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GREEN FLUORESCENT PROTEIN-BASED ENHANCER TRAPS FOR RICE FUNCTIONAL GENOMICS

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Corrections page

1. P15, L15 Shimomura et al. 1962


2. P19, L13 Replace ‘transfected’ with ‘introduced’


4. P28, L20 Hoekema et al, 1983 (missing year)

5. P45, L8 Sanger et al., 1977


6. P66, L7 Approximate number of plants obtained: 500

7. The following citations can be found in the references: Chilton et al., 1974 (P50); Chu et al., 1975 (P53); Omirulleh et al., 1993 (P74); Wilson et al., 1995 (P70)

8. FU01 297 had GFP pattern AEOPRS. In Chapter 7 (Genetic test and confirmation of the trans activator’s functionality), only AEO (root, stomata, lodicule) with relatively strong signal were investigated in the F1 populations.
STATEMENT

I hereby declare that the results presented in this thesis are original work, except where acknowledged, conducted by myself in CAMBIA during the course of my enrolment as a PhD student at the Australian National University. No materials in this thesis have been previously submitted for an academic record at any other university.

Xiqin Fu

11/02/09
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ABSTRACT

The enhanced green fluorescent protein (EGFP) reporter was cloned into 26 binary vectors including enhancer traps and positive/negative controls, and successfully used for tracing dynamic gene expression in the transformation process of rice, and for screening enhancer trap pattern lines. GUS::EGFP and GUSPlus::EGFP fusion reporters were also created to make good use of the advantage of GFP as a vital marker for dynamic monitoring of gene expression and the advantage of GUS for its high sensitivity in histochemical staining.

An *Agrobacterium*-mediated transformation system for the japonica rice variety Millin was well established. In a case study with enhancer trap constructs harboring GUSPlus::EGFP fusion reporter, a total of 1,021 transgenic rice lines were obtained in a single transformation experiment starting with 1,000 scutellum-derived calli. Further improvement of the transformation efficiency was achieved by decreasing the temperature to 22°C during co-cultivation of rice calli with agrobacteria. The efficiency can fulfill the prerequisite for the TransGenomics project in which large populations of transgenic lines are needed.

Several sets of enhancer traps were constructed and tested through transformation of rice calli. GFP expression of the enhancer traps, along with corresponding Gal4-deletion constructs, was studied extensively during the callus stage and at plant level. Constructs carrying the CaMV 35S promoter exhibited serious within T-DNA cis-activities, imposing significant background problem for screening genomic enhancers being trapped. The problem was in a great extent solved by replacing the CaMV 35S promoter with Ubi-1 promoter in the selection cassette of enhancer trap constructs. Out of 393 enhancer trap lines obtained with the improved constructs, 129 (32.8%) lines were found GFP-positive with diverse expression patterns, which were valuable genetic resources for functional studies of gene of interest in rice.

Sexual crosses, between enhancer trap lines with EGFP reporter and target gene lines harboring 6xUAS-MP-GUS, were made to verify functionality of the
transactivator in the system. Clear co-expression of GFP and GUS in F₁ progenies were observed, implying that the transactivator performed its functions as theoretically expected and the system established could be used for creating gain-of-function mutagenesis in rice. This is the first genetic testing of the Gal4-UAS system in rice, providing solid evidences for a successful establishment of the Transcriptional Activator-Facilitated Enhancer Trap (TAFET) system in rice. Potential usage of the system for innovative rice breeding was also discussed.
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Chapter 1 Introduction and literature review

1.1 Rice as a model system for cereal genomics

1.1.1 Rice as an important crop plant

The word “rice” generally indicates a plant and a crop of the species *Oryza sativa* L. The genus *Oryza* L. is classified under the tribe Oryzeae, subfamily Oryzoideae, of the grass family Poaceae (Gramineae) (Lu, 1999). This genus has two cultivated species (*O. sativa* L. and *O. glaberrima* Steud.) and more than 20 wild species distributed throughout the tropics and subtropics. The so-called Asian cultivated rice (*O. sativa*) is actually cultivated worldwide, while *O. glaberrima* is only cultivated in a few countries in West and Central Africa.

Rice is the world’s single most important food crop that is the staple food for more than one-half of the world’s population. It is a nutritious grain crop which contains carbohydrates, proteins, lipids, minerals, etc. Rice is used for food in various forms. Grains are heated in water to become cooked rice. Rice flour is usually kneaded with water, boiled and used for various rice products. The bran is an important source of oil for food and manufacturing. Husks are used for fertilizers and animal feed, and rice straw is used as an important animal feed and for making various wrapping materials and mats.

Rice is planted on about 150 million hectares annually. According to FAO data, 603 million tons of rice was harvested from plantings of 154 million hectares worldwide (FAO, 2003). More than 91% of world rice production comes from Asia, 5% from the Americas, 3% from Africa, and another 1% from Europe and Oceania. Rice is the predominate staple food for fifteen countries in Asia and the Pacific, ten countries in Latin America and the Caribbean, one country in North Africa and seven countries in Sub-Saharan Africa (FAO, 1999). In developing countries, rice accounts for 715 kcal/capita/day which corresponds to 27% of dietary energy supply, 20% of dietary protein and 3% of dietary fat. Countries in
Southeast Asia are heavily reliant upon rice; in Bangladesh, Laos, Viet Nam, Myanmar and Cambodia, rice supplies more than fifty percent of per capita dietary energy and protein supply and 17-27% of dietary fat. Rice is also an important staple food for several countries in Africa. In Guinea, Guinea-Bissau, Gambia, Liberia, Senegal and Cote d'Ivoire, rice supplies between 22-40% of dietary energy and 23-39% of dietary protein.

### 1.1.2 Challenges for rice breeding in the future

The population of rice consumers is increasing at the rate of 1.8% annually. The present annual rice production of 560 million tons must be increased to 850 million tons by 2025 (FAO, 2002b). There are no additional lands available for rice cultivation. In fact, the area planted to rice is going down in several countries due to pressures of urbanization. Thus, we need the rice varieties with higher yield potential and yield stability for meeting the challenges of increased rice production.

Major advances have been made in increasing rice production worldwide as a result of large-scale adoption of modern high-yielding rice varieties and improved cultural practices. The rapid rice production increase was mainly achieved through the application of principles of classical Mendelian genetics and conventional plant breeding methods, including the hybrid rice technology.

Hybrid rice is the most significant technology since the identification of dwarf plant-types. Hybrids have consistently shown a 15-20% increase in yield compared to conventional varieties. Scientists in China initiated hybrid rice breeding efforts in the late 1960s and the first commercial hybrid was introduced in 1974. In year 2000, it was estimated that hybrid rice was cultivated on approximately 16 million hectares in China; 300,000 hectares in Vietnam; 150,000 hectares in India and 30,000 hectares in Bangladesh (IRRI, 2000).

Because of consistent efforts by plant breeders, rice production has doubled between 1966 and 1990, but it must increase further by 60% by 2025 in order to
feed the additional rice consumers (Khush, 1997). However, the contribution of traditional breeding methods to increased rice yield has reached a plateau, for the average rice yield per unit area has been stagnated for several years according to the FAO data (FAO, 2003). Yield-stagnation also appears to be a problem even with hybrid rice (Yuan, 1998). The growth in rice yields has decelerated to slightly more than 1% per year or approximately equal to population growth (FAO, 2002a).

Rice demand in year 2030 is projected to be approximately 533 million tons of milled rice (FAO, 2002b). To meet this goal, rice varieties with higher yield potential, durable resistance to diseases and insects, and tolerance to abiotic stresses are needed. Sustainability in rice production can only be achieved by addressing the major yield constraints imposed by drought, floods, salinity, soil degradation and pests and diseases. Addressing these problems requires a coordinated approach using the best available research tools at molecular level. These include use of transgenics, isolation and transfer of novel genes that confer tolerance or resistance to particular abiotic/biotic stresses as well as the use of molecular markers in breeding programs to achieve faster genetic improvement.

1.1.3 Rice as a model system for cereal genomics

Apart from its economic significance, rice has been also an important model plant for genetic and genomic studies. Rice has relatively small genome (430 Mb) compare to other cereal crops, such as maize (2,400 Mb), barley (4,900 Mb) and wheat (16,000 Mb) (Bennetzen, 2002). Rice researchers have developed important tools for genetic analysis, including high-density molecular genetic maps and efficient genetic transformation techniques. Comparative genetic maps within the grass family indicate the existence of conserved gene content and gene order among grasses genomes (Ahn et al., 1993; Devos and Gale, 1997, 2000).

In rice, the first publication of a molecular genetic map using RFLP was in 1988 by S. McCouch et al. (McCouch et al., 1988), and subsequently, higher resolution maps were published by Kurata et al. (Kurata et al., 1994), Causse et
More recently, a sophisticated linkage map has been developed by Yano et al. (Yano, 2000) in which 3267 DNA markers were located and many of them were converted from RFLP to CAPS (Cleaved Amplified Polymorphic Sequence) markers (Glazebrook et al., 1998). CAPS facilitates the identification of polymorphisms within a PCR product using a restriction enzyme as an alternative to the laborious Southern hybridization technique. The quality and number of markers on this map are undoubtedly the most outstanding among all organisms genetically analyzed by DNA markers. The total number of co-dominant markers such as RFLP, SSR or CAPS published to date is about 5000. This means that the average marker density within the rice genome is one marker in every 80 kb.

In April 2002, a rough draft sequence of indica rice variety 93-11 was published by an academic institute, the Beijing Genomics Institute (Yu et al., 2002). In the same month, a draft sequence of japonica variety ‘Nipponbare’ was published by a private company, Syngenta (Goff et al., 2002). Both draft sequences were obtained by a whole-genome shotgun approach. In November 2002, a few month later, two members of the International Rice Genome Sequencing Project (IRGSP), namely the Rice Genome Research Program (RGP, Japan) and the National Center for Gene Research (NCGR, China) published high-quality phase-3 sequences of chromosome 1 and chromosome 4, respectively (Feng et al., 2002; Sasaki et al., 2002). Both sequences were obtained using complete sequencing of BAC/PAC contigs. On December 18th 2002, the IRGSP announced the completion of a high-quality draft with at least phase-2 sequences of the 12 rice chromosomes (Sasaki and Sederoff, 2003).

The number of cereal genes that are estimated based on partial genomic sequencing and EST clusters ranges from 44,700 for rice (Wu et al., 2002) to 55,000 for maize (Brendel et al., 2002). Two independent analyses that were based on the shotgun genomic sequencing of the rice genome estimate the number of predicted rice genes to be 32,000–50,000 (Goff et al., 2002) and 46,022–55,615 (Yu et al., 2002). More recent estimates, obtained by
extrapolating the annotation of the finished rice chromosomes 1 and 4, predict a slightly higher gene number of 57,000–62,500 (Feng et al., 2002; Sasaki et al., 2002). Together, these estimates suggest that rice has a transcriptome that contains nearly twice the number of genes in Arabidopsis (Arabidopsis Genome Initiative, 2000) and humans (Lander et al., 2001), and more than three times the number in Caenorhabditis elegans (The C. elegans Sequencing Consortium, 1998) and Drosophila (Adams et al., 2000).

Whole-genome analyses from Arabidopsis and rice suggest that higher estimate of gene number in cereals can be explained by a combination of factors that includes gene amplification and inaccurate gene predictions (Ware and Stein, 2003). These findings are supported by reports from the International Rice Genome Sequencing Project (IRGSP) on rice chromosomes 1 and 4 (Feng et al., 2002; Sasaki et al., 2002).

A finished rice genome will provide a complete index of potential rice genes but will not tell us which of these genes are important in providing desired traits in cereal crops. Genome sequences are just a beginning: they provide a necessary resource for powerful methods for proteome analysis that require sequence knowledge. In the future, techniques such as gene, protein and metabolic profiling will provide insights into the function and expression patterns of genes and into how these genes ultimately contribute to a crop’s ability to react to an environment and reproduce (Ware and Stein, 2003).

1.2 Functional genomics - approaches and limitations

The raw data produced by genome sequencing projects currently provides little insight into the precise workings of an organism at the molecular level. Therefore, the goal of functional genomics is to complement the genomic sequence by assigning useful biological information to every gene. Through this, we can aim to improve our understanding of how the different biological molecules contained within the cell (i.e., DNA, RNA, proteins, and metabolites) interact to
make the organism viable. Clearly, the main challenge is the elucidation of all molecular, cellular, and physiological functions of each gene product.

1.2.1 Gene expression by defined promoter

Ectopic misexpression of a single gene in Drosophila is often sufficient to disrupt normal development, generating dominant phenotypes that may provide important clues regarding gene function. The simplest method for controlled gene expression is to use transcriptional regulatory elements from a well-characterized promoter. With the regulatory elements, the gene of interest will express in a defined tissue or stage-specific manner.

A good example of this technique is the use of the sevenless promoter to drive gene expression in a subset of cells in the eye imaginal disc of Drosophila (Basler et al., 1991). Gain-of-function mutants were obtained by overexpressing the sevenless gene with duplicated transcriptional regulatory elements in the promoter. This approach has proven invaluable in analyzing signal transduction and cell fate determination in the Drosophila eye.

The Drosophila Rh1 promoter contains a pentamer of binding sites for the GLASS transcriptional activator. Hay et al. (Hay et al., 1994) subcloned the regulatory sequence from Rh1 into the GMR (glass multimer reporter) vector and studied ectopic gene expression in the developing Drosophila eye. GLASS expression is largely restricted to the developing eye and the larval photoreceptor organs, showing a tight spatial regulation on expression of the P35 gene concerned.

This method is the easiest way for studying ectopic gene expression since only a single or a few transgenic lines are needed. A great limitation of the method is the availability of well-characterized promoters. Moreover, constitutive promoters cannot be used to investigate genes whose constant over- or under-expression has deleterious effects on the plant. Furthermore, when the gene
product to be expressed is toxic to the organism, it becomes impossible to establish stable transgenic lines carrying the gene.

1.2.2 Enhancer trap mutagenesis

Enhancer traps are reporter gene constructs that can respond to cis-acting transcriptional signals when inserted in the genome of certain organism. The concept of enhancer trap has been successfully used in Drosophila (O'Kane and Gehring, 1987; Bellen et al., 1989), mouse (Allen et al., 1988) and Arabidopsis (Klimyuk et al., 1995; Sundaresan et al., 1995; Campisi et al., 1999), leading to the isolation of genes important in development whose phenotypes would have been missed in conventional mutagenesis screens.

1.2.2.1 The concept of enhancer traps

Transcriptional enhancers are DNA sequences that control expression of nearby genes and direct tissue-specific, positive regulation of those genes (Karp, 2002). The general mode of action of these sequences is that they associate with specific activator proteins that are found in only certain tissues and this association of enhancer and activator facilitates transcription initiation at the target gene's promoter. Enhancers might be located upstream, downstream or even within the transcription unit that they control. Under the influence of one or more enhancers, a gene can be abundantly expressed in certain tissues of the organism where the activator protein is found and expressed weakly or not in other tissues. By identifying and studying enhancers, we can study how genes are regulated in time and space within multicellular organisms and we are able to use enhancers to tag tissues or even individual cells (Karp, 2002).

Conventional mutagenesis approaches are dependent on the presence of a recognizable phenotype when a particular gene is mutated. These approaches have at least two limitations. First, many genes play multiple roles and phenotypic screens may fail to detect such genes that exhibit discrete functions at different stages in development. Second, if a gene is functionally redundant,
disruption of the gene may result in only subtle phenotype or no phenotype at all unless all genes specify that function are mutated simultaneously.

In recent years, a better method called enhancer trap mutagenesis has been developed for detecting and studying enhancers. This method utilizes a recombinant molecule construct that can be inserted at essentially random sites within the genome and that, when inserted near an enhancer, will express a reporter gene product within the tissue or cells containing activator protein for that enhancer. The enhancer trap transposon contains a minimal promoter element linked to a reporter gene that is inactive, except when influenced by a nearby enhancer. Because most enhancers direct tissue-specific gene expression (Karp, 2002), insertion of the enhancer trap molecule near an enhancer usually results in reporter gene expression within the particular tissue or group of tissues in which that enhancer normally functions. By mobilizing the transposon to diverse, essentially random chromosomal sites, one can detect diverse enhancers at those sites and study the tissue-specific gene control that they direct. Furthermore, enhancer trap constructs have been engineered to permit cloning of genomic DNA adjacent to the site of any particular insertion, enabling the isolation of the enhancer and its target gene from genomic DNA. This has proved to be a powerful system for studying enhancers and promoters that direct highly specific gene expression patterns.

1.2.2.2 Development of enhancer trap systems

The first cis-acting enhancer trap was established in *Drosophila* from transposable P-element (O'Kane and Gehring, 1987) primarily due to high efficiency of the transformation system. In these constructs, the β-galactosidase gene (*lacZ*) has been placed under the control of a weak promoter so that there should be no detectable *lacZ* expression without enhancer activity. In integrated copies of the P-lacZ, the *lacZ* gene acts as a reporter for genomic transcriptional enhancer elements that are located sufficiently close to the detector to activate the P-element promoter.
Bellen et al. (Bellen et al., 1989) generated and characterized more than 500 *Drosophila* strains that carry single copies of a P-element enhancer trap. A remarkable diversity of spatially and temporally regulated staining patterns was observed in embryos carrying different insertions. The expression patterns evident in those early enhancer studies showed that 5-10% of the lines with single P-element insertions expressed lacZ in very specific tissues or cells, providing many excellent new tissue and cell markers (Bellen, 1999). These markers are very useful in studying the embryonic development of these tissues and cells.

The enhancer piracy approach developed by Noll et al. (Noll et al., 1994) is a modification of enhancer detection technique. In their enhancer piracy vectors, the *Drosophila* rhomboid (rho) gene was substituted for the lacZ reporter gene present in enhancer trap vectors. Upon introducing the modified construct into the *Drosophila* genome, rho expression came under the influence of neighboring genomic enhancer elements. A variety of dominant rho alleles were generated through the enhancer piracy approach, providing important insight into the developmental function of the rho gene. Enhancer piracy may prove to be a general strategy for obtaining dominant alleles of a gene of interest in diverse insects, worms, plants and potentially in vertebrates (Noll et al., 1994).

Enhancer trap mutagenesis approach has also been employed in plants initially in *Arabidopsis* (Klimyuk et al., 1995; Sundaresan et al., 1995; Campisi et al., 1999) and recently in rice (Wu et al., 2003). A well studied enhancer trap system has been established by Sundaresan et al. (Sundaresan et al., 1995) based on the two component Ac/Ds transposable elements (Bancroft et al., 1992). The system uses a modified Ds element carrying a β-glucuronidase gene fused to a 35S minimal promoter as enhancer trap. Various starter lines, each containing a single stable DsE insertion, are generated by *Agrobacterium*-mediated transformation. Transposition of the DsE is achieved by crossing the DsE starter lines with lines expressing the Ac transposase. Since parental Ac transposase gene is linked to a gene coding for the indole acetic acid hydrolase (IAAH), which confers sensitivity to naphthalene acetamide (NAM), progenies that are free of
transposase activity can be selected so that each selected DsE insertion is stable. The IAAH gene is also linked to the DsE in the T-DNA donor site to act as a counter-selectable marker for eliminating the progenies retaining the T-DNA donor site in the presence of NAM. At the same time, plants with a transposition event are selected by the kanamycin resistance gene nptII contained in the DsE element. This selection procedure ensures that only the plants with transposition to more distal sites can survive. In this way, the genome can be saturated with random DsE insertions.

1.2.3 The Gal4 system

The yeast transcriptional activator Gal4 provides the most powerful method currently available for directing tissue- or cell-specific ectopic expression (Brand and Perrimon, 1993). The Gal4 protein can activate transcription of any target genes bearing Gal4 binding site UAS (upstream activation sequence) and a minimal promoter near the UAS. The Gal4 system is designed to generate lines that express a transcriptional activator, rather than a target gene, in numerous specific expression patterns. Two approaches have been used to generate different patterns of Gal4 expression. The first is to drive Gal4 expression using characterized promoters (e.g., a tissue-specific or heat shock promoter). The second is based on enhancer trapping, in which the Gal4 expression pattern is directed by flanking genomic enhancer elements proximal to insertion site of the transactivator construct.

The UAS-target gene lines are generated separately. A cassette of UAS-minimal promoter-target gene is constructed and used for generating target transgene lines. Usually several independent insertion lines should be tested for expression experiments due to the fact that expression of UAS transgenes is subject to position effects (differences in expression potential of various chromatin domains).

Rorth (Rorth, 1996) developed a method of screening for phenotypes caused by misexpression of endogenous genes. In this method, a minimal promoter under
the control of UAS elements is introduced into multiple sites in the *Drosophila* genome to drive endogenous genes flanking downstream the inserted UAS-regulated promoter. When combined with a specific Gal4 line, the flanking endogenous gene will express ectopically in a pattern directed by the Gal4 activator. Rorth et al. (Rorth et al., 1998) generated 2300 independent UAS lines that were screened for dominant phenotypes in combination with various Gal4 pattern lines. Dominant phenotypic abnormalities were detected in 2-7% of the UAS lines depending on which Gal4 line was used. These gain-of-function screens seems to be an important approach to study functions of genes, for mis- or overexpression phenotypes are much less likely to arise by traditional loss-of-function mutagenesis.

The key feature of the Gal4 system is that the Gal4 gene and UAS-target gene are initially separated into two distinct transgenic lines. This ensures that the generated parental lines are viable since in Gal4 pattern lines, expression of Gal4 only has no detectable effects on development of organisms; in UAS lines, the target gene is silent in the absence of Gal4 activator. Only on crossing these two lines will the target gene be activated in the progeny of the cross, and can the phenotypically defective or lethal consequences of misexpression be conveniently studied.

Another feature of the system is the potential to create numerous distinct expression patterns of target gene by crossing target lines with a range of pattern lines.

### 1.2.4 Chemical-inducible transcription factors

The ability to tightly regulate gene expression in plant cells is an effective tool for the elucidation of gene function and manipulating complex developmental pathways. Chemical-inducible gene expression systems have advantages over other systems as heterologous transcription activators are used and transgene induction can be regulated in a spatial and temporal fashion using tissue or developmental specific promoters to express the activator, and by applying
inducer at a desired time and location. Gene expression can also be restricted to a particular window by constructing transactivators that can be switched on with one chemical and switched off with another chemical. In addition, transgenes can be expressed at physiologically relevant levels by varying the inducer concentration.

The chemical-inducible systems developed for plants so far include: Tet repressor-based, tetracycline de-repressible (Gatz et al., 1992; Love et al., 2000); tTA-based, tetracycline inactivatable (Weinmann et al., 1994); glucocorticoid receptor-based, dexamethasone inducible (Aoyama and Chau, 1997); AlcR-based, ethanol inducible (Caddick et al., 1998; Roslan et al., 2001); Ecdysone receptor (EcR)-based, EcR agonist inducible (Martinez et al., 1999); estrogen receptor-based, β-estradiol inducible (Bruce et al., 2000; Zuo and Chua, 2000); and Tet and glucocorticoid receptors-based, dual control (Bohner et al., 1999).

An ideal inducible gene expression system should have the following desirable properties (Zuo and Chua, 2000): low basal expression levels, high inducibility, specificity to inducer, high dynamic range to inducer concentrations, fast response, switch-off after removal of inducer, and low toxicity. Among the systems reported, the following two systems make use of the function of Gal4.

