true isogenic background (Reynolds and Figures, 1999).

A heat shock-based gene expression system using a β -glucuronidase (GUS) for gene expression detection was able to reveal differences in a heat shock response during tobacco and *Arabidopsis* plant growth and development (Prandl et al., 1995). However, limited genes were affected by the heat shock system, and prolonged exposure to temperature elevation generates pleiotropic gene expressions (Ainley and Key, 1990).

A Tet repressor (TetR)-based gene expression system uses the tetracycline-responsive Tet repressor (TetR) which binds to the *tet* operator in the absence of tetracycline to regulate a target gene expression driven by a modified 35S promoter. The TetR regulates *tet* genes (A-E, G, H, J, Z, 30, 33) (Levy et al., 1999; Tauch et al., 2000, 2002) at the level of transcription (Berens and Hillens, 2003). In this system, one and two copies of the *tet* operator were placed upstream and downstream from the TATA-box, respectively. This so called tetracycline de-repression system worked in tobacco, tomato and potato, but did not work in *Arabidopsis* (Gatz et al., 1992). Besides the tetracycline-repressing system, a tetracycline-inactivation system was also developed by fusing the *Tet* repressor to the Herpes simplex virus VP16 activation domain, activating “target gene” expression driven by a target promoter containing seven *tet* operators upstream of a minimal promoter in the absence of tetracycline (Weinmann et al., 1994; Reynolds and Figures, 1999). Since tetracycline must be applied continuously for generating a negative control, this was considered a non-practical system for gene expression.

Another inducible system is the GVG (glucocorticoid-based system), which uses the rat glucocorticoid receptor hormone-binding domain (GR) as a regulatory domain

and a chimeric transcription domain (a fusion between a GAL4 binding domain and a VP16 activating domain). This system activates a transcription of the gene of interest when a glucocorticoid or dexamethasone (DEX), a strong synthetic GR ligand, is present (Aoyama and Chau, 1997; Reynolds and Figures, 1999). This system provides flexibility, where the 35S promoter driving the GVG can be replaced by a tissue-specific promoter, and both transcription factor and hormone binding domain are also interchangeable (Aoyama and Chau, 1997). Although it is considered simple, and the glucocorticoid did not cause any pleiotropic effect in plants, in some cases the GVG system created a DEX-dependent toxic effect. In rice this system was able to induce GUS activities to levels comparable to those conferred by a 35S promoter, when a relative low concentration of DEX (1-10 μ M) was applied (Ouwerkerk et al., 2001).

The *Tet* repressor combined with the glucocorticoid receptor and the VP16 activating domain produced a TGV that is subject to dual regulation, by tetracycline and DEX. In a DEX-dependent fashion, TGV activates the expression of a reporter gene driven by a synthetic promoter consisting of multiple copies of the modified *Tet* placed upstream of a 35S minimal promoter (Bohner et al., 1999). When DEX is removed and tetracycline is applied, the system is switched off as the association of tetracycline renders the chimeric factor incapable of binding DNA (Zuo and Chua, 2000). The system has been applied in tobacco, and was able to induce stable expression over several generations. Expression levels were comparable to a plasmid containing the CaMV35S promoter, with only slightly elevated background activity (Bohner et al., 1999).

1.5.4 ACTIVATION TAGGING

Plant functional genomics has also been approached using the activation of genes to identify gene function. Two ideas have motivated this approach: firstly, genes that are not absolutely required for a certain pathway can still be identified through a mutant allele, if such a gene is sufficient to activate that pathway, and, secondly, genes that are essential for early survival might be identified if ectopic activation of the pathways they regulate is compatible with survival of the organism (Weigel et al., 2000). The activation tagging system was developed to deal with the gene redundancy issue, through miss- or over-expressing endogenous genes inducing *Gain-of-Function* (GoF) phenotypes (Weigel et al., 2000). In contrast to the GoF is the *Loss-of-Function* (LoF), inactivation or alteration the expression of plant genes that can be due to an integration of insertional sequences into protein coding regions of the nuclear genome.

The T-DNA constructs used in activation tagging contained four copies of the transcriptional enhancer from the CaMV 35S promoter (35Se) at the right border (Hayashi et al., 1992). The 35Se was spliced into the plant genome at random sites in *Arabidopsis* (Kardailsky et al., 1999; Borevitz et al., 2000; Ito and Meyerowitz, 2000; Weigel et al., 2000) and rice (Jeong et al., 2002). In each independent line, the 35Se strongly activated the plant genes which, by chance, lay adjacent, causing dominant phenotypes that appeared in the T₁ generation. The efficiency of creating GoF mutations through this activation tagging system was low, since most of the mutants observed in the T₁ generation segregated in a manner suggesting they were primarily caused by LoF mutations. Genes identified using this system were a dominant allele of the flowering locus T (*FT*) that caused early flowering in *Arabidopsis* (Kardailsky et al., 1999) and the *iso-1D* and *iso-2D* allelic mutants of the *AS2* gene causing leaf morphology changes (Nakazawa et al., 2003).

1.6 GENE TRAPPING

An alternative approach for plant functional genomics is to reveal spatial (cell- or tissue-specific) and/or temporal (developmental stage) gene expression patterns. Three systems have been developed: the gene trap, the promoter trap and the enhancer trap (Topping and Lindsey, 1995; Campisi et al., 1999; Jeong et al., 2002). Each type has advantages and disadvantages over the other types.

1.6.1 GENE TRAP

Gene trap constructs contain 3' splice acceptor sites adjacent to a reporter gene and no promoter. Expression can therefore only be produced when the molecule is inserted into the transcriptional unit (transcribed region) and only if the orientation is correct (Springer, 2000). One or more splice acceptor sequences preceding a reporter gene allow expression if insertions are in the intron site (Springer, 2000). Apart from transcriptional fusions, this trap can also create translational fusions, which may provide information about protein localisation. However, gene trap insertions are more likely to lead to gene disruption.

In the *Ds-G* (transposable element-based gene trap) construct, the GUS reporter gene is preceded by an intron and two consensus splice acceptor sequences, so a splice acceptor appears in every reading frame. If the *Ds-G* transposes into a chromosomal intron with the GUS in the correct orientation, it will get a splicing from the splice donor of the chromosomal intron to the splice acceptor in front of the GUS gene, resulting in expression of the reporter gene (Jeon et al., 2000b). In T-DNA-based gene trap constructs, the reporter gene is cloned near to the T-DNA border (Maes et al., 1999). Plasmids containing a promoterless GUS gene sited immediately next to the right border have been transformed into rice (Jeon et al., 2000b).

It has been reported that only half of the gene trap insertions in genes were in the appropriate orientation resulting in reporter gene expression (Gu et al., 1998).

From 2000 *Arabidopsis* gene trap lines screened, 32% of gene trap insertions exhibited expression in seedlings and 10% exhibited expression in floral and reproductive tissues (P. Springer, Q. Gu, D. Bush, C. Yordan, and R.A. Martienssen, unpublished results) (Gu et al., 1998). In rice, the maximum GUS tagging efficiency would be about 25% (Jeon et al., 2000b).

1.6.2 PROMOTER TRAP

Promoter trap constructs contain a reporter gene, the beta-glucuronidase (GUS), either fused to a minimal promoter or without a promoter at the T-DNA left border (Topping et al., 1991; 1994). Similar to the gene trap, promoter trap expression can only be produced when a construct is inserted into a transcribed region (Topping et al., 1991; 1994). As in the gene trap system, translational fusion and gene disruption can result. The system was first developed in tobacco, *Arabidopsis* and potato. Similar percentages of lines with expression in leaf and/or root (73% and 78%), were displayed by transgenics transformed with either a minimal promoter fused to GUS or a promoterless GUS construct (Topping et al., 1991).

1.6.3 ENHANCER TRAP

Enhancer trap constructs typically contain a minimal promoter; a TATA box and transcription start site, fused to a reporter gene which is activated by chromosomal enhancer elements, resulting in expression of the reporter gene.

In producing gene expression, the enhancer trap does not suffer from the same constraints as the two previous systems, and a high frequency of reporter gene expression is demonstrated (Springer, 2000). Since transcription is affected by position, expression levels of transgenes at different chromosomal locations may vary

greatly. However, expression patterns of reporter genes in enhancer trap lines frequently resemble the expression patterns of endogenous trapped genes (Wilson et al., 1990; Sundaresan et al., 1995a). Enhancer trap lines will almost always be viable even when the enhancer trap inserts in the middle of an essential gene, because the enhancer trap element is dominant and patterns can be detected when insertions are in the hemizygous state (Campisi et al., 1999).

The use of enhancer traps in gene expression studies exploits the fact that enhancers are essential for gene transcription in eukaryotic cells (Bonifer, 2000; Martin, 2001), and such traps have been used in *Drosophila* (Bellen, 1989; Bellen, 1999) and in *Arabidopsis* (Sundaresan et al., 1995a). A high efficiency of trapping, from 25% to 59%, was displayed by rice enhancer trap lines (Wu et al., 2002).

1.6.3.1 FIRST DEVELOPMENT OF ENHANCER TRAPS

The enhancer trap was first developed in *Escherichia coli*, using bacteriophage Mu carrying a promoterless *lacZ* gene. Insertion of the construct into a gene under constitutive control produced constitutive *lacZ* gene expression, whereas the insertion into a regulated gene led to *lacZ* induction only under specific conditions (temporal) (Casadaban and Cohen, 1979). Ten years later the system was applied to the eukaryote, *Drosophila melanogaster* (Bellen, 1989) using constructs containing a translation fusion between the *P*-transposase gene and the promoter-less *Escherichia coli* β -galactosidase (*LacZ*) gene. This system cannot drive expression in the absence of a transcriptional enhancer. The transgene can only be activated if the transposon integrates into the genome close to a genomic enhancer, which occurred in about 65%-70% of cases (O'Kane and Gehring, 1987). The *P*-element system was also incapable of inducing gene regulation in a specific biological pathway, as the transposon tends to insert non-randomly (Bellen, 1999).

Ds or *Ac*-based enhancer trap constructs were designed containing the shortest 5' end and the core sequence of the CaMV 35S promoter, so expression of the reporter gene was dependent on insertions near the chromosomal enhancer sequence in plants (Sundaresan et al., 1995a). *DsE* has been applied in *Arabidopsis* and rice (Fedoroff and Smith, 1993; Klimyuk et al., 1995; Sundaresan et al., 1995a; Chin, 1999), resulting in about 48% of transpositions associated with reporter gene expression patterns in various organs (Sundaresan et al., 1995a), and patterns which were stably inherited in subsequent generations (Klimyuk et al., 1995). The higher frequency of staining obtained with *DsE* is expected, as enhancers can act over a long distance and *DsE* insertions into both transcribed and non-transcribed regions of genes resulted in GUS reporter gene expression (Sundaresan et al., 1995a).

A T-DNA-based enhancer trap construct containing a minimal CaMV 35S promoter fused to the GUS reporter gene has been developed, where the reporter gene is located immediately next to the right T-DNA border. Enhancer trap lines in rice and *Arabidopsis* generated with these constructs exhibited tissue specific expression patterns (Campisi et al., 1999; Jeon et al., 2000b).

1.6.3.2 FURTHER DEVELOPMENT OF TRANSCRIPTIONAL ACTIVATOR-BASED ENHANCER TRAP

Because of the limitations of the *P*-element system, a new enhancer trap system, using a transcriptional activator was developed and employed first in *Drosophila*. The yeast transcription factor GAL4 activates transcription from a promoter containing a GAL4 binding site (Fischer et al., 1988). This activity was shown in yeast, *Drosophila*, and mammalian cells (Fasano and Kerridge, 1988; Kakidani and Ptashne, 1988; Webster et al., 1988). The GAL4 transcriptional activator functions through the recognition of a DNA binding domain of the upstream activating sequence of the GAL

gene (UAS_G), allowing transcription of any gene linked to the UAS (Fischer et al., 1988).

The GAL4/UAS enhancer trap system offers advantages over other enhancer trap systems. 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 as a “receptor” or target (Brand and Perrimon, 1993; Castelli-Gair et al., 1994). Targeted expression of toxin genes can also be used to kill or inactivate specific cells under investigation (Brand and Dormand, 1995).

The GAL4/VP16 transcriptional activator that comprises a DNA-binding domain from the yeast GAL4 gene and the activating domain VP16 of the Herpes Simplex virus was utilised in the more recent enhancer trap system to replace the GAL4. Similar to the GAL4, the GAL4/VP16 activates genes adjacent to the UAS (Fields, 1989). This system is also able to direct cell- or tissue-specific gene expression patterns in *Drosophila* (Brand and Perrimon, 1993) which may generate *Gain-of-function* phenotypes (Castelli-Gair et al., 1994; Brand and Dormand, 1995; Phelps and Brand, 1998). In addition, it activates gene expression about 10- to 100-fold higher than the GAL4 system (Sadowski et al., 1988). Until now, the use of the transcriptional activator facilitated enhancer trap system has been mostly limited to *Drosophila* and *Arabidopsis* (Brand and Perrimon, 1993; Haseloff, 2002).

1.7 THE PROJECT: DEVELOPMENT AND VALIDATION OF THE GAL4/VP16 TRANSCRIPTIONAL ACTIVATOR-FACILITATED ENHANCER TRAP SYSTEM FOR RICE FUNCTIONAL GENOMICS

1.7 THE PROJECT: DEVELOPMENT AND VALIDATION OF THE GAL4/VP16 TRANSCRIPTIONAL ACTIVATOR-FACILITATED ENHANCER TRAP SYSTEM FOR RICE FUNCTIONAL GENOMICS

This thesis reports the development of transcriptional activator facilitated enhancer trap (TAFET) constructs to reveal expression patterns in rice, *Oryza sativa* var. Millin and Nipponbare and validation of the system in rice.

During preparation of the thesis, a paper on a similar system applied in rice was published by Wu et al (2003). Indeed, the single construct applied by Wu et al (2003) in *Indica* rice variety 9311, was developed by CAMBIA, as part of the Rice Trans-Genomics Project. Contrary to what the paper described, the construct actually used was a reporter fusion of β -glucuronidases GUSPlus (Nguyen, 2002) and enhancer Green Fluorescence protein (EGFP) (Fu, 2004). Importantly, most data in this thesis were generated prior to the development of enhancer trap lines reported by Wu et al (2003). Instead eight transactivator constructs and two transactivator deletion constructs that were developed and tested to facilitating of enhancer trap in *japonica* rice variety Nipponbare and Millin, were presented in this thesis.

The project was carried out at the Center for the Application of Molecular Biology to International Agriculture (CAMBIA), and had the following purposes:

- to develop the GAL4/VP16 transcriptional Activator-facilitated enhancer trap (TAFET) system for rice functional genomics;
- to validate the functionalities of the TAFET system in rice and
- To produce GAL4/VP16-facilitated enhancer trap rice lines for rice functional genomics.

Chapter 2

MATERIALS AND METHODS**2.1 MATERIALS**

Almost all chemicals, amino acids and hormones used in the experiments were Sigma-Aldrich product, unless it was stated differently.

2.1.1 Bacterial media**LB (1 L H₂O)**

Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

SOC (1 L H₂O)

Bacto yeast extract	5 g
Bacto tryptone	2 g
NaCl	5 g
MgCl ₂ · 6H ₂ O	2 g
KCl	0.2 g
Glucose	3.6 g

AB (1 L H₂O)

Glucose	5 g
Bacto agar	15 g
H ₂ O	900 mL

Autoclave at 120° C for 20 minutes, then add

20X AB buffer	5 mL
20X AB Salts	50 mL

AB buffer, 20X (500 mL H₂O)

K ₂ HPO ₄ ·3H ₂ O	30 g
--	------

NaH ₂ PO ₄	10 g
----------------------------------	------

AB Salts, 20X (500 mL H₂O)

NH ₄ Cl	10 g
--------------------	------

MgSO ₄ ·7H ₂ O	3 g
--------------------------------------	-----

KCl	1.5 g
-----	-------

CaCl ₂ ·2H ₂ O	0.15 g
--------------------------------------	--------

FeSO ₄ ·7H ₂ O	0.025 g
--------------------------------------	---------

2x YT Medium (1L H₂O)

Tryptone	16 g
----------	------

Yeast Extract	10 g
---------------	------

NaCl	5 g
------	-----

Adjust to pH 7.0 and autoclaved

2.1.2 Rice tissue culture media (Hiei et al., 1994)**2N6 (Callusing medium) (1 L H₂O)**

N6 salts 10X (Sigma)	100 mL
----------------------	--------

2,4-D (1 mg/mL)	2 mL
-----------------	------

Chu's vitamins (100X)	10 mL
-----------------------	-------

Casamino acids	1 g
----------------	-----

Glutamine	0.5 g
-----------	-------

Proline	0.5 g
---------	-------

Sucrose	30 g
---------	------

Add H₂O to dissolve and adjust to pH 5.8

Add 2.5 g of Phytagel and adjust volume to 1 L

2N6-AS (Co-cultivation medium) (1 L H₂O)

N6 salts 10X	100 mL
--------------	--------

2,4-D (1 mg/mL)	2 mL
Chu's vitamins (100X)	10 mL
Casamino acids	1 g
Sucrose	30 g

Add H₂O to dissolve and adjust to pH 5.2.

Add 2.5 g of Phytigel and adjust volume to 1 L.

Autoclave, and after cooling to about 60°C add 1 mL of 100 µM acetosyringone.

2N6-TCH (selection medium) (1 L H₂O)

N6 salts 10X	100 mL
2,4-D (1 mg/mL) (sigma)	2 mL
Chu's vitamins (100X)	10 mL
Casamino acids	1 g
Sucrose	30 g

Add H₂O to dissolve and adjust to pH 5.2.

Add 2.5 g of Phytigel and adjust volume to 1 L.