1.2.4.1 Glucocorticoid receptor-based, dexamethasone inducible system (GVG)

GVG is a chimeric transcription factor consisting of the DNA-binding domain of the yeast transcription factor Gal4, the transactivation domain of the herpes virus VP16, and the receptor domain of the rat glucocorticoid receptor (GR). The GVG system is quite similar to the Gal4 system in its components with the only difference that the hormone-binding domain (HBD) of the glucocorticoid receptor is fused to the Gal4/VP16 transactivator, rendering the system controllable by HBD. The system is based on the rationale that HBD of vertebrate steroid hormone receptors has repressive effects on covalently linked, neighboring domains in the absence of their cognate ligands and de-repression occurs when appropriate ligands bind to the HBD (Picard, 1993).
Aoyama and Chua (Aoyama and Chau, 1997) reported the first GVG system for transcription induction in tobacco. In their system, the GVG chimeric transactivator is driven by the constitutive promoter CaMV 35S. The gvg gene is introduced into tobacco together with the Luc receptor gene which is under the control of a minimal promoter with 6 copies of Gal4-binding sequence (UAS). In the absence of the chemical inducer dexamethasone (DEX), activity of the transactivator is repressed due to the effect of GR. When transgenic tobacco plants are treated with DEX in the concentration range from 0.1 to 10 μM, gvg is activated and expression of the reporter gene is stimulated even over 100-fold depending on the concentrations of DEX applied. The transcription induction by DEX is rapid (60 min after DEX addition) and can be maintained for several days after removal of the inducer (Aoyama and Chau, 1997).

Several studies have demonstrated the utility of the GVG-based glucocorticoid-inducible gene expression system in transgenic tobacco and Arabidopsis plants (McNellis et al., 1998). Their results indicate that the GVG system should be suitable for studying the effects of expression of most genes of interest, including those that are extremely toxic or deleterious to the plants.

Using the GVG system, Kunkel et al. (Kunkel et al., 1999) developed a method for marker-free transformation. The ipt gene of Agrobacterium Ti plasmid is known to cause cytokinin production in transformed cells leading to shoot regeneration, though the uncontrolled production of cytokinins causes developmental abnormalities, and the transgenic shoots were unable to produce roots and their flowers were infertile. Kunkel et al. (Kunkel et al., 1999) placed the ipt gene under the control of the GVG system (Aoyama and Chau, 1997). Agrobacteria carrying the GVG–ipt construct were used to inoculate tobacco leaf disc in a medium without auxin and cytokinin. No shoot regeneration was observed in the absence of dex. In the presence of the inducer many shoots regenerated though they were developmentally abnormal. These shoots were then transferred to a medium without the inducer, and after several weeks normal marker-free transgenic plants developed.
However, some shortcomings of GVG system were also reported (Kang et al., 1999). In transgenic Arabidopsis, some severe developmental and growth defects were related with the strong gvg expression, and the inducer chemical DEX caused non-specific induction of defense-related genes in control plants.

1.2.4.2 Tet and glucocorticoid receptors-based, dual control system (TGV)

The TGV system is in a way a combination of TetR de-repression system (Gatz et al., 1992; Gatz, 1997; Gatz and Lenk, 1998) and DEX-mediated GVG system (Aoyama and Chau, 1997). Gal4, the DNA-binding element of the GVG transactivator, is replaced by TetR and correspondingly, the Gal4-binding site (UAS) is replaced by tet operator sequence for the binding of TetR. The feasibility for TetR’s binding to the operators is controllable by tetracycline, while the activity of the transactivator is conditioned by whether the hormone inducer DEX is added or not. Therefore, the TGV system is a dual-control inducible system, where target gene expression can be switched on with DEX and switched off with tetracycline, allowing precise control of induced expression of a transgene.

In this system, the chimeric transcription activator TGV consists of TetR, the regulatory region of the rat GR and the VP16 transactivating sequence. The system has been established and characterized with tobacco plants (Bohner et al., 1999). In their experiments, the TGV transactivator is constitutively driven by the CaMV 35S promoter. The target promoter, consisting of 7 tet operators upstream of a TATA-box, is used to control expression of the GUS reporter gene.

When the plants were treated by DEX at a concentration of 30 μM for 14 days, GUS activity reached to 2000U, which was the same inducing activity as in the tTA system and about 20% of the GUS activity driven by CaMV 35S promoter (10000U). After 14 days of DEX feeding, GUS activity could maintain at maximal levels for a further 6 weeks under DEX-free conditions, leading to quasi-constitutive levels of gene expression (Bohner et al., 1999).
On the other hand, application of tetracycline caused efficient shut down of the induced promoter. When GUS expression reached its maximal level and DEX was removed, application of tetracycline to the plants led to a decline of GUS mRNA levels after 6 h, and no gus expression being detectable after 24 h (Bohner et al., 1999).

However, there are also some potential problems in this system. First, the induction level is not desirably high and no induction is detected in flowers. Second, tetracycline causes browning of roots and reduction of root growth, and affects photosystem II efficiency by 5-15%. Third, the target promoter construction is still questionable. The one used in the tTA system causes silence of the target gene over time, while the modified target promoter in the TGV system leads a higher background expression.

### 1.3 Use of the green fluorescent protein as a reporter

#### 1.3.1 Discovery of the green fluorescent protein

The green fluorescent protein (GFP) was discovered by Shimomura et al (1962) as a companion protein to aequorin, the famous bioluminescent protein from jellyfish *Aequorea victoria*. GFP was first isolated in 1974 and has two excitation peaks, one at 395 nm and a second, smaller peak at 475 nm. A single emission peak is found at 508 nm (Morise et al., 1974). The fluorescence in jellyfish is generated by sequential excitation of the two photoproteins (aequorin and GFP). The binding of calcium to aequorin causes the emission of a blue chemiluminescence. The blue light produced by aequorin is absorbed by GFP, which then emits green fluorescence efficiently without the assistance of a cofactor (Chalfie et al., 1994). The crucial breakthroughs in GFP studies came with the cloning of the gene by Prasher et al (Prasher et al., 1992) and the demonstrations by Chalfie et al (Chalfie et al., 1994) and Inouye and Tsuji (Inouye and Tsuji, 1994) that expression of the gene in other organisms creates fluorescence. In just a few years, GFP has become one of the most widely studied and exploited reporter proteins in biochemistry, cell and molecular
biology. The number of published papers involving GFP reporter or marker has since risen to several thousands (5728 based on a PubMed search).

![Image of GFP structure](image)

Figure 1.1 The overall shape of GFP. Eleven strands of β-sheet (green) form the walls of a cylinder. Short segments of α-helices (blue) cap the top and bottom of the “β-can” and also provide a scaffold for the fluorophore which is near geometric center of the can. Two monomers are associated into a dimer in the crystal and in solution at low ionic strengths (Yang et al., 1996a).

### 1.3.2 Molecular structure of GFP

GFP is a small protein containing 238 amino acids (Prasher et al., 1992). From crystallographic data (Figure 1.1), GFP has been shown to exist as a barrel-like cylinder, composed of 11 closely-packed β-sheets (the slats of the barrel) with an α-helix inside (Yang et al., 1996a). The ends of the cylinder are capped by short helical segments of the polypeptide, forming an internal environment comprised of only the protein and some structured interior water molecules (Boxer, 1996). The structural motif of GFP is unique and has been termed the β-can (Yang et al.,
1996a; Phillips, 1997). It is inside this ‘can,’ in the center of the internal α-helix, where the GFP chromophore is located. The chromophore is a p-hydroxybenzylideneimidazolinone (Prasher et al., 1992; Cody et al., 1993) formed from residues 65–67.

Figure 1.2 Mechanism proposed by Cubitt et al (Cubitt et al., 1995) for the intramolecular biosynthesis of the GFP chromophore, with rate constants estimated for the Ser65Thr mutant by Reid & Flynn (Reid and Flynn, 1997) and Heim et al (Heim et al., 1995).

1.3.3 Structure of the GFP fluorophore

Figure 1.2 shows the currently accepted mechanism (Heim et al., 1994; Cubitt et al., 1995; Reid and Flynn, 1997) for chromophore formation of GFP. The fluorophore is derived from three consecutive amino acids, which are Ser65,
Tyr66 and Gly76 in the native protein. Following synthesis of GFP, these three amino acids undergo autocatalytic cyclization and oxidation to form the functional fluorophore. The carbonyl group (C=O) of Ser65 forms a covalent bond to the amino group (NH$_2$) of Gly67 and finally the $\alpha$-$\beta$ bond of Tyr66 is oxidized to a double bond to put its aromatic group into conjugation with the imidazolinone. Only at this stage does the chromophore acquire visible absorbance and fluorescence.

1.3.4 Basic characteristics of GFP

GFP is an extremely stable protein (Prasher et al., 1992). The photostability of GFP is thought to be due to the tight packing of the fluorochrome within the protein core, which shields it from solvent and from reactive oxygen species responsible for photobleaching. The fluorescent properties of the protein are unaffected by prolonged treatment with 6M guanidine HCl, 8M urea or 1% SDS, and two day treatment with various proteases such as trypsin, chymotrypsin, papain, subtilisin, thermolysin and pancreatin at concentrations up to 1 mg/ml fail to alter the intensity of GFP fluorescence (Bokman and Ward, 1981). GFP is stable in neutral buffers up to 65°C, and displays a broad range of pH stability from 5.5 to 12. The protein is intensely fluorescent, with a quantum efficiency of approximately 80% and molar extinction coefficient of $2.2 \times 10^4$ cm$^{-1}$ M$^{-1}$ (Morise et al., 1974).

1.3.5 GFP as a reporter

The use of GFP as a reporter molecule has several advantages over current reporter systems such as $\beta$-galactosidase, luciferase, alkaline phosphatase, chloramphenicol acetyltransferase, and GUS. These reporters all require exogenous substrates, co-factors, or antibodies for detection, while GFP only requires oxygen and illumination by the proper wavelength of light (Prasher, 1995; Sheen et al., 1995), thereby facilitating non-invasive, in vivo detection. GFP’s relatively small size (27kDa), monomeric structure, and unusual stability allow fusion proteins to be made without disturbing GFP’s native structure or
function (Boxer, 1996; Mitra et al., 1996; Plautz et al., 1996). In fact, Roger Tsien's group has produced constructs where a protein of interest is genetically inserted into one of the exterior β-sheets, such that the GFP maintains proper folding and is fully fluorescent (Tsien, 2000).

GFP retains fluorescence when fused to another protein at either the N- or C-terminus (Leffel et al., 1997). This property makes it an ideal fluorescent tag to monitor subcellular localization of proteins, organelles, and biochemical processes. GFP was fused to organelle targeting tags. This resulted in GFP localized to subcellular structures including the ER (Haseloff and Amos, 1995), nucleus (Grebenok et al., 1997), mitochondria (Kohler, 1998), cytoskeleton (Katz et al., 1998), Golgi apparatus (Boevink et al., 1998), chloroplasts (Kohler et al., 1997), and peroxisomes (Jedd and Chua, 2002).

GFP has been successfully transfected and expressed in a number of heterologous plant systems including rice, corn, wheat, oat, sugarcane, tobacco, soybean, and Arabidopsis (Hu and Cheng, 1995; Casper and Holt, 1996; Zhang et al., 1996; Elliott et al., 1999; Jordan, 2000; Kaeppler et al., 2000; Upadhya et al., 2000). Microprojectile bombardment of GFP fusion constructs into onion epidermal peels as well as roots from intact Arabidopsis seedlings has proved effective in transient transfection experiments (Sheen et al., 1995; Scott et al., 1999), while Agrobacterium-mediated transformation of Arabidopsis is now routinely used to establish stable transgenic plants expressing GFP (Pang et al., 1996; Cutler et al., 2000; Hasezawa et al., 2000). Several uses of GFP include measurement of gene expression in vivo, monitoring dynamic cellular processes in real time, easy visual identification of transformants, and monitoring protein subcellular location and protein-protein interactions (Prasher, 1995).

1.3.6 Important variants of GFP

Cubitt et al. (Cubitt et al., 1995) created the first important mutation of wild-type GFP, which shifted the primary excitation peak from 395 nm to 488 nm, combining the area under the two peaks into a single larger peak. This change
was the result of a single mutation in the chromophore, mutating serine 65 to a threonine (Cubitt et al., 1995). The mutation had several other beneficial effects, including six-fold brighter fluorescence, faster oxidation, and little or no photobleaching of the chromophore (Cubitt et al., 1995). The S65T derivatives of GFP are ideal for confocal microscopy because they are very efficiently excited by the 488 nm argon laser illumination.

The synthetic S65T gene with the cryptic intron removed and humanized codon-usage was called sGFP (S65T) (Haas et al., 1996). Codon engineering conferred a significant increase in expression efficiency and the combination of the S65T mutation and codon optimization resulted in a DNA segment encoding a highly visible mammalian marker protein. Haas et al. (Haas et al., 1996) reported that there was a net improvement in fluorescence per cell of between 40–120 folds, depending on detection conditions. Chiu et al. (Chiu et al., 1996) demonstrated that the Ser65 to Thr (S65T) mutation provided a fluorescence gain of up to 100-fold in plant cells after human codon optimization was performed. The sGFP-S65T mutation variant, which has a single blue excitation peak (489 nm optimum) and a redshifted excitation peak (511 nm optimum), has been used very often in monocots (Elliott et al., 1999; Jordan, 2000; Kaeppler et al., 2000; Upadhyaya et al., 2000).

Another GFP variant popularly used for expression in plants (frequently dicots) is the mutant mGFP5 created by Jim Haseloff (Cambridge, U.K.). Haseloff et al. (Haseloff et al., 1997) optimized wild-type GFP for expression in plants by demonstrating that an 84-nt cryptic intron was removed from GFP cDNAs between nucleotides 400 and 483 in Arabidopsis (between residues 380-463 of the GFP coding sequence). The resultant protein was non-fluorescent (Haseloff and Amos, 1995). A modified version of the gene was created that mutated the splice sites and altered codon usage to reduce the AU content of the mRNA. Transgenic plants stably expressing the modified GFP (mGFP series) were created, demonstrating proper expression of the protein in plants (Siemering et al., 1996; Haseloff et al., 1997). mGFP5, however, is similar in spectral properties to wild-type GFP, save a larger excitation shoulder at 475 nm.
(excitation = 395/475 nm; emission = 509 nm). Functionally, this means that mGFP5 can be excited equally well by UV and blue spectral components (DAPI and FITC filter settings), while the native GFP from *Aequorea victoria* is preferentially excited by UV radiation (395 nm). Excitation of mGFP5 in living plant tissues by blue light is preferable to UV due to the greater incidence of photodamage induced by the latter.

The enhanced green fluorescent protein (EGFP) is a popular humanized (Yang et al., 1996b) and bright commercial variant from Clontech (Clontech Laboratories, Palo Alto, CA). The characteristics of EGFP are as follows:

- The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (Haas et al., 1996)
- A mutation of Ser65 to Thr to promote chromophore ionization and a mutation of Phe64 to Leu to improve folding at 37°C, contributing to a greatly increased (100-fold) fluorescence intensity compared to the wild-type (Cormack et al., 1996)
- A mutation of His231 to Leu, which was probably inadvertent and neutral
- Upstream sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (CGCCACCATGG) (Kozak, 1987) to further increase the translation efficiency in eukaryotic cells, including insertion of a new codon GTG (Val) right after the start codon ATG. The inserted amino acid Val is often numbered 1a to maintain correspondence with the wild-type numbering.
- Excitation maximum = 488 nm; emission maximum = 507 nm

Many other groups optimized and reshaped GFP, creating spectral variants altered in both excitation and emission maxima. The most distinguishable mutant form of GFP features a tyrosine to histidine change at residue 66, resulting in blue fluorescence rather than green (Palm et al., 1997). The existence of two spectrally distinguishable fluorescent proteins made dual-labeling experiments a
possibility and spurred the development of additional variants (Davis and Vierstra, 1998).

In a comprehensive review paper, Tsien (Tsien, 1998) summarized the factors affecting the detectability of GFP as the following four categories, which are practically helpful in utilization of GFP reporter.

Total amount of GFP

- Number of copies of gene, duration of expression
- Strength of transcriptional promoters and enhancers
- Efficiency of translation including Kozak sequence and codon usage
- Absence of mRNA splicing, protein degradation and export

Efficiency of posttranslational fluorophore formation

- Solubility vs. formation of inclusion bodies
- Availability of chaperones
- Hindrance to folding because of unfortunate fusions to host proteins
- Time, temperature, availability of O2, and intrinsic rate of cyclization/oxidation

Molecular properties of mature GFP

- Wavelengths of excitation and emission
- Extinction coefficient and fluorescence quantum yield
- Susceptibility to photoisomerization/bleaching
- Dimerization

Competition with noise and background signals

- Autofluorescence of cells or culture media at preferred wavelengths
- Location of GFP, diffuse vs. confined to small subregions of cells or tissues
- Quality of excitation and emission filters and dichroic mirrors
- Sensitivity, noise, and dark current of photodetector
1.4 Agrobacterium-mediated T-DNA transformation

*Agrobacterium tumefaciens* is a Gram-negative soil pathogenic bacterium that causes crown galls or tumours on a wide range of dicotyledonous plant species (Zhu et al., 2000). It has played a major role in the development of plant genetic engineering and the basic researches in molecular biology. Initially, it was believed that only dicots, gymnosperms and a few monocot species could be transformed by this bacterium, but recent achievements totally changed this view by showing that many “recalcitrant” species not included in its natural host-range such as monocots and fungi can now be transformed (Chan et al., 1993; Hiei et al., 1994; Bundock et al., 1995; Dong et al., 1996; Ishida et al., 1996; Rashid et al., 1996; Cheng et al., 1997; Tingay et al., 1997; Toki, 1997). In addition, the transformed cells usually carry single or low copy number T-DNA integrated in their genome with less rearrangement, and very large DNA segments can be transformed into the plants (Hamilton et al., 1996; Liu et al., 1999). Twenty-five years ago, the concept of using *Agrobacterium tumefaciens* as a vector to create transgenic plants was viewed as a prospect and a “wish.” Today, many agronomically and horticulturally important species are routinely transformed using this bacterium. Plant transformation mediated by *Agrobacterium tumefaciens* has become the most used method for the introduction of foreign genes into plants (Gelvin, 2003).

1.4.1 Characteristics of *Agrobacterium tumefaciens*

Virulent strains of *Agrobacterium tumefaciens*, when interacting with susceptible plant cells, induce diseases known as crow gall. These strains contain a large megaplasmid which is more than 200 kbp in size (Goodner et al., 1999; Goodner et al., 2001; Wood et al., 2001) and plays a key role in tumor induction and for this reason it was named (tumor-inducing) Ti plasmid. The Ti plasmid is a large, circular double stranded DNA molecule, existing as an autonomous replicating unit within the bacteria.
The transferred DNA (T-DNA) is referred to as the T-region when located on the Ti plasmid. T-regions on native Ti plasmids are approximately 10 to 30 kbp in size (Zambryski et al., 1980; Suzuki et al., 2000).

T-regions are defined by T-DNA border sequences. These borders (right border and left border) are 25 bp in length and highly homologous in sequence (direct repeats). They flank the T-region in a directly repeated orientation (Wang et al., 1984). The right border is generally considered to be more important than the left border (Wang et al., 1984; Jen and Chilton, 1986; Sen et al., 1989).

The Ti plasmid also contains other functional parts for virulence (vir), conjugation (con) and the origin of its own replication (ori). Proteins responsible for T-DNA processing and transfer are encoded by the vir region of the Ti plasmid. Approximately twenty genes in this region are essential for wild-type levels of pathogenesis on most host plants and are expressed in six operons, virA, virB, virC, virD, virE, and virG (Sheng and Citovsky, 1996; Zhu et al., 2000).

1.4.2 *Agrobacterium*-mediated T-DNA transfer process

The mechanism of T-DNA processing and transfer during *Agrobacterium* infection has been subjected to a number of excellent reviews (Zambryski, 1992; Zupan and Zambryski, 1995; Zhu et al., 2000; Gelvin, 2003). The process of gene transfer from *Agrobacterium tumefaciens* to plant cells implies several essential steps: (1) bacterial colonization (2) induction of bacterial virulence system, (3) generation of T-DNA transfer complex (4) T-DNA transfer and (5) integration of T-DNA into plant genome. A brief description of the process is given below (Figure 1.3).
Figure 1.3 Two-way exchange of chemical signals between *Agrobacterium tumefaciens* and host plants. Wound-released chemical stimuli are perceived by the VirA to VirG proteins, which leads to transcription of vir promoters. T-DNA is processed by the VirD2 protein, and single-stranded linear T strands are formed by strand displacement. T strands and VirE2 are translocated from the bacteria via a pore encoded by the virB operon and form a T complex within the plant cytoplasm. T complexes are transported into the nucleoplasm via the host protein karyopherin alpha, and the T-DNA is integrated into genomic DNA. Transferred genes encode phytohormone synthases that lead to plant cell proliferation and opine synthases that provide nutrients to the colonizing bacteria. Opines are released from the plant cell, enter the bacteria via dedicated opine permeases, and are catabolized via opine-specific catabolic proteins. Opine permeases and catabolic enzymes are encoded by the Ti plasmid (Zhu et al., 2000).

- Bacterial colonization is an essential and the earliest step in tumor induction and it takes place when *Agrobacterium tumefaciens* is attached
to the plant cell surface. This attachment is achieved by a group of genes located within the bacterial chromosome. The precise mechanism for attachment is still unknown.

- The infection begins at the wounded sites. The injured plant cells release signaling molecules. *Agrobacterium* can recognize plant compounds such as acetosyringone, sinapinic acid, coniferyl alcohol, caffeic acid and methylsyringic acid which induce the bacteria’s virulence (Bolton et al., 1986).

- Signaling molecules are recognized by the dimeric transmembrane receptor complex VirA-ChvE, where ChvE is a sugar binding protein; VirA is activated by a way of autophosphorylation.

- Phosphorylation of cytoplasmic VirG by autophosphorylated VirA.

- Activated VirG binds to the regulatory regions of other *vir* operons, acting as a transcriptional activator; and inducing expression of *vir* genes. There are at least six *vir* operons (*virA, virB, virC, virD, virE* and *virG*) which mediate the actual transfer (Zambryski, 1992). The *virA* and *virG* are required for overall *vir* expression, indicating they are *vir* master control genes.

- The VirD1 and VirD2 are responsible for the T-strand (a single-stranded copy of the T-DNA) generation by recognizing the 25bp T-DNA borders and specifically cutting the bottom strand at the two borders (endonucleolytic cleavages between the third and fourth base of the 25bp border repeats). After cutting, VirD2 remains covalently attached to the 5’-end of the T-strand, forming a complex with polar character, i.e. the 5’-end is the leading end in subsequent steps of the transfer. Once the T-strand is generated, it remains as single strand linear DNA and must be protected from degradation by bacterial endo- and exonucleases. Protection from endo- and exonucleases is thought to be mediated by the VirE2 protein by binding the T-strand, preventing degradation both in the plant cytoplasm and perhaps in the nucleus (Rossi et al., 1993).

- The T-strand-VirD2 complex and another single strand binding protein VirE2 are believed to be transferred separately into plant cells through a
pilus-like structure made of a complex set of VirB proteins (Zupan and Zambryski, 1995; Baron and Zambryski, 1996; Fullner et al., 1996; Sundberg et al., 1996). There are 11 VirB proteins (Christie, 1997), each except VirB1 is essential for the transfer and tumorigenesis.