Autoclave, and after cooling to about 60°C add:

Timentin 100 mg/mL (GllaxoSmithKline)	1 mL
Cefotaxime (Claforan) 250mg/mL (Hoechst Marion Roussel)	1 mL
Hygromycin B 50mg/mL (Boehringer Mannheim)	1 mL

RGH6 (regenerating medium) (1 L H₂O)

N6 salts 10X	100 mL
2,4-D (1 mg/mL)	2 mL
Chu's vitamins (100X)	10 mL
Casein enzymatic hydrolysate	1 g
Glutamine	0.5 g
Proline	0.5 g

BAP (1mg/mL)	3 mL
NAA (1mg/mL)	0.5 mL
Sucrose	30 g

Add H₂O to dissolve and adjust to pH 5.8.

Add 2.5 g of Phytigel and adjust volume to 1 L.

Autoclave, and after cooling at about 60°C add 1 mL of hygromycin B (50 mg/mL).

½MS-H (1 L H₂O)

MS salts 10X	50 mL
Chu's vitamins (100X)	5 mL
NAA (1 mg/mL)	0.5 mL
Sucrose	10 g

Add H₂O to dissolve and adjust to pH 5.8.

Add 2.5 g of Phytigel and adjust volume to 1 L.

Autoclave and after cooling at about 60°C add 1 mL of hygromycin B (50 mg/mL).

2,4-Dichlorophenoxyacetic acid (2,4-D, Sigma) 1 mg/mL

Dissolve 100 mg 2,4-D in 100 mL of methanol. Do not autoclave. Keep the solution at 4°C.

Chu's vitamins 100X (500 mL) (Chu, 1978)

Nicotinic acid	25 mg
Pyridoxine	25 mg
Thiamine-HCl	50 mg
Myo-inositol	5 mg

Filter sterilize.

N6 Salts (1 L) (Chu, 1978)

$(\text{NH}_4)_2\text{SO}_4$	463 mg
KNO_3	2.83 g
CaCl_2	125.3 mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.85 mg
MgSO_4	90.37 mg
KH_2PO_4	400 mg
MnSO_4	3.33 mg
H_3BO_3	1.6 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.5 mg
KI	0.8 mg
$\text{Na}_2\text{-EDTA}$	37.25 mg

MS Salts (1 L H_2O) (Murashige and Skoog, 1962)

NH_4NO_3	1.65 g
H_3BO_3	6.2 mg
CaCl_2	332.2 mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025 mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025 mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.85 mg
$\text{Na}_2\text{-EDTA}$	37.25 mg
MgSO_4	180.7 mg
MnSO_4	16.9 mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25 mg
KI	0.83 mg
KH_2PO_4	170 mg
KNO_3	1.9 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6 mg

AAM medium (1 L H₂O) (Hiei et al., 1994)

AA amino acid	10 mL
AA macro	100 mL
AA micro	1 mL
AA iron	10 mL
AA-ms Vit	10 mL
CA	0.5 g
Sucrose	68.5 g
Glucose	35 g
pH 5.2, dispense to 4 x 250 mL and autoclave	

Acetosyringone (100 µM)

16.6 mg of acetosyringone (3'.5'-dimethoxy-4-hydroxy-acetophenone) in 1 mL of dimethyl sulfoxide (DMSO). Do not autoclave.

2.1.3 Soil Mix

Potting mix	75%
Perlite	25%
Osmocote	1 g/L

Potting Mix

River loam	60%
Peat moss	20%
River sand	20%

2.1.4 Binary plasmid for rice transformation (pCAMBIA1201)

Plasmid pCAMBIA1201 (Fig. 2.1) is a binary vector derived from the pPZP vector (Hajdukiewicz et al., 1994). It contains the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985) driving the selectable Hygromycin phosphotransferase (*hpt II*) gene (Gritz and Davies, 1983), a pUC18 multiple cloning site (MCS) and the β -glucuronidase (*gus A*) reporter gene (Jefferson et al., 1987), located between left and right T-DNA borders. The N-terminus of the *gus A* coding sequence contains the castor bean catalase intron (Tanaka, 1990) for optimal *gus A* expression and to prevent the expression of *gus A* in bacterial cells.

2.1.5 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain EHA 105 is a kanamycin sensitive strain derived from *A. tumefaciens* strain EHA 101 (Hood et al., 1986; 1993) for use in rice transformation.

2.1.6 Rice seeds

Most experiments were done with the Australian Japonica rice variety Millin, provided by Dr. Russell Reinke from the Yanco Experimental Station of the New South Wales Department of Agriculture. The Japanese Japonica rice variety Nipponbare was also used and the seed provided by Dr Sasaki from The RGP, Tsukuba, Japan.

2.1.7 GUS Staining Solution (100 mL H₂O)

0.1 M sodium phosphate buffer	50 mL
EDTA 0.5 M pH 8.0	2 mL
Triton X-100	100 μ l

Potassium ferrocyanide, $K_4[Fe^{II}(CN)_6]$	90 mg
Potassium ferricyanide, $K_3[Fe^{III}(CN)_6]$	66 mg
X-GlcA (Duchefa Biochemie)	
(XGlcA stock 50 mg/mL, dissolved in DMSO)	100 mg

2.2 METHODS

2.1 Restriction digestion

All restriction endonucleases used were purchased from New England Biolabs (NEB) and were used according to the manufacturer's instructions.

2.2.2 Phosphorylation

DNA phosphorylation was conducted at 37°C for 1 hour by addition of 2 units of polynucleotide kinase in the presence of 1X T4 ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/mL BSA, pH 7.5). The reaction was terminated by heat inactivation at 65°C for 20 minutes. The DNA was purified by column purification (Qiagen Nucleotide Removal Kit) and eluted in 25 µL TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) (Sambrook et al., 1989).

2.2.3 Dephosphorylation

DNA dephosphorylation was conducted in a 25 µL reaction containing 2 units of shrimp alkaline phosphatase (SAP, Boehringer Mannheim) in the presence of 1X dephosphorylation buffer (10X buffer contains 0.5 M Tris-HCl and 50 mM $MgCl_2$, pH 8.5) at 37°C for 1 hour. The reaction was terminated by heat inactivation at 65°C for 20 minutes.

2.2.4 Blunt-Ending and Phosphorylation

The DNA fragment was added to a mixture containing:

2 units of T4 DNA polymerase

5 units of T4 polynucleotide kinase in 20 μ L reaction mixture

1X T4 ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP, 25 μ g/mL BSA, pH 7.5)

0.5 mM dNTPs (Amersham Pharmacia).

The reaction was carried out at 37°C for 1 hour, and then terminated by heat inactivation at 65°C for 20 minutes. The DNA was purified by column purification (Qiagen Nucleotide Removal Kit) and eluted in 25 μ L TE buffer (pH 8.0).

2.2.5 DNA purification

2.2.5.1 Gel purification

DNA fragments were isolated and purified from agarose gels using a Qiaquick gel extraction kit (Qiagen). Samples were separated by electrophoresis in a 1.0% agarose gel in 1X TAE containing 0.5 μ g/mL ethidium bromide. The DNA was excised from the gel and placed in a 1.5 mL eppendorf tube. The tube was weighed and the gel was dissolved with 3 mL/1mg of QC buffer added to the tube. This mixture was incubated at 50°C for a few minutes until the agarose had completely dissolved. The solution was then applied to a Qiaquick spin column which was placed in a 2 mL collection tube, and centrifuged at 10,000 xg for 1 minute using a Hermle benchtop centrifuge. The flow-through was discarded and the column was placed back in the collection tube. The column was washed by applying 0.750 mL of PE buffer, and was centrifuged at 10,000 xg for 1 minute. The flow-through was discarded and the column centrifuged again for an additional minute. The column was then placed into a clean 1.5 mL eppendorf tube and 50 μ L of EB buffer added to the centre of the column. The

column was incubated at room temperature for 1 minute and then centrifuged at 10,000x g for 1 minute. Composition of buffers used is not printed in the products catalog.

2.2.5.2 Nucleotide removal

DNA fragments were purified using a Qiaquick Nucleotide Removal kit (Qiagen). Ten volumes of PN buffer were added to 1 volume of sample and mixed. The mixture was then placed into a Qiaquick spin column, which was then placed in a 2 mL collection tube, and centrifuged at 5,000 xg for 1 minute. The flow-through was discarded and the column was placed back in the collection tube. The column was washed by applying 0.75 mL of PE buffer, and then centrifuged at 5,000 xg for 1 minute. The flow-through was discarded and the column centrifuged once more at 10,000 xg for 1 minute to remove residual ethanol. The column was placed into a clean 1.5 mL eppendorf tube and 50 µl of EB buffer added to the centre of the column. The column was incubated at room temperature for 1 minute and centrifuged at 10,000 xg for 1 minute (Qiagen product catalog). Composition of buffers used is not printed in the products catalog.

2.2.6 Ligation

DNA ligation was performed in a 15 µl reaction comprising 100 units of T4 DNA ligase (NEB), 1X T4 ligation buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 µg/mL BSA, pH 7.5), and incubated at 16°C overnight. T4 DNA ligase was inactivated by incubation at 65°C for 10 minutes.

2.2.7 Preparation of electrocompetent cells

A single bacterial colony of *Escherichia coli* strain DH5 α and *Agrobacterium tumefaciens* strain EHA105 were inoculated into 3 mL of LB medium (refer to 2.1.1) and grown overnight at 37°C and 29°C, respectively. One mL of the culture was inoculated into 1L of 2YT (refer to 2.1.1) medium in a sterile 2 L flask. The culture was grown at 37°C (or 29°C for *Agrobacterium*) by shaking at 200 xg to reach 0.7 to 0.9 values at an optical density (OD) 600nm. Cells were chilled on ice for 10 minutes. The culture was then centrifuged at 5,000 xg for 10 minutes at 4°C using a JLA rotor in a Beckman centrifuge. The supernatant was discarded and the pellet was re-suspended in 2.5 mL ice-cold sterile H₂O by pipetting it in and out several times. The re-suspended cells were washed with 100 mL ice-cold sterile H₂O by inverting the tube several times. The cells were centrifuged as above and the pellet washed again. Washing was repeated two times. After the third wash cells were centrifuged at 6,000 xg for 10 minutes at 4°C. The supernatant was poured off and the pellet re-suspended in 40 mL of 10% glycerol. Cells were centrifuged at 6,500 xg for 10 minutes at 4°C, the supernatant was poured off and the pellet was re-suspended in 2 mL of 10% glycerol. The cells were left overnight at 4°C on ice. The cells were then divided into 150 μ L aliquots and snap frozen in liquid nitrogen.

2.2.8 Electroporation

Transformation was done using a Gene Pulser apparatus (Biorad). One to 2 μ L of plasmid solution or ligation mix was mixed gently with 50 μ L of DH5 α competent cells which had been on ice. This mixture was then transferred into a 0.2 mL pre-chilled Gene Pulser cuvette. The transformation was carried out by applying an electric pulse using the following settings: 200 Ω resistance, 25 μ F capacitance and 2.5 K voltage. Half a millilitre of SOC (refer to 2.1.1) was added to the cell mixture and the mixture

incubated at 37°C for 30 minutes. Selection for transformants was conducted by plating 50–100 µL of the mixture onto solid media containing the appropriate antibiotic. This procedure was also applied for *Agrobacterium* transformation.

2.2.9 Small scale plasmid DNA isolation

Small scale plasmid DNA isolation was carried out by using the CTAB (cetyltrimethylammonium bromide) method (Del Sal et al., 1989). About 10 to 20 colonies were picked from solid media and inoculated into test tubes containing 2 mL liquid media with the appropriate antibiotic. The cultures were grown overnight in a shaker incubator at 200 xg at 37°C. Cells were collected by centrifugation at 800 xg for 10 minutes using a Hermle benchtop centrifuge. Supernatants were aspirated completely and pellets were resuspended by vortexing in STET buffer (8% sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 0.1% Triton-X-100). Four microlitre of lysozyme (Sigma) solution (10 mg/mL in 10 mM Tris-Cl pH 8.0) and 4 µL of RNase A were added into re-suspended cells (50 mg/mL in STET) and mixed by quick vortexing. The mixture was incubated at room temperature for 10 minutes and then put into boiling water for 45 seconds, centrifuged at 13,500 xg for 10 minutes and pellets removed using toothpicks. 10 µL of 5% (w/v) CTAB was added into tubes, vortexed and incubated at room temperature for 10 minutes. DNA was collected by centrifugation at 13,500 xg for 10 minutes and the supernatant was aspirated. Pellets were re-suspended in 300 µL of 1.2M NaCl by vortexing. The plasmid DNA was precipitated by adding 750 µL of 96% ethanol and followed by incubation at -80°C for 10 minutes. The DNA pellets were collected by centrifugation at 13,500 xg for 15 minutes. Supernatants were aspirated and pellets were washed with 700 µL of 70% ethanol. After air drying the pellets were dissolved in 20-50 µL TE buffer or sterile water.

2.2.10 Isolation of rice total genomic DNA

Approximately 0.5 g of rice tissue was ground in a mortar with liquid nitrogen. The finely ground tissue was added to 50mL tubes (Oakridge) containing 14 mL preheated (65°C) S-buffer (110 mM Tris-HCl pH8.0, 55 mM EDTA pH8.0, 1.54 M NaCl and 1.1% CTAB). The mixture was vortexed vigorously for a few seconds. 700 µL of 20% SDS (sodium dodecyl sulfate) was added and mixed carefully. The mixture was incubated at 65°C for 2 hours. During incubation the tube was shaken lightly for a few seconds by hand. After cooling at room temperature, 7 mL of chloroform and iso-amyl-alcohol (24:1) was added. The tube was shaken gently using a rotor for about 15 minutes at room temperature to form an emulsion. It was then centrifuged in a Beckman centrifuge at 4500 xg for 20 minutes at 4°C to pellet the debris. The upper phase was transferred into a clean Oakridge tube and 0.7 volumes of 2-propanol (prop-2-nol) was added. The tube was immediately inverted, gently and repeatedly, until DNA precipitation occurred. After DNA precipitation was observed, gentle mixing was continued for another minute. The mixture was then centrifuged at 4500 xg for 20 minutes to pellet the DNA. The pellet was re-suspended with 96% ethanol and transferred to 1.5mL eppendorf tube. The mixture was centrifuged at 13,500 xg for 10 minutes. The supernatant was aspirated and the pellet was washed with 70% ethanol. The tube was centrifuged at 13,500 xg for 5 minutes. The ethanol was poured off and the DNA was dissolved in 0.5 mL of TE pH 8.0 (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). To remove RNase, 0.5 µL of 10 mg/mL RNase A (Sigma) that dissolved in 10 mM Tris-Cl and 15 mM NaCl and boiled at 95°C for 15 minutes was added and the tube was incubated at 55°C for 2 hours.

2.2.11 DNA Quantification (Sambrook et al., 1989)

DNA was quantified by measuring its absorbance at 260 nm and the concentration was calculated from reference values ($A_{260} = 1 = 50 \mu\text{g/mL dsDNA}$).

2.2.12 DNA sequencing

Sequencing was conducted using the ABI PRISM Dye Terminator Cycle Sequencing Ready kit (Perkin Elmer) with half the recommended concentration. Five hundred nanograms of plasmid or 100 ng of PCR product was used as a template in the presence of 3.2 pmole of primer and 2 μ L of dye terminator reaction mix in a 10 μ L reaction in 0.1 mL PCR strip tubes (Sarstedt). The reaction was carried out for 30 cycles in a Corbett FTS-960 thermal sequencer (Corbett Research) under the following conditions: 95°C denaturation for 10 seconds, 50°C annealing for 10 seconds and 60°C extension for 4 minutes. Following the sequence reaction, the mixture was transferred into 1.5 mL eppendorf tubes and the DNA was precipitated by adding 1 μ L of 3 M sodium acetate pH 3.8 and 20 μ L absolute ethanol. The reaction was mixed by tapping the tubes which were then incubated at -80°C for 10 minutes. The DNA was pelleted by centrifugation at 13,500 xg in a bench top centrifuge for 10 minutes. The supernatant was removed and the pellet was washed twice with 50 μ L of 70% ethanol. The pellet was air-dried and the tube covered with aluminum foil. The sample was sent to The Australian Genome Research Facility (AGRF, Brisbane) for gel separation. The result was retrieved from the AGRF file transfer protocol site using the file transfer program WS_FTP LE version 5.8 and was viewed using the Chromas software (version 1.45).

2.2.13 PCR amplification

A hundred picograms per μ L DNA was used as a template in a PCR reaction containing 1 μ M of each forward and reverse primers in the presence of 200 μ M dNTP mix, 1X RedTaq PCR buffer (containing $MgCl_2$) (Sigma) and 0.05 unit/ μ L RedTaq DNA polymerase (Sigma). Amplification was carried out over 30 to 35 cycles depending on the requirements, under the following conditions: denaturation at 95°C for 30 seconds, annealing temperature at 50 to 55°C (depended on GC content of primers) for 30

seconds and extension at 72°C for 2 minutes. An initial denaturation step at 95°C for 2 minutes before cycling and an extension at step 72°C for 5 minutes after cycling were applied. The PCR amplification reaction was terminated at 4°C.

2.2.14 Southern Hybridisation

2.2.14.1 Preparation of target DNA

Digested DNAs were separated in 0.7% 20x20cm agarose gels in 1X TAE running buffer at 0.5 V/cm. The DNA fragments were then transferred overnight onto a positively charged nylon membrane (Boehringer Mannheim) by an alkaline transfer method using 0.4 N NaOH and 0.6 N NaCl as the transfer buffer (Sambrook et al., 1989). Following the transfer, the membrane was washed briefly in 2X SSC to remove excess salt, and the DNA was fixed on the membrane by baking at 80°C for 2 hours.

2.2.14.2 Pre-hybridisation and hybridization

The baked membrane was pre-hybridised in 50mL pre-warmed (65°C) pre-hybridisation solution containing 1X HSB and 1X Denhardt's solution for 5 hours at 65°C in a plastic container in an oven. After pre-hybridisation membranes were hybridised in 20 mL hybridisation solution containing 1X HSB (5X HSB contains 0.1 M PIPES pH 6.8, 3 M NaCl and 20 mM EDTA pH 8.0), 1X Denhardt's solution [100X Denhardt's contains 2% (w/v) bovine serum albumin (Sigma), 10% (w/v) SDS, 2% (w/v) Ficoll (type 400, Sigma), 2% (w/v) PVP (Polyvinylpyrrolidone), 5 mM Na₄P₂O₇·10 H₂O] (Sambrook et al., 1989) and 2 mL (10 mg/mL) denatured herring sperm DNA and a radioactive labeled probe for about 18 – 24 hours at 65°C in the plastic container in the oven. The radioactively labelled probe was prepared about 20 minutes before pre-hybridisation finished. It was added to the hybridisation solution last and after it had been denatured in boiling water (about 95°C) for 5 minutes.

2.2.14.3 Preparation of radioactively labelled probe

A template for the probe was prepared either from a digested DNA or a PCR product. About 20-30 ng denatured DNA was mixed in a 50 μ L solution containing 500dCTP, 500dTTP, 500dGTP, 4 μ L α P³²-dATP, 20 μ L buffer mixed, 1 μ L Klenow polymerase enzyme and H₂O. The DNA template was denatured in water at 95°C for 5 minutes. The reaction was carried out at 37°C for 10 minutes.

2.2.14.4 Washing

After hybridisation, the membrane was washed in about 300 mL of low stringency washing solution (2X SSC and 1% (w/v) SDS) (20X SSC contains 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H₂O, then adjust the volume to 1 L with H₂O), followed by a wash in 300 mL of medium stringency (1X SSC and 0.5% SDS) and lastly in 300 mL of high stringency solution (0.5X SSC and 0.25% SDS). Each wash was conducted for 20 minutes at 65°C.

2.2.14.5 Autoradiography

Hybridisation signal on the membrane was detected by using either a Biorad GS-250 Molecular Imager or X-ray film. The membrane was wrapped in vinyl wrap and taped either on the lid of the sample loading dock of the imager or on the reflection screen of the X-ray cassette. The molecular imaging screen B1 was exposed to the membrane overnight or longer depending on the strength of the signal detected by a Geiger counter. A Geiger counter was also used to determine a time to store the X-ray cassette at -80°C. The signal was analysed using the molecular analysis program of the GS-250 Molecular Imager in 100 or 200 μ m resolution mode. The signal from the

membrane in the cassette was captured directly on Kodak autoradiography film placed between the membrane and the reflection screen.

2.2.14.6 Stripping

The ^{32}P -labelled probe was removed from the membrane by placing the membrane in 200 mL of pre-heated stripping buffer (0.1X SSC and 0.1% SDS) in a plastic container at 85°C for about 30 minutes.

2.2.15 Computer analysis

2.2.15.1 Plasmid DNA constructions and annotations

Construction and annotation of all plasmids and DNA fragments were conducted using Vector NTI version 4.5 software (Informax). Plasmids were named after the creator or the investigator, the notebook number and the page of that particular notebook on which the plasmid was first confirmed. For example, pSKC66.1 was generated by Sri Koerniati and was documented in notebook C, page 66 and from the colony in lane 1 of the gel confirming the intensity of the molecule.

2.2.15.2 BLAST homology search

Confirmation of known DNA sequences was conducted using the Genbank (www.ncbi.nlm.nih.gov) BLAST version 2.0.

2.2.16 Rice Transformation (Hiei et al., 1994)

Agrobacterium tumefaciens mediated transformation in rice used in this experiment was as developed by Hiei et al. (1994).

2.2.16.1 Transformation of binary plasmid to *Agrobacterium tumefaciens*

Binary plasmids were introduced into *A. tumefaciens* by electroporation. One microgram of plasmid was used for the electroporation. Transformants were selected on LB medium containing 50 µg/mL kanamycin. Successful transformation was confirmed by suspending 5 colonies in 10 µl of water and then using 1 µL for PCR amplification of the insert with XL-polymerase.

2.2.16.2 Seed sterilisation and plating

Twenty grams of de-husked rice seeds were used as starting material for one transformation. Seeds were sterilised using a solution containing 16 mL of 70% (v/v) bleach (NaOCl), 4 mL sterilised water, 2 drops Tween-20, and incubated on a rotating wheel at room temperature for 20 minutes. Seeds were first washed with 70% ethanol and then with sterilised water 2 to 3 times. After incubation, seeds were washed several times with sterilised water until the smell of bleach disappeared (5 to 6 times, performed in a laminar flow cabinet). Seeds were then dried for about 20-30 minutes before they were plated on 2N6 medium.

2.2.16.3 Embryonic callus induction

Seeds plated on 2N6 medium were incubated in the dark at 25°C for about 4 weeks. Calli produced were cut into pieces approximately 5 mm in diameter, plated on fresh 2N6 and incubation was continued under the same condition as before for 4 days, after which they were ready to be used for *A. tumefaciens* mediated transformation (co-cultivation).

2.2.16.4 Co-cultivation

Three days before co-cultivation, *A.tumefaciens* EHA105 containing the plasmid to be transformed into rice was streaked onto AB solid medium containing 50 µl/mL of ampicillin. The *Agrobacterium* was grown at 29°C. After 3 days incubation, it was scraped from the AB plate, re-suspended in a 50 mL tube of AAM liquid (Hiei et al., 1994) medium containing 100 µM acetosyringone, and left at RT for 1 hour until an OD₆₀₀ of approximately 1.0 was reached. The 4 day-old calli were then added to this suspension, mixed by swirling, and left at RT for 1 hour to allow contact to occur. Calli were then placed onto sterile filter paper (3M Whatman no.1 paper) to remove excess medium without allowing the calli to become dry. Calli were then transferred to co-cultivation medium (2N6-AS) and co-cultivated for 3 days at 25°C in the dark.

2.2.16.5 Selection, sub-culturing and regeneration

After 3 days of co-cultivation, the *Agrobacterium* was removed from the calli. Calli were washed using sterile water containing of 250 mg/mL cefotaxime (Claforan, Hoechst Marion Roussel) and mixing well. Washing was conducted 3 to 4 times until the washing solution turned clear. Calli were left for about 0.5-1 hour in the solution between washes. Calli were transferred onto sterile filter paper (Whatman no.1 paper, 3M) to remove the excess medium without allowing the calli to become dry. The calli were then transferred onto selection media 2N6-CH plates containing 50 µl/mL of hygromycin, and incubated at 25°C in the dark. Calli were sub-cultured regularly onto fresh medium every 2 weeks, until good sized proliferating calli were obtained.

After proliferating calli reached a size of about 0.5-1 cm in diameter, the lines were transferred to regeneration medium RGH6, and incubated in the dark at 25°C for 7 days. The lines were then transferred to light at 25°C until plantlets were obtained.

Plantlets were transferred onto 1/2MS-H medium containing 50 µl/mL of hygromycin. After roots were well developed, the plants were transferred into the greenhouse. Growing conditions were set to a maximum of 29°C and minimum of 18°C and conditions were monitored both by HOBO data logger (Onset Computer Corp.) and computer in The Australian National University's Research School of Biological Science (RSBS) transgenics greenhouse.

2.2.17 Histological assay

Various plant tissues (roots, leaves and flowers) were freshly collected from several developmental stages (early, medium and old) for the histological assay. The tissues were placed into GUS solution and a vacuum applied for 5 minutes. The vacuum was released slowly to allow the solution to get into the tissue, and the tissue/GUS solution mixture was then incubated at 37°C for 24 hours. The GUS solution was drained from the tissue and 70% ethanol added to remove chlorophyll. Reporter gene expression was observed under the microscope after 3 days of ethanol treatment. The GUS stain in the tissue is stable for at least one year.

2.2.18 Microscopy

Samples were observed using a Leica Wild M8 microscope and a Leitz Diaplan microscope with bright-field optics setting. Images were obtained with a Nikon CoolPix Digital photo camera. Expression of Green Fluorescent protein (GFP) was analysed with a Leica MZFLIII using a Leica GFP3 filter set with 480/40 nm excitation setting, and images obtained with a Nikon N-2000 photo camera. Some samples were also observed with a Hitachi 4500 Field Emission Scanning Electron Microscope (Vesk et al., 1994).

2.2.19 Statistical analysis

ANOVA and regression analysis of the histological reporter gene expression data from the T_0 generation tissues were carried out using Genstat version 6.2 software. χ^2 (Chi-square) tests were carried out to define heterogeneity of plants in families of the T_1 and T_2 generation.

Chapter 3

DEVELOPMENT OF GAL4/VP16 TRANSCRIPTIONAL ACTIVATOR-FACILITATED ENHANCER TRAP SYSTEM IN RICE

3.1 INTRODUCTION

Enhancer trap systems have been generated by several laboratories in the past few years (Klimyuk et al., 1995; Sundaresan et al., 1995a; Campisi et al., 1999; Jeon et al., 2000a). Transposable element- (Klimyuk et al., 1995; Sundaresan et al., 1995a) or T-DNA-based (Campisi et al., 1999; Jeon et al., 2000b) enhancer trap constructs have been applied in *Arabidopsis* and rice, respectively. Enhancer trap constructs consisting of a minimal promoter fused to a reporter gene have been applied and various cell- or tissue-specific expression patterns have been detected in the enhancer trap lines through GUS reporter gene expression (Klimyuk et al., 1995; Sundaresan et al., 1995a; Jeon et al., 2000b).

More recent enhancer trap constructs have contained the transcriptional activator GAL4 or GAL4/VP16. These constructs have been previously used in *Drosophila* (Brand and Perrimon, 1993; Brand and Dormand, 1995) and their application in plants is still limited to *Arabidopsis* (Kiegle et al., 2000; Haseloff, 2002).

The main aims of the project were two fold: to develop a transcriptional activator-facilitated enhancer trap system, and to test whether this system would be able to reveal gene expression in a tissue-specific (spatial) fashion and/or during developmental stages (temporal) in rice.

In general, gene expression in eukaryotic cells is regulated when transcriptional activators (*trans*-acting proteins) directly interact with one or more general transcription factors (GTFs), facilitating the recruitment of basal factors to *cis*-acting elements (TATA box and enhancers) in promoters (Alberts et al., 1994). Gene transcription by RNA polymerase II in eukaryotic cells requires the assembly of a pre-initiation complex, comprising the general transcription factors: a TATA binding protein (TBP), a transcriptional factor IID (TFIID), a TFIIB, a TFIIE and a TFIIH. The transcriptional activator binds directly to the TATA-binding protein (TBP), a component of TFIID *in vitro* (Stringer et al., 1990; Ingles et al., 1991).

Cis-acting elements (TATA boxes), which are components of gene promoters (about 100 base pairs) lie immediately upstream of the transcription units, upstream activating sequences (UAS) and only operate in close proximity to transcription initiation sites (Martin, 2001). In contrast, enhancers which link *cis* to promoters (upstream or downstream) and are binding sites for transcriptional activators, act from several hundred base pairs away from the transcription start sites (Bohmann et al., 1987; Martin, 2001). When an enhancer is placed close to a promoter, it is difficult to distinguish it from the UAS, because both are bound by the same factor. The binding of an enhancer to a UAS produces much closer distances between regulatory proteins to the second critical *cis*-acting element, the UAS in the TATA box (Guarente, 1988; Alberts et al., 1994), and in eukaryotic cells, enhancers are necessary for gene transcription (Guarente, 1988).

The most comprehensively studied transcriptional activator is the yeast *Gal* gene transcriptional activator GAL4. GAL4 has two protein domains with distinct functions required for its activity: domain 1 is a DNA binding domain located in the N-terminal 147 amino acids of the GAL4 activator protein, directing a sequence-specific

binding, while domain 2 is an activation region which interacts with components of the basal transcription complex. The GAL4 DNA binding domain 1 recognises and binds to specific sequences in the upstream activating region (UAS_{GAL4}). Domain 2 activates gene transcription when it binds to the DNA-binding domain (Fischer et al., 1988). This domain activates a minimal promoter bearing only the UAS and TATA box in *Drosophila* and plants (Fasano and Kerridge, 1988; Fischer et al., 1988) and is an acidic region with an amphipathic α -helix located in the carboxyl end of the protein (Giniger and Ptashne, 1987).

The transcriptional activator VP16 has also been studied thoroughly. A component of the virion of Herpes Simplex Virus (HSV), its role is to activate the expression of the viral immediate early (*IE*) genes. This activator comprises two domains, one located within the carboxyl-terminal 80 amino acids of the VP16 polypeptide, which is acidic, and the other in the N-terminal region of the protein (Friedman et al., 1988). The acidic region is necessary and sufficient for transcriptional activation when it is fused to the DNA binding domain of a gene from another organism (Friedman et al., 1988; Sadowski et al., 1988). Disruption of the acidic region, especially a phenylalanine residue, affects the ability of this transcriptional activator to activate transcription (Friedman et al., 1988; Cress and Triezenberg, 1991). Mutants of VP16 with reduced negative charges appear to be more affected in their DNA binding than in activation ability (Ingles et al., 1991). Moreover, these changes affect the ability of the VP16 mutant to form open promoter complexes (Jiang et al., 1994) which are common targets for transcriptional activators in a bacterial system.

The GAL4/VP16 transcriptional activator is a fusion between the VP16 activating domain and the GAL4 DNA binding domain (Sadowski et al., 1988). This protein fusion works the same way as the GAL4 which is a universal transactivator (Ptashne and Gann, 1990). However, the GAL4/VP16 fusion protein activates

transcription especially well, stimulating the transcription of a promoter bearing GAL4 sites (the UAS_{GAL4}) 10- to 100-fold higher than the native GAL4 transcription rate (Sadowski et al., 1988). Due to the high efficiency of GAL4/VP16 in the activation of gene transcription, it has been employed in yeast, plant, insect and animal cell systems (Aoyama and Chau, 1997; Koster and Fraser, 2001).

Among the most interesting applications of the GAL4/VP16 transactivator is its use in novel enhancer trap systems. Initially a transactivator-based enhancer trap system was developed and applied in *Drosophila* (Brand and Dormand, 1995) and has recently been extended to other systems. In plants, it has been used to generate about 10,000 *Arabidopsis* enhancer trap lines (Haseloff, 2002).

This chapter describes research aimed at the development and analysis of a population of pattern lines with special emphasis on the functional evaluation of transactivator-facilitated enhancer trap system components. Two reporter gene cassettes and two transactivator cassettes were employed in these studies. The effects of the relative position of various elements within the T-DNA on reporter gene expression in rice vegetative and floral tissues are also reported.

3.2 MATERIALS AND METHODS

3.2.1 DESIGN AND CONSTRUCTION OF TRANSCRIPTIONAL ACTIVATOR-FACILITATED ENHANCER TRAP BINARY VECTORS.

In order to develop an efficient Transcriptional Activator-Facilitated Enhancer Trap (TAFET) system, initial efforts were directed towards the evaluation of the principal components of the system and their interactions. The main component of an enhancer trap system is the reporter gene. We therefore attempted to evaluate two reporter genes: β -glucuronidases GUS and GUSPlus (Figure 3.2 and Table 3.1). The

GUS reporter gene is a widely used β -glucuronidase from *Escherichia coli* (Jefferson et al., 1987), whereas GUSPlus is a newly developed β -glucuronidase from *Staphylococcus* sp (Nguyen, 2002).

In the TAFET system, the transactivator is also a critical element. Since there were no reports of the use of GAL4/VP16 in a transactivator system at the start of the project, two different cassettes were evaluated: one cassette with the castor bean *catalase* gene intron 1 and another one without. In order to maximise the trapping ability with the GAL4/VP16, a minimal promoter (mp) of the transcriptional activator cassettes was always positioned at the right (5') border of the T-DNA.

The F1 replication origin, a coding sequence of *bla* and a replication origin of the pMB1 mutant of pBlue-Script backbone (total 3.2kb) are sequences providing a separation between the UAS-reporter gene and transactivator in plasmids of pSKC59.1 (Fig. 3.2), pSKC66.1 (Fig. 3.4), pSMRJ18 (Fig. 3.2) and pSMRJ17 (Fig. 3.4). Transactivator plasmids were also constructed to position a mGFP5ER gene closer to the Cauliflower Mosaic Virus (CaMV) 35S promoter, and UAS- β -glucuronidase reporters were relatively close (1.6kb) to or distant (7kb) from the CaMV35S promoter driving a hygromycin resistance gene (*hpt II*), for example the plasmid of pSKC59.1 (Fig. 3.2) and the plasmid pSKD76-1 (Fig. 3.3), respectively. The GAL4/VP16 transcriptional activator with a 108bp deletion of the GAL4 binding domain replaced a full-length GAL4/VP16 in plasmids pSKC59.1 and pSKC66.1 to generate pSKD15.1 and pSKD15.2, respectively (see Fig. 3.6 and Fig 3.7). The nomenclature and description of the binary vectors produced are shown in Table 3.1. All TAFET constructs also contained the mgfp5-ER reporter gene, but this reporter gene proved to have very low sensitivity and was observed only in the context of transactivation tests (presented in Chapter 6) to ensure lack of Green-Fluorescence background in TAFET lines used for crossing.