- Once into the plant cell, many VirE2 molecules bind cooperatively to the T-strand, forming T-complex (Sundberg et al., 1996; Gelvin, 1998), which is then targeted into the nucleus by the nuclear target signals (NLSs) of its associated VirD2 and VirE2, where the T-DNA randomly integrates into the plant genome as single or multiple copies.

- The integration is shown to preferentially occur in the transcription active and/or repetitive regions of the genome through a process of illegitimate recombination (Mayerhofer et al., 1991; Zambryski, 1992; De Buck et al., 1999), requiring short homology between the T-DNA and the target site of the genome, producing various forms of rearrangement such as small duplications, deletions and fillings. The mechanisms of integration into the host genome are still relatively unknown. However, it is known that the T-complex polarity is maintained upon integration. It is believed that the VirD2-bound 5'-end of the T-complex may help unwind the host DNA and form a gap. Next, the 5'-end of the T-complex is bound to the gap in host DNA and 3'-end is incorporated down-stream. The plant repair and recombination mechanisms may then replace the DNA using the T-strand as a template and ligate the ends together (Zupan and Zambryski, 1995).

1.4.3 T-DNA binary vectors

The native Ti plasmids are very large and T-DNA regions do not generally contain unique restriction endonuclease sites not found elsewhere on the Ti plasmid. Therefore, one cannot simply clone a gene of interest into the T-region. Scientists therefore developed a number of strategies to introduce foreign genes into the T-DNA.
The early method employed to produce a delivery vector from Ti plasmids used cointegration to make the necessary changes to the Ti-plasmid. An intermediate shuttle vector containing T-DNA (excluding the repeat borders), a multiple cloning site, an NPTII gene (for Kanamycin resistance selection in plants) and a selectable marker for Agrobacterium was prepared. The gene of interest was ligated into the multiple cloning site, transformed into E. coli. and AmpR colonies were selected and mated with Agrobacterium, allowing the shuttle vectors to be transferred into Agrobacterium. The T-DNA of the shuttle vector recombined with the T-DNA of the Ti plasmid between the border repeats, incorporating the gene of interest, the NPTII gene and the selectable marker for Agrobacterium. Bacteria containing only the modified Ti plasmid and not the intermediate shuttle vector were selected for and propagated in culture. This recombination step was critical in that it removed the tumor inducing genes from the T-region and replaced them with the desired genes. Thus, the Agrobacterium has only the ability to integrate T-strands into the host genome and lacks oncogenic activity. The recombinant Agrobacterium was allowed to infect plant cells and integrate its T-strand into the host’s nuclear genome.

As the technology progressed, the long and cumbersome process of cointegration was replaced by the binary system, which is now the standard for Agrobacterium vector production. This strategy was based on findings of Hoekema et al. that the T-region and the vir genes could be separated into two different replicons. When these replicons were within the same Agrobacterium cell, products of the vir genes could act in trans on the T-region to effect T-DNA processing and transfer to a plant cell. Hoekema et al. (Hoekema et al., 1983) called this a binary-vector system; the replicon harboring the T-region constituted the binary vector, whereas the replicon containing the vir genes became known as the vir helper. The vir helper plasmid generally contained a complete or partial deletion of the T-region, rendering strains containing this plasmid unable to incite tumors.

The binary vector is produced in vitro. The vector contains a bacterial selectable marker, a bacterial origin of replication and an integration region. The integration region is bordered by the T-region left and right border sequences and contains a
multiple cloning site and a resistant gene for selection in plants. The gene of interest is inserted into the multiple cloning site.

T-DNA binary vectors revolutionized the use of *Agrobacterium* to introduce genes into plants. Scientists without specialized training in microbial genetics could now easily manipulate *Agrobacterium* to create transgenic plants. These plasmids are small and easy to manipulate in both *E. coli* and *Agrobacterium* and generally contain multiple unique restriction endonuclease sites within the T-region into which genes of interest could be cloned. Moreover, the use of two separate plasmids in the binary system enhances the production of VirG and VirE, which increases the effectiveness of transformation. Furthermore, the binary system can accommodate foreign inserts up to 150kb, where the cointegration system can only contain up to 20kb of foreign DNA (Birch, 1997). An excellent review and guideline on binary vectors, *Agrobacterium* strains and their use was published recently by Hellens et al. (Hellens et al., 2000). The most comprehensive and updated review is the White Paper (*Agrobacterium*-Mediated Transformation of Plants) compiled by C. Roa-Rodríguez and C. Nottenburg in CAMBIA (http://www.cambiaip.org/Whitepapers/Transgenic/AMT/books/whole.html).

1.4.4 Methods of transformation

According to Hansen and Wright (Hansen and Wright, 1999), successful transformation of plants demands that certain criteria be met. Among the requirements for transformation they listed are:

- Target tissues competent for propagation or regeneration.
- An efficient DNA delivery method.
- Agents to select for transgenic tissues.
- The ability to recover fertile transgenic plants at a reasonable frequency.
- A simple, efficient, reproducible, genotype-independent and cost-effective process.
• A tight timeframe in culture to avoid somaclonal variation and possible sterility.

The Agrobacterium-mediated transformation techniques appear to fulfill these criteria. Several methods have been successfully used for Agrobacterium-mediated transformation of plants, including co-culture of agrobacteria with various explants, such as protoplasts, leaf discs and the tissue in whole plants, and other methods integrating non-Agrobacterium factors such as the systematic virus infection (agroinfection) and the biolistic method for gene transfer (agrolistics). The choice of the methods depends on the plant species to be transformed and mostly relies on in vitro regeneration of the transformed tissues. Typically, Agrobacterium-mediated transformation of dicots is performed using sterile leaf pieces, cotyledons, stem segments, callus suspension cultures and germinating seeds. For Arabidopsis however, efficient in planta transformation protocols were developed (Bechtold et al., 1993; Clough and Bent, 1998; Richardson et al., 1998). These in planta simplified transformation protocols have allowed the large scale production of transgenic plants necessary for T-DNA tagging strategies.

1.4.5 Agrobacterium-mediated transformation in monocots

The first demonstrated monocot transgenic plant mediated by Agrobacterium was Asparagus officinalis (Bytebier et al., 1987). Before that, nearly all the early evidence showing the transformation of monocot cells by Agrobacterium failed to provide sufficient molecular data to confirm the stable T-DNA integration and inheritance (Smith and Hood, 1995). The major breakthrough occurred in rice, for which two independent groups reported unequivocal molecular and genetic evidence for its transformation (Chan et al., 1993; Hiei et al., 1994). Molecular analysis confirmed that the characteristic features of T-DNA integration were basically similar to those in dicots, and the transformation frequency was also equally high (30%) (Hiei et al., 1994). These greatly stimulated further studies on using Agrobacterium to transform other cereal crop species including maize.
(Ishida et al., 1996), wheat (Cheng et al., 1997), barley (Tingay et al., 1997) and sugarcane (Enriquez-Obregon et al., 1998).

It was 10 years after the first dicot plants were transformed (Barton et al., 1983), that transgenic rice plants were obtained from immature embryos infected with *Agrobacterium* (Chan et al., 1993). One reason for the delay in monocot transformation by *Agrobacterium* was the weak, or lack of wounding response, from injured monocot tissues. This response is essential for successful infection of dicot plants as factors, such as acetosyringone (AS), released by damaged dicot cells induce expression of virulence genes located on the Ti-plasmid. The wounding response of monocots is very different. Monocot cells usually tend to be lignified or sclerified without active cell division. This is one of the major reasons accounting for the insusceptibility of most monocots to *Agrobacterium* infection (Hiei et al., 1997), and the widely used methods based on wounding response such as leaf-disc transformation are obviously unsuitable for monocots.

Despite the weak wounding induction response from monocot cells, many attempts were made to adapt the *Agrobacterium* system for monocot transformation. There are many factors affecting T-DNA transfer into monocot cells, and recent studies have identified several key factors for successful transformation of monocots by *Agrobacterium*, which are listed below.

1.4.5.1 Target cells with high division activity and strong regeneration potential

Hiei (Hiei et al., 1994) reported, for the first time in monocot plants, an efficient *Agrobacterium*-mediated transformation system for rice. They examined various tissues, namely, shoot apices and segments of roots from young seedlings, scutella, immature embryos, calli induced from young roots and scutella, and cells in suspension cultures induced from scutella, as donor for *Agrobacterium tumefaciens*. The greatest production of transgenic cells was obtained from scutellum callus. In addition, scutellum callus have high ability of propagation and regeneration, which showed scutellum callus was suitable for a donor material of *Agrobacterium*-mediated transformation.
Using the agro-infection technique in which the genome of the maize streak virus (MSV) was cloned into T-DNA, Grimsley et al. (Grimsley et al., 1987) found that the symptom of viral infection is much more serious by infecting the meristematic tissues than that by infecting the non-meristematic tissues. In maize, the tissues with more dividing cells, such as scutellum node and mesocotyl, were also more susceptible to be transformed by Agrobacterium (Graves and Goldman, 1986). The use of the actively proliferating embryogenic cells especially the immature embryos or the embryogenic callus derived from the immature or mature embryos, which currently are the ideal cells for monocot plant regeneration, plays key roles in the successful transformation by Agrobacterium (Chan et al., 1993; Hiei et al., 1994; Aldemita and Hodges, 1996; Hiei et al., 1997; Tingay et al., 1997; Zhang et al., 1997). In a few experiments, calli cultured on solid media are shown to be better than the suspension cultures (Hiei et al., 1994; Hiei et al., 1997).

1.4.5.2 Increasing vir gene expression

Significant modifications to the virulence of Agrobacterium have expanded the range of plant species that are susceptible to T-DNA transformation by improving the frequency of T-DNA transfer, most notably to the cereals. The main modification enhancing virulence has been to boost the expression of virG. Because virG is a transcriptional activator of the vir operons (Stachel and Zambryski, 1986), expression of additional copies of this regulatory vir gene was thought to enhance the expression of VirE2 and other Vir proteins involved in T-DNA transfer (Gelvin, 2003). Several laboratories have determined the effect of additional copies of wild-type virG genes on vir gene induction and plant transformation. Rogowsky et al. (Rogowsky et al., 1987) showed that additional copies of nopaline-type virG resulted in increased vir gene expression. Liu et al. (Liu et al., 1993) showed that multiple copies of virG altered the pH response profile for vir gene induction. Normally, vir gene induction is very poor at neutral or alkaline pH or in rich medium; additional copies of virG permitted a substantial degree of induction in rich medium even at pH 8.5. Additional copies
of virG also increased the transient transformation frequency of rice, celery, and carrot tissues (Liu et al., 1992).

Extensive genetic studies resulted in the identification of a number of mutations that render the VirG protein active in the absence of phenolic inducing compounds (Han et al., 1992; Pazour et al., 1992). These altered proteins contain mutations that converted either asparagine-54 to aspartic acid (virGN54D) or isoleucine-106 to leucine (virGI106L). Both of these mutant proteins stimulated a high level of vir gene expression, especially when expressed from a high-copy-number plasmid (Gubba et al., 1995). When tested in transient tobacco and maize transformation assays, strains containing the virGN54D mutant effected a higher level of transformation than did strains encoding the wild-type virG gene (Hansen et al., 1994). An even greater effect was seen when the virGN54D allele was harbored on a high-copy-number plasmid; the presence of this mutant gene in Agrobacterium increased the transient transformation of rice and soybean two-to sevenfold (Ke et al., 2001).

1.4.5.3 Bacterium strains and T-DNA vectors

Among many Agrobacterium strains, only a few have been used successfully in monocot transformation. Use of the super-virulent strain A281 which harbors the pTiBo542 plasmid (Jin et al., 1987) partly circumvented the low vir gene induction occurring during monocot infection and resulted in production of transgenic rice (Chan et al., 1993). The virB, virC and virG genes of the pTiBo542 plasmid encode the super-virulent phenotype of strain A281, and have been used to assemble super-binary vectors (Komari, 1990; Hiei et al., 1994). In this vector system, the virulence of Agrobacterium strains harboring a disarmed Ti-plasmid is increased by the extra copies of virB, virC and virG provided by the super-binary vector. The development of super-binary vector systems contributed to the breakthrough of Agrobacterium-mediated transformation of rice (Hiei et al., 1994) and maize (Ishida et al., 1996).
However, the super-virulent *Agrobacterium* strain does not appear to be a prerequisite for monocot transformation, since transgenic barley (Tingay et al., 1997) and wheat (Cheng et al., 1997) were later obtained using non-super-virulent *Agrobacterium* strains.

Different combinations of bacterial strains and vectors have great effects on transformation efficiency. In rice, Hiei et al. (Hiei et al., 1994) tested different combinations between 2 strains and 3 binary vectors, showing that LBA4404 (pTOK233) was the most efficient. Surprisingly, they found that the combination of the super-virulent strain EHA101 with the super-binary vector pIG121Hm was even less efficient than the common strain LBA4404 with common binary vector (pIG121Hm). Apparently, in the transformation of monocots the bacterial strain and the vector need to be carefully tested and selected.

### 1.4.5.4 Other influencing factors

The function of the pilus in T-DNA transfer remains unclear. One aspect of pilus biology that may be important for transformation is its temperature lability. Although *vir* gene induction is maximal at approximately 25 to 27°C (Turk et al., 1991; Jin et al., 1993), the pilus of some *Agrobacterium* strains is most stable at lower temperatures (approximately 18 to 20°C) (Fullner and Nester, 1996; Lai et al., 2000; Baron et al., 2001). Thus, one may consider co-cultivating *Agrobacterium* with plant cells at lower temperatures during the initial few days of the transformation process.

### 1.5 Aims of the project

The use of GFP as a reporter molecule has several advantages over other reporter systems, especially the feasibility of GFP for non-invasive, in vivo detection. Using GFP reporter to monitor dynamic performances of the components in T-DNA constructs and the efficiency of different steps in the rice transformation process may well lead to insights into improving the whole system.
The Gal4-UAS binary system has become the system of choice for enhancer trapping and targeted misexpression experiments in *Drosophila* (Fischer et al., 1988; Brand and Perrimon, 1993; Phelps and Brand, 1998), and for similar studies in other organisms, such as zebrafish and mouse (Rowitch et al., 1999; Scheer and Campos-Ortega, 1999; Koster and Fraser, 2001; Scheer et al., 2001). It was reasoned that the same system would be beneficial for studying gene function in rice, as it would overcome a number of the limitations of the transgenic strategies currently being utilized and offer potential usage of gain-of-function for innovative breeding.

The present study embedded in the rice TransGenomics project at the Center for the Application of Molecular Biology to International Agriculture (CAMBIA), has several purposes:

- To introduce and make use of the green fluorescent protein (GFP) reporter for studies of the enhancer trap system in rice.
- To develop a new genetic tool, the GFP-based transactivator-facilitated enhancer trap system, for rice functional genomics and molecular breeding.
- To produce a transgenic mutagenesis population of rice for further studies of functional genomics.
Chapter 2 Materials and methods

2.1 GFP detection

Expression of the green fluorescent protein was monitored regularly using a Leica MZ FLIII fluorescence stereomicroscope, usually with magnifications of 25x for callus and 40x for tissues/organs. The light source was a 100W high-pressure mercury vapor lamp with a heat-absorbing filter. The filter set used was the Leica GFP-Plant fluorescence filter set (also called GFP3, excitation filter: 470/40 nm, barrier filter: 525/50 nm, Figure 2.1). The use of the GFP-Plant filter set eliminated background chlorophyll fluorescence that has been observed with other filter sets (Elliott et al., 1999) and allowed GFP-expressing green leaf tissue to fluoresce bright green. Non-expressing leaf tissue appeared dark and did not fluoresce.

Figure 2.1 Wavelengths of the filters used for detecting EGFP expression.

A possible complication of GFP-based studies is a green autofluorescence typical of dead plant cells (Garabagi and Strommer, 2000). In my study, most such cells
could be readily identified by their lack of red autofluorescence in green tissues and gross appearance. Based on my experiences, drying tissues also generated significant green autofluorescence. Great care was taken to keep the samples from drying. GFP-like green autofluorescence from tissues (such as roots and nodes) with high lignin content was identified by their blue fluorescence observed using a BFP filter (390-420-430LP, CHROMA 31022).

Images were taken either on a Nikon Coolpix 900 digital camera or using Fuji chrome 400F film mounted in a Minolta camera. For optimum exposure of the film with relatively weak GFP signal from plant tissues/organs, the camera was set at ASA25 and exposure adjustment at plus 2.

2.2 GUS histochemical assay

As described by Jefferson et al. (Jefferson et al., 1987), the colourless substrate X-Gluc (5-Brom-4-chlor-3-Indolyl-β-D-glucuronide) can be converted into 5-Brom-4-chlor-3-Indolyl and glucuronide by the catalysis of the enzyme β-glucuronidase (GUS). Dimers of 5-Brom-4-chlor-3-Indolyl are subsequently formed as blue 5,5’-Dibrom-4,4’-Dichlor-Indolyl by oxidation. Tissues such as leaves of transgenic plants with GUS expression can be directly immersed into the GUS staining solution (Table 2.1) for in vivo reaction at 37°C. After a certain amount of time, blue spots or blocks may appear in the tissue and become more obvious after extraction of the chlorophyll.

Rice tissues (callus, root, leaf, spikelet etc.) were examined for GUS activity based on the procedure described by Jefferson et al. (Jefferson, 1987; Jefferson et al., 1987) with some modifications. Fresh samples were immersed in GUS staining solution, vacuum infiltrated for 5-10 min and incubated at 37°C overnight. The GUS stained tissues (except for callus) were then cleared by incubating in 70% ethanol at RT or 60°C overnight or longer with alterations of the ethanol for several times. The cleared tissues were observed under a Leica stereomicroscope. Images were recorded using a Nikon Coolpix 900 digital camera.
Table 2.1 Components of the GUS staining solution.

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock solutions</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM phosphate buffer, pH7.0</td>
<td>0.1M sodium phosphate, pH7.0</td>
<td>200 ml</td>
</tr>
<tr>
<td>10 mM Na$_2$EDTA</td>
<td>0.5 M Na$_2$EDTA</td>
<td>8 ml</td>
</tr>
<tr>
<td>2 mM potassium ferrocyanide</td>
<td>-</td>
<td>360 mg</td>
</tr>
<tr>
<td>2 mM potassium ferricyanide</td>
<td>-</td>
<td>264 mg</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>Triton X-100</td>
<td>400 ml</td>
</tr>
<tr>
<td>1.0 mg/ml X-Gluc</td>
<td>50 mg/ml</td>
<td>8 ml</td>
</tr>
<tr>
<td>100 µg/ml chloramphenicol</td>
<td>100 µg/ml chloramphenicol</td>
<td>400 µl</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>-</td>
<td>to 400 ml</td>
</tr>
</tbody>
</table>

2.3 DNA manipulation and cloning

The techniques used for DNA manipulation and cloning were generally as described by Sambrook et al. (Sambrook et al., 1989). Restriction endonucleases were mainly purchased from New England Biolabs and the digestions were normally carried out in a 20-30 µl reaction using the buffers provided with the enzymes. Isolation of plasmid DNA was done using the miniprep protocol described below or a simple and fast method using the QIAPrep Miniprep-Kit (Qiagen).

2.3.1 Plasmid DNA isolation (miniprep protocol)

Plasmid DNA isolation was routinely done as described in Sambrook et al. (Sambrook et al., 1989) with some modifications. Details of the procedure and chemical preparations are as follows.

- Inoculated a single bacterial colony into 3-5 ml of LB medium containing the appropriate antibiotics in a loosely capped 15-ml tube.
- Incubated the culture at 37°C with vigorous shaking (about 200 rpm) for 12-16 hours.

- Transfer the culture into a centrifuge tube and spin down the bacterial cells at 4500 rpm for 2 min.

- Remove the supernatant (medium) → add 250 µl of cold Buffer P1 → vortex briefly to resuspend the cells → transfer the cell suspension into a 1.5-ml microfuge tube.

- Add 250 µl of Buffer P2 (newly prepared) → mix by gentle inversion → incubate at RT for 3 min. (Do not vortex. Do not allow the lysis reaction to proceed for more than 5 min.)

- Add 250 µl of Buffer P3 (pre-chilled) → mix by gentle inversion → incubate on ice for 5 min.

- Spin at 12000 ×g for 10 min → transfer the supernatant into a fresh microfuge tube.

- Extract by adding 750 µl of phenol/chloroform (1:1), mix by inversion.

- Spin at 12000 ×g for 10 min → carefully transfer the supernatant (about 750 µl) into a fresh microfuge tube.

- Add 750 µl of cold 100% ethanol → mix by inversion → let stand at -20 °C for 20 min.

- Spin at 12000 ×g for 15 min → very carefully remove the supernatant.

- Add 750 µl of cold 70% ethanol → Spin at 12000 ×g for 3 min → very carefully remove the supernatant.

- Vacuum dry for 2 min.

- Add 30-50 µl of Buffer EB to dissolve DNA/RNA pellet.

- Add 1.5 µl of RNase A (10 mg/ml stock) and mix, incubate at 50 °C for 30 min → 65 °C for 15 min, then chill on ice.

*Preparation of the chemicals:*
• Buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA): Dissolve 1.212 g Tris base, 0.744 g EDTA·2H₂O in 160 ml distilled water. Adjust the pH to 8.0 with HCl. Adjust the volume to 200 ml with distilled water

• Buffer P2 (200 mM NaOH; 1% SDS): Prepare 2N NaOH stock (16 g NaOH in 200 ml distilled water) and 10% SDS stock. Working solution: 1 ml of 2N NaOH, 1 ml of 10% SDS, 8 ml distilled water

• Buffer P3 (3.0 M potassium acetate, pH 5.5): Dissolve 58.9 g potassium acetate in 100 ml distilled water. Adjust the pH to 5.5 with glacial acetic acid (about 22 ml). Adjust the volume to 200 ml with distilled water.

• Buffer EB (10 mM Tris-Cl, pH 8.5)

2.3.2 Quantitation of DNA

The amount of DNA in a preparation was routinely determined by measuring OD₂₆₀ on a spectrophotometer. An OD₂₆₀ of 1.0 equal to approximately 50μg/ml for double-stranded DNA and 33μg/ml for single-stranded DNA. The concentration of DNA solution was calculated accordingly.

2.3.3 DNA purification

2.3.3.1 Purification from the agarose gel

DNA fragments were separated by electrophoresis in an agarose gel in 1x TAE buffer containing 0.5 μg/ml ethidium bromide. DNA purification from agarose gels was done using the QIAquick gel extraction kit as described in the user manual (Qiagen). In the last step, the DNA was typically eluted in 30 ml distilled water from the column.

2.3.3.2 Purification from other sources

The QIAquick PCR purification kit (Qiagen) was used to purify DNA from other applications such as restriction enzyme digestion, DNA ligation, PCR amplification and rude preparation. The protocol described in the user manual
(Qiagen) was followed. To elute DNA, 30 ml distilled water was loaded in the center of the column membrane and let stand for at least 1 min, with a following centrifugation for 1 min at 14000 rpm.

### 2.3.4 Fill-in reaction

T4 DNA Polymerase has both a template-directed DNA polymerase activity, which extends a DNA primer in the $5' \rightarrow 3'$ direction, and a potent $3' \rightarrow 5'$ exonuclease activity. The following is the protocol used for blunting the ends on double-stranded DNA molecules with $5'$- or $3'$- protruding termini.