No	Binary vector description	GUS	No	GUSPLUS
1	35S_HPTII-5UAS_mGFP5ER-6UAS_βGlucuronidase-pBS_GAL4/VP16	pSKC59.1	5	pSMRJ18
2	35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_βGlucuronidase_GAL4/VP16	pSKD76.1	6	pSMRJ18R
3	35S_HPTII-5UAS_mGFP5ER-6UAS_βGlucuronidase-pBS-intron_GAL4/VP16	pSKC66.1	7	pSMRJ17
4	35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_βGlucuronidase-intron_GAL4/VP16	pSKD76.2	8	pSMRJ17R
9	35S_HPTII-5UAS_mGFP5ER-6UAS_βGlucuronidase-pBS_delGAL4/VP16	pSKD15.1		
10	35S_HPTII-5UAS_mGFP5ER-6UAS_βGlucuronidase-pBS-intron_delGAL4/VP16	pSKD15.2		

Table 3.1 TAFET constructs containing GUS and GUSPlus reporter genes. 35S: the CaMV35 promoter, HPTII: *hpt* II Hygromycin resistance gene, mGFP5ER: modified Green Fluorescent Protein, 5UAS, 6UAS: Upstream activation sequence of the GAL4, pBS: plasmid Blue-script, intron: the castor bean *catalase* intron, GAL4/VP16: transcriptional activator fusion.

3.2.1.1 Cloning of the GUS binary vectors: pSKC59.1 and pSKD76.1.

A pTG113 vector backbone containing the castor bean *Catalase-1* gene intron and the GUS gene, and the pDAMSNS-Luc L plasmid containing 6 repeats of the Upstream Activating sequence (6xUAS_{GAL4}) of the GAL gene were digested with *Hind* III and *Nco* I. The UAS sequence recognised by the GAL4 DNA binding domain in pDAMSNS-Luc L is GGAAGACTCTCCTCCG. The fragments were then excised from agarose gels, purified using the Qiagen gel extraction kits, and DNA fragments were subsequently ligated using T4 DNA ligase enzymes and transformed into DH5-α competent-cell. After incubating for 30 minutes in a 37°C shaker, aliquots of cells were plated on solid medium contained ampicillin¹⁰⁰ (100 µg mL⁻¹ AMP) and grown in a 37°C incubator overnight. Some individual colonies were cultured in LB liquid medium containing ampicillin¹⁰⁰ overnight. Plasmid DNAs was isolated using a CTAB method

(Del Sal et al., 1989) and identified by digestion with restriction enzymes *Hind* III/*Nco* I and *Spe* I. These experiments produced pSKC2.1 that has a 6xUAS_{GAL4}-catalase intron-GUS reporter gene, as shown in Figure 3.1.

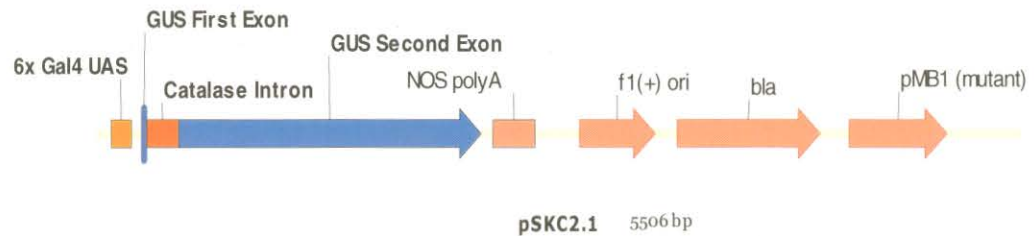


Figure 3.1 The plasmid pSKC2.1, containing 6xUAS_{GAL4}, the catalase intron and GUS reporter gene.

Plasmids pSKC2.1, containing the 6xUAS_{GAL4}-catalase intron-GUS reporter gene, and pFX-B61-1, containing the GAL4/VP16, were digested with *Hind* III restriction enzyme. DNA purification and ligation were performed using procedures described in Chapter 2, page 30-32. After incubation for 30 minutes at 37°C, cells were plated on LB solid medium with chloramphenicol¹⁰⁰, and incubated overnight at 37°C. Some individual colonies were cultured in LB liquid media containing chloramphenicol¹⁰⁰ overnight. DNA was isolated using a CTAB method (Del Sal et al., 1989) and candidate clones were identified by digestion with *Mfe* I restriction enzyme (NEB). This experiment produced pSKC59.1 and pSKD76.1 plasmids. Plasmid pSKC59.1 has the 6xUAS_{GAL4}-catalase intron-GUS 1.6kb away from to the 35S promoter driving *hpt* II (Fig. 3.2 and Fig. 3.3), whereas pSKD76.1 has the 6xUAS_{GAL4}-catalase intron-GUS gene, separated by 7kb from the 35S promoter driving *hpt* II.

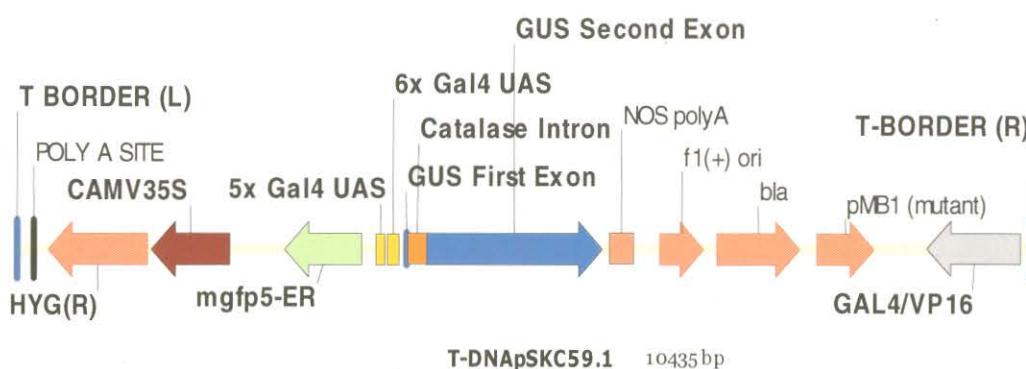


Figure 3.2 The 35S_HPTII-5UAS_mGFP5ER-6UAS_βGlucuronidase-pBS_GAL4/VP16 plasmid.

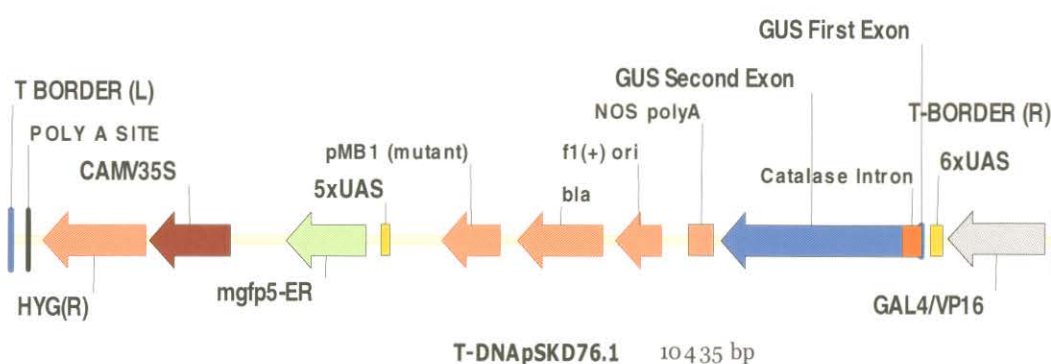


Figure 3.3 The 35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_βGlucuronidase_GAL4/VP16 plasmid.

3.2.1.2 Cloning of the GUS binary vectors: pSKC66.1 and pSKD76.2 binary vectors.

Plasmids pSKC2.1, containing the castor bean *Catalase-1* gene intron (catalase intron)-GUS fragments, and pFX-B751-1, containing the GAL4/VP16 and the catalase intron, were digested with *Hind* III, heated at 65°C for 20 minutes and purified using QIAquick buffer removal kits and then ligated as described in Chapter 2 page 30-32. Plasmid pSKC66.1 (Fig. 3.4) and pSKD76.2 (Fig. 3.5) were produced. These plasmids contain a catalase intron upstream from the GAL4/VP16 and different orientations of the 6xUAS_{GAL4}-catalase intron-GUS gene. Plasmid pSKC66.1 contains the 6xUAS_{GAL4}-catalase intron-GUS gene that is 1.6kb away from the 35S promoter driving *hpt* II. In

plasmid pSKD76.2 the 6xUAS_{GAL4}-catalase intron-GUS gene is 7kb distant from the 35S promoter driving *hptII*.

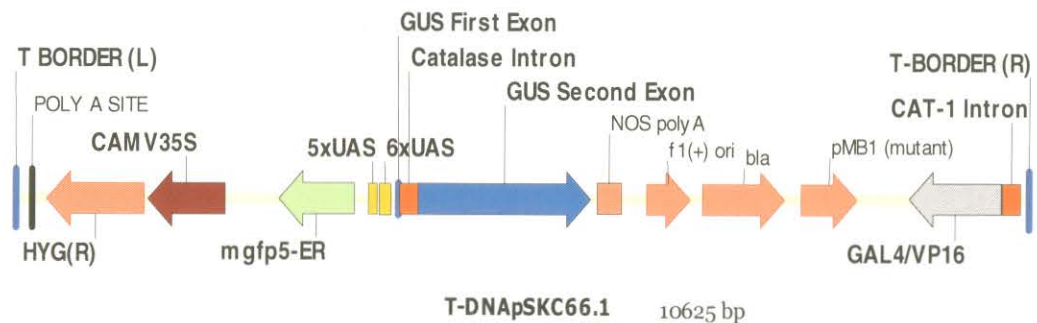


Figure 3.4 The 35S_HPTII-5UAS_mGFP5ER-6UAS_βGlucuronidase-pBS-intron_GAL4/VP16 plasmid.

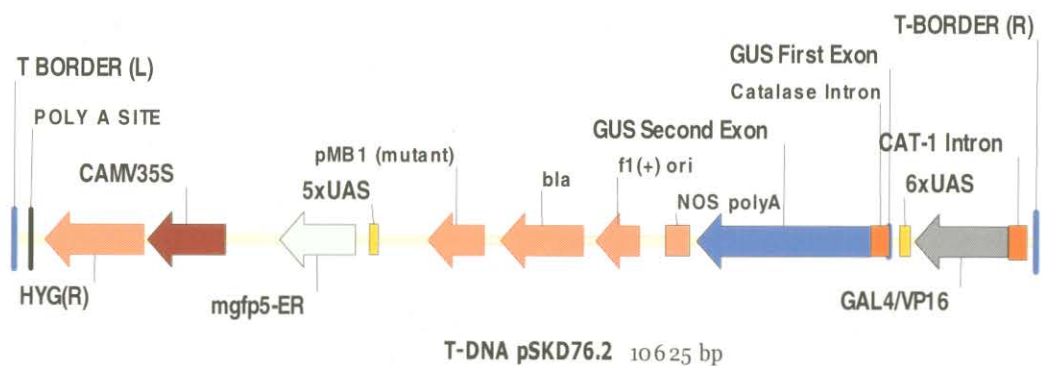


Figure 3.5 The 35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_βGlucuronidase-intron_GAL4/VP16 plasmid.

3.2.1.3 Cloning of the GUSPlus binary vectors: pSMRJ18 and pSMRJ18R.

Plasmids pSKC2.1 and pTANH114 containing the GUSPlus reporter gene were digested with *Nco* I and *Afl* II. A fragment 3.6kb in length from pSKC2.1 and the GUSPlus fragment from pTANH114 were excised from an agarose gel purified using a Qiagen gel extraction kit and ligated. This produced the pSKE15.11 plasmid containing the 6xUAS_{GAL4}-catalase intron fragment and the GUSPlus. Plasmids pSKE15.11 and pFX-B61-1, containing the GAL4/VP16, were digested with *Hind* III, the pFX-B61-1 was dephosphorylated using SAP (shrimp alkaline phosphatase) (Boehringer Mannheim) to

prevent self-ligation of the backbone and then both pFX-B61-1 and pSKE15.11 fragments were ligated. These cloning steps produced two plasmids, pSMRJ18 and pSMRJ18R, which had different orientations of the 6xUAS_{GAL4}-catalase intron-GUSPlus gene, and different distances from the 35S promoter. Plasmid pSMRJ18 (Fig. 3.6) has the 6xUAS_{GAL4}-catalase intron-GUSPlus gene 1.6kb away from the 35S promoter driving *hpt* II, whereas pSMRJ18R (Fig. 3.7) has 6xUAS_{GAL4}-catalase intron-GUSPlus 7kb away from the 35S promoter driving *hpt* II gene.

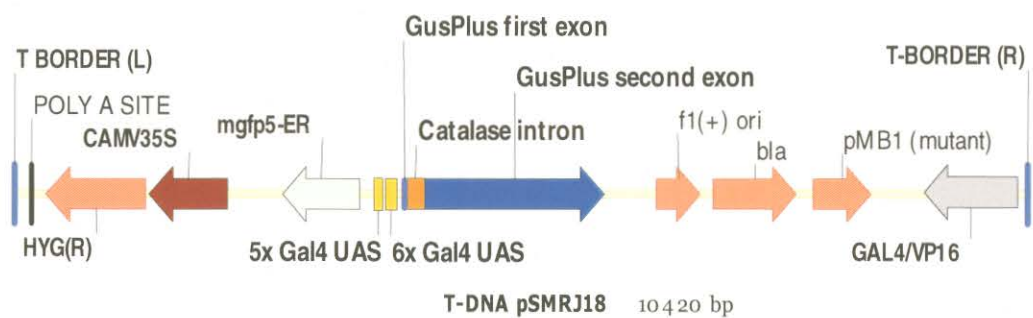


Figure 3.6 The 35S_HPTII-5UAS_mGFP5ER-6UAS_βGlucuronidase (GUSPlus)-pBS_GAL4/VP16 plasmid.

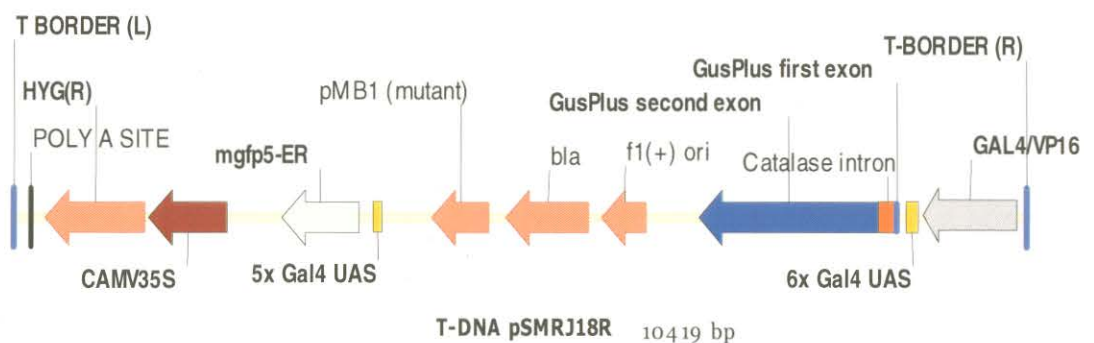


Figure 3.7 The 35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_βGlucuronidase (GUSPlus)_GAL4/VP16 plasmid.

3.2.1.4 Cloning of the GUSPlus binary vectors: pSMRJ17 and pSMRJ17R binary vectors.

Plasmids pSKE15.11 and pFX-B75.1-2 containing the GAL4/VP16 and the castor bean *Catalase-1* gene intron were digested with *Hind* III. These two plasmids

were dephosphorylated using SAP to prevent self-ligation then both pFX-B75.1-2 and pSKE15.11 fragments were ligated. These generated plasmid pSMRJ17 and pSMRJ17R which were different in their 6xUAS-catalase intron-GUSPlus gene orientations and their distance from the 35S promoter. Plasmid pSMRJ17 (Fig. 3.8) has the 6xUAS_{GAL4}-catalase intron-GUSPlus gene 1.6kb away from the 35S promoter driving *hptII*, whereas pSMRJ17R (Fig. 3.9) has 6xUAS_{GAL4}-catalase intron-GUSPlus 7kb away from the 35S promoter driving *hpt II* gene.

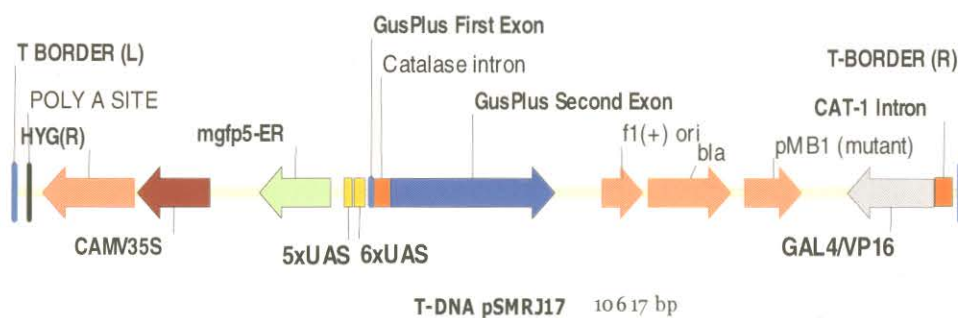


Figure 3.8 The 35S_HPTII-5UAS_mGFP5ER- 6UAS_βGlucuronidase (GUSPlus)-pBS_intron_GAL4/VP16 plasmid

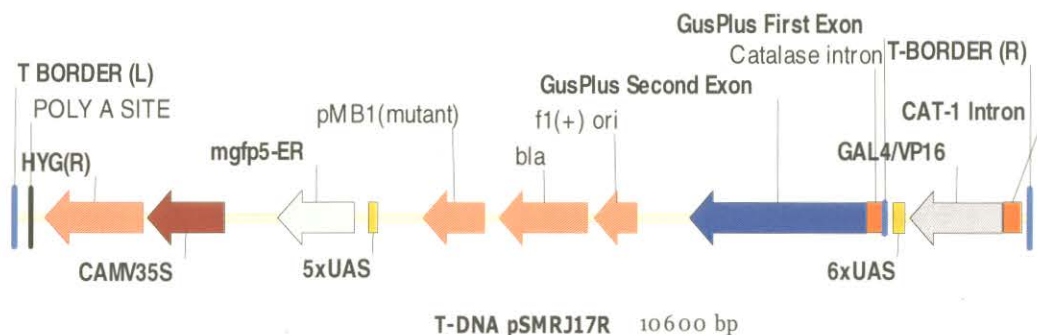


Figure 3.9 The 35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_βGlucuronidase (GUSPlus)_intron_GAL4/VP16 plasmid

3.2.1.5 Cloning of pSKD15.1 and pSKD15.2 binary vectors.

Plasmids pSKC59.1 and pSKC66.1 were digested with *Eco47 III* and *Stu I*, purified and then re-ligated. These double digestions deleted 108 bp of the GAL4 DNA binding domain, and this was confirmed through digestion of the plasmids; firstly by

were dephosphorylated using SAP to prevent self-ligation then both pFX-B75.1-2 and pSKE15.11 fragments were ligated. These generated plasmid pSMRJ17 and pSMRJ17R which were different in their 6xUAS-catalase intron-GUSPlus gene orientations and their distance from the 35S promoter. Plasmid pSMRJ17 (Fig. 3.8) has the 6xUAS_{GAL4}-catalase intron-GUSPlus gene 1.6kb away from the 35S promoter driving *hptII*, whereas pSMRJ17R (Fig. 3.9) has 6xUAS_{GAL4}-catalase intron-GUSPlus 7kb away from the 35S promoter driving *hpt II* gene.

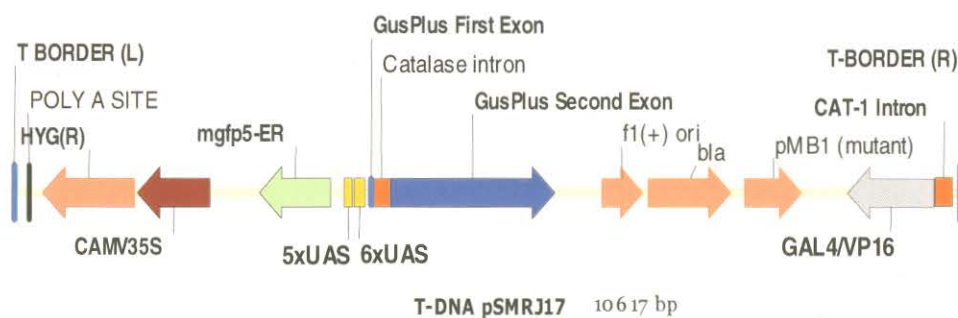


Figure 3.8 The 35S_HPTII-5UAS_mGFP5ER- 6UAS_βGlucuronidase (GUSPlus)-pBS_intron_GAL4/VP16 plasmid

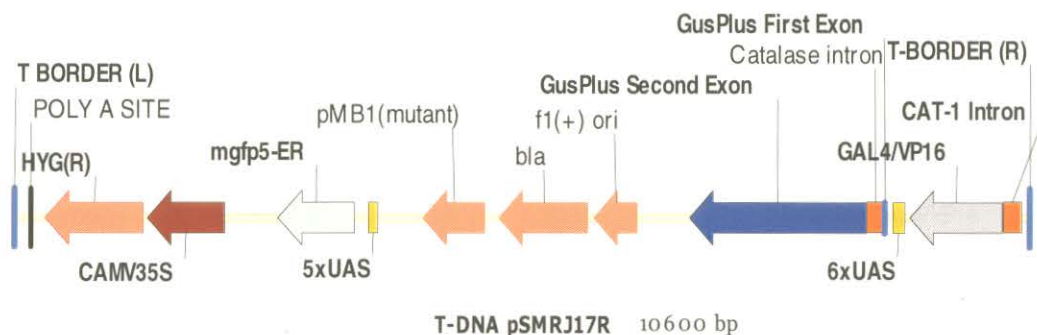


Figure 3.9 The 35S_HPTII-5UAS_mGFP5ER-pBS-6UAS_βGlucuronidase (GUSPlus)_intron_GAL4/VP16 plasmid

3.2.1.5 Cloning of pSKD15.1 and pSKD15.2 binary vectors.

Plasmids pSKC59.1 and pSKC66.1 were digested with *Eco47 III* and *Stu I*, purified and then re-ligated. These double digestions deleted 108 bp of the GAL4 DNA binding domain, and this was confirmed through digestion of the plasmids; firstly by

transformation procedure used was a modification of the rice transformation method from Hiei et al. (1997), described in Chapter 2, page 38-40. All transgenic rice plants were regenerated on 100 mg/L hygromycin B-containing medium. The regenerated plants were grown in the greenhouse with 28°C during daylight hours and 20°C at night.

3.2.3 GUS and GUSPlus ASSAYS

Histochemical detection of GUS (β -glucuronidase) and GUSPlus was performed using transformed calli and fresh plant organs from vegetative and floral parts of transgenic lines as previously described by Jefferson et al. (1987) and described here in Chapter 2, page 40-41. Histochemical analysis of the vegetative tissues (root, shoot and leaves) was conducted at the plantlet stage, just before transfer to the greenhouse. Floral parts were analysed at three stages of flower development; young (early booting stage), medium (full booting) and mature flower (just before flower dehiscence). 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.

3.2.4 MOLECULAR ANALYSIS

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

3.2.4 STATISTICAL ANALYSIS

To determine the effect of the construct components and their relative positions on expression patterns in the T_0 generation of TAFET lines, an ANOVA was carried out. Regression analysis was also performed to determine whether each component and/or an interaction between components, independently affected the intensity and/or complexity of the observed expression patterns. The ANOVA and regression analyses were performed using GenStat Release 6.1 software.

3.3 RESULTS

3.3.1 TRANSFORMATION AND GENERATION OF RICE TAFET LINES

Eight different transcriptional activator facilitated enhancer trap constructs, two negative controls (pSKD15.1 and pSKD15.2) and one positive control, pCAMBIA1201 containing the CaMV35S promoters driving GUS reporter gene and the CaMV35S promoter with double enhancers driving an *hpt* II gene (Fig. 3.12), were transformed into rice calli (var. Nipponbare and var. Millin) using *Agrobacterium tumefaciens* strain EHA-105.

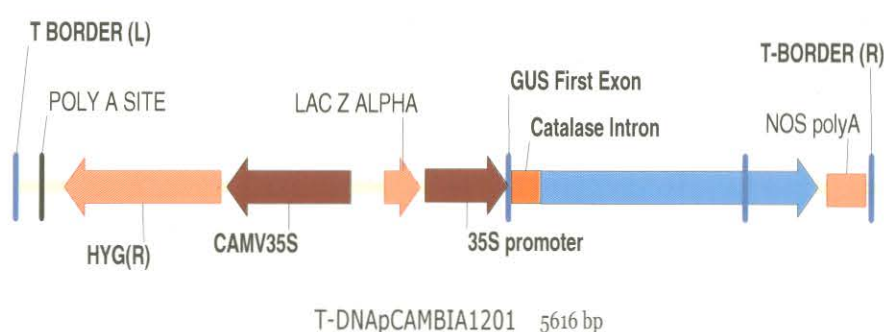


Figure 3.12 The pCAMBIA1201 plasmid as a positive control for the transactivator constructs.

About 1,000 TAFET lines and control lines were generated using *Agrobacterium* transformation; 330 lines transformed with GUS reporter gene, 663 lines with GUSPlus

reporter gene, 3 lines with pSKD15.1 and 64 lines with pCAMBIA 1201 gene. These were considered as T₀ TAFET lines.

Initial observation of reporter gene expression was performed on calli 3 days after co-cultivation with *Agrobacterium* carrying the various binary vectors described above. Both EGFP and β -glucuronidase expression were analysed. However, the signal from mGFP5ER was very weak and difficult to discriminate from callus autofluorescence. Therefore, I focused my attention on the glucuronidase histochemical assay and did not analyse GFP expression in calli and plants obtained using these TAFET constructs. The average percentage of calli exhibiting blue foci of GUS expression ranged from 14.4% to 54.3% for various GUS TAFET constructs, from 42% to 71.5% with GUSPlus TAFET constructs (Fig. 3.13), whereas calli with pCAMBIA 1201 (positive control) had 60.6% displaying blue foci of GUS expression. In contrast, calli transformed with both TAFET constructs that contained a deletion of the GAL4/VP16 DNA-binding domain (pSKD15.1 and pSKD15.2) displayed no blue foci of reporter gene expression. Transformation of these two deletion constructs were replicated three times and observation on each were carried out on 3, 7 and 14 days after co-cultivation. These results were interpreted as a clear indication of the ability of GAL4/VP16 to act as a transactivator in rice.

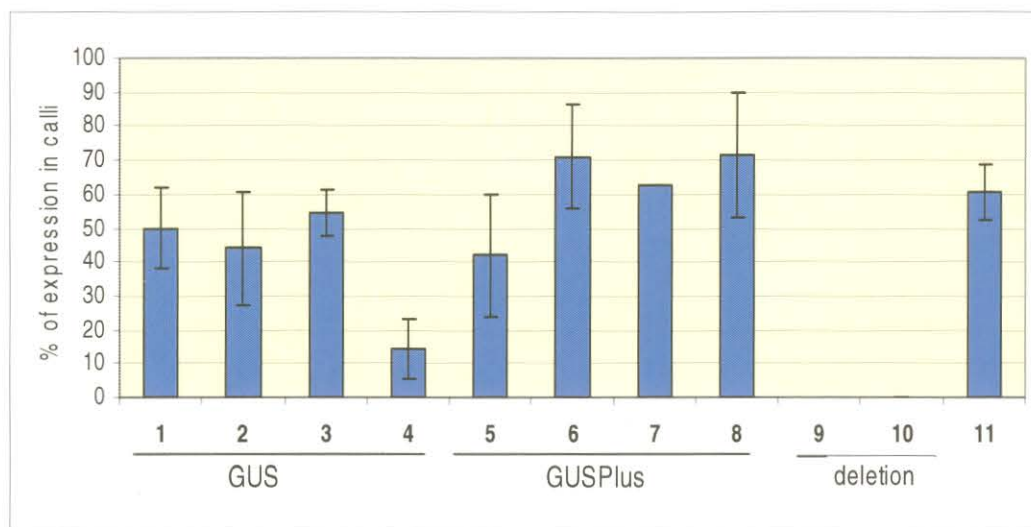


Figure 3.13 Expression of enhancer trap constructs in callus stage. 1-8: transactivator constructs; 9-10: GAL4/VP16 deletion constructs (refer Table 3.1), 11: pCambia 1201 (a positive control).

Based upon the percentage of stained calli with different constructs, there were no obvious differences between transactivator cassettes with or without the catalase intron or those with different relative distances between transactivator cassettes and the CaMV35S driving *hptII* gene (7kb and 1.6kb), and with two different reporter gene cassettes. Interestingly, calli transformed with the GUS reverse_intron (6xUAS_{GAL4}_GUS was 7kb away from 35S) construct (construct number 4 in Figure 3.13 or Table 3.1), displayed a significantly lower percentage of calli reporter gene expression. Moreover, in general the percentages of reporter-positive calli transformed with transactivator constructs were similar in comparison to calli transformed with pCambia1201 (Fig. 3.13).

3.3.2 GENE EXPRESSION IN T₀ PLANTS OF TAFET LINES

A total of 745 T₀ TAFET lines were observed for reporter gene expression. Details of the lines are available at <ftp://farm.cambia.org.au>. It can be opened using the username: nia and the password: thesis.

About 5% of lines did not show any gene expression and of those expressing the reporter gene, about 34%, 36% and 25% of TAFET lines had weak, medium and strong intensity of expression, respectively (Fig. 3.14). GUS lines had a higher percentage of weak expression than GUSPlus (47% and 30%, respectively). The converse was true for lines with strong expression, since GUSPlus constructs produced a higher percentage of lines with strong expression (36%) than GUS constructs (12%) (Fig. 3.14). This may indicate GUSPlus is a more sensitive reporter gene than GUS. Only three deletion lines were produced and observed for gene expression and these plants produced no GUS staining at all. These results give a confirmation of the ability of GAL4/VP16 to act as a transactivator in rice.

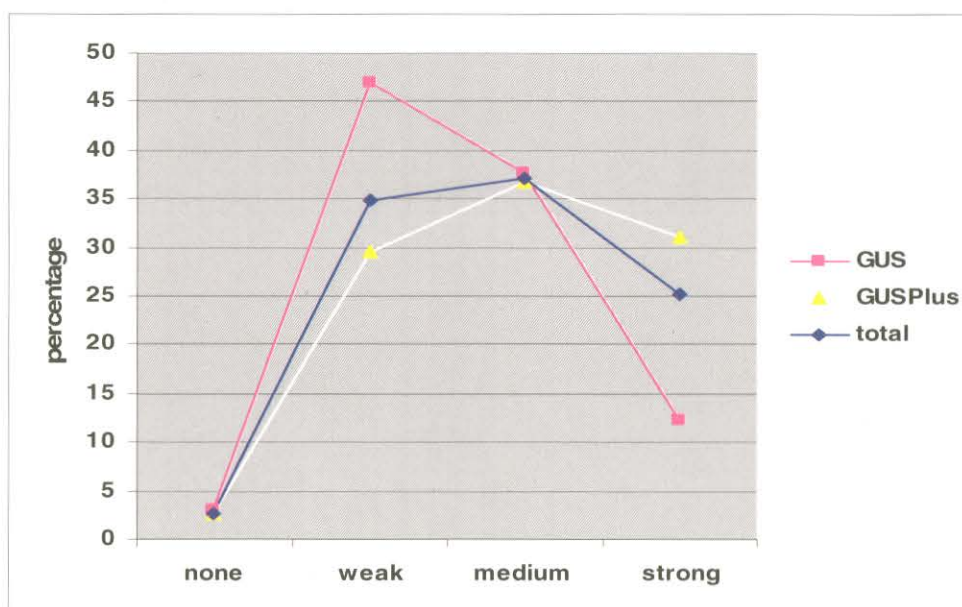


Figure 3.14 The percentage of TAFET lines with different intensities of reporter gene expression. **none**: lines without expression; **weak, medium and strong**: intensities of expression observed among 745 of GUS and GUSPlus lines.

3.3.3.1 Gene Expression Patterns in Vegetative Tissues

T₀ plants were observed for their expression patterns in vegetative tissues, during the initial growth phase before flowering. Of 1000 TAFET lines, only 222 GUS TAFET lines and 321 GUSPlus TAFET lines were observed for gene expression in vegetative parts, in the root, shoot and leaf tissues.

The percentage of GUS lines showing expression in vegetative tissues is lower than for GUSPlus lines. This was suggested from a higher percentage of total unique patterns divided by total lines with staining compared to a percentage of total patterns divided by total lines analysed in each transactivator GUS construct (Fig. 3.15). In contrast, the percentages of the two calculations for each transactivator GUSPlus construct were similar. These results are due to the fact that GUSPlus constructs produced more lines with expression than GUS constructs confirming the observation in the callus stage, that GUSPlus is a more sensitive reporter gene than GUS.

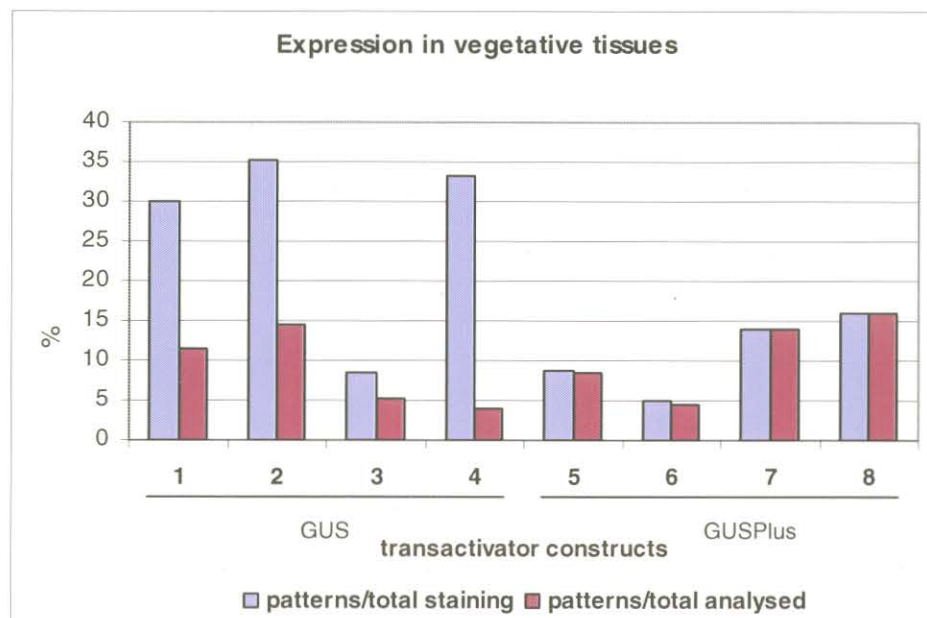


Figure 3.15 The percentage of number of patterns/total lines staining and number of patterns/total lines analysed for each transactivator construct in vegetative tissues. For details of constructs refer to Table 3.1

To analyze the diversity of patterns which were observed, it was necessary to classify the observations. The patterns were scored for the combination of staining that was observed in root (a), shoot (b) and leaf (c) tissues. In theory this could lead to 7 patterns which were; a, b, c, ab, ac, bc and abc.

Analysis of the three most abundant patterns of expression in vegetative tissues showed that while there were clear differences in the distribution of those patterns among constructs, the main difference was between GUS and GUSPlus constructs (Fig. 3.16). The number of lines analysed for each construct was obviously different, but this factor is unlikely to have affected the results obtained. The number of lines analysed for the GUSPlus reverse_intron construct (construct 8) was higher than that for the GUS reverse_intron construct (construct 4), 44 compared to 25 lines, respectively. However, the GUS reverse_intron construct showed only one of the three dominant patterns in vegetative tissues, and at a low percentage (12%), whereas the GUSPlus reverse_intron construct had all dominant patterns (Fig. 3.16).