- Prepare reaction mixture by adding the following:
  
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x T4 DNA Polymerase buffer</td>
<td>3 µl</td>
</tr>
<tr>
<td>Digested DNA</td>
<td>1 µg (or less)</td>
</tr>
<tr>
<td>2 mM dNTP Mix</td>
<td>1.5 µl (0.1 mM final conc.)</td>
</tr>
<tr>
<td>T4 DNA Polymerase (3 U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 30 µl</td>
</tr>
</tbody>
</table>

- Incubate the mixture at $12^\circ$C for 20 min.
- Stop the reaction by heating at $70^\circ$C for 10 min.

### 2.3.5 SAP treatment of vector DNA

Alkaline phosphatases are used to prevent recircularization and religation of linearized cloning vehicle DNA by removing phosphate groups from both $5'$-termini. Shrimp Alkaline Phosphatase (SAP) catalyzes the dephosphorylation of $5'$ phosphates from DNA and is active on $5'$ overhangs, $5'$ recessed and blunt ends. Unlike Calf Intestinal Alkaline Phosphatase, SAP can be completely and irreversibly inactivated by heating at $65^\circ$C for 15 min. The SAP treatment procedure is as follows:

- Prepare reaction mixture by adding the following:
  
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Dephosoylation Buffer</td>
<td>3 µl</td>
</tr>
<tr>
<td>Digested DNA</td>
<td>0.1 µg (or less)</td>
</tr>
</tbody>
</table>
Shrimp Alkaline Phosphatase (1 U/µl) 1 µl
Distilled water to 30 µl

- Incubate the mixture at 37°C for 20 min.
- Stop the reaction by heating at 65°C for 15 min.

2.3.6 Rapid DNA ligation

DNA ligation was performed in a 20-µl reaction using the Rapid DNA Ligation kit (BOEHRINGER MANNHEIM, Cat. No. 1635 379). The kit contains:

- Vial 1: T4 DNA ligation buffer (2x conc.), 0.5 ml
- Vial 2: DNA dilution buffer (5x conc.), 0.5 ml
- Vial 3: T4 DNA ligase, 5 units/µl, 40 µl
  • It is absolutely necessary to thoroughly mix separately the contents of each vial 1 and 2 directly prior to use.

Ligation reaction for DNA to be inserted into plasmid vectors (sticky- or blunt-end ligation), recircularization for electroporation into *E. coli* cells.

Procedure:

- 10 µl DNA (vector + insert) in 1x DNA dilution buffer: Dissolve vector DNA and insert DNA in 1x DNA dilution buffer (diluted from the stock in vial 2). The molar ratio of vector DNA and insert DNA in a total volume of 10 µl should be 1+3 (with equal length of vector DNA and insert DNA, 50 ng linearized dephosphorylated vector DNA and 150 ng insert DNA). Other molar ratios 1+1, 1+2 are possible when the vector DNA and insert DNA are not similar in length.
- Add 10 µl of T4 DNA ligation buffer (2x conc., vial 1). Mix thoroughly.
- Add 1 µl of T4 DNA ligase (vial 3). Mix thoroughly.
- Incubate for 30 min at RT.
- Purify the ligation mixture by Qiagen PCR Purification kit (Qiagen) and elute DNA with 30 ml of distilled water.

2.3.7 Electroporation

All plasmids were maintained and propagated in the *E. coli* strain DH5α. Plasmid was introduced into DH5α by electroporation.

2.3.7.1 Transformation of *E. coli* through electroporation

Thaw 50 µl of electrocompetent cells (DH5α) on ice for 10 min. Add 2-7 µl of purified ligation reaction mixture (or 10-50 ng of plasmid DNA) into the cells and mix by pipetting gently. Load into a 2-mm pre-chilled electroporation cuvette, and keep on ice for 1 min. Apply the pulse to the cuvette on the electroporation unit Gene Pulser apparatus (Bio-Rad) with the following settings: 2.45 kv (voltage), 25 µF (capacitance), 200 ohms (resistance), 5 msec (calculated pulse time).

Transfer the cuvette quickly to ice in less than 15 seconds, then add 500 µl of SOC medium to suspend the electroporated cells by gentle pipetting. Transfer all cell suspension to a 1.5-ml Eppendorf tube and incubate at 37°C for 30 min. Selection for transformants was conducted by plating the mixture onto solid LB media containing the appropriate antibiotics (for example, 10 µg/ml chloramphenicol).

2.3.7.2 Transformation of *Agrobacterium* through electroporation

The above procedure was also applied for electroporation of *Agrobacterium* competent cells with plasmid DNA, in which case, the electroporated cells suspended in SOC medium was incubated at 28°C for 1-2 hours. From the LB plate containing selection antibiotic (100 µg/ml chloramphenicol), 4 colonies were picked, identified by PCR and one of the confirmed colonies was propagated for plant transformation.
• SOC Medium (1 liter)
  Bacto tryptone 20 g
  Bacto yeast extract 5 g
  NaCl 0.5 g
  MgCl$_2$·6H$_2$O 2 g
  KCl 0.2 g
  Glucose 3.6 g
  pH 7.0

• LB Medium (1 liter)
  Bacto tryptone 10 g
  Bacto yeast extract 5 g
  NaCl 0.5 g
  Bacto agar 15 g (for solid media)

2.3.8 Polymerase chain reaction (PCR)

Presented below are standard PCR conditions for detection of genes in plant genome or confirmation of plasmid existence in Agrobacterium strains. PCRs were done using either FTS-960 thermal sequencer or PC-960 thermal cycler (Corbett Research, Australia) and chemicals from PCR Kit for RedTaq DNA polymerase (Sigma). In the case of difficulties to obtain expected amplification products, which could be due to a low specificity of used primers, experiments on optimization of PCR conditions, mainly different melting temperatures (Tm), were performed.

**Steps**
- Denaturation
- 3-step cycling (30-35 cycles):
  - Denaturation
  - Annealing
  - Extension
- Final extension

**Reaction conditions**
- 2 min at 94°C
- 30 sec at 94°C
- 30 sec at 50-60°C
- 1 min/kbp at 72°C
- 5 min at 72°C

**Components**
- Template DNA
- PCR buffer
- MgCl$_2$
- Primer 1

**Concentration in reaction**
- 0.1-10 ng
- 1x
- 1.5 mM
- 0.5 µM
2.3.9 Oligo synthesis and DNA sequencing

All oligonucleotides for PCR and sequencing were synthesized either in Division of Plant Industry, CSIRO or GENSET Pacific Company (Australia).

DNA sequencing was based on the dideoxynucleotide chain termination method (Sanger et al., 1977) but using automatic sequencing machine. Plasmid DNA and PCR fragments were used as sequencing template. Sequencing reactions were carried out using the Big Dye Terminator Ready Reaction Mix (PE Applied Biosystems) with cycle sequencing program set at 25-30 cycles of 10 sec at 95°C, 5 sec at 50°C, and 4 min at 60°C. The products were then precipitated by ethanol and sent to Australian Genome Research Facility, University of Queensland for gel separation. Sequencing results were retrieved from the AGRF file transfer protocol site using the file transfer program WS_FTP LE version 5.8 and were viewed using the Chromas software version 1.45 (Conor McCarthy, School of Health Science, Griffith University).

2.4 Preparation of electrocompetent cells

2.4.1 Preparation of electrocompetent Agrobacterium cells

The protocol is adapted from Current Protocols in Molecular Biology, with modifications based on my experiences in making various electrocompetent cells for the lab at CAMBIA.

1. Inoculate a single EHA105 colony into 5 ml of LB medium containing 10 µg/ml of rifampicin and culture at 28°C for about 48 hours, shaking at 240 rpm.
2. Inoculate 1 ml of the LB culture into 1 liter of 2xYT medium in a sterile 3-liter flask. Grow at 28°C with vigorous shaking at 240 rpm until the OD$_{600}$ reaches 0.8-0.9.

3. Chill cells on ice-water bath 10 to 15 min. Split the 1-liter culture into sterile pre-chilled 500-ml centrifuge bottles. Cells should be kept at low temperature for all subsequent steps.

4. Centrifuge cells at 7000 rpm for 10 min at 4°C.

5. Resuspend the pellet in ice-cold sterile water using a pasteur pipet. Once this is achieved, add sufficient water to fill each centrifuge bottle and mix by vigorous shaking.

6. Centrifuge cells at 7000 rpm for 10 min at 4°C, then pour off supernatant immediately.

7. Repeat steps 5 and 6.

8. Resuspend the pellet in sterile ice-cold 10% glycerol using a pasteur pipet. Transfer these suspensions to one centrifuge bottle. Fill the bottle with 10% glycerol, mix and centrifuge at 7000 rpm for 15 min. Pour off supernatant immediately being careful to stop before the bacterial pellet comes fully loose.

9. Add about 2 ml of ice-cold sterile 10% glycerol and mix by swirling. Generally from every 1-liter culture, a final volume of 4 ml cells (in 10% glycerol ) can be obtained to be aliquot out for storage. This should give final cell density of about 5.0 x 10$^{10}$/ml.

10. Keep the cells on ice overnight, aliquot 150 µl of the cells into 0.5-ml Eppendorf tubes, freeze immediately in liquid nitrogen or dry-ice in ethanol and store at -80°C.

### 2.4.2 Preparation of electrocompetent E. coli cells

The procedure for preparation of electrocompetent E. coli cells is similar to that of Agrobacterium with only a little difference in steps 1 and 2.
1. Inoculate a single DH5α colony into 5 ml of LB medium containing 10 µg/ml of nalidixic acid and culture at 37°C overnight, shaking at 240 rpm.

2. Inoculate 1 ml of the LB culture into 1 liter of 2xYT medium in a sterile 3-liter flask. Grow at 37°C with vigorous shaking at 240 rpm until the OD$_{600}$ reaches 0.8-0.9.

- 2xYT Medium (1 liter)
  - Bacto-tryptone 16 g
  - Bacto-yeast extract 10 g
  - NaCl 5 g
  - pH 7.0

2.5 Agrobacterium-mediated transformation of rice callus

The protocol for Agrobacterium-mediated transformation of rice was established in CAMBIA (Canberra, Australia), based on the work by a Japanese group (Hiei et al., 1994; Hiei et al., 1997). For all plant transformation experiments, the Agrobacterium strain EHA105 (Hood et al., 1993) was used.

2.5.1 Callus Induction from rice seeds

- Dehusk seeds (Millin seed obtained from Yanko Rice Research Institute, NSW, Australia), usually 20-30 grams are required for an experiment

- Treat dehusked seeds with 70% ethanol for 1 min, wash thoroughly with distilled water.

- Sterilize seeds with a solution of 70% commercial bleach (4.5% active chlorine) and 1 drop of Tween 20, shake gently for 30 min at RT.

- Wash the bleached seeds in the laminar flow till can no longer smell the bleach. Usually about 300 ml of distilled water is needed.

- Blot dry seeds on sterile filter paper.

- Plate seeds on 2N6 medium, approximately 15-20 seeds per plate.

- Keep the plate at 26°C in the dark for 3 weeks for callus induction.
2.5.2 Callus subculture

After 3 weeks of callus induction, the proliferated embryogenic calli derived from scutella of the seeds are selected, cut into pieces around 5 mm in size, and placed on fresh 2N6 medium for a further 7 days before transformation.

2.5.3 Growing agrobacteria for transformation

Two days before the transformation, streak out agrobacteria containing plasmid of interest on solid AB medium with appropriate antibiotics (usually chloramphenicol at 100 µg/ml). Keep the agrobacteria culture at 28°C for two days.

2.5.4 Transformation

- Scrap off agrobacteria from the solid AB medium using an inoculation loop and resuspend the agrobacteria in AAM medium containing 100 µM acetosyringone (19.62 mg/l).

- Adjust the concentration of the suspension to an OD$_{600}$ reading of 0.7-1.0. In every experiment, the OD$_{600}$ readings for different constructs are adjusted to more or less the same value. Keep the suspension at RT for 2-3 hours.

- Transfer the suspension to a larger container. Add the callus in, swirl, and leave them to sit for 25-30 min at RT.

- Blot dry the callus on sterile filter paper and plate them onto 2N6-AS medium.

- Co-cultivate the callus and agrobacteria at 26°C in the dark for 3 days.

- After co-cultivation the agrobacteria is removed completely from the callus by several times of washing:
  - Prepare a washing solution of 100 mg/l timentin and 250 mg/l cefotaxime in autoclaved distilled water.
  - Transfer the callus to the washing solution, swirl and leave for 30 min. (During this time most of the agrobacteria is released from the callus.)
• Pour off the solution and wash the callus again a few more times until the solution is clear.

- Blot dry the washed callus on sterile filter paper and transfer them to 2N6-TCH medium which contains timentin and cefotaxime for eliminating agrobacteria and hygromycin for selection of transformed cells. Usually 25 calli are transferred into a 9-cm petri dish containing 2N6-TCH medium. The callus is cultured at 26°C in the dark and transferred to fresh 2N6-TCH medium every 2 weeks. Tiny brownish translucent protuberances will start appearing from the callus surface after two to three weeks on selection.

- Lines are made with small proliferating globular calli from the initial callus after about 3-4 weeks on 2N6-TCH medium. The proliferating calli are carefully picked out and placed in lines on fresh 2N6-TCH medium. All of the proliferating hygromycin resistant calli originating from a single co-cultured embryogenic callus represent a line, and each individual proliferation from this initial callus is called a sub-line. Proliferations that grow in a cluster are grouped into a single sub-line.

- Such proliferating callus lines are allowed to continue proliferating for another 2 weeks on new 2N6-TCH plates under the same incubation conditions.

- After two weeks or when proliferating callus has increased in size, the compact, opaque and yellowish callus is transferred to RGH6 regeneration medium for plant generation. Leave the callus on the RGH6 medium in the dark for one week at 26°C, and then transfer to the light.

- The calli on RGH6 medium are maintained at 26°C for 3-5 weeks under a 12/12-h (day/night) photoperiod with light provided at an intensity of 30 µmol m⁻² s⁻¹. After 5-10 days under the light, calli will be found turning green and in about 14 days shoots will start differentiating.

- The generated shoots are separated from the callus and transferred to the rooting medium ½MS-H to promote vigorous tiller and root development before being transferred to soil pots.
2.5.5 Media used for transformation

- 2N6, callus induction medium (1 liter)
  - N6 salts and vitamins (Table 2.2)
  - Casamino acids 1 g
  - Proline 500 mg
  - Glutamine 500 mg
  - Sucrose 30 g
  - 2,4-D 2 mg
  - pH 5.8
  - Phytagel 2.5 g

- AB medium (Chilton et al., 1974)

Prepare and autoclave the following stock solutions:

20x AB buffer (1 liter)

- K$_2$HPO$_4$ 30 g
- NaH$_2$PO$_4$ 4 g

20x salts (1 liter)

- NH$_4$Cl 20 g
- MgSO$_4$·7H$_2$O 6 g
- KCl 3 g
- CaCl$_2$·2H$_2$O 3 g
- FeSO$_4$·7H$_2$O 50 mg

To prepare 1 liter of AB medium, add 5 g glucose and 15 g Bacto-agar to 900 ml of distilled water and autoclave. Cool the medium to RT and add 50 ml each of sterilized 20x AB buffer and 20x AB salts.

- AAM medium (1 liter)
  - AA Macro-elements 100 ml
  - AA Micro-elements 1 ml
  - AA Iron 10 ml
  - AA Amino acids 10 ml
  - MS vitamins 10 ml
  - Casamino acids 0.5 g
  - Sucrose 68.5 g
  - Glucose 35 g
  - pH 5.2
  - Acetosyringone (AS) 100 µM
Dispense into four 250 ml bottles and autoclave. Usually 4.9 mg acetosyringone is dissolved in 1 ml DMSO and added to 250 ml of autoclaved AAM medium in the laminar flow.

Stock solutions:

**AA Macro-elements: (10x, 1 liter)**

- Na$_2$HPO$_4$·2H$_2$O 1.696 g
- MgSO$_4$·7H$_2$O 5 g
- KCl 29.5 g
- CaCl$_2$·2H$_2$O 1.5 g

**AA Micro-elements: (1000x, 100 ml)**

- MnSO$_4$·4H$_2$O 1 g
- NaMoO$_4$·2H$_2$O 25 mg
- H$_3$BO$_3$ 300 mg
- ZnSO$_4$·7H$_2$O 200 mg
- CuSO$_4$·5H$_2$O 3.87 mg
- CoCl$_2$·6H$_2$O 2.5 mg
- KI 75 mg

**AA Iron: (100x, 100 ml):** 280 mg of FeSO$_4$·7H$_2$O

**AA Amino acids: (100x, 100 ml)**

- Glycine 75 mg
- Arginine 1.74 mg
- Glutamine 8.76 mg

- 2N6-AS, co-cultivation medium (1 liter)
  
  - N6 salts and vitamins (Table 2.2)
  - Sucrose 30 g
  - 2,4-D 2 mg
  - Casamino acids 1 g
  - Glucose 10 g
  - pH 5.2
  - Phytagel 3.5 g
  - Acetosyringone (AS) 100 µM

Preparation of AS: 19.62 mg acetosyringone is dissolved in 1 ml of dimethyl sulphoxide (DMSO) and added to the medium after autoclaving.
• 2N6-TCH, selection medium (1 liter)
  - N6 salts and vitamins (Table 2.2)
  - Sucrose 30 g
  - 2,4-D 2 mg
  - Casamino acids 1 g
  - pH 5.2
  - Phytagel 2.5 g

After autoclaving cool the medium to hand temperature and add to each liter of the medium the following antibiotics:

- 0.5 ml of 200 mg/ml timentin
- 1 ml of 250 mg/ml cefotaxime
- 1 ml of 50 mg/ml hygromycin

• RGH6, regeneration medium (1 liter)
  - N6 salts and vitamins (Table 2.2)
  - Sucrose 30 g
  - Glutamine 500 mg
  - Proline 500 mg
  - Casein enzymatic hydrolysate 300 mg
  - 6-benzyl aminopurine (BAP) 3 mg
  - 1-naphthalene acetic acid (NAA) 0.5 mg
  - pH 5.8
  - Phytagel 6 g

After autoclaving cool the medium to hand temperature and add to each liter of the medium the following antibiotic:

- 1 ml of 50 mg/ml hygromycin

_Preparation of BAP:_ Weigh 100 mg BAP, and add 1N KOH drop-wise until the powder is dissolved. Make up the volume to 100 ml using distilled water to achieve 1 mg/ml solution.

_Preparation of NAA:_ Dissolve 100 mg NAA in 1 ml of absolute ethanol. Add 3 ml of 1N KOH and approximately 80 ml of water. Adjust pH to 6.0 with 1N HCl and make up the volume to 100 ml using distilled water to achieve 1 mg/ml solution.

• ½ MS-H, rooting medium (1 liter)
  - 0.5x MS salts and 1x vitamins (Table 2.3)
- Sucrose 10 g
- pH 5.8
- Phytigel 2.5 g

After autoclaving cool the medium to hand temperature and add to each liter of the medium the following antibiotic:

- 1 ml of 50 mg/ml hygromycin

Table 2.2 Components of N6 salts and vitamins (Chu et al., 1975).

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Final conc.</th>
<th>Stock solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N6 Macro-elements</strong></td>
<td>mg/l (1x)</td>
<td>g/l (20x)</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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<td>KNO₃</td>
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<td><strong>N6 Micro-elements</strong></td>
<td>mg/l (1x)</td>
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<td>4.4</td>
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<tr>
<td>H₃BO₃</td>
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<td>1.6</td>
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<tr>
<td>ZnSO₄·7H₂O</td>
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<td>0.8</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
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<td>0.25</td>
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<td>CuSO₄·5H₂O</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Fe₂EDTA solution</strong></td>
<td>mg/l (1x)</td>
<td>g/l (200x)</td>
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<td>FeSO₄·7H₂O</td>
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<tr>
<td>Na₂EDTA</td>
<td>37.25</td>
<td>7.45</td>
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<tr>
<td><strong>N6 Vitamins</strong></td>
<td>µg/l (1x)</td>
<td>mg/l (100x)</td>
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<tr>
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<td>50</td>
</tr>
<tr>
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<td>50</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>1000</td>
<td>100</td>
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<td>200</td>
</tr>
<tr>
<td>Myo-inositol</td>
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<td>10000</td>
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</table>
Table 2.3 Components of MS salts and vitamins (Murashige and Skoog, 1962).

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Final conc.</th>
<th>Stock solutions</th>
</tr>
</thead>
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<tr>
<td><strong>MS Macro-elements</strong></td>
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<tr>
<td>CuSO₄·5H₂O</td>
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<td>0.25</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.025</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Fe₂EDTA solution</strong></td>
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</tr>
<tr>
<td>FeSO₄·7H₂O</td>
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</tr>
<tr>
<td>Na₂EDTA</td>
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<td>7.45</td>
</tr>
<tr>
<td><strong>MS Vitamins</strong></td>
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</tr>
<tr>
<td>Nicotinic acid</td>
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<td>50</td>
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<tr>
<td>Pyridoxine·HCl</td>
<td>500</td>
<td>50</td>
</tr>
<tr>
<td>Thiamine·HCl</td>
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<td>50</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100000</td>
<td>10000</td>
</tr>
</tbody>
</table>

2.6 Rice genomic DNA isolation

- Collect plant leaves on ice. The samples can be left in -70°C freezer till extraction.

- Preheat S buffer at 65°C. Add 7 ml per tube (50 ml) and keep at 65°C.
- Grind leaf material with mortar and pestle, pouring liquid nitrogen over sample before grinding. Pour material into preheated tube with S buffer.

- Shake tube vigorously, then add 0.7 ml of 20% SDS and shake again. Put back in oven at 65°C to shake gently (20 rpm) for 2 hours.

- Take samples out after 2 hours and leave sit for 5 min at RT.

- Add 7 ml of chloroform (chloroform/iso-amyl alcohol, 24:1, v/v); shake very gently at RT for 15 min.

- Centrifuge at 7500 rpm (10395 ×g, BECKMAN Avanti J-25, JS7.5) for 25 min at 4°C.

- Transfer off carefully supernatant, into new tube (15 ml).

- Dispose of remaining pellet into chloroform waste.

- Add 0.6 volumes of isopropanol to the yellow supernatant and shake gently. Should be able to see DNA.

- Let the tube stand for 20 min at RT.

- Centrifuge at 5000 rpm (9000 ×g, HERMLE Z382K) for 15 min at 4°C.

- Pour off supernatant carefully into beaker and let tube dry a little upside down.

- Add 2 ml of 70% ethanol to wash pellet, careful not to wash pellet down.

- Centrifuge at 5000 rpm (9000 ×g) for 5 min at 4°C.

- Pour off ethanol carefully and tip tube upside down to dry.

- Add approximately 100 μl of TE and dissolve pellet at 55°C for 1 hour.

- Add 3 μl RNase A (10 mg/ml stock) and leave overnight at 37°C.

- Run on gel to check DNA quality.

**S buffer, 400 ml**

- Distilled water 200 ml
- NaCl 35.06 grams
- 1 M Tris (pH 8.0) 44 ml
- 0.5 M EDTA (pH 8.0) 44 ml
- 10% CTAB 44 ml
- Distilled water to 400 ml

**Preparation of stock solutions**

- **1 M Tris·Cl** [tris (hydroxymethyl) aminomethane] (pH 8.0): Dissolve 121.1 g Tris base in 800 ml distilled water. Adjust the pH to 8.0 by adding about 42 ml of concentrated HCl. Allow the solution to cool to RT before making the final adjustments to the pH. Make the volume to 1 liter by adding distilled water. Dispense into aliquots and sterilize by autoclaving (121°C for 21 min).