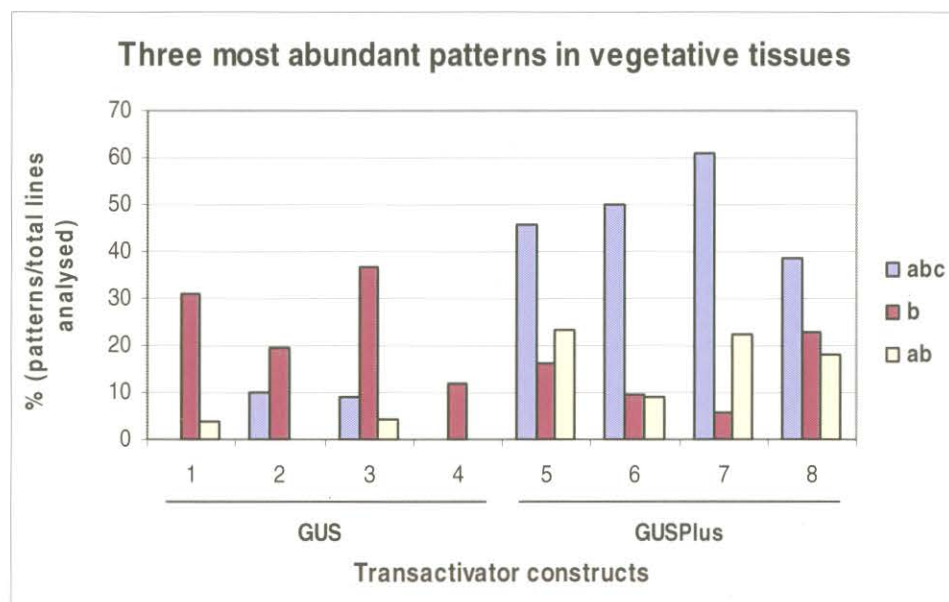


Figure 3.16 The three most abundant patterns expressed by TAFET lines in vegetative tissues. **abc**: root, shoot and leaves **b**: shoot, **ab**: root and shoot. For details of the constructs refer to Table 3.1.

When the three unique patterns produced by each different construct were analysed, the most abundant pattern of all GUS constructs (the first) was expression in the shoots (b), the second was expression in root, shoot and leaves (abc) or in the root and shoot (ab), and the third one either in the shoot and leaf (bc) or root and shoot (ab) or only in the root (c). Interestingly, the GUS reverse_intron construct (4) (pSKD76.2, see Table 3.1 or Fig. 3.5) had only one of the dominant patterns as described above, that being the shoot expression (b) (Fig. 3.17).

In contrast, the most dominant pattern of GUSPlus constructs (the first unique pattern) was expression of the reporter gene in the root shoots and leaves (abc). The second and the third ones were ab, bc, or b patterns (Fig. 3.17). This may indicate that GUSPlus is a more sensitive reporter gene. The patterns mentioned above are shown pictorially in Figure 3.18.

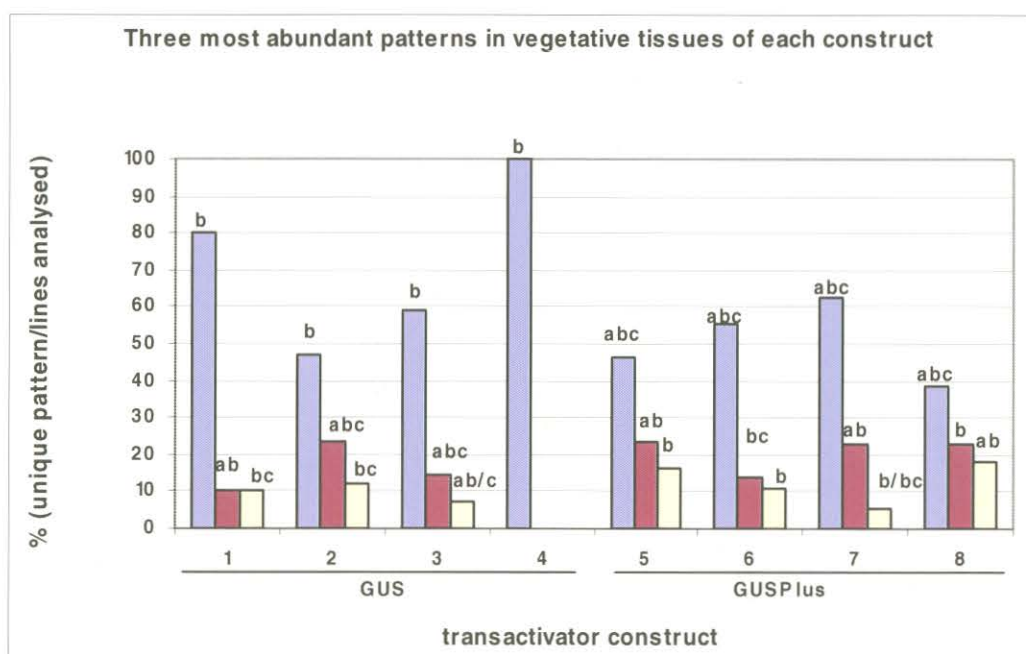


Figure 3.17 Three most abundant patterns expressed by each transactivator construct in vegetative tissue. 1-4: Transactivator constructs with GUS, 5-8: Transactivator constructs with GUSPlus. **abc**: root, shoot and leaves, **ab**: root and shoot, **bc**: shoot and root, and **b**: shoot. For details of the constructs refer to Table 3.1.

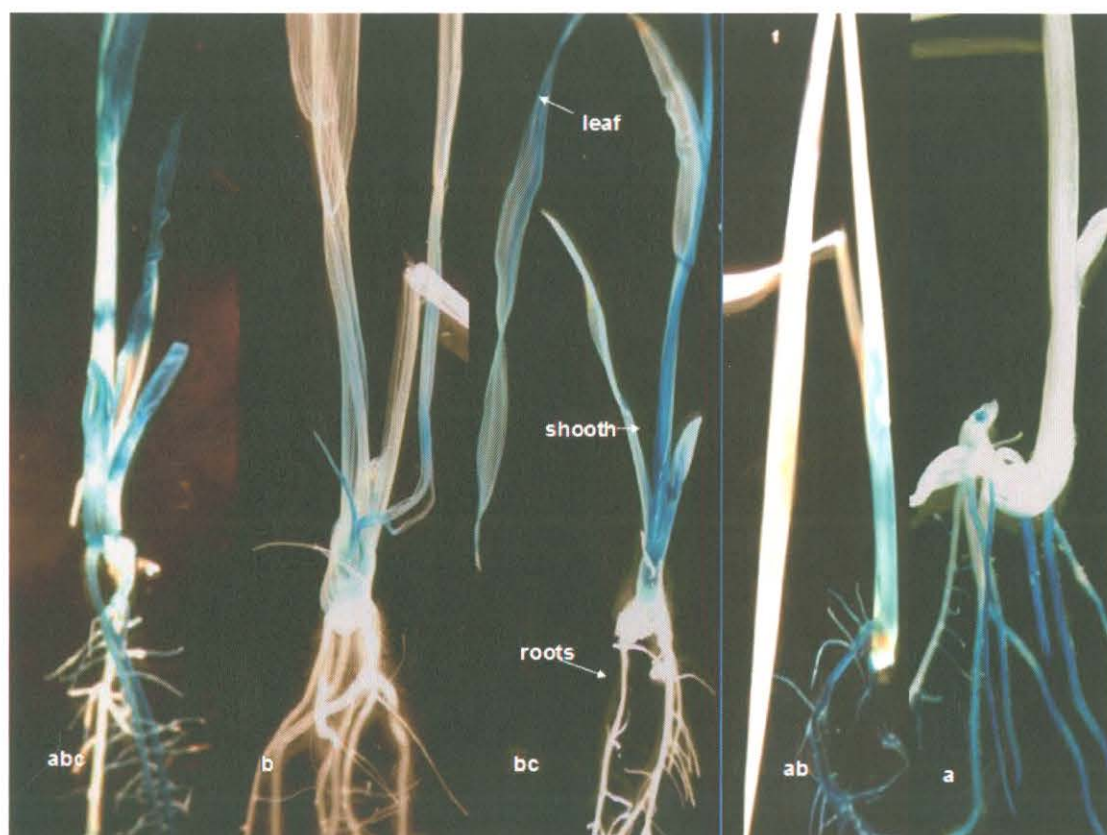


Figure 3.18 Four most abundant patterns expressed in vegetative tissues of TAFET lines. abc: root, shoot and leaves; **b:** shoot; **bc:** Shoot and leaves; **ab:** root and shoot or **a:** only in the root.

Tissue-specific patterns produced in the roots were either in initiation sites of secondary roots (Fig. 3.19A and Fig. 3.19B), in cap roots (Fig. 3.19C), in elongation zones (Fig. 3.19D) or everywhere in the root (Fig. 3.19E), whereas patterns of expression in the leaves were observed either in the vascular bundle (Fig 3.20C) and in the guard cells of stomata (Fig. 3.20D). Some lines had strong expression in the vascular bundle of the ligule (Fig. 3.20A and Fig. 3.20B).

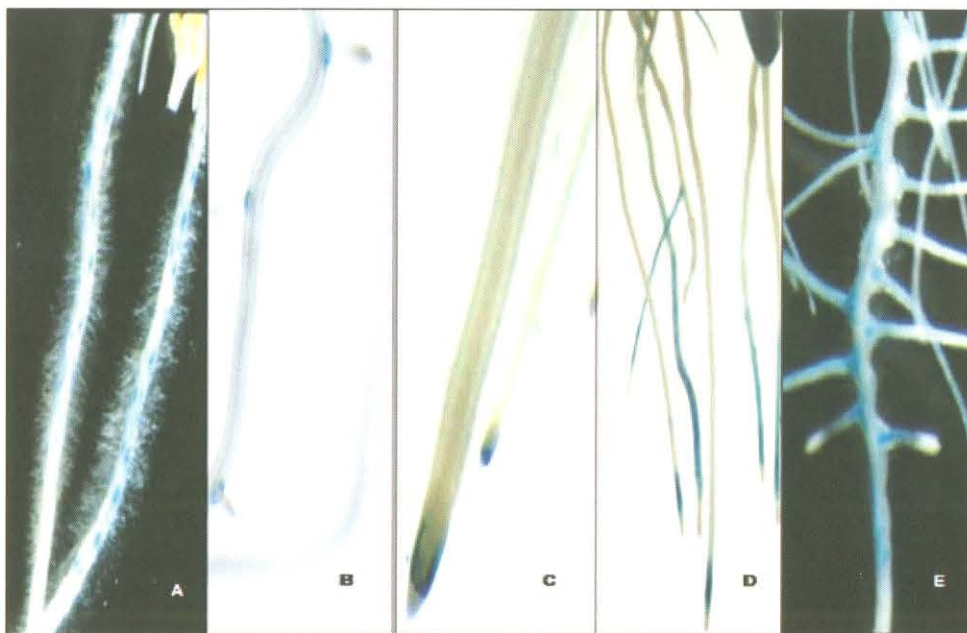


Figure 3.19 Expression patterns in the root of TAFET lines. **A & B.** in initiation sites of secondary roots, **C.** in cap roots, **D.** in elongation and **E.** the whole roots.

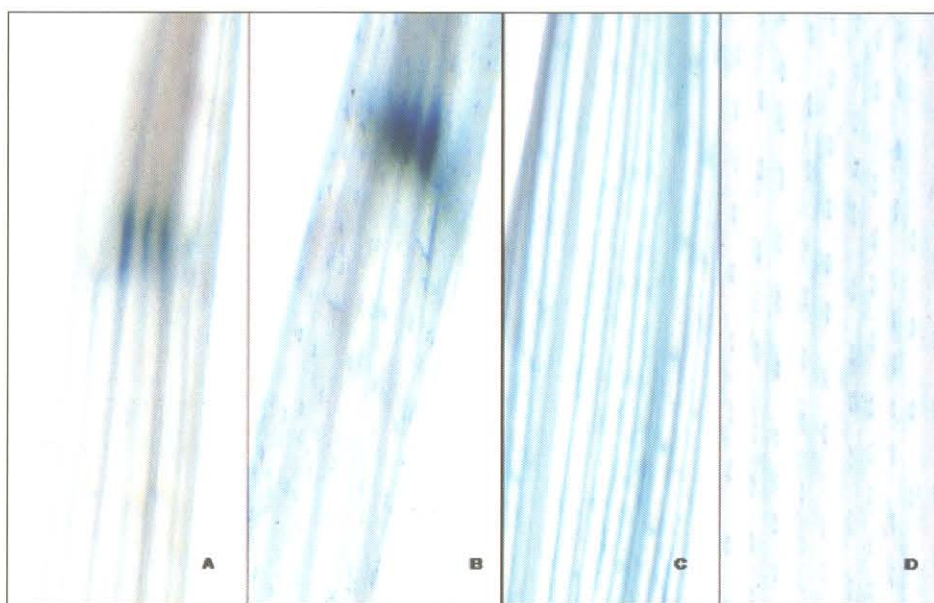


Figure 3.20 Expression patterns in the leaves of TAFET lines. **A.** in vascular bundle of ligule, **B.** guard cells of stomata and vascular bundle of ligule, **C.** vascular bundle and **D.** guard cells of stomata.

3.3.3.2 Gene Expression Patterns in Floral Tissues

Four hundred and twenty lines were observed for reporter gene expression in various floral tissues: lemma, palea, lodicules, filament, anther sac, pollen, stigma, style and ovule. The total percentage of lines has expression in any of the above tissues induced by different transactivator constructs ranged from about 3% to 66%. More than 60% of lines showed expression in male parts (anther sacs and/or pollen), 21% in lodicules, more than 30% in stigmas and styles, but only 3% of lines showed expression in ovules.

Tissue specific expression patterns in floral parts were displayed by 17.3% of TAFET lines. The percentages of lines that had expression only in one, two or three tissues, were about 2.14%, 10.5% and 4.76%, respectively. Moreover, the percentage of lines that had expression in the ovule and another tissue was only about 0.5% (2 of 420 lines observed), while the lines that had gene expression only in the lodicule, and the lodicule and another floral tissue was about 6.4% (27 of 420 lines observed) (Table 3.2).

Tissues	Percentage (of 420 lines)
Palea and lemma	4.00
filament	0.95
lodicules	6.42
stigma	2.86
style	1.20
Ovule	0.48
anther sac	1.40
Total	17.31

Table. 3.2 Percentage of lines with tissues-specific expression patterns in TAFET population.

To analyze expression patterns in the floral tissues, staining was observed in floral whorl tissues starting from the most outside tissue (palea and lemma) to the most inside (ovule). Nine tissues were scored, being palea (d), lemma (e), lodicule (f), filament (g), anther wall (h), pollen (i), style (j), stigma (k) and ovule (l).

Using this analysis, a total of 108 different patterns were produced by both GUS and GUSPlus transactivator constructs, in which 41 patterns were unique for GUSPlus and 22 were unique for GUS constructs. The number of patterns with expression in the palea (d) and/or lemma (e) was 46, whereas there were 26 patterns starting with expression in the lodicule (f) (i.e. had no expression in the palea and lemma). Sixteen patterns started with expression in the filament (g), 10 patterns started with expression in the anther wall (h), 7 patterns started with expression in the pollen (i) and two patterns started with expression in the stigma and/or style (j and/or k). The GUSPlus constructs produced more patterns than GUS constructs.

When a percentage between the total unique patterns divided by the total lines staining was compared with the total patterns divided by the total lines analysed for each transactivator construct was calculated, percentage of those two were almost similar for 6 constructs, except that of for GUSPlus and GUSPlus reverse constructs (Fig. 3.21). This means that not all lines with GUSPlus and with GUSPlus reverse constructs which were analysed had reporter gene expression (staining), whereas all lines of two GUSPlus constructs (construct 7 and 8) analysed showed gene expression (Fig. 3.21).

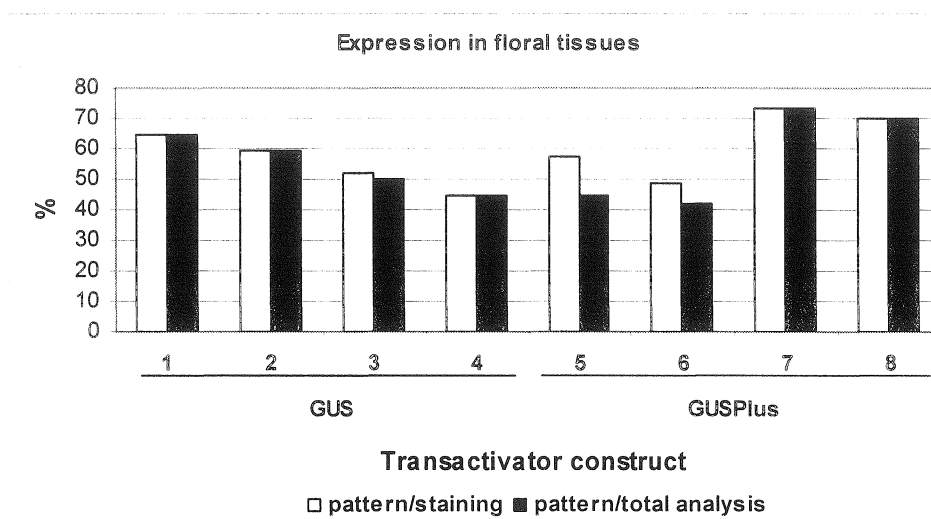


Figure 3.21 The percentage number of patterns/total lines staining and a number of patterns/total lines analysed, for each transactivator construct in floral tissues. For details of the constructs refer to Table 3.1.

When the five most abundant patterns in floral tissues were analysed among, the GUS lines had similar patterns to GUSPlus lines, except the GUS reverse_intron construct (4) (pSKD76.2, see Table 3.1 or Fig. 3.5) and GUSPlus_intron (7) (pSMRJ17, see Table 3.1 or Fig.3.4) which had less than the five patterns, 2 of 5 and 1 of 5, respectively (Fig. 3.22). Similar to observations with vegetative tissues, differences in numbers of lines analysed in each construct were encountered. The GUS construct 1 (pSKC59.1, see Table 3.1 or Fig. 3.2) with 17 lines had higher numbers of patterns than those produced by GUS construct 4 (pSKD76.2, see Table 3.1 or Fig. 3.5) (22 lines) and GUSPlus construct 7 (pSMRJ17, see Table 3.1 or Fig. 3.4) (30 lines) (Fig. 3.22).

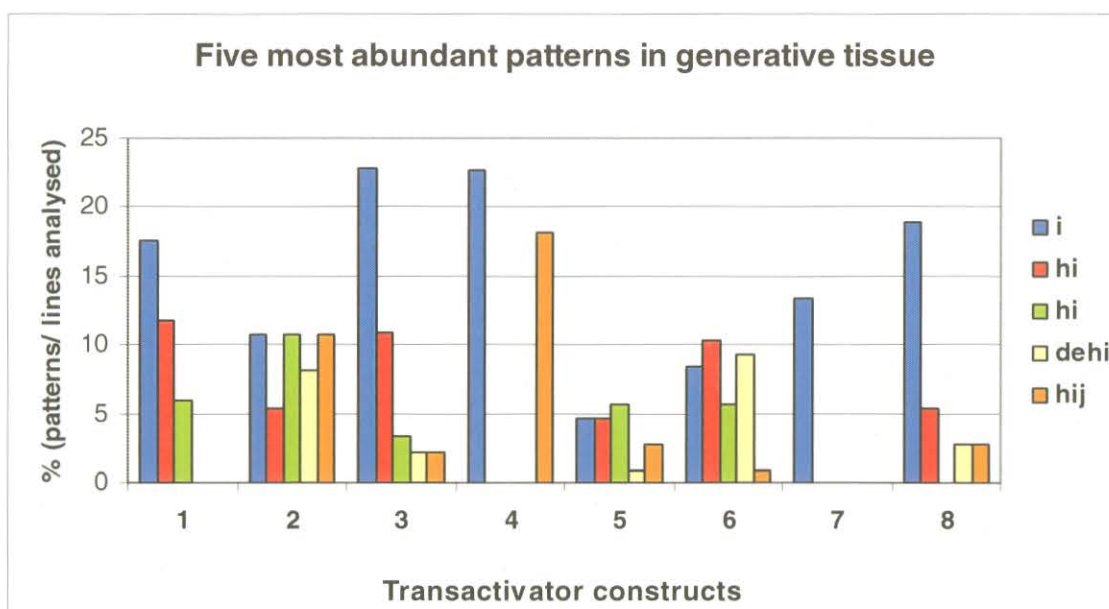


Figure 3.22 Five most abundant patterns produced by transactivator constructs. i: palea and/or lemma, hi: anther wall and pollen, h: anther wall, dehi: palea and lemma, anther wall and pollens, hij: anther wall, pollen and stigma and/or style. For details of the constructs refer to Table 3.1.

In examining the pattern distribution produced by each construct, it appeared that GUSPlus lines had a greater distribution of expression pattern. When the five most abundant patterns in the floral tissues of each different construct were analysed, 25.3 to 40.1% of GUSPlus lines were represented in the five most abundant pattern classes (Fig. 3.23), whereas about 45.9 to 58.9% of GUS lines were in the five most abundant pattern classes (Fig. 3.15). These percentages were summed for the five most abundant pattern classes for each constructs. For example, lines of GUSPlus reverse which had the five most abundant pattern classes was about 25.3% of total lines of GUSPlus reverse. This may indicate that GUSPlus constructs tended to produce a greater variety of pattern than that of GUS. Interestingly, constructs containing the *catalase-1* intron upstream of GAL4/VP16 (constructs number 3, 4, 7 and 8, refer to Fig. 3.10) had only one or two dominant patterns, which expressed in higher numbers of lines over the rest of the patterns. For example, the first and the second dominant

patterns were displayed by 33.7 of those 46.6% of GUS_intron (2) lines and by 40.9% of these 58.9% of GUS reverse_intron (4) lines (Fig. 3.23).

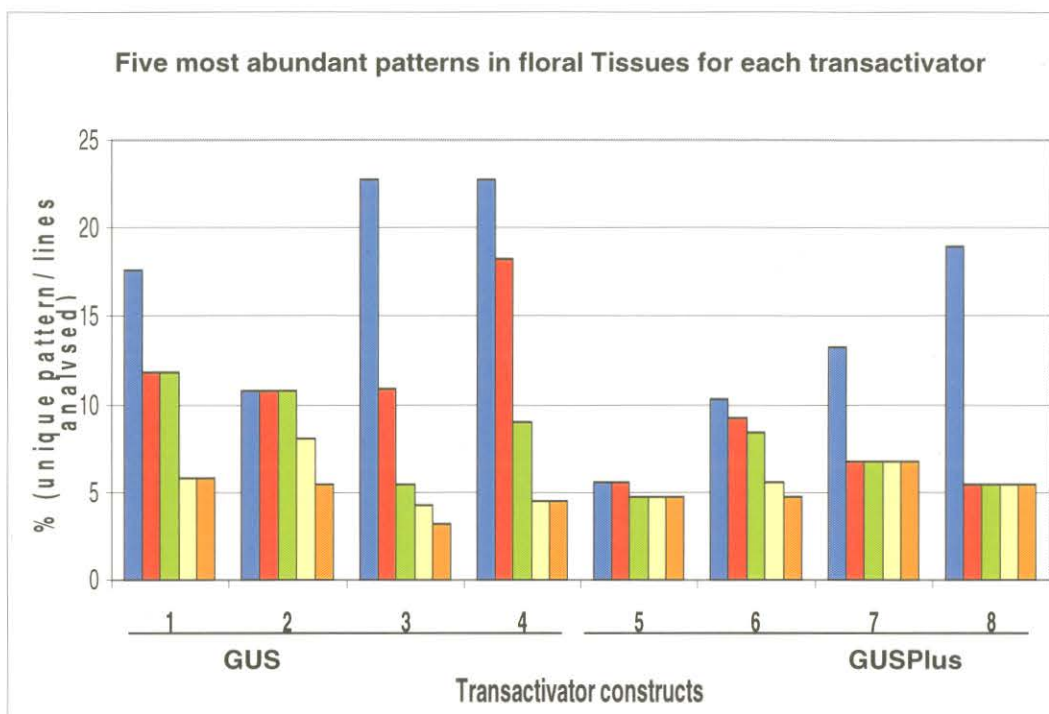


Figure 3.23 The five most abundant patterns expressed by each transactivator construct in floral tissues. **Blue:** 1st pattern, **red:** 2nd, **green:** 3rd, **yellow:** 4th, and **orange:** 5th pattern. For detail of patterns produced by each construct refer to Table 3.3 below. For details of constructs refer to Table 3.1.

Patterns	GUS constructs				GUSPlus constructs			
	1	2	3	4	5	6	7	8
1st	i	i	i	i	h	hi	l	l
2nd	dehi	h	hi	hij	deh	dehi	deghek	Hi
3th	hi	hij	dei	defghijk	hi	i	dei	Hij
4th	ghij	dehi	hijk	degghijk	i	h	fgi	lj
5th	h	hi	h	hijk	dehijk	deh/dehj	Fi	Degghik

Table 3.3 The five most abundant patterns expressed by each transactivator construct in floral tissues. Patterns produced by each construct can be a combination several tissues, which noted **d:** for palea; **e:**

lemma; **f**: Lodicule; **g**: filament; **h**: anther wall; **i**: Pollen; **j**: Style; **k**: stigma and **l**: ovule. **i**. For details of the constructs refer to Table 3.1.

The five most abundant patterns in the floral tissues displayed by TAFET lines had expression in 1) the pollen (Fig. 3.24, number 1); 2) the anther wall and pollen (Fig. 3.24, number 2); 3) the anther wall (Fig. 3.24, number 3); 4) the palea and lemma, anther wall and pollen (Fig. 3.24, number 4) and 5) in the anther wall, pollen and stigma and/or style (Fig. 3.24, number 5). Three additional patterns in the floral tissues had expression in 6) the palea and lemma, anther wall, pollen (Fig. 3.24, number 6); 7) in the palea and lemma, lodicule, anther wall, pollen, style (Fig. 3.24, number 7) and 8) palea and lemma, lodicule, filament, anther wall, style, stigma and ovule (Fig. 3.24, number 8).

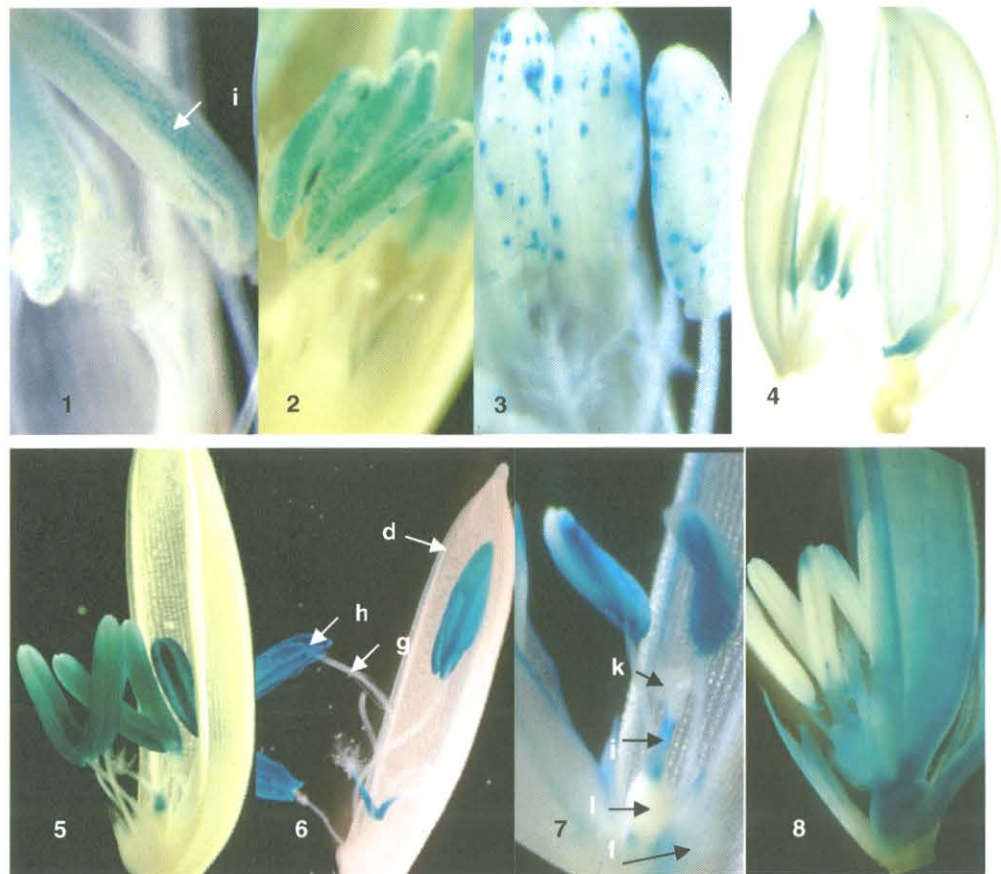


Figure 3.24 The most abundant patterns expressed in GUS or GUSPlus TAFET lines. 1: pollen, 2: pollen and anther wall, 3: anther wall, 4: palea and lemma, anther wall, pollen, 5: anther wall, pollen and style, 6: palea and lemma, anther wall, pollen, style, 7: palea and lemma, lodicule, anther wall, pollen, style, and 8: palea and lemma, lodicule, filament, anther wall, style, stigma and ovule. **d**: palea, **e**: lemma, **f**: lodicule, **g**: filament, **h**: anther wall, **i**: pollen, **j**: style, **k**: stigma, and **l**: ovule

Most plants transformed with pCAMBIA1201 had expression in almost all floral tissues (Fig 3.25, a, b and c) and many had strong expression of the GUS reporter gene in the ovule. Some had expression in palea and lemma or in anther and pollen (d) (Fig. 3.25) were similar to one of the five most abundant patterns of TAFET lines.

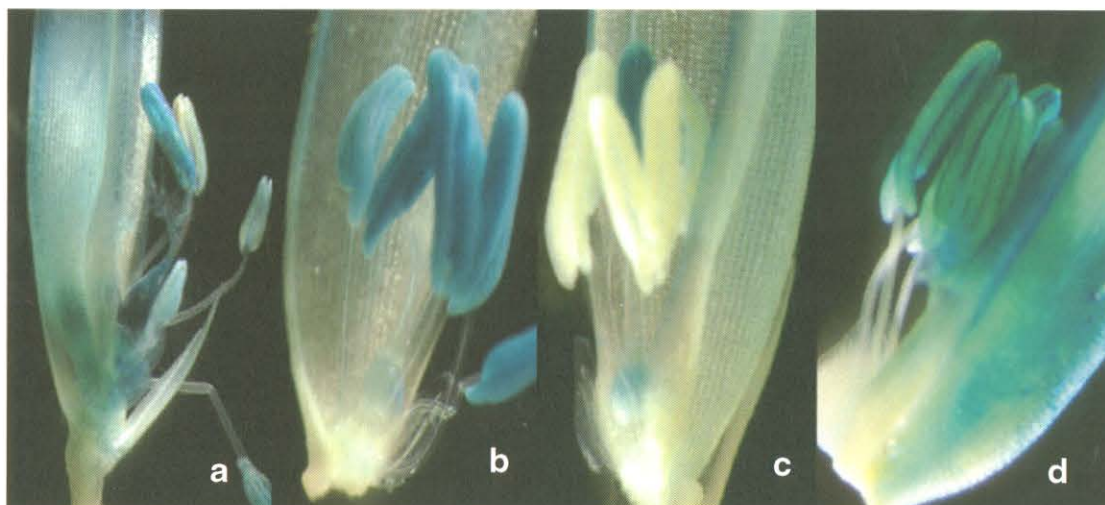


Figure 3.25. Expression patterns in generative tissues of pCAMBIA1201 lines. **a & b.** Reporter gene expressed in palea and lemma, filament, lodicule, anther wall, pollen, style, stigma and in ovule, **c.** midrib of palea, lodicule, one anther (anther and pollen), and ovule, and **d.** palea and lemma, anther wall and pollen.

3.3.3 STATISTICAL ANALYSIS OF COMPONENTS WITHIN TAFET CONSTRUCTS

Statistical analysis to define whether two different β -glucuronidase reporter genes, two GAL4/VP16 transactivator cassettes and their relative position have affected the performance of the transcriptional Activators Facilitated Enhancer Trap (TAFET) in inducing gene expression was carried out. A total of 603 lines produced by eight transactivator constructs with three different intensity of expression (weak, medium and strong) were analysed (Table. 3.4).

When weak, medium and strong expression were converted into values of 1, 2 and 3, respectively, ANOVA analysis showed that intensity of expression was influenced by reporter genes at a highly significant level ($p < 0.001$) and by the intron at a significant level ($p = 0.003$) (Table. 3.4, A). When weak, medium and strong intensities were not given numerical values, the ANOVA analysis showed that only the reporter gene had a highly significant effect on gene expression ($p < 0.001$) (Table. 3.4, B).

ANOVA				
A. Weak=1 medium=2 strong=3				
	d.f.	m.s.	v.r.	p.
+ reporter	1	18.03	32.82	<0.001**
+ position	1	1.31	2.31	0.122
+ intron	1	4.99	8.81	0.003*
+ reporter.position	1	1.77	3.12	0.078
+ reporter.intron	1	0.02	0.05	0.826
+ position.intron	1	3.85	6.81	0.009
+ reporter.position.intron	1	0.21	0.37	0.541
Residual	596	337.82	0.57	
Total	603	368.03	0.61	
B. Weak<medium<strong				
	d.f.	m.s.	v.r.	p.
+ reporter	1	18.03	31.90	<0.001**
+ position	1	2.19	3.88	0.049
+ intron	1	4.11	7.27	0.007
+ reporter.position	1	3.815	6.75	0.010
+ position.intron	1	1.84	3.25	0.072

Table 3.4 The ANOVA of components of TAFET constructs against three levels of the intensity of expression. A. Weak, medium and strong expressions were converted into numerical values 1, 2 and 3, B. Weak, medium and strong expressions were converted into weak is smaller than medium, medium is smaller than strong. Level of significance was defined at highly significant at $P=0.001(**)$ and significant at $P=0.01 (*)$.

Elements of constructs and the intensity of expression were analysed against the complexity of patterns in vegetative and in floral tissues. The ANOVA showed the reporter genes influenced the complexity of patterns in vegetative tissues (402 lines) at a highly significant level ($p<0.001$) and GUSPlus was correlated highly significant ($p<0.001$) with complexity of patterns in the vegetative tissues (Table. 3.5). This result is consistent with the result obtained when the three most abundant patterns in vegetative tissues were analysed among the constructs (Fig. 3.16). GUSPlus constructs induced expression of the reporter gene in all vegetative tissues (the abc pattern) (Fig. 3.16).

ANOVA				
With Intensity				
change	d.f.	m.s.	v.r.	p
+ reporter	1	35.9042	74.79	$<0.001^{**}$
residual	396	0.4801		
Total	401	0.7314		
Without intensity				
change	d.f.	m.s.	v.r.	F pr.
+ reporter	1	56.5246	92.42	$<0.001^{**}$
Regression analysis				
		Estimation	s.e.	t (396) T pr.
Constant		1.4828	0.0726	20.42 $<0.001^{**}$
Reporter GUSPlus		0.8209	0.0854	9.61 $<0.001^{**}$

Table 3.5 The ANOVA and regression analysis of components of constructs and/or intensity of expressions against expression patterns in vegetative parts of TAFET lines. Level of significant was defined at $P=0.01 (*)$ and highly significant at $P=0.001(**)$.