- **0.5 M EDTA** (pH 8.0) [ethylenediamine tetraacetic acid]: Add 186.1 g of Na₂EDTA·2H₂O [ethylenediamine tetraacetic acid di-sodium salt] to 800 ml distilled water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with (about 20 g of) NaOH (The di-sodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH). Dispense into aliquots and sterilize by autoclaving (121°C for 21 min).

- **10% CTAB** [cetyltrimethylammonium bromide]: Dissolve 20 g of CTAB in 160 ml distilled water, Stirring vigorously on a magnetic stirrer at 50°C. Make the volume to 200 ml by adding distilled water. Store the solution at 37°C.

- **20% SDS** [sodium dodecyl sulfate]: Dissolve 50 g of SDS [C₁₂H₂₅O₄SNa, FW 288.4] in 200 ml distilled water, Stirring vigorously on a magnetic stirrer. Make the volume to 250 ml by adding distilled water.

### 2.7 Southern hybridization

Southern hybridization of rice genomic DNA was done based on the protocol described by Southern (Southern, 1975) but using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals). Positively charged nylon membranes (Boehringer Mannheim) were used.
2.7.1 Enzyme digestion of genomic DNA

About 5 µg of rice genomic DNA was digested completely with the restriction enzyme EcoRI at 37°C for 4 hours or overnight.

*Digestion mixture:*

- DNA 5 µg
- 10x Buffer RE (EcoRI) 3 µl
- 10x BSA 3 µl
- EcoRI 2 µl
- Distilled water to 30 µl

2.7.2 DNA gel electrophoresis and blotting

Prepare 1% agarose gel containing 0.5 µg/ml ethidium bromide for Southern blotting. Load the DNA digestion sample in a mixture with 6x DNA loading buffer in the gel slot and run at 30-40V overnight for optimal separation, then document the gel with a transparent ruler under UV illumination.

For transferring the fractionated DNA fragments from the gel to a positively charged nylon membrane, capillary transfer was then carried out as described in Sambrook et al. (1989) using the alkali transfer solution.

Transfer DNA to membrane (alkali transfer):

- Prepare the alkali transfer solution: 0.4 M NaOH + 0.6 M NaCl (2 liters).
- Cut the gel to 10 cm, mark the gel by cutting off the upper left corner, put into the transfer solution (0.4 M NaOH + 0.6 M NaCl), denature for 20 min
- Cut the filter paper (3MM from Whatman) to 35 cm x 15 cm (1 piece), 15 cm x 10 cm (3 pieces).
- Cut the membrane to 15 cm x 10 cm (1 piece), cut off the upper left corner and write down the code and date in the upper right corner.
- Lay down the filter paper (35 cm) on the grass plate in a tray with 500 ml of transfer solution, make it perfectly flat.

- Put the gel on the filter paper (upside down), put the membrane on the gel, rolling gently with a 5-ml pipette.

- Put 3 pieces of the filter paper on top of the membrane, and then a stack of paper towels. Put some weight of 0.5-1 kg on top. Seal the gel with parafilm strips.

- Keep over night.

- Fixation
  - After over night transfer, wash the membrane briefly in 2x SSC.
  - Bake the membrane at 80°C for 2 hours.

2.7.3 Probe preparation

- Add 100 ng template DNA (Gal4/VP16 or HPT fragment) and autoclaved, double distilled water to a final volume of 16 µl to a 0.5-ml Eppendorf tube.

- Denature the DNA by heating in a boiling water bath for 10 min and quickly chilling on ice. Complete denaturation is essential for efficient labeling.

- Mix DIG-High Prime (vial 1) thoroughly and add 4 µl to the denatured DNA, mix and centrifuge briefly. Incubate for 1 hour or longer at 37°C. Longer incubations (up to 20 hours) will increase the yield of DIG-labeled DNA.

- Stop the reaction by heating to 65°C for 10 min.

2.7.4 Prehybridization and hybridization

- Roll the membrane and transfer to a glass tube with a thermostable plastic cap. Add 15 ml of prehybridization solution (DIG Easy Hyb, preheated at 37°C) to the tube and soak the membrane gently, avoiding bubbles. Screw the cap of the tube securely and cling to the low-speed rotor of an oven at 37°C with gentle agitation (12 rpm) for 1-2 hours.

- Prepare DIG-Labeled DNA probe:
  
  150 ng labeled DNA (Gal4/VP16 or HPT fragment)
3 ng λ DNA (labeled)
Distilled water to a total of 50 µl (mixture)
Denature the probe mixture at 96°C for 10 min, rapidly cooling on ice.

- Prepare hybridization solution: put the denatured probe mixture into 5 ml of DIG Easy Hyb (preheated at 37°C); mix well.
- Pour off the prehybridization solution; add hybridization solution to the tube.
- Incubate at 68°C at 20 rpm over night.
- Pour off and save hybridization solution. DIG Easy Hyb containing DIG-labeled probe can be stored at -20°C and be reused for several times when freshly denatured at 68°C for 10 min before use.

2.7.5 Immunological detection

- Wash the membrane in 200 ml of stringency wash solution (2x SSC + 0.1% SDS) under constant agitation, twice, 5 min each, at RT.
- Wash the membrane in 200 ml of stringency wash solution (0.5x SSC + 0.1% SDS, preheated to wash temperature) under constant agitation, twice, 15 min each, at 65-68°C.
- Rinse the membrane briefly in Washing buffer, 2 min, at RT.
- Incubate the membrane in 100 ml Blocking solution (DNA side down), 2 hours, at RT.
- Incubate the membrane in 20 ml Antibody solution, 30 min, at RT (Do not prolong the antibody incubation)
- Wash the membrane in 100 ml Washing buffer, twice, 15 min each, at RT.
- Equilibrate the membrane 5 min in 20 ml Detection buffer, at RT.
- Place the membrane (DNA side up) on a development folder (or hybridization bag), add 2 ml of ready-to-use CSPD (vial 5) onto the membrane, immediately cover the membrane with the second sheet to spread the substrate over the membrane without air bubbles. Incubate for 5 min at RT. Squeeze out excess liquid and seal the edges of the developing folder.
- Incubate the damp membrane for 15 min at 37°C to enhance the luminescent reaction.

- Expose X-ray film (with Kodak's BioMax MR film) at RT. Exposure time is 30 min-4 hours; light emission intensity increases for 7-8 hours after application of CSPD, and is maximal for about 12 hours.

**Stripping and re-probing of DNA blots**

- Rinse the membrane thoroughly in distilled water.
- Wash the membrane in 0.2 M NaOH containing 0.1% SDS to remove the DIG-labeled probe, twice, 15 min each, at 37°C.
- Rinse the membrane thoroughly in 2x SSC for 5 min.
- Prehybridize and hybridize with a second probe. Once the membrane is stripped, it can be stored in Maleic acid buffer or 2x SSC until used again.

### 2.7.6 Chemicals for Southern hybridization:

The kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Molecular Biochemicals, Cat. No. 1 585 614) contains:

- Vial 1: DIG-High Prime, 50 µl (5x conc.)
- Vial 2: DIG-labeled Control DNA, 20 µl
- Vial 3: DNA Dilution Buffer, 1 ml
- Vial 4: Anti-Digoxigenin-AP Conjugate, 50 µl (750 U/ml)
- Vial 5: CSPD ready-to-use, 50 ml
- Vial 6: Blocking solution, 400 ml (10x conc.)
- Vial 7: DIG Easy Hyb granules, 100 ml

- 2x SSC + 0.1% SDS, 1000 ml
  - 20x SSC
  - 20% SDS
  - Distilled water
  - 100 ml
  - 5 ml
  - 895 ml

- 0.5x SSC + 0.1% SDS, 1000 ml (preheated)
  - 20x SSC
  - 20% SDS
  - Distilled water
  - 25 ml
  - 5 ml
  - 970 ml

- Washing buffer, 1000 ml
  - Maleic acid buffer
  - 0.3% (v/v) Tween 20
  - 1000 ml (autoclaved)
  - 3 ml
• Blocking solution, 130 ml (fresh)
  Maleic acid buffer 117 ml
  Blocking Solution (vial 6) 13 ml

• Antibody solution, 30 ml (fresh, 1:10,000, 75 mU/ml)
  Blocking solution 30 ml
  Antibody (vial 4) 3 µl

• Detection buffer, 1000 ml
  0.1 M Tris-HCl 12.114 g
  0.1 M NaCl 5.844 g
  Adjust pH to 9.5

• 0.2 M NaOH + 0.1% SDS, 1000 ml
  5 M NaOH 40 ml
  20% SDS 5 ml
  Distilled water 955 ml

• Maleic acid buffer, 4000 ml
  0.1 M Maleic acid 46.44 g
  0.15 M NaCl 35.06 g
  Adjust pH to 7.5 with NaOH c.a. 32 g

• 20x SSC (sodium chloride/sodium citrate), 1000 ml
  Distilled water 800 ml
  3 M NaCl 175.3 g
  0.3 M Na3Citrate·2H2O 88.2 g
  Adjust pH to 7.0 with 1 M HCl
  Distilled water add to 1000 ml
Chapter 3  Construction and expression of GFP and its fusion reporter systems

3.1 Introduction

Two important marker proteins used in plant gene expression studies are green fluorescent protein (GFP) and β-glucuronidase (GUS). GUS has been very widely used as a reporter gene in plants (Jefferson et al., 1987). One of the advantages with GUS reporter is its sensitivity for detecting weak expression of the gene and suitability for quantitative assay (Jefferson and Wilson, 1991; Mantis and Tague, 2000). Transformed tissues or patterns of gene expression can be identified histochemically, but this is generally a destructive test and is not suitable for assaying primary transformants, nor for following the time course of gene expression in living plants, nor as a means of rapidly screening segregating populations of seedlings. GFP from the jellyfish *Aequorea victoria* shares none of these problems, and there has been much interest in using the protein as a genetic marker in transgenic plants.

GFP was first discovered in the mid-1970s. Breakthroughs in GFP studies came with the cloning of the gene by Prasher et al (1992) and the demonstrations by Chalfie et al (1994) and Inouye and Tsuji (1994) that expression of the gene in other organisms creates fluorescence. In just a few years, GFP has become one of the most widely studied and exploited biological markers in biochemistry, cell and molecular biology.

Several key advantages are true for all native and engineered GFPs: (i) no substrate is needed. GFP only requires oxygen and illumination by the proper wavelength of light (Prasher, 1995; Sheen et al., 1995), thereby facilitating non-invasive, in vivo detection. Using GFP reporter to monitor the efficiency of different steps in the cereal transformation process may well lead to insights into improving the process (Upadhyaya et al., 1998); (ii) the proteins are very stable and function in virtually any fixed or living cell or tissue (Bokman and Ward,
1981; Plautz et al., 1996); (iii) GFP retains fluorescence when fused to another protein at either the N- or C- terminus (Leffel et al., 1997). Since its cloning, the heterologous expression of GFP cDNA has triggered its widespread use as a reporter molecule for gene expression, and protein localization and trafficking studies in a broad variety of organisms including bacteria, yeast, slime mold, plants, *Drosophila*, zebrafish, and mammalian cells (Cubitt et al., 1995; Prasher, 1995). The expression of GFP has not been reported to disturb normal function of any organism in which it has been expressed.

However, for screening enhancer trap lines in plants the usefulness of GFP may be limited due to the high levels of autofluorescence in some plant organs. For this purpose, the reporter gene GUS remains unsurpassed in its sensitivity. It is expected that creating GUS::GFP fusion reporters make it possible to combine the advantage of GUS, its high sensitivity in histochemical staining, with the advantages of GFP as a vital marker.

![Figure 3.1 The pEGFP plasmid introduced from CLONTECH](image)
3.2 Construction of the enhanced green fluorescent protein (EGFP) reporters

The egfp gene used is originally from the pEGFP plasmid (Figure 3.1) which is a gift from Jason Li (CLONTECH Laboratories, Inc., Palo Alto, California, USA; Catalogue number 6077-1, GenBank accession number U76561).

3.2.1 Comparison between EGFP and mgfp5-ER

The mgfp5-ER had been using in CAMBIA before the introduction of EGFP from CLONTECH. The advantage of EGFP over mgfp5-ER was studied through a simple comparison in their expression in E. coli (DH5α).

The fluorescence strength of EGFP and mgfp5-ER was compared based on their identical backbone. An NcoI-SpeI fragment harboring the egfp gene was removed from the plasmid pEGFP and replaced by the mgfp5-ER in an NcoI-NheI fragment from pLAD-F65.4 (CAMBIA), resulting in a new plasmid pFX-B104.1 which had the same backbone as pEGFP but the reporter gene replaced by mgfp5-ER.

Transformed E. coli cells of pFX-B104.1 and pEGFP were streaked on the same LB plates and kept at 37°C for 16 hours. GFP expression was observed under the stereomicroscope using the GFP3 filter. It was found that the fluorescence strength of pEGFP was at least 10-fold stronger than that of pFX-B104.1. Based on this experiment and other information from the research group about poor expression of mgfp5-ER in transgenic rice calli and plants (data not shown), EGFP other than mgfp5-ER was chosen as the green fluorescent protein reporter for all the experiments with rice.
3.2.2 Construction of the binary vector pFX-B114.1 with EGFP reporter

A good positive control binary vector is needed for either testing fusion reporters or monitoring transformation efficacy of rice with enhancer trap constructs. Practically, the GFP reporter can be more useful than GUS for transformation experiments where dynamic information of transgene expression is under consideration.

The binary vector pCAMBIA 1201 was used as the backbone. An Ncol-PmlII fragment harboring gus gene was removed from pCAMBIA 1201. The egfp gene was taken from pEGFP as an Ncol-Stul fragment and inserted into the cut pCAMBIA 1201 backbone. The resulting new binary vector was named pFX-B114.1 (Figure 3.2).

![Figure 3.2 The binary vector pFX-B114.1 harboring egfp gene driven by CaMV 35S promoter](image)

3.2.3 Performance of the binary vector pFX-B114.1

The binary vector pFX-B114.1 has been extensively used as a positive control for various transformation experiments in CAMBIA. When rice calli were
transformed with pFX-B114.1, expression of GFP was easily detected immediately after co-cultivation, and dynamics of the transgene expression was conveniently studied based on repeated observation of GFP expression at different steps of callus growth and differentiation. Rice calli with high-level expression of the egfp gene produced very strong green fluorescence that was readily detectable even by naked eye.

Hundreds of transgenic rice plants were obtained with pFX-B114.1 and no specific abnormalities were found relevant to this construct. GFP expression of transgenic plants was observed under a fluorescent microscope, with either the whole plant or fresh samples taken from the plant. Expression patterns of pFX-B114.1 were apparently conferred by characteristics of the CaMV 35S promoter (Battraw and Hall, 1990; Terada and Shimamoto, 1990; Cornejo et al., 1993). GFP expression was detected widespread in the whole plant, and most frequently in root, stem base, collar, leaf, pollen, stomata and vascular bundles (Figure 3.3).
Figure 3.3 Expression of GFP in transgenic rice tissues/organs obtained with the binary vector pFX-B114.1. A-callus, B-root, C-stem base, D-young shoot, E-stomata, F-stigma, G-collar, H-vascular bundle, I-young spikelet (negative control on the right).
3.3 Construction of the GUS::EGFP and GUSPlus::EGFP fusion reporters

The GUS::EGFP and GUSPlus::EGFP fusion reporters were constructed through several steps:

1. **pFX-C8-1**: a plasmid with GUS reporter which can express in bacterium. It is a ligation product of an Ncol-EcoRI fragment of pEGFP (Clonetech) as the backbone of a bacterium expression plasmid (with lac promoter), and an Ncol-EcoRI fragment franking the gus gene from pTB99 (CAMBIA).

2. **pFX-C10-4**: an intermediate plasmid prepared for fitting the reading frame in future N-terminal fusion. pEGFP was cut with AgeI and Xmal and re-ligated so that 7 bp were removed in the franking sequence before the egfp gene.

3. **pFX-C20.1-2**: an intermediate plasmid prepared for removing the stop codon and fitting the reading frame in future C-terminal fusion with EGFP. pFX-C8-1 was cut with Stul and Pmll and re-ligated so that 65 bp including the stop codon of the gus gene were removed in the franking sequence after the gus gene.

4. **pFX-C27.2-4**: a plasmid with GUS::EGFP fusion reporter which can express in bacterium (driven by lac promoter). The plasmid pFX-C20.1-2 (with gus gene) was used as the backbone. It was cut open at the Spel site and a Xbal-XbaI fragment flanking the egfp gene from pFX-C10-4 was inserted and re-ligated.

5. **pFX-C39.1-10**: a binary vector with GUS::EGFP fusion reporter driven by the CaMV 35S promoter. The binary vector pCAMBIA 1201 was used as the backbone. pCAMBIA 1201 has a GUS reporter (with catalase...
intron) and a hygromycin resistance gene (both are driven by the CaMV 35S promoter). pCAMBIA 1201 was cut with SnaBI and PmlII so that most portion of the gus gene was removed but the Ncol-SnaBI portion of the gus gene (including the catalase intron) was kept in the backbone. The insert fragment of the construct was from pFX-C27.2-4 which contributed the GUS::EGFP fusion reporter, not including the Ncol-SnaBI fragment. pFX-C27.2-4 was firstly cut with NotI and filled in using T4 DNA polymerase. A second cut with SnaBI generated the insert fragment. The backbone and insert fragments were ligated to form the binary vector.

6. pFX-D18.1-1: a plasmid with GUSPlus reporter which can express in bacterium. It is a ligation product of an NcoI-Stul fragment of pFX-C8-1 as the backbone of a bacterium expression plasmid (with lac promoter), and an NcoI-PmlII fragment of pTANG86.1 (CAMBIA). This is simply a process of replacing GUS with GUSPlus reporter.

7. pFX-D18.2-3: an intermediate plasmid with GUSPlus reporter which can not correctly express in bacterium but for constructing a binary vector with GUSPlus::EGFP fusion reporter. It is a ligation product of an NcoI-Stul fragment of pFX-C8-1 and an NcoI-PmlII fragment of pTANH114 (CAMBIA). This construct is similar to pFX-D18.1-1 but the GUSPlus gene from pTANH114 has a catalase intron in pFX-D18.2-3.

8. pFX-D88-12: a plasmid with GUSPlus::EGFP fusion reporter which can express in bacterium (driven by lac promoter). The plasmid pFX-D18.1-1 (with GUSPlus gene) was used as the backbone. It was cut open at the SpeI (2138) site by partial digestion and an XbaI-XbaI fragment flanking the egfp gene from pFX-C10-4 was inserted and re-ligated.

9. pFX-D95-3: an intermediate plasmid with GUSPlus::EGFP fusion reporter which can not correctly express in bacterium but for constructing a binary vector with GUSPlus::EGFP fusion reporter. It is a ligation product of a HindIII-MfeI fragment of pFX-D88-12 and a HindIII-MfeI
fragment of pFX-D18.2-3. This construct is similar to pFX-D88-12 but the \textit{GUSPlus} gene fragment from pFX-D18.2-3 brought a catalase intron to pFX-D95-3.

10. **pFX-D102-9**: A binary vector with \textit{GUSPlus::EGFP} fusion reporter driven by the CaMV 35S promoter (Figure 3.4). The binary vector pFX-C39.1-10 was used as the backbone. pFX-C39.1-10 has the GUS::EGFP fusion reporter and a hygromycin resistance gene (both are driven by the CaMV 35S promoter). An NcoI (9541)-NcoI (11615) fragment franking the \textit{gus} gene and the linker between \textit{gus} and \textit{egfp} was removed from pFX-C39.1-10, and replaced by an NcoI (288)-NcoI (2362) fragment from pFX-D95-3 which franks the \textit{GUSPlus} gene and the linker between \textit{GUSPlus} and \textit{egfp}.

![Figure 3.4](image)

Figure 3.4 The binary vector pFX-D102-9 harboring GUSPlus::EGFP fusion driven by CaMV 35S promoter.

### 3.4 Co-expression of GUS/GUSPlus and GFP in KW1

The \textit{E. coli} strain KW1 [\textit{metB strA purB(aad-uid-man) hsr hsm}^{+} \textit{gusA}^{-}] is a strain without intrinsic GUS expression (Wilson et al., 1995) and therefore can be used for testing GUS expression. It is reasonable to test these fusion reporters first in KW1 before they are promoted to the binary vectors for transformation
and expression in plants. The KW1 competent cells were prepared using the general competent cell preparation protocol.

KW1 cells were transformed through electroporation using the four plasmids constructed: pFX-C8-1 (GUS), pFX-C27.2-4 (GUS::EGFP), pFX-D18.1-1 (GUSPlus), pFX-D88-12 (GUSPlus::EGFP). The transformed colonies were selected based on GUS or GFP expression and co-expression of the fusion reporters was further examined by plating the cells on LB medium with X-gluc.

Blue colonies were generated on all plates, showing GUS expression of the four plasmids (pFX-C8-1, pFX-C27.2-4, pFX-D18.1-1 and pFX-D88-12). The blue colonies of pFX-C27.2-4 and pFX-D88-12 also showed strong GFP expression when observed under a fluorescent microscope. The results indicated that both GUS::EGFP and GUSPlus::EGFP were fused correctly and could co-express in E. coli.

3.5 Co-expression of GUS/GUSPlus and GFP in rice calli

Scutellum-derived embryonic rice calli were co-cultured with Agrobacterium tumefaciens EHA105 carrying the binary vector pFX-B114.1 (EGFP, positive control), pFX-C39.1-10 (GUS::EGFP fusion), pFX-D102-9 (GUSPlus::EGFP fusion), respectively. Two hundreds calli were used for each of the vectors in each of the two replications. After the co-culture, every 25 calli were put in lines in a 9 cm Petri dish with 2N6TCH selection medium. The transformation efficiency was relatively high, nearly 90% of the co-cultured calli produced hygromycin-resistant cells, with proliferating calli generated after selection for seven weeks.

After transformation, GFP expression was observed every week under a fluorescent microscope with the GFP3 filter set and a magnification of 25x. During the observation, calli were kept untouched and covered inside the plate. Percentages of calli with GFP expression were calculated based on the total number of calli used for transformation. At the same time, a small sample of 15
calli with individual GFP data was taken for GUS staining, so that co-expression of GUS/GUSPlus and GFP was monitored all the way during selection.

For studying co-expression of the two reporter genes in a fusion, every individual sample callus was first observed for its GFP expression and number of spots and pattern were recorded. The same callus was undergone GUS staining and the blue spots and patterns were compared with the previous GFP result. Co-expression of GUS/GUSPlus and GFP was noticed all the way during the selection process. Either GUS or GUSPlus expression was well coincident with GFP expression in their number and pattern of spots on each individual callus (Figure 3.5).

Figure 3.5 Co-expression of GFP (left) and GUS (right) in the same rice callus transformed with pFX-D102-9.

On 90% of the calli transformed with the positive control vector pFX-B114.1 harboring EGFP reporter, numerous GFP spots were observed right after transformation. The percentage of calli with GFP expression reached it peak in a week after transformation and dropped down gradually by a margin of 25 percent points (Table 3.1; Figure 3.6). This phenomenon was found in all transformation experiments, suggesting unsuccessful integration of the T-DNA or silencing of the genes integrated.
Table 3.1 Comparison of GFP expression among different reporters, showing percentage of calli with GFP expression in replication I and II.