When intensity was taken out of the analysis against complexity of patterns, the ANOVA showed that complexity was influenced significantly by the spacing of the 6xUAS_{GAL4} element to the 35S promoter driving the *hpt* II (hygromycin resistance gene) ($p=0.006$) (Table 3.6). Constructs with a proximate distance (pSKC59.1, pSKC66.1, pSMRJ18 and pSMRJ17) produced lines with more complex of pattern. Regression analysis also showed that complexity was correlated negatively with the proximate spacing (1.6 kb) of the UAS minimal promoter and 35S promoter at a significant level ($p=0.006$) (Table 3.6).

With Intensity ANOVA					
Change	d.f.	s.s.	m.s.	v.r.	p
+ 35S proximate	1	2.964	2.964	1.12	0.29
Residual	380	1004.043	2.642		
Total	382	1141.713	2.989		
Without intensity ANOVA					
Change	d.f.	s.s.	m.s.	v.r.	p.
+ 35S	1	22.38	22.387	7.58	0.006
+intron	1	5.22	5.22	1.77	0.184
Regression analysis		Estimation	s.e.	t (396)	p
Constant		3.365	0.118	28.41	<0.001**
35S proximate		-0.499	0.181	-2.75	0.006

Table 3.6 The ANOVA and regression analysis components of constructs and or intensity of expressions against expression patterns in floral parts of TAFET lines. Level of significant was defined at $P=0.01$ (*) and highly significant at $P=0.001$ (**).

These results may indicate that transactivator constructs containing a distal spacing between the UAS minimal promoter and 35S promoter induced a greater intensity of expression as well as a greater complexity of patterns than constructs containing a proximate spacing. These results support the finding that reporter genes affected the intensity of gene expression, with GUSPlus producing more lines with

strong intensity (see section 3.3.3 and Fig. 3.14) as well as with more complex patterns in vegetative tissues (see section 3.3.3.1 and 3.3.3.2). Overall, the analysis of expression patterns and statistical analysis indicate that GUSPlus revealed more patterns and more lines with strong intensity of expression and more complexity of patterns than GUS.

3.3.4 MOLECULAR ANALYSIS

Insertion of the GAL4/VP16 enhancer trap molecule was analysed by Southern blot analysis (Fig. 3.26) in 253 of T₀ TAFET lines. Based upon a pattern of DNA fragments hybridised with a P³²-labelled GAL4/VP16 fragment as a probe, it appeared that TAFET T-DNA inserted randomly into the rice genome (Fig. 3.26).

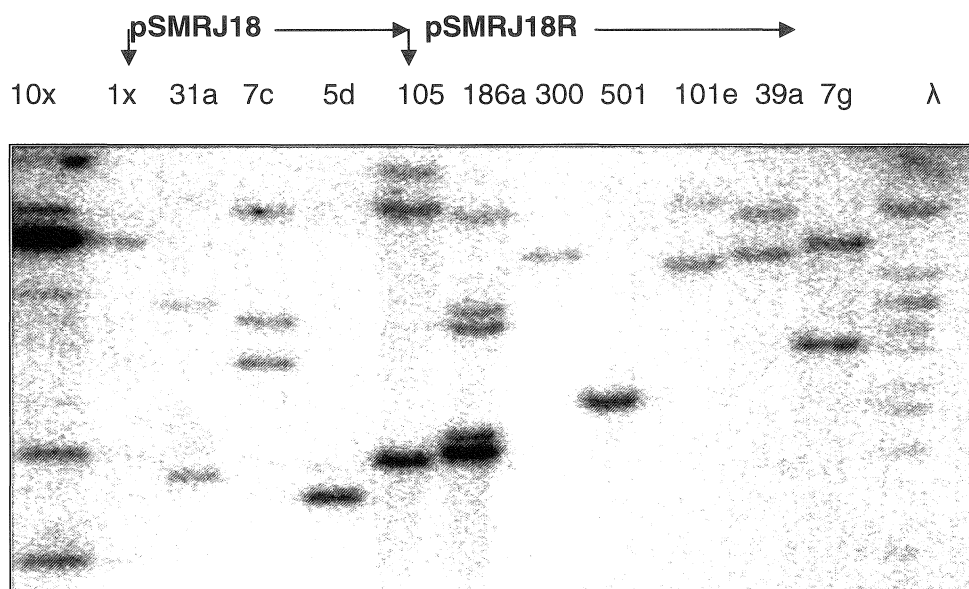


Figure 3.26 Southern blot hybridisations of pSMRJ18 and pSMRJ18R lines with the GAL4/VP16 fragment as a probe. Plant DNA(s) was digested with *EcoR* I restriction enzyme and hybridised with ³²P-labelled GAL4/VP16 probe. Lanes marked **10X** and **1X** contained 10 and 1 copy equivalent of pSMRJ18 as positive controls. The line numbers are shown on the top. DNA of phage λ digested with *BstE* II restriction enzyme served as molecular size marker.

The number of T-DNA insertions ranged from 1 to 7 copies with an average of 2.0 copies per line. About 49% of lines have a single T-DNA insert and less than 30% have 3 or more T-DNA copies. Only 1% of lines have 7 copies (Table 3.7).

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

Table 3.7 T-DNA copy number insertion in T₀ generation of TAFET lines.

In addition, plants of a deletion line (pSKD15.2-1e) transformed with a transactivator deletion construct (see Table 3.1, Fig. 3.7) were analysed for the GAL4/VP16 enhancer trap molecule by Southern blot analysis (data is presented in Chapter 4, Fig. 4.23). T₂ plants contained two T-DNA insertions, but did not show any GUS expression. These results were interpreted as a clear indication of the ability of GAL4/VP16 to act as a transactivator in this rice system.

3.4 DISCUSSION

In eukaryotic promoters, a TATA-box (which is a component of a gene promoter about of 100 base pairs in size), operates in close proximity to the transcription initiation site (Martin, 2001), whereas an enhancer which links in *cis* to a promoter, acts from several hundred base pairs away from the transcription start site (Bohmann et al., 1987, Martin, 2001). Enhancers are binding sites for transcriptional activators (regulatory proteins). When an enhancer is placed close to a promoter, it is difficult to distinguish it from a UAS (the second critical *cis*-acting element), because both are

bound by the same factor. The binding of an enhancer to a UAS produces much closer distances between regulatory proteins and the UAS in the TATA box (Guarente, 1988; Alberts et al., 1994). These characteristics were exploited in development of the enhancer trap systems.

Ten Transcriptional Activator Facilitated Enhancer Trap (TAFET) constructs, including two negative controls of Transcriptional activator (transactivator) constructs were developed and tested to facilitate enhancer trapping in rice (see Table 3.1, Fig. 3.2, Fig. 3.3, Fig. 3.4, Fig. 3.5, Fig. 3.6, Fig.3.7, Fig. 3.8, Fig. 3.9). These 10 constructs including the two negative controls (Fig. 3.10 and Fig. 3.11) and pCAMBIA1201 (a positive control) (Fig. 3.12), were transformed into scutellum-derived rice calli.

The average reporter gene expression observed at the calli stage ranged between 14.4 to 54.6% with transactivator-GUS constructs, between 42 to 71.5% with transactivator-GUSPlus constructs and 0% with pSKD15.1 and pSKD15.2 (negative controls). This was a clear indication that the GAL4/VP16 was operating as a transcriptional activator and that deletion of 108bp in the binding domain of pSKD15.1 and pSKD15.2 disrupted the binding of GAL4 to the UAS_{GAL4} element and stopped transcription of the reporter gene fused to the UAS_{GAL4}, as previously described (Sullivan et al., 1998). It has also been previously reported (Fischer et al., 1988; Ma et al., 1988) that GAL4 amino-acids 1-147, the amino terminal portion of the 881 amino-acids GAL4 protein, is a DNA-binding domain. The GAL4 DNA binding domain recognises and binds to the UAS_{GAL4} (Fischer et al., 1988), but in the absence of an activating domain, fails to activate transcription in yeast and mammalian cells (Ma et al., 1988). In addition, the average reporter gene expression observed at the calli stage also showed no significant differences among constructs, except for the GUS reverse_intron which had much lower reporter gene expression than other constructs.

A wide variety of expression patterns were observed among 750 TAFET lines and some patterns displayed tissue-specific expression in the vegetative or floral tissues. About 95% of the TAFET lines showed gene expression in the roots, leaves or floral tissues, which was higher than previously reported (Klimyuk et al., 1995; Sundaresan et al., 1995a; Wu et al., 2003). About 48 to 66% of *Arabidopsis* *Ds*-based enhancer trap lines (Klimyuk et al., 1995; Sundaresan et al., 1995a) and about 70% of rice transactivator-based enhancer trap lines (Wu et al., 2003) displayed gene expression in various tissues. 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.

In addition to those described above, 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). Expression in 1, 2 or 3 organs in floral tissues was about 2%, 10% and 5%, respectively. However, the percentage of lines showing expression in the ovule was only about 0.5%. This 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).

In contrast to gene expression produced by the 8 different TAFET constructs in the calli stage, analysis of reporter gene expression observed at the plant stage showed that there were differences between transactivator GUSPlus constructs and transactivator GUS constructs in inducing gene expression in rice. This was supported by four results derived from observation of gene expression patterns in vegetative and floral tissues. Firstly, the analysis of reporter gene expression in over 750 TAFET lines

showed that constructs with GUSPlus induced more lines with strong expression (36%) than those with GUS (12%) (Fig.3.14). Secondly, analysis of staining patterns in vegetative tissues showed that the constructs with GUSPlus produced more lines with staining than those with GUS (Fig. 3.15). Thirdly, GUSPlus constructs also induced more lines with a high complexity of pattern than GUS constructs (Fig. 3.15 and Fig 3.16). The GUSPlus constructs had a dominant abc pattern, while the GUS constructs had the b pattern (Fig. 3.18). Fourthly, although analysis of the five most abundant patterns in floral tissues showed that GUS constructs produced similar patterns of expression to GUSPlus constructs (Fig 3.22), the pattern distribution produced by each construct showed that GUSPlus had a greater spread of pattern distribution (Fig 3.23). In addition, GUSPlus constructs also induced more patterns of expression than GUS constructs, producing 41 and 22 unique patterns, respectively, of a total of 108 patterns of expression (phenotype) produced by TAFET lines in floral tissues. These differences appear to be related to sensitivity differences between the two reporter genes. Sensitivity of the reporter gene is an important factor for gene expression and attempts have been made to develop better and more sensitive reporter genes (Jefferson et al., 1987; Haseloff et al., 1997). The newly developed β -glucuronidase (GUSPlus) from *Staphylococcus* sp has been codon optimised for high expression in plants and has better catalytic activity for more rapid detection of GUS activity (Nguyen, 2002).

In addition to the results described above, the GUS reverse_intron (construct number 4 in Table 3.1) behaved rather differently than the other transactivator constructs, displaying only 1 of the 3 dominant patterns in vegetative tissues (Fig. 3.19) and only 2 of the 5 dominant patterns in the floral tissues (Fig. 3.23). Besides that, the GUSPlus_intron (construct number 7 in Table 3.1) also displayed 1 of the 5 dominant patterns in the floral tissues (Fig. 3.23). This is not due to limited numbers of lines analysed, but is perhaps related to the nature of the construct, as a similar

phenomenon was displayed in the calli stage (Fig. 3.9). The reason for the phenomenon in these constructs is not known and needs further investigation, although it might be speculated that a random mutation within T-DNA was occurred. The random mutation can not be ruled out, since sequencing was not carried out in confirming of the construction product. Vectors resulted from construction were only confirmed using restriction enzyme digestions.

In comparing the patterns of transactivator constructs in floral tissues to those of pCAMBIA1201, some patterns were found to be similar. For example, patterns with expression in all floral tissues or with expression in the palea, lemma, anther wall and pollen. The most obvious pattern difference between TAFET lines and pCAMBIA1201 lines was that a higher percentage of pCAMBIA1201 lines showed strong expression patterns in the whole ovule (Fig 3.25), something rarely observed in TAFET lines (Fig. 3.24).

Other patterns of TAFET lines similar to those previously reported as being induced by the CaMV35S promoter in rice were the expression in the vascular bundles of the roots and leaves (Fig. 3.19, a and b; Fig. 13.20, a, b and d) (Battraw and Hall, 1990; Terada and Shimamoto, 1990), and expression in the basal part of the ovule (Terada and Shimamoto, 1990). Benfey et al. (1989; 1990) reported that the expression in the vascular bundles, in the lateral roots and in the pericycle (the cell layer from which lateral roots develop) of tobacco were tissue-specific expressions induced by the CaMV35S promoter. These expression patterns were also displayed in rice (Terada and Shimamoto, 1990; Battraw and Hall, 1990) and in maize (Omirulleh et al., 1993). Since the number of pCAMBIA1201 lines observed was relatively limited and most patterns previously reported (Battraw and Hall, 1990; Benfey et al., 1990; Terada and Shimamoto, 1990) were expressed in the roots and leaves, a further investigation of reporter gene expression in the floral tissues is needed.

Hobbs et al. (1990) reported that a position effect might not always be a major cause of inter-transformant variability; but that expression depended on the kind of T-DNA introduced into plant genomes. In addition, transgenic expression pattern is affected by the transcriptional regulation signal (Breyne et al., 1992). In our TAFET T-DNAs design, the activity of the GAL4/VP16 transcriptional activator was expected to magnify any signal from the chromosomal transcriptional regulation sequences (enhancers) and, by binding to 6xUAS_{GAL4}, produce expression patterns. Ranish and Hahn (1996) reported that the rate of transcription increases dramatically when the general transcription machinery was activated by a transcriptional activator. As expression patterns were affected by chromosomal transcriptional activator sequences, TAFET T-DNA insertion sites within the rice genome cannot be ignored as a potential source of influence on the patterns displayed.

Results from statistical analysis were comparable to those obtained from analysis of patterns related to constructs. For example, gene expression was affected by reporter genes highly significantly ($p < 0.001$) and by intron significantly ($p = 0.003$) (Table. 3.3). The complexity of patterns in vegetative tissues was affected highly significantly by reporter genes ($p < 0.001$) (Table 3.4), while the complexity of patterns in floral tissues was affected slightly significantly by a distance between UAS_{GAL4} and the 35S promoter driving *hpt* II ($p = 0.006$). Regression analysis also showed that the presence of the GUSPlus reporter gene correlated in a highly significant way to a complexity of patterns in vegetative and floral tissues, but when intensity was removed from analysis, it correlated negatively with a proximate (1.6kb) UAS_{GAL4} to the CaMV 35S promoter driving the *hpt* II gene (Table 3.6). Overall, analysis of expression patterns and statistical analysis indicate that the GUSPlus reporter gene revealed more patterns and more lines with strong intensity of expression and more complexity of pattern than GUS, because of its higher sensitivity.

The number of T-DNA insertions ranged from 1 to 7 copies and the average was 2.0 T-DNA copies per line (Table 3.8) which is similar to that reported by Hiei et al. (1997), Wu et al (2003) and Muthukalianan et al. (2003), but a slightly higher than that reported by Jeon et al. (2000a). About 49% of lines have a single T-DNA which is rather similar to the results of Wu et al. (2003) (42%) and higher than previously analysed in *Arabidopsis* (25%) and in rice (35%) (Campisi et al., 1999; Jeon et al., 2000a). Southern blot analysis also showed that lines transformed with transactivator-deleted constructs that showed no reporter gene expression, contained T-DNA insertions. This gave a confirmation that gene expression was due to the activation of the reporter gene by the GAL4/VP16 transcriptional activator.

Based upon the above results, it may be concluded that the different reporter gene and intron upstream GAL4/VP16 combinations used have some effect on the patterns produced. It is also apparent that 35S enhancers affect the expression pattern in a number of lines. However, abundant patterns are likely to represent primarily the effect of endogenous enhancers. Complex interaction between rice genomic enhancers and those from the T-DNA are the likely explanation for the results obtained.

In relation to the behaviour of the 35S promoter within the transactivator construct, these results may suggest that a further development of the transactivator construct should avoid the use of such a strong promoter like 35S to drive the *hpt II* gene. An attempt to do this by replacing the CaMV 35S promoter with the ubiquitin-1 promoter has been conducted by Fu (2004). An alternative solution might be to develop two separate constructs: a transactivator construct and a selection construct containing the 35S promoter driving the *hpt II* gene, and to apply co-transformation, as previously reported by Komari et al. (1996).

3.5 CONCLUSION

Five conclusions can be drawn from these experiments, as follows.

- First, Transactivator-facilitated enhancer trap constructs containing the GAL4/VP16 transcriptional activator and 6xUAS_{GAL4} were able to reveal patterns of expression both in vegetative and floral tissues, in rice.
- Second, expression patterns were due to the activity of the GAL4/VP16 transcriptional activator.
- Third, the different reporter genes and intron upstream GAL4/VP16TAFET in the combinations used had some effect on the patterns produced. It is also apparent that 35S enhancers affected the expression patterns in numbers of lines. However, patterns are likely to represent primarily the abundant effect of endogenous enhancers. Complex interaction between rice genomic enhancers and those from the T-DNA are likely to be the explanation of the results obtained.
- Fourth, transactivator GUSPlus constructs affected patterns of gene expression more than transactivator GUS, as they produced more lines with staining, strong expression and with complexity of patterns, produced more patterns and a greater spread of pattern distribution than transactivator GUS. These were due to differences in reporter gene sensitivity.
- Fifth, the GUSPlus gene proved to be a more sensitive reporter gene than GUS for revealing gene expression patterns.