<table>
<thead>
<tr>
<th>Days after transformation</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
<th>42</th>
<th>49</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFX-B114.1 (I)</td>
<td>89.0</td>
<td>92.5</td>
<td>86.0</td>
<td>84.0</td>
<td>81.5</td>
<td>80.0</td>
<td>78.6</td>
<td>73.4</td>
</tr>
<tr>
<td>pFX-B114.1 (II)</td>
<td>90.0</td>
<td>94.1</td>
<td>86.0</td>
<td>83.0</td>
<td>82.0</td>
<td>79.0</td>
<td>76.0</td>
<td>65.0</td>
</tr>
<tr>
<td>pFX-C39.1-10 (I)</td>
<td>0.0</td>
<td>0.0</td>
<td>2.4</td>
<td>3.8</td>
<td>5.6</td>
<td>11.2</td>
<td>17.0</td>
<td>30.0</td>
</tr>
<tr>
<td>pFX-C39.1-10 (II)</td>
<td>0.0</td>
<td>0.0</td>
<td>3.0</td>
<td>4.0</td>
<td>6.0</td>
<td>17.0</td>
<td>25.2</td>
<td>32.0</td>
</tr>
<tr>
<td>pFX-D102-9 (I)</td>
<td>0.0</td>
<td>2.0</td>
<td>17.5</td>
<td>21.0</td>
<td>33.0</td>
<td>35.5</td>
<td>36.0</td>
<td>41.0</td>
</tr>
<tr>
<td>pFX-D102-9 (II)</td>
<td>0.0</td>
<td>3.2</td>
<td>20.0</td>
<td>20.0</td>
<td>37.0</td>
<td>41.7</td>
<td>43.2</td>
<td>45.0</td>
</tr>
</tbody>
</table>

Figure 3.6 Dynamics of GFP expression with the positive control (pFX-B114.1 harboring EGFP) and GUS/GUSPlus::EGFP fusion reporters.

GFP expression in calli transformed with either pFX-C39.1-10 (GUS::EGFP fusion) or pFX-D102-9 (GUSPlus::EGFP fusion) was much poorer than the positive control (non-fusion EGFP) in the early stage of selection. The dynamics
of GFP expression were also quite different from the positive control (Table 3.1; Figure 3.6). There was almost no GFP (and GUS as well, data not shown) expression in the first one week with the GUSPlus::EGFP fusion, and in the first two weeks with the GUS::EGFP fusion. However, the percentages of calli with GFP expression were increasing gradually with time, though finally still lower than the non-fusion control. Nonetheless, the GUSPlus::EGFP fusion is apparently better than the GUS::EGFP fusion in terms of their reporter gene expression.

3.6 Co-expression of GUS/GUSPlus and GFP in rice plants

A total of 186 transgenic lines were obtained, among which, 73 lines were transformed with pFX-B114.1 (EGFP), 39 lines with pFX-C39.1-10 (GUS::EGFP) and 74 lines with pFX-D102-9 (GUSPlus::EGFP). More than 92% of these lines were normal in fertility and set seeds. No morphological mutants were detected among these lines. GFP expression of the T₀ plants was investigated in different developmental stages, including the young transgenic plantlet stage, tillering stage and flowering stage. GUS staining was also done in late growth stages for samples transformed with the fusion reporters.

It was found that plant samples generated dramatic autofluorescence upon drying. To prevent such autofluorescence, samples were kept fresh in small plastic tubes with 2-3 ml of water and stored at low temperature (4°C) before observation for GFP expression. After GFP observation, samples were cut into smaller pieces and put into GUS staining following the general protocol.

Expression patterns of these reporters were found following the characteristics of the CaMV 35S promoter (Omirulleh et al., 1993) which was used in either EGFP or GUS/GUSPlus::EGFP fusion constructs in the experiment. In plants transformed with the positive control harboring EGFP reporter, GFP expression was detected in root, stem, leaf, collar, auricule and stomata at the young plantlet and tillering stages, and in almost all parts of the spicklet especially palea, lemma, stamen and lodicules at the flowering stage. Similar patterns were also found in
plants transformed with GUS/GUSPlus::EGFP fusion reporters. Co-expression of the fusion reporters was confirmed through simultaneous studies of the samples with both fluorescent microscopy and GUS staining.

The expression levels of the fusion reporters were compared with the non-fusion positive control based on GFP observations of transgenic rice plants. GFP signal strength was artificially divided into trace, weak, medium, strong and very strong. The tendency of whole plant GFP expression differences among the three reporter constructs was quit similar to that found in calli. The single EGFP reporter showed stronger expression level than either GUS::EGFP or GUSPlus::EGFP fusion reporter (Table 3.2).

Table 3.2 Levels of GFP expression in transgenic rice plants with different reporters.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Total No. of lines</th>
<th>Very strong</th>
<th>Strong</th>
<th>Medium</th>
<th>Weak</th>
<th>Trace</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFX-B114.1 (EGFP)</td>
<td>73</td>
<td>42</td>
<td>15</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>57.5%</td>
<td>20.5%</td>
<td>1.4%</td>
<td>2.7%</td>
<td>17.8%</td>
<td></td>
</tr>
<tr>
<td>pFX-C39.1-10 (GUS::EGFP)</td>
<td>39</td>
<td>1</td>
<td>13</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.6%</td>
<td>33.3%</td>
<td>15.4%</td>
<td>5.1%</td>
<td>2.6%</td>
<td>41.0%</td>
</tr>
<tr>
<td>pFX-D102-9 (GUSPlus::EGFP)</td>
<td>74</td>
<td>8</td>
<td>21</td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.8%</td>
<td>28.4%</td>
<td>20.3%</td>
<td>4.1%</td>
<td>36.5%</td>
<td></td>
</tr>
</tbody>
</table>

3.7 Discussion

In my experiments with the purpose of evaluating performance of different constructs, normal transformation efficiency was necessary for collecting unbiased and reliable data. GFP expression of the positive control pFX-B114.1 had been very stable over experiments. Rate of GFP-positive calli in pFX-B114.1 was used as an indication of transformation efficiency in the repeated
experiments, for the rate of hygromycin resistant calli was found to be tightly correlated with the percentage of calli showing GFP expression (data not shown). The judgment could be done at any time during hygromycin selection but the parameters are different with time points. For example, 6 weeks after transformation, if the GFP-positive calli in pFX-B114.1 is about 35-55%, transformation is usually normal level; higher than 55% is a indication of high transformation efficiency; lower than 35% is considered a failure of the transformation.

The performance of GUS::EGFP and GUSPlus::EGFP fusion reporters driven by the CaMV 35S promoter was found not as good as the non-fusion single EGFP reporter. In transformed calli, there was a significant delay for the fusion reporter gene activities to reach a detectable level. The overall plant expression level of these fusion reporters was also lower than that of the non-fusion version. Molecular mechanisms of the reduced expression of fusion proteins are not clear.

Detailed mutational analysis revealed that the structure of GFP is extremely sensitive to molecular or biochemical modifications (Dopf and Horiagon, 1996). For example, deletion of the first N-terminal amino acid (methionine) or the last seven amino acids from the C-terminus results in a total loss of fluorescence activity (Dopf and Horiagon, 1996). Based on the crystallographic structure of GFP, Ormo et al. (1996) hypothesized that there are no large segments that could be deleted while preserving the structural integrity of the protein (Ormo et al., 1996). In both pFX-C39.1-10 (GUS::EGFP) and pFX-D102-9 (GUSPlus::EGFP), the first N-terminal amino acid of EGFP was maintained and no deletion was made from the C-terminus.

GFP retains fluorescence when fused to another protein at either the N- or C-terminus. This property makes it an ideal fluorescent tag to monitor subcellular localization of proteins, organelles, and biochemical processes. In fact, both GFP and GUS can tolerate N- or C-protein fusions (Cubitt et al., 1995; Gerdes and Kaether, 1996; Heim and Tsien, 1996; Leffel et al., 1997). However, there is possibility that the fusion of GFP to a protein of interest (POI) alters the
conformation from that of the native structure such that loss of enzymatic activity, anomalous subcellular localization, or accelerated degradation results (Katz et al., 1998; Thomas and Maule, 2000), and the same is true with GUS or GUSPlus. This concern is what prompts researchers to produce fusions of GFP to both the N- and C-termini of POIs when employing such methodology (Timmons et al., 1997; Quaedvlieg et al., 1998). It was due to the limitation of time, fusions of GFP to the N-terminus of GUS/GUSPlus were not tried in my experiment.

Based on the fact that the fusion reporters, especially the GUSPlus::EGFP fusion, were functional in rice plants, the GUSPlus::EGFP fusion reporter was utilized for constructing enhancer trap vectors. The resulting pFX-E9 series (with GUS::EGFP fusion reporter) and pFX-E24 series (with GUSPlus::EGFP fusion reporter) enhancer trap constructs have been used in CAMBIA and other research institutes. Using the binary vector pFX-E24.2-15R (Figure 3.7) to transform rice calli from japonica variety Zhonghua 11 and Zhonghua 15, Wu et al. (2003) generated 31,443 independent enhancer trap lines and obtained various enhancer patterns based on reporter gene analysis (Wu et al., 2003). Though not perfect, the GUSPlus::EGFP fusion reporter is apparently useful in practices.

![Figure 3.7 T-DNA components of the enhancer trap pFX-E24.2-15R.](image-url)
3.8 Conclusion

A binary vector pFX-B114.1 with the enhanced green fluorescent protein (EGFP) reporter was successfully constructed and tested in rice. Bright green fluorescence was observed with a fluorescent stereomicroscope in virtually all examined tissues of transgenic rice. The expression signal of EGFP was found much stronger than that of mGFP5ER. In certain transgenic rice calli, high-level expression of the egfp gene under the enhanced CaMV 35S promoter produced green fluorescence that was readily detectable even by naked eye. Such high-level expression of GFP was not found to disturb normal function of any tissues/organs in which it had been expressed.

Hundreds of transgenic rice plants were obtained with pFX-B114.1 and no specific abnormalities were found relevant to this construct. The binary vector pFX-B114.1 has been extensively used as a positive control for various transformation experiments in CAMBIA.

By fusing the genes encoding GFP and GUS/GUSPlus, I have created a set of 10 bifunctional reporter constructs that are suitable for use in stable expression studies in rice plants. Co-expression of GFP and GUS/GUSPlus has been surveyed in rice callus and whole plant level. The fusion reporters make good use of the advantage of GFP as a vital marker for dynamic monitoring of gene expression and the advantage of GUS for its high sensitivity in histochemical staining.
Chapter 4  Optimization of a high throughput
Agrobacterium-mediated transformation
system

4.1 Transformation of rice callus: a case study

Large-scale and high efficient transformation is necessary for the TransGenomics project launched at CAMBIA. The Agrobacterium-mediated transformation system has been established (Hiei et al., 1994) but there is still no standard protocol in rice that is suitable for all varieties. The present study is to test and further improve a protocol established at CAMBIA for its efficiency in large-scale transformation experiments.

4.1.1 Rice cultivar, bacterium strain and binary vectors used in the experiment

The japonica rice variety Millin (Oryza sativa L.) was used in this study. Millin is a commercial semi-dwarf medium grained variety bred and released in Australia in 1995.

The Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) was chosen for almost all the transformation experiments in my studies. EHA105 is a Km(S) derivative of EHA101 (genotype C58 pTiBo542) that carries a plasmid derived from the super-virulent Ti plasmid pTiBo542. Both EHA101 and EHA105 exhibit broader host-range and higher transformation efficiency. Many recalcitrant plants such as rice (Rashid et al., 1996), wheat (Cheng et al., 1997) and barley (Tingay et al., 1997) have been transformed using EHA101 and EHA105.

A series of binary vectors were constructed for testing elements of enhancer traps. In this transformation experiment, four enhancer trap constructs were used (Figure 4.1), all of them harbor the enhancer trap cassette (minimal 35S-
Gal4/VP16), the reporter cassette (UAS-minimal 35S-GUSPlus::EGFP) and the selection cassette (CaMV 35S-Hygromycin).

Figure 4.1 Enhancer trap constructs used for the case study of Agrobacterium-mediated transformation.
4.1.2 Induction and pre-culture of calli derived from the scutellum of rice seeds

Dehulled Millin seeds were sterilized, inoculated on 2N6 medium and incubated for 24 days in the dark at 26°C as described in Chapter 2. Scutellum-derived calli were separated from the seeds and cut into small pieces (3-5 mm diameter). These calli were transferred onto fresh 2N6 medium and incubated for an additional 9 days under the same condition.

4.1.3 Transformation and selection of transgenic calli

Agrobacterium strains harboring each of the four pFX-E24 series plasmids were streaked on AB solid medium containing chloramphenicol (100 mg/l) and incubated at 28°C for 3 days to grow the Agrobacterium strains into a lawn.

The Agrobacterium strains were suspended in AAM liquid medium supplemented with 100 µM acetosyringone by scraping Agrobacterium from plates with an inoculation loop. The suspensions with OD$_{600}$ values adjusted to 0.55-0.58 were left at room temperature for 2 hours for pre-activating the Agrobacterium cells before transformation.

Rice calli and Agrobacterium suspensions were mixed and co-cultured for 30 minutes at room temperature. The rice calli were then blotted dry on sterile filter papers and transferred onto 2N6-AS plates. The calli and Agrobacterium strains plated on 2N6-AS medium were co-cultivated for 3 days in the dark at 26°C.

After co-cultivation, the calli were washed thoroughly with sterile water containing 500 mg/l cefotaxime and 200 mg/l timentin to remove the bacteria. The calli were blotted dry and transferred onto 2N6-TCH solid medium which contained hygromycin (50 mg/l) for selection of transformed tissues and cefotaxime (250 mg/l) and timentin (100 mg/l) for eliminating Agrobacterium. During the selection period, the calli were kept in the dark at 26°C and were transferred onto fresh 2N6-TCH every 2 weeks.
After 2 weeks of selection on 2N6-TCH medium, the original calli turned dark brown but tiny brownish translucent protuberances started arising throughout the callus surface. Six weeks after co-culture, hygromycin-resistant cells proliferated into round shaped, compact, opaque and yellowish calli. The proliferating calli were carefully picked out, placed on the fresh 2N6-TCH medium and incubated for another 2 weeks in the dark at 26°C. Proliferating calli apparently from different positions of the original callus were considered as putatively independent transgenic callus lines while as the proliferating calli grew together were not separated and considered as a single line.

4.1.4 Regeneration of transgenic plantlets

Eight weeks after transformation and selection, the putatively transgenic callus lines were transferred to RGH6 medium for plant regeneration. The calli were kept in the dark for one week, then maintained for 3-5 weeks under a 12/12-h (day/night) photoperiod with illumination (about 30 µmol m⁻² s⁻¹).

Calli started turning green after culturing under light for one week and shoots differentiation was noticed in 2 weeks. Shoots regenerating from an embryogenic callus were dissected and transferred to the rooting medium 1/2MS-H. The shoots were cultured for 2-3 weeks to promote vigorous tiller and root development. Plants with well differentiated roots were transferred to soil pots directly.

4.1.5 Summary of the single transformation experiment

The summary of results at every key steps of the transformation process is shown in Table 4.1. From this single transformation experiment, a total of 1,021 transgenic rice lines were obtained (including 151 lines from the positive control pFX-B114.1). It took about 5 months from the initiation of callus to the transfer of regenerated plants to soil. The efficiency is relatively high and may fulfill the prerequisite for the TransGenomics project in which large populations of transgenic lines are required.
Table 4.1 Results of a single transformation experiment.

<table>
<thead>
<tr>
<th>Construct</th>
<th>No. of scutellum calli used</th>
<th>No. of Hyg-resistant callus lines generated</th>
<th>No. of calli transferred to regeneration medium</th>
<th>No. of transgenic lines obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFX-E24.1-4</td>
<td>200</td>
<td>1085</td>
<td>520</td>
<td>215</td>
</tr>
<tr>
<td>pFX-E24.1-12R</td>
<td>200</td>
<td>1330</td>
<td>656</td>
<td>230</td>
</tr>
<tr>
<td>pFX-E24.2-3</td>
<td>200</td>
<td>875</td>
<td>384</td>
<td>189</td>
</tr>
<tr>
<td>pFX-E24.2-15R</td>
<td>200</td>
<td>1400</td>
<td>656</td>
<td>287</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>800</strong></td>
<td><strong>4690</strong></td>
<td><strong>2216</strong></td>
<td><strong>921</strong></td>
</tr>
</tbody>
</table>

4.2 Improvements in transformation efficiency

In classical experiments performed in 1926, A. J. Riker showed that development of tumors on plants following infection by *Agrobacterium tumefaciens* was optimal at temperatures around 22°C and was absent at temperatures greater than 28°C (Riker, 1926). The most obvious decrease in tumorigenesis occurred in the narrow temperature range between 25 and 28°C. It has been shown that the primary cause of tumor suppression by high temperature is the T-DNA transfer machinery which does not function efficiently (Fullner and Nester, 1996).

Using *Phaseolus* calli and *Nicotiana tabacum* leaves as experimental materials and with different *Agrobacterium* strains, Dillen et al. (Dillen et al., 1997) studied the effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer. It was found that the levels of transient *gus* gene expression decreased notably when the temperature was raised above 22°C. Expression was low at 27°C and undetectable at 29°C. Dillen et al. anticipated that the efficiency of many published transformation protocols could be improved by reconsidering the factor of temperature (Dillen et al., 1997).
To confirm previous findings about temperature effects and further improve transformation efficiency, a transformation experiment was done with 5 binary vectors and 2 temperature settings (26°C, 22°C). The 5 binary vectors included the positive control (pFX-B114.1) and 4 constructs originally designed for testing cis-activity (Figure 4.2). The whole procedure described above was followed except for the temperature setting during co-cultivation of rice calli with *Agrobacterium* strains.

Rice calli were soaked in each of the five *Agrobacterium* suspensions (for the 5 binary vectors used) and co-cultured for 30 minutes at room temperature. The rice calli were then blotted dry on sterile filter papers. Calli for each of the 5 binary vectors were divided into two portions (to be co-cultured at the 2
temperature settings) and transferred onto 2N6-AS plates, with 25 calli in each plate. The two sets of calli were co-cultivated for 3 days in the dark at 26°C and 22°C respectively.

The summary of results obtained in this experiment is shown in Table 4.2. Though the number of embryogenic callus lines obtained in this experiment was relatively low compared to other experiments (especially the case study described above), the difference between the two temperature treatments was very clear, showing a two-fold advantage in transformation efficiency with 22°C co-cultivation temperature.

Table 4.2 Effects of co-cultivation temperature on transformation efficiency.

<table>
<thead>
<tr>
<th>Construct</th>
<th>26°C, 3 days</th>
<th>22°C, 3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of calli used</td>
<td>No. of embryogenic callus lines obtained</td>
</tr>
<tr>
<td>pFX-J57.8</td>
<td>150</td>
<td>82</td>
</tr>
<tr>
<td>pFX-J55.7R</td>
<td>150</td>
<td>71</td>
</tr>
<tr>
<td>pFX-H84.3</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>pFX-H84.4</td>
<td>150</td>
<td>80</td>
</tr>
<tr>
<td>pFX-B114.1</td>
<td>150</td>
<td>76</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>750</strong></td>
<td><strong>369</strong></td>
</tr>
</tbody>
</table>

The lower temperature advantage was also shown by the result of dynamic GFP expression, for the reporter gene expression could indicate directly the transformation events. Right after transformation, GFP observation on all the 1,500 calli was done every three days and the percentages of calli showing GFP expression were reordered. A two-fold advantage of 22°C treatment over 26°C was found in the positive control pFX-B114.1 (CaMV 35S-EGFP) during the whole period of observation. The same was true in other 4 constructs at the time of 4-5 weeks after transformation. Dynamic GFP expression of the positive
control (pFX-B114.1) and one of the cis-acting constructs (pFX-J55.7R) is shown in Figure 4.3.

![Graph showing dynamic GFP expression](image)

Figure 4.3 Dynamic GFP expression in calli of two constructs (pFX-B114.1 in triangle and pFX-J55.7R in square), transformed with 2 different co-cultivation temperatures (26°C in dotted lines and 22°C in lines).

The dynamic curve of GFP expression in pFX-J55.7R with 26°C treatment is typical for such kind of constructs. There is an apparent delay for their GFP signal to be detectable because the gfp gene there is not driven directly by a complete promoter but dependent on cis-activities from neighboring enhancer elements. The early GFP expression in pFX-J55.7R with 22°C treatment is believed to be a very strong transient expression that has never been detected in such kind of constructs with 26°C co-cultivation temperature. This phenomenon suggests that with lower co-cultivation temperature, much more T-DNAs can be transferred into plant cells though not all these T-DNAs may be integrated into the genome.
4.3 Discussion

The present transformation experiment shows that the protocol followed in the experiment is feasible for large-scale production of transgenic plants in the TransGenomics project. On average, one transgenic line can be obtained from one scutellum callus used. The efficiency is relatively high compared to those reported by Hiei et al. where the highest transformation frequency is 28.6% (Hiei et al., 1994). Based on the experiment with different co-cultivation temperatures, a lower co-cultivation temperature (22°C, 3 days) may further double the transformation efficiency. Other potential areas for improvement were also found through the present experiments, including a precise and effective control of callus quality and an optimal timing for separation of independent transgenic calli.

The studies on transformation of rice have shown that numerous factors including genotype of plants, types and ages of tissues inoculated, kind of vectors, strains of Agrobacterium, selection methods, and various conditions of tissue culture including pH of the media and co-cultivation temperature, are important factors in the Agrobacterium-mediated transformation of rice. The most critical factors necessary for successful transformation by Agrobacterium are (i) the activation and proper expression of vir genes and (ii) the active division of the infected cells.

For the first factor, the results of present study in rice support previous findings from the studies of Phaseolus calli and Nicotiana tabacum leaves (Dillen et al., 1997). Dillen et al. reported that the levels of transient gus gene expression decreased notably when the temperature was raised above 22°C. Our knowledge about pilus biology has shown that the temperature lability of pilus could be important for successful transformation. Although vir gene induction is maximal at approximately 25 to 27°C (Turk et al., 1991; Jin et al., 1993), the pilus of some Agrobacterium strains is most stable at lower temperatures (approximately 18 to 20°C) (Fullner and Nester, 1996; Lai et al., 2000; Baron et al., 2001). Therefore,
it should be reasonable to consider co-cultivating *Agrobacterium* with rice calli at lower temperatures (22°C) during the initial 3 days of the transformation process.

Modifications of binary vectors may also contribute to increased transformation efficiency. A significant improvement is the construction of the superbinary vector pTOK233 (Hiei et al., 1994) by cloning the *virB*, *virG* and *virC* genes of pTiBo542 in pGA472. This led to the first successful transformation of japonica rice. The same strategy was used for the transformation of indica rice (Aldemita and Hodges, 1996; Mohanty et al., 1999), javanica rice (Dong et al., 1996), maize (Ishida et al., 1996), Sorghum (Zhao et al., 2000) and Allium cepa (Zheng et al., 2001). Recently, pTOK233 was also used to transform a pulse, mungbean (Jaiwal et al., 2001).

Another consideration is to find a proper concentration of the *vir* gene inducer. Co-cultivation of explants with *Agrobacterium* in the presence of acetosyringone has become a routine measure in the transformation of recalcitrant crops such as rice (Hiei et al., 1994), maize (Ishida et al., 1996), barley (Tingay et al., 1997) and wheat (Cheng et al., 1997). Aldemita and Hodges (Aldemita and Hodges, 1996) reported that pre-induction of *Agrobacterium* strain At656 with 400 μM acetosyringone prior to co-cultivation with immature embryos is important in rice transformation, which is four times of the concentration (100 μM) used in the present experiments and by several other research groups.

The second critical factor necessary for successful transformation by *Agrobacterium* is the use of actively growing, embryogenic calli. Though scutellum callus cultures are excellent sources of cells for the production of transgenic rice, only appropriate quality calli should be selected based on their appearance. Transformation efficiencies were found dramatically different with different batches of calli in many transformation experiments in my studies (ranging from 24% to 100% plus).
With the help of a GFP reporter, dynamic development of transgenic calli was easily surveyed. It was found that each responsive co-cultured callus could generate a large number of independent hygromycin-resistant calli, many of them were not be easily separated when the calli proliferated into larger sizes. Many of the callus lines transferred to regeneration media might be a mixture of several independent calli growing near each other. GFP observations revealed existence of this phenomenon. When all the plantlets arising from a callus line were surveyed for GFP expression, totally different expression patterns were often observed. If these independent transgenic calli could be separated earlier from the co-cultured scutellum callus, the number of transgenic lines generated in a given experiment may increase by several folds.

A highly efficient transformation procedure for japonica rice was reported recently by a French group (Sallaud et al., 2003). In their experiments, from 4 to 10 independent transformants per co-cultivated callus were obtained with various japonica cultivars. The efficiency of the reported procedure resulted from a high frequency (75-98%) of co-cultured calli forming hygromycin-resistant cell lines and the generation of multiple (10 to more than 20) resistant cell lines per co-cultured callus. Though their procedure was also developed out of CAMBIA’s standard protocol, such dramatically increased transformation efficiency was achieved apparently through precise control of callus quality and in time separation of independent transgenic calli.

### 4.4 Conclusion

An *Agrobacterium*-mediated transformation system for the japonica rice variety Millin was well established. A case study was made to establish a large-scale and high efficient transformation protocol for the TransGenomics project. From a single transformation experiment with 1,000 scutellum calli, a total of 1,021 transgenic rice lines were obtained. It took about 5 months from the initiation of callus to the transfer of regenerated plants to soil. The efficiency can fulfill the
prerequisite for the TransGenomics project in which large populations of transgenic lines are needed.

Efforts to further improve transformation efficiency were also made and the results were exciting. By decreasing the co-cultivation temperature from 26°C to 22°C, the transformation efficiency was doubled. Other factors and relevant measures for increasing transformation efficiency were discussed.
Chapter 5 Testing elements of enhancer trap constructs

5.1 The TAFET system and its elements

Since Casadaban and Cohen (Casadaban and Cohen, 1979) first developed enhancer trapping for use in *Escherichia coli*, approaches have been developed for a large number of organisms, including *Drosophila* (O'Kane and Gehring, 1987), mouse (Allen et al., 1988) and *Arabidopsis* (Sundaresan et al., 1995).

The core component of early enhancer trap constructs is a minimal promoter fused to a reporter gene that is only expressed when inserted near cis-acting chromosomal enhancers. Insertional mutagenesis using these enhancer traps involves generating a large number of individuals that have the reporter gene integrated at different sites throughout the genome. These individuals and/or their progeny are examined for (i) expression of the reporter gene; and (ii) mutant phenotypes caused by insertion. In lines in which the reporter gene is inserted within or near a chromosomal gene, reporter gene expression pattern mimics that of the chromosomal gene. Enhancer traps have proved successful in detecting novel genes in *Arabidopsis* (Sundaresan et al., 1995).

In the last few years, several modifications have been made to the basic system of enhancer traps. A novel and much more powerful enhancer trap strategy has been developed in *Drosophila* (Brand and Perrimon, 1993), where the enhancer trap construct carries a gene encoding a transcriptional activator protein that activates gene expression via its recognition of, and binding to, a specific DNA target recognition sequence (Brand and Perrimon, 1993; Ferveur et al., 1995). As with the previous approach, this gene is under the control of a minimal promoter, so that it is only expressed when the T-DNA integrates near genomic regulatory sequences. The element typically also carries a reporter gene adjacent to the target sequence of the transcriptional activator, to allow detection of the expression of the transcriptional activator. The transcriptional activator gene that
has been used in this strategy encodes the Gal4/VP16 fusion protein. The Gal4 DNA binding domain recognizes and binds to a specific sequence element, called the Upstream Activator Sequence (UAS), which functions as a Gal4-responsive enhancer; and the VP16 transcriptional activator domain effects the transcriptional activation of any gene that is adjacent to the UAS. Use of this transactivator gene versus a reporter gene to trap genomic enhancer sequences, results in the immediate production of lines that can be used to express any gene of interest with the same expression pattern as that exhibited by the enhancer trap. Populations of enhancer traps using Gal4/VP16 system have been developed and extensively used in Drosophila (Brand and Perrimon, 1993; Ferveur et al., 1995). In plants similar system was developed in Arabidopsis (Haseloff, 1999; Kiegle et al., 2000).

Based on the strategies developed in Drosophila and Arabidopsis, a Transcriptional Activator-Facilitated Enhancer Trap (TAFET) system was designed to generate enhancer trap populations in rice. The enhancer trap vectors in the TAFET system were constructed including the three elements: (1) a transactivator cassette; (2) a reporter cassette; and (3) a selection cassette (Figure 5.1).

![Figure 5.1 Components of the TAFET system.](image-url)
The transactivator cassette is the trapping element instead of the reporter cassette used for this purpose in the earlier strategies. The Gal4/VP16 is driven by a minimal promoter. There is no detectable expression of Gal4/VP16 except for its integration into the vicinity of genomic enhancers. Therefore, this cassette is the sensor responding to enhancers and is usually located close to the right border of T-DNA. Through the sensing, and often amplifying, the enhancer signal, transactivator is capable of transferring the enhancer signal onto genes linked to UAS sites elsewhere in the genome.

The reporter cassette is composed of a few copies of UAS, a minimal promoter and a reporter gene. In my experiments I used 6 copies of UAS and the enhanced green fluorescent protein (EGFP) as a reporter. It is a target of the transactivator and the reporter gene expression is under the control of Gal4/VP16 protein, serving as an indicator of Gal4/VP16 expression and therefore reporting the enhancer activity detected by the transactivator cassette.

The selection cassette is of a hygromycin resistance gene (hygromycin phosphotransferase, \( hpt \)) driven by a constitutive promoter for the purpose of selecting transgenic individuals. The CaMV 35S promoter was used in this experiment.

5.2 Design and construction of the testing vectors

5.2.1 Design and construction of enhancer trap vectors

The sources of various elements for cloning enhancer trap vectors were all from CAMBIA. Details of the cloning processes are described below.

5.2.1.1 The intermediate plasmid \( pVALE15.5 \)

The Gal4/VP16 fragment (964 bp BamHI-EcoRI) was inserted into pLITMUS38 (BamHI-EcoRI) and gave rise to pSKB30.1, then, a PCR fragment of 35S minimal promoter (69 bp HindIII-PstI) was further inserted into pSKB30.1
(HindIII-PstI) and the intermediate plasmid pVALE15.5 with the 35S MP-Gal4/VP16 component was generated.

5.2.1.2 The intermediate binary vector pFX-B61-1

A 5xUAS-MP-mGFP5ER fragment (1322 bp HindIII-XmnI) was cut from pBinmGal4VP16mGFP5HDEL and inserted into pLITMUS39 (HindIII-EcoRV), resulting in the intermediate plasmid pFX-A109.1-17. From this intermediate plasmid, a 1384 bp fragment (BspEI-NgoMI) containing the 5xUAS-MP-mGFP5ER component was inserted into pVALE15.5 (cut open by BspEI) and generated the plasmid pFX-B46.1-23. A Pmel-BsrGI fragment was cut from pFX-B46.1-23 and ligated to the pCAMBIA1200 vector cut by Pmel and Acc65I, leading to the enhancer trap construct pFX-B61-1. This enhancer trap construct was not used for large-scale transformation experiments since the GFP signal by mGF5PER was found not strong enough for enhancer trapping in rice based on the preliminary studies.

5.2.1.3 The intermediate plasmid pFX-C70-6

A HindIII-NcoI fragment (204 bp) containing the 6xUAS-MP component was cut from pDAM-SNS/LUCr and ligated to a fragment of pTG113 (HindIII-NcoI, with gusA gene), leading to generation of pSKC2.1. To replace gusA with an EGFP reporter, a two-step cloning was carried on: (i) a SnaBI-AflII fragment was cut off from pSKC2.1 and replaced by a SnaBI-AflII fragment containing EGFP from pFX-C39.1-10 (a GUS::EGFP fusion reporter, Chapter 3); (ii) the intermediate plasmid pFX-C52-15 generated in the first step was cut with NcoI and re-ligated so that the gusA was removed from the GUS::EGFP fusion. The final product of this two-step cloning was pFX-C70-6.
5.2.1.4 Binary enhancer trap vectors \( pFX-C90.1-12R \) and \( pFX-C97.1-2 \)

Both \( pFX-B61-1 \) and \( pFX-C70-6 \) was cut open by HindIII and ligated together in two different orientations respectively, resulting in two binary enhancer trap vectors: \( pFX-C90.1-12R \) and \( pFX-C97.1-2 \).

5.2.1.5 Binary enhancer trap vector \( pFX-C90.2-17 \)

The PCR fragment of CAT-1 intron (190 bp PstI-PstI) was inserted into pVALE15.5 which was cut open by PstI, forming the intermediate plasmid pVALE15.5+CAT-1. Then, a Pmel-BgII fragment from pVALE15.5+CAT-1 was cloned into the vector \( pFX-B61-1 \) at the Pmel and BgII sites, leading to generation of another vector \( pFX-B75.1-2 \) (similar to \( pFX-B61-1 \), not used in formal transformation experiments). In the next step, both \( pFX-B75.1-2 \) and \( pFX-C70-6 \) was cut open by HindIII and ligated together in two different orientations respectively, resulting in two binary enhancer trap vectors: \( pFX-C90.2-17 \) and \( pFX-C90.2-2R \) (\( pFX-C90.2-2R \) was not used for transformation experiments because unwanted recombination in the T-DNA was detected).

Arrangement and components of T-DNAs of the preliminary designed enhancer trap vectors are shown in figure 5.2.

Major differences among these enhancer trap vectors are listed below:

- **\( pFX-C90.1-12R \) vs \( pFX-C97.1-2 \):** (1) the distance between the CaMV 35S promoter (for HPTII) and the minimal promoter (for EGFP) is small (1609 bp) in \( pFX-C97.1-2 \) but large (5644 bp) in \( pFX-C90.1-12R \); and (2) opposite orientations of the reporter cassette 6xUAS-MP-EGFP. The purpose of this design is to evaluate a possible cis-activation of the minimal promoter by the enhancer elements carried by the CaMV 35S promoter.
Figure 5.2 Arrangement of elements on T-DNAs of enhancer trap vectors.

- **pFX-C97.1-2 vs pFX-C90.2-17**: the only difference is that pFX-C90.2-17 contains a castor bean CAT-1 gene intron (190 bp) just upstream of the Gal4/VP16 coding region, while pFX-C97.1-2 does not contain an intron.
• pFX-C90.2-17 vs pFX-C90.1-12R: similar to the case of pFX-C90.1-12R vs pFX-C97.1-2; one more difference is that pFX-C90.2-17 contains a CAT-1 intron (190 bp) upstream of the Gal4/VP16 while pFX-C90.1-12R does not.

5.2.2 Design and construction of Gal4-deletion vectors

To verify the functionality of Gal4/VP16 in the enhancer trap vectors, two Gal4-deletion vectors (pFX-C103.2-3 and pFX-C103.1-2) were constructed, serving as negative controls for the pFX-C90.2-17 and pFX-C90.1-12R, respectively. Both pFX-C90.2-17 and pFX-C90.1-12R were cut by Eco47III and Stul, and then re-ligated, leading to a deletion of 108 bp in the Gal4 binding domain (Figure 5.3).

Figure 5.3 T-DNA structures of the Gal4-deletion vectors pFX-C103.1-2 (based on pFX-C90.1-12R) and pFX-C103.2-3 (based on pFX-C90.2-17).
5.3 Reporter gene expression in rice calli transformed with enhancer trap constructs

5.3.1 Transformation and GFP expression of rice calli in enhancer trap vectors

Scutellum-derived rice calli were transformed with the *Agrobacterium* strain EHA105 harboring the enhancer trap vectors (pFX-C90.1-12R, pFX-C97.1-2 and pFX-C90.2-17) and the positive control vector pFX-B114.1 (CaMV 35S promoter driven EGFP reporter, Chapter 3). The OD$_{600}$ values of the *Agrobacterium* suspensions were adjusted to 0.8 and 200 calli were transformed with each of the vectors. Starting immediately after co-cultivation transformation, the dynamics of GFP expression during hygromycin selection was analyzed in weekly intervals by observing all the calli under the Leica MZ FLIII fluorescence stereomicroscope equipped with GFP3 filter set.

At the callus stage, dynamics of GFP expression in the enhancer traps were different from that of the positive control pFX-B114.1 as shown in Figure 5.4. In the first week after transformation, transient expression of the reporter gene in enhancer traps also showed a peak though percentage of calli showing GFP expression was lower compared to pFX-B114.1. Two or three weeks after transformation, percentage of the GFP-positive calli reached a bottom of the curve and then increased gradually, while the curve in pFX-B114.1 went down all the way.

In terms of gene expression, most probably expression of the Gal4/VP16, the CAT-1 intron (Figure 5.2) showed an apparent negative effect. The effect should be indicated by the expression of GFP reporter gene since GFP expression was under the control of Gal4/VP16. The enhancer trap construct pFX-C90.2-17 with the CAT-1 intron showed much lower GFP expression (Figure 5.4). This phenomenon was found in repeated experiments with the same construct (Figures
5.4 and 5.5) or other similar sets of enhancer trap vectors (pFX-E24 and pFX-E9 series, data not shown).

![Graph showing dynamics of GFP expression in rice calli transformed with enhancer trap vectors.](image)

Figure 5.4 Dynamics of GFP expression in rice calli transformed with enhancer trap vectors. pFX-B114.1 (dotted line) is the positive control containing EGFP driven by the CaMV 35S promoter.

### 5.3.2 Transformation and GFP expression of rice calli in Gal4-deletion vectors

Another transformation experiment was done using the enhancer trap vectors pFX-C90.1-12R and pFX-C90.2-17 and their corresponding Gal4-deletion vectors pFX-C103.1-2 and pFX-C103.2-3 (Figure 5.3). Number of scutellum-derived calli used was 200 for each of the constructs and OD\(_{600}\) values of the *Agrobacterium* suspensions were adjusted to 0.76. GFP expression was surveyed in weekly intervals starting immediately after transformation. Observation was
made using the Leica MZ FLIII fluorescence stereomicroscope equipped with GFP3 filter set.

Two weeks after transformation, there was basically no GFP expression detected in calli transformed with the two Gal4-deletion constructs while between 32 to 42 % of the calli transformed with enhancer trap vectors containing non-deleted transactivator showed clear GFP expression (Figure 5.5). These results provided evidence that Gal4/VP16 was functional in the enhancer trap vectors.

Figure 5.5 Dynamics of GFP expression in rice calli transformed with enhancer traps and their corresponding Gal4-deletion vectors.

In three weeks after transformation, however, GFP expression was detected in the two Gal4-deletion constructs and the number of calli showing GFP expression increased dramatically after 4 weeks from Agrobacterium co-cultivation. Such expression of GFP was apparently not due to enhancer trap mechanism since one of the necessary elements was deleted (Figure 5.3). The other unwanted ways which would lead GFP expression in these Gal4-deletion constructs could be: (i) cis-activities of enhancer elements from the CaMV 35 promoter, which acted on the minimal promoter of the reporter cassette 6xUAS-
MP-EGFP; (ii) other unknown regulatory proteins produced at late callus stage, which either acted on the minimal promoter fused to the GFP reporter or banded to the UAS and mimicked the function of Gal4/VP16.

The difference of GFP expression levels between pFX-C103.1-2 and pFX-C103.2-3 might imply that GFP expression in these Gal4-deletion constructs could be due to cis-activation of reporter gene by the enhancer elements located in the CaMV 35 promoter (Figures 5.3 and 5.5). Rice calli transformed with pFX-C103.2-3 showed higher rate of GFP expression since the 6xUAS-MP-EGFP cassette was located very close to the CaMV 35S promoter (1.6 kbp), whereas in pFX-C103.1-2 showing much lower rate of GFP expression, the distance between the 6xUAS-MP-EGFP cassette and the CaMV 35S promoter was relatively larger (5.6 kbp). However, the parallel sharp increase for both deletion controls might suggest other factors were also responsible for the observed results.

Figure 5.6 Constructs designed for testing cis-activities of enhancer elements in the CaMV 35S promoter.
5.3.3 GFP expression of rice calli with more negative control vectors

The cis-activities of enhancer elements in the CaMV 35S promoter were also detected in another experiment conducted for testing temperature effects on transformation efficiency (Chapter 4). The constructs used in this experiment were similar to the Gal4-deletion vectors described above. In both pFX-J55.7R and pFX-J57.8, the Gal4/VP16 was also deleted. The CaMV 35S promoter-HPT cassette was set in different orientation (Figure 5.6).

Transformation efficiency in this experiment was very high because of the temperature effects during co-cultivation (22°C, 3 days). There was no noticeable difference between the two constructs in the rates of hygromycin resistant calli, but the percentages of calli showing GFP expression was apparently different due to different orientations of the CaMV 35S-HPT cassette. Dynamics of GFP expression was traced and the result is shown in Figure 5.7. In pFX-J55.7R, where the location of CaMV 35S promoter was very close to the 6xUAS-MP-EGFP cassette, higher rate of GFP expression was detected.

![Graph showing GFP expression over time]

Figure 5.7 Comparison of GFP expression in Gal4-deletion constructs with different orientation of the CaMV 35S promoter-HPT cassette.
5.4 GFP expression in T₀ rice plants transformed by the enhancer trap and Gal4-deletion constructs

5.4.1 Frequency and strength of GFP expression in transgenic lines

A total of 590 transgenic lines (including the lines obtained with the positive control pFX-B114.1) were generated. Before transplanted into soil pots, all the plantlets were surveyed for GFP expression using the Leica MZ FLIII fluorescence stereomicroscope equipped with GFP3 filter set, usually at 40x magnification. The GFP signals were scored as strong, medium and weak, and patterns of GFP expression were recorded.

The percentage of transgenic lines showing GFP expression was relatively high in all three enhancer trap constructs, 83% in pFX-C90.1-12R, 84% in pFX-C97.1-2, and 70% in pFX-C90.2-17 (Table 5.1). Like the situation in callus stage, pFX-C90.2-17 showed weaker GFP expression, most probably due to the negative effect of CAT-1 intron in the Gal4/VP16 cassette.

Table 5.1 Number and percentage of transgenic lines showing different strength of GFP expression.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Total number of lines</th>
<th>Strong (%)</th>
<th>Medium (%)</th>
<th>Weak (%)</th>
<th>Negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFX-C90.1-12R</td>
<td>109</td>
<td>38 (34.86)</td>
<td>26 (23.85)</td>
<td>26 (23.85)</td>
<td>19 (17.43)</td>
</tr>
<tr>
<td>pFX-C97.1-2</td>
<td>138</td>
<td>41 (29.71)</td>
<td>45 (32.61)</td>
<td>30 (21.74)</td>
<td>22 (15.94)</td>
</tr>
<tr>
<td>pFX-C90.2-17</td>
<td>123</td>
<td>22 (17.89)</td>
<td>22 (17.89)</td>
<td>42 (34.15)</td>
<td>37 (30.08)</td>
</tr>
<tr>
<td>pFX-C103.1-2</td>
<td>43</td>
<td>10 (23.26)</td>
<td>12 (27.91)</td>
<td>9 (20.93)</td>
<td>12 (27.91)</td>
</tr>
<tr>
<td>pFX-C103.2-3</td>
<td>59</td>
<td>13 (22.03)</td>
<td>17 (28.81)</td>
<td>9 (15.25)</td>
<td>20 (33.90)</td>
</tr>
</tbody>
</table>
Importantly, GFP expression in the plantlets with Gal4-deletion constructs (pFX-C103.1-2 and pFX-C103.2-3) was fairly high (Table 5.1). In both pFX-C103.1-2 and pFX-C103.2-3, the percentage of lines with strong and medium expression strength of GFP was nearly as high as in the two enhancer trap constructs (pFX-C90.1-12R and pFX-C97.1-2). The lines obtained with the negative control constructs were actually expressing GFP at a similar strength as those obtained with pFX-C90.2-17. The results obtained in callus stage indicated that Gal4NP 16 was not functional in the Gal4-deletion constructs and it was mostly the cis-activity from enhancer elements of the CaMV 35S promoter that caused the reporter gene expression in the later stages of hygromycin selection. The same mechanisms could be also suggested as an explanation of the results obtained at the plantlet stage.

5.4.2 Frequency of GFP expression in different tissues and organs

In order to confirm the influence of cis-acting enhancer elements within the T-DNAs on the reporter gene expression, both the enhancer trap lines and Gal4-deletion constructs derived lines were analyzed for GFP expression in a number of tissues and organs. In addition, this analysis was expected to yield a better characterization of the possible cis-activities of the CaMV 35S promoter and the interplay between cis- and Transactivation processes.

The frequencies of GFP expression in various tissues and organs of rice plants obtained using any of the three enhancer trap constructs are listed in Table 5.2. In all three constructs, GFP expression was frequently detected in collar, stem base, root, leaf and stomata, which were also typical expression patterns of the CaMV 35S promoter (Battraw and Hall, 1990; Terada and Shimamoto, 1990; Cornejo et al., 1993). The frequencies of GFP expression in various tissues and organs in pFX-C97.1-2 were quite similar to those of pFX-C90.2-17. These two constructs had the same arrangements of elements along the T-DNA except for an extra CAT-1 intron in pFX-C90.2-17. The result indicated that the CAT-1 intron did not influence expression patterns.
GFP expression in pFX-C90.1-12R was somewhat different from the other two constructs. There were much more lines (44.0% of all transgenic lines) showing GFP expression in stomata, compared to other two constructs (5.1% and 4.9%). The reason for this difference is not clear but it could be related to the position of the 6xUAS-MP-EGFP cassette within the T-DNA. In pFX-C90.1-12R, the cassette (minimal promoter) was located away from the CaMV 35S promoter (5644 bp) but very close in other two constructs (1609 bp).

Table 5.2 Expression frequencies of GFP in different tissues/organs of enhancer trap lines.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Tissue with GFP expression</th>
<th>Number of lines</th>
<th>Percentage of all GFP lines</th>
<th>Percentage of all transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFX-C90.1-12R</td>
<td>Root</td>
<td>33</td>
<td>36.7</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td>Root branch</td>
<td>5</td>
<td>5.6</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Stem base</td>
<td>60</td>
<td>66.7</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>49</td>
<td>54.4</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td>Stomata</td>
<td>43</td>
<td>47.8</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>Vascular band</td>
<td>2</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Collar</td>
<td>78</td>
<td>86.7</td>
<td>71.6</td>
</tr>
<tr>
<td></td>
<td>Auricle</td>
<td>7</td>
<td>7.8</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>Ligule</td>
<td>4</td>
<td>4.4</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Trichome</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pFX-C97.1-2</td>
<td>Root</td>
<td>69</td>
<td>59.5</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>Root branch</td>
<td>3</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Stem base</td>
<td>84</td>
<td>72.4</td>
<td>60.9</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>46</td>
<td>39.7</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>Stomata</td>
<td>7</td>
<td>6.0</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>Vascular band</td>
<td>2</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Collar</td>
<td>111</td>
<td>95.7</td>
<td>80.4</td>
</tr>
<tr>
<td></td>
<td>Auricle</td>
<td>16</td>
<td>13.8</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>Ligule</td>
<td>3</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Trichome</td>
<td>1</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>pFX-C90.2-17</td>
<td>Root</td>
<td>39</td>
<td>45.3</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>Root branch</td>
<td>2</td>
<td>2.3</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Stem base</td>
<td>75</td>
<td>87.2</td>
<td>61.0</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>22</td>
<td>25.6</td>
<td>17.9</td>
</tr>
<tr>
<td></td>
<td>Stomata</td>
<td>6</td>
<td>7.0</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Vascular band</td>
<td>1</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Collar</td>
<td>85</td>
<td>98.8</td>
<td>69.1</td>
</tr>
<tr>
<td></td>
<td>Auricle</td>
<td>4</td>
<td>4.7</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Ligule</td>
<td>1</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Trichome</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
5.4.3 GFP expression in Gal4-deletion lines

For easy comparison, data of GFP expression patterns in all three enhancer traps were pooled together, and so did in Gal4-deletion constructs (Table 5.3), for there were no significant differences within each of the two categories.

Table 5.3 GFP expression in various tissues and organs of rice enhancer trap lines and Gal4-deletion control lines.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Tissue with GFP expression</th>
<th>Number of lines</th>
<th>Percentage of all GFP lines</th>
<th>Percentage of all transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enhancer trap</td>
<td>Root</td>
<td>141</td>
<td>48.3</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>Root branch</td>
<td>10</td>
<td>3.4</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Stem base</td>
<td>219</td>
<td>75.0</td>
<td>59.2</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>117</td>
<td>40.1</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>Stomata</td>
<td>56</td>
<td>19.2</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>Vascular band</td>
<td>5</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Collar</td>
<td>274</td>
<td>93.8</td>
<td>74.1</td>
</tr>
<tr>
<td></td>
<td>Auricle</td>
<td>27</td>
<td>9.2</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>Ligule</td>
<td>8</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Trichome</td>
<td>1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Gal4-deletion</td>
<td>Root</td>
<td>35</td>
<td>50.0</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td>Root branch</td>
<td>4</td>
<td>5.7</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Stem base</td>
<td>60</td>
<td>85.7</td>
<td>58.8</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>21</td>
<td>30.0</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>Stomata</td>
<td>2</td>
<td>2.9</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Vascular band</td>
<td>2</td>
<td>2.9</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Collar</td>
<td>66</td>
<td>94.3</td>
<td>64.7</td>
</tr>
<tr>
<td></td>
<td>Auricle</td>
<td>6</td>
<td>8.6</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Ligule</td>
<td>2</td>
<td>2.9</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Trichome</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

It was found that GFP expression in Gal4-deletion constructs was not only similar in strength to that of enhancer traps, but also had nearly the same frequencies of lines with GFP expression in various tissues and organs analyzed (Table 5.3). The tissues/organs with most frequent GFP expression among the Gal4-deletion transgenic lines were collar (64.7%), stem base (58.8%), root (34.3%) and leaf (20.6%), whereas among the enhancer trap lines, they were similarly collar (74.1%), stem base (59.2%), root (38.1%) and leaf (31.6%). Also,
a similar tendency in the distribution of expression patterns was observed for negative control lines and the enhancer trap lines (Table 5.3).

There were some differences in GFP expression patterns between enhancer trap and Gal4-deletion lines. The enhancer trap lines showed higher frequency in stomata expression, mainly due to one of the constructs (pFX-C90.1-12R). The molecular mechanisms behind those differences were not further studied, for the research focused on developing and testing new, improved enhancer traps.

5.5 Discussion

A typical eukaryotic promoter consists of a minimal promoter and other upstream cis elements (Lam et al., 1989; Benfey and Chua, 1990). The minimal promoter is essentially a TATA box region where RNA polymerase II, TATA binding protein (TBP), and TBP-associated factors (TAFs) bind to initiate transcription (Zawel and Reinberg, 1995), but minimal promoters alone have no transcriptional activity (Benfey and Chua, 1990). The cis elements, to which tissue-specific or development-specific transcription factors bind, individually or in combination, determine the spatio-temporal expression pattern of a promoter at the transcriptional level. This is the principle used in designing enhancer trap constructs, and bidirectional promoters as well.

A recent report described a strategy to make the CaMV 35S polar promoter bidirectional by fusing a minimal promoter at its 5’ end in opposite orientation, so that one promoter could direct the expression of two genes (CaMV 35S-npt II and MP-gusA), one on each end of the promoter (Xie et al., 2001). In Arabidopsis, the GUS activities directed by the bidirectionalized 35S promoter were comparable to those directed by the conventional 35S promoter (Xie et al., 2001).

The Gal4-deletion constructs designed for the present experiments are similar to the 35S bidirectional promoter (Figures 5.3 and 5.6), with the differences in that the minimal promoter and the gene under its control (MP-EGFP) in Gal4-
deletion constructs is located away from the CaMV 35S promoter and in both orientations. In pFX-J55.7R and pFX-J57.8, there is even another minimal promoter-gene cassette (MP-GUSPlus) located further away from the CaMV 35S promoter (Figure 5.6). Significant GUS expression was observed in rice calli transformation with either pFX-J55.7R or pFX-J57.8, though the strength of GUS expression was weaker than GFP (data not shown).

It has been known that enhancers can affect expression of genes located several kbp away. Atchison and Perry (1986) studied the interaction of enhancer and promoter elements by transfecting plasmacytoma cells of mouse with genes that had tandem kappa promoters located next to a single kappa enhancer and assaying those genes for transient or stable transcription. It was found that the promoters located proximal and distal to the enhancer functioned identically whether they were located 1.7 or 7.7 kbp away from the enhancer. The results suggested that the enhancer exerted its influence uniformly over large distances and independently of the presence of intervening promoters (Atchison and Perry, 1986).

Weigel et al. (2000) transformed Arabidopsis plants with activation-tagging vectors containing CaMV 35S enhancers and generated several tens of thousands of transformed plants. From these, over 30 dominant mutants with various phenotypes were isolated. Analysis of the mutants revealed that overexpressed genes were almost always found immediately adjacent to the inserted CaMV 35S enhancers, at distances ranging from 380 bp to 3.6 kbp (Weigel et al., 2000).

The present transformation experiments with the Gal4-deletion constructs revealed strong cis-activities of enhancer elements within the T-DNA. This activity causes a serious background problem in expression patterns of the enhancer trap lines generated with those constructs. As a result, the enhancer trap constructs initially developed would have very limited use. It could be very difficult to distinguish whether the reporter gene expression is due to genomic enhancers or due to the T-DNA enhancer elements, making any functional influences about the chromatin regions surrounding T-DNA insertions tenuous.
Even more importantly, the efficiency of developing a comprehensive collection of diverse transactivator’s expression patterns fairly inefficient.

The experiment results have shown that cis-activities could not be avoided by changing the orientation of the CaMV 35S promoter-HPT cassette, neither by setting a distance of up to 5.7 kbp as seen in the case of the Gal4-deletion construct pFX-C103.1-2. To make the TAFET system in practical use, new enhancer trap constructs had to be designed by replacing the CaMV 35S promoter with other promoter showing no or minimal cis-acting effects on other T-DNA elements.

5.6 Conclusion

A series of preliminary enhancer traps were constructed and tested through transformation of scutellum-derived calli. GFP expression of the enhancer traps, along with corresponding Gal4-deletion constructs, was studied extensively during the callus stage. Comparisons in dynamics of GFP expression between enhancer traps and Gal4-deletion constructs revealed that (1) the Gal4/VP16 cassette functioned as a transactivator in these enhancer trap constructs, apparently performing better without CAT-I intron; and (2) there were strong cis-activities imposed by enhancer elements of the CaMV 35S promoter on the minimal promoter (MP) of the reporter gene and apparently also the MP of Gal4/VP16 in these constructs.

A total of 590 transgenic lines were generated and patterns of GFP expression studied. Analysis of GFP expression in various tissues and organs in the transgenic lines indicated considerable cis-activities of enhancers within the CaMV 35S promoter. Such cis-activities were a significant barrier for a practical usage of the enhancer traps tested. It was deemed necessary to eliminate the CaMV 35S promoter in the enhancer trap constructs.
Chapter 6  Improvement of the TAFET system

6.1 Requirement for the new TAFET constructs’ design

Previous experiments have shown that with the preliminary designs of enhancer trap constructs, there were strong cis-activities imposed by enhancer elements of the CaMV 35S promoter on the minimal promoter (MP) of the reporter gene and the MP of Gal4/VP16 in these constructs. This background enhancer activity constrained the usefulness of the enhancer trap constructs, for it could require determining that it was not only the MP of reporter gene which was influenced. Moreover, it would be practically impossible to determine whether the reporter gene expression is due to genomic enhancers or cis-activity of the enhancers within the T-DNA. New enhancer trap constructs, were therefore, designed by replacing the CaMV 35S promoter with the maize ubiquitin promoter (Ubi-1) isolated by Christensen et al. (Christensen et al., 1992).

6.2 Characteristics of the Ubi-1 promoter

Ubiquitin is a eukaryotic protein consisting of 76 highly conserved amino acid residues and found in most cell types either as free monomers or conjugated to a variety of cytoplasmic, nuclear or membrane proteins (Cornejo et al., 1993). Ubiquitin has been implicated in multiple cellular functions, including protein turnover, chromatin structure, cell cycle control, DNA repair (Rechsteiner, 1987) and response to heat shock and other stress (Christensen et al., 1992; Wang et al., 2000).

Several ubiquitin genes have now been isolated from higher plants. These genes contain three to seven ubiquitin repeats. An intron is commonly found in the 5′UTR of plant ubiquitin genes (Christensen et al., 1992; Norris et al., 1993; Genschik et al., 1994; Rollfinke and Pfitzner, 1994; Callis et al., 1995). This intron has been implicated as a quantitative determinant of expression in transient assays in Arabidopsis (Norris et al., 1993). In contrast, in similar
experiments using tobacco ubiquitin constructs, deletion of the intron had no effect on expression levels (Genschik et al., 1994).

Christensen et al. (1992) identified two out of the 8 to 10 loci encoding ubiquitin in maize. Ubi-1 and Ubi-2 genes, the characterized genes, both contain an open reading frame of 1599 bp arranged as seven tandem, head-to-tail repeats of 228 bp encoding ubiquitin. S1 mapping and northern analysis of the Ubi-1 gene revealed a structure consisting of a 5’ exon of about 82 bp, an intron of about 1010 bp, a coding region of 1599 bp and about 140 bp of 3’ untranslated region (Christensen et al., 1992). Several sequence elements common to promoters of many other eukaryotic genes are also found in the Ubi-1 5’ flanking sequence. A TATA box sequence is located at -30, and two overlapping sequences that are similar to the consensus heat shock element found in heat inducible genes are found at -214 and -204 bases from the transcription start site (Christensen et al., 1992). These appear to be functional heat shock elements as expression of ubiquitin is increased upon a temperature shock (Christensen et al., 1992).

The Ubi-1 promoter had a stronger activity compared to the CaMV 35S promoter in several studies of rice (Cornejo et al., 1993; Bassie et al., 2000). Cornejo et al. (1993) reported that the luciferase-specific activity obtained from Ubi-1 promoter (average value 2537 light units per µg protein) was 13-fold higher than the activity obtained from the CaMV 35S promoter. GUS-specific activity from Ubi-1 promoter (average value 2 pmol MU per minute per µg protein) was 10.3-fold higher than the activity from the CaMV 35S promoter (Cornejo et al., 1993). Bassie et al. (2000) analyzed molecularly and biochemically a series of transgenic rice lines expressing the oat *adc* (arginine decarboxylase) cDNA under the control of the constitutive maize Ubi-1 promoter. They found that the maize Ubi-1 promoter resulted in significantly higher levels of expression of *adc* compared to the CaMV 35S promoter (Bassie et al., 2000).

Investigations in the expression pattern of Ubi-1 suggest that the Ubi-1 promoter is most active in cells with high metabolic activity and differs from other promoter activities that have also been characterized by GUS fusions in
transgenic rice. The CaMV 35S promoter expression is widespread, exhibiting activity in and around the vascular tissue (Terada and Shimamoto, 1990) and in most cell types of roots and leaves (Battraw and Hall, 1990).

Earlier studies on the Ubi-1 promoter at CAMBIA suggested Ubi-1 a good candidate to replace CaMV 35S, for Ubi-1 was found not acting on proximal MP as strongly as CaMV 35S.

6.3 Construction of enhancer trap vectors and Gal4-deletions

6.3.1 Construction of pFX-G74.1 and pFX-G85.2

In the enhancer trap vector pFX-G74.1, the MP-Gal4/VP16 cassette and the 6xUAS-MP-EGFP cassette were from pFX-C90.1-12R; whereas the Ubi-1 promoter-ubi intron-HPT cassette was from pWSA60.1 (CAMBIA). A two-step digestion was conducted to create blunt ends for compatible ligation. Firstly, pFX-C90.1-12R was cut open with Sacl, and pWSA60.1 with HindIII, and then all the sticky ends were filled-in by T4 DNA polymerase. Secondly, the blunt-end fragments of both pFX-C90.1-12R and pWSA60.1 were cut with BsiWI and the expected fragments were isolated in 0.8% agarose gel. Recovered fragments from the gel were ligated to generate the construct pFX-G74.1 (Figure 6.1).

Figure 6.1 T-DNA components of the enhancer trap pFX-G74.1.

To remove the intron between the Ubi-1 promoter and hygromycin resistance gene, an Mfel-RsrII fragment was eliminated from pFX-G74.1 and replaced by the Mfel-RsrII fragment from pWSA45.1 (CAMBIA) carrying the intron-less
Ubi-1 promoter-HPT cassette. Resulting enhancer trap without the ubi intron was generated and named pFX-G85.2 (Figure 6.2).

Figure 6.2 T-DNA components of the enhancer trap pFX-G85.2.

The only difference between pFX-G74.1 and pFX-G85.2 is that there is an ubi intron in the Ubi-1 promoter-HPT cassette for pFX-G74.1, but not for pFX-G85.2.

Figure 6.3 T-DNAs of the Gal4-deletion vectors pFX-G99.3 and pFX-H13.3.
6.3.2 Construction of Gal4-deletion vectors

Gal4-deletion vectors were created by replacing a SacI-Eco47III fragment (339 bp) of the enhancer trap construct pFX-G85.2 with the SacI-SnaBI fragment (362 bp) from the plasmid pFX-A109.1-17 (CAMBIA). This replacement destroyed almost the whole sequence of Gal4 DNA-binding domain and a small portion of the VP16 activation domain, and resulted in the Gal4-deletion construct pFX-G99.3 (Figure 6.3). Another Gal4-deletion construct, pFX-H13.3, was generated by replacing an MfeI-RsrII fragment from pFX-G99.3 with the MfeI-RsrII fragment from pFX-G74.1, creating a molecule with an intron between the Ubi-1 promoter and hygromycin resistance gene (Figure 6.3). The Gal4-deletion constructs pFX-H13.3 and pFX-G99.3 are corresponding negative controls of pFX-G74.1 and pFX-G85.2, respectively.

6.4 Construction of the positive control vector

A positive control binary vector, pFX-J99.2, was constructed for surveying expression patterns of GFP reporter driven by the Ubi-1 promoter. In this construct, the Ubi-1 promoter-intron-HPT cassette was from pWSA60.1 (CAMBIA); the EGFP reporter was from pFX-B114.1; and the promoter (ubi-intron) for driving EGFP reporter was from pSAN L12.1 (CAMBIA).

The positive control vector was made through a three-step cloning process. Firstly, a BamHI-AflIII fragment harboring Ubi-1 promoter-intron-GUS reporter was cut from pSAN L12.1 and inserted into pLITMUS38 which was cut open with BamHI and AflIII, resulting in the intermediate plasmid pFX-J58.2. Secondly, the GUS reporter was replaced by EGFP. An NcoI-AflIII fragment was removed from pFX-J58.2 and replaced by an NcoI-AflIII fragment harboring EGFP reporter from pFX-B114.1, generating the intermediate plasmid pFX-J83.2. Thirdly, a HindIII-AflIII fragment was removed from the binary vector pWSA60.1 and replaced by the HindIII-AflIII fragment harboring a cassette of Ubi-1 promoter-intron-EGFP reporter from pFX-J83.2. The resulting binary
vector was named as pFX-J99.2 (Figure 6.4). pFX-J99.2 is similar to the frequently used positive control pFX-B114.1 except for the promoters driving HPT and EGFP. In pFX-B114.1, it is the CaMV 35S promoter that drives both HPT and EGFP.

Figure 6.4 T-DNA components of the positive control binary vector pFX-J99.2.

6.5 Reporter gene expression in rice calli transformed with enhancer trap and Gal4-deletion constructs

Scutellum-derived rice calli were transformed with the Agrobacterium strain EHA105 harboring the enhancer trap vectors (pFX-G74.1 and pFX-G85.2) and their corresponding Gal4-deletion negative controls (pFX-H13.3 and pFX-G99.3). In this experiment, the binary vector pFX-B114.1 was used as a positive control and also for comparing effects of hygromycin selection controlled by different promoters (CaMV 35S and Ubi-1). The OD_{600} values of the Agrobacterium suspensions were adjusted to 0.75 and 175 calli were transformed with each of the vectors. Previous experiments had shown that the dynamics of GFP expression in rice calli during hygromycin selection provided useful information about performance of various elements and the constructs. Therefore, GFP expression of rice calli was regularly investigated after co-cultivation with agrobacteria. Observations were done in weekly intervals using the Leica MZ FLIII fluorescence stereomicroscope equipped with GFP3 filter set, usually at 25x magnification.
6.5.1 GFP expression of rice calli in the positive control

Dynamics of GFP expression in the positive control pFX-B114.1 followed the usual way (Figure 6.5). In the first week after transformation, percentage of the calli showing GFP expression reached a peak (74.3%) and dropped down gradually thereafter. Six weeks after transformation, rate of the GFP-positive calli was 44.5%. Transformation efficiency of this experiment was on the average level based on GFP expression of the positive control used in several experiments (Discussions in Chapter 3).

The peak of GFP expression in the positive control in the first week after co-cultivation could be the result and effect of transient expression, and therefore, there was a significant drop of GFP expression after the peak, most probably due to the hygromycin selection pressure. Cells without integrated T-DNA would stop growth and die under the selection. During the period of 1-3 weeks under selection, a great portion of calli showing GFP expression could be due to the remaining GFP protein generated earlier.

GFP is unusually resistant to proteolysis (Tsien, 1998) and very stable (Tombolini et al., 1997), with a half-life time reported as >1 day in vivo (Andersen et al., 1998). This means that once made, GFP will persist in a cell even after the promoter that drives its expression is shut down. Andersen et al. also reported that both colonies and liquid cultures of *E. coli* MV1190 (λ-pir) expressing GFPmut3 remained green fluorescent after several weeks of incubation without inducer (Andersen et al., 1998).

6.5.2 GFP expression of rice calli in the enhancer traps

During the hygromycin selection stage, dynamics of GFP expression in the enhancer traps (pFX-G74.1 and pFX-G85.2) were similar to that of the positive control as shown in Figure 6.5. In the first week after transformation, transient expression of the reporter gene in enhancer traps also showed a peak with percentages of calli showing GFP expression even higher than that of the positive
Such strong transient expression of the reporter gene in enhancer traps could not be completely due to trapped enhancers of rice genome, with the same reasoning as described above for the positive control.

The enhancer trap pFX-G74.1 showed much higher GFP expression than both the enhancer trap pFX-G85.2 and the positive control pFX-B114.1 (Figure 6.5), which was most probably attributed to the Ubi-1 promoter driving the hygromycin resistance gene. The percentage of calli showing GFP expression had been found tightly correlated with the rate of hygromycin-resistant calli (Discussions in Chapter 3). The result suggested that Ubi-1 (in pFX-G74.1) was more active than the CaMV 35 promoter (in pFX-B114.1) when driving expression of the hygromycin resistance gene in rice calli.

Figure 6.5 Dynamics of GFP expression in rice calli transformed with enhancer trap constructs and their corresponding Gal4-deletion vectors. pFX-B114.1 was used as a positive control in the experiment.

The only difference between pFX-G74.1 and pFX-G85.2 was the ubi intron in the Ubi-1 promoter. Since the intron was removed in pFX-G85.2, expression
level of the hygromycin resistance gene was much weaker and some of the transgenic cells might have died under the selection pressure.

### 6.5.3 GFP expression of rice calli in the Gal4-deletion constructs

Two weeks after transformation, there was basically no GFP expression detected in calli transformed with the two Gal4-deletion constructs (pFX-G99.3 and pFX-H13.3) while 51.8% to 73.6% of calli transformed with the vectors containing non-deleted transactivator (pFX-G74.1 and pFX-G85.2) showed clear GFP expression (Figure 6.5). These results provided evidences that Gal4/VP16 was functional in the enhancer trap vectors.

Three weeks after transformation, however, GFP expression was detected in the two Gal4-deletion constructs and the number of calli showing GFP expression increased dramatically after 4 weeks from *Agrobacterium* co-cultivation. The phenomenon was similar to what was observed with CaMV 35 promoter constructs (Figure 5.5). The molecular mechanisms of such phenomenon were not well understood. Therefore, cis-activities of enhancer elements from the Ubi-1 promoter could not be ruled out based on present results.

There was a small difference in GFP expression levels between pFX-G99.3 (38.2%) and pFX-H13.3 (27.8%). The difference should be larger when the influence of ubi intron (in pFX-H13.3 but not pFX-G99.3) on hygromycin resistance gene was considered. The observed difference in GFP expression could be due to the distance between the MP-EGFP cassette and the Ubi-1 promoter. In pFX-G99.3, the minimal promoter fused to GFP reporter was located 1 kb closer to the Ubi-1 promoter compared to pFX-H13.3, therefore, the MP in pFX-G99.3 might be affected more easily by cis-activation of enhancer elements from the Ubi-1 promoter.
6.6 GFP expression in T₀ rice plants transformed by the enhancer trap and Gal4-deletion constructs

6.6.1 Frequency of GFP expression in transgenic lines

A total of 661 transgenic lines were generated, including 251 and 142 lines obtained with the enhancer trap vector pFX-G74.1 and pFX-G85.2, respectively; 130 lines with positive control vectors pFX-B114.1 and pFX-J99.2; and 138 lines with the Gal4-deletion vectors pFX-G99.3 and pFX-H13.3.

Before being transplanted into soil pots, all the transgenic plantlets were surveyed for GFP expression at the vegetative stage. For surveying GFP expression at the generative stage, samples of young panicles were taken from each of the transgenic lines at the beginning of flowering stage. For each transgenic line, 15-20 spikelets were carefully dissected under a stereomicroscope and GFP observations were done using the Leica MZ FLIII fluorescence stereomicroscope equipped with GFP3 filter set.

Table 6.1 Number and percentage of transgenic lines showing GFP expression.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Total number of transgenic lines (A)</th>
<th>No. of lines with GFP expression (B)</th>
<th>% (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFX-G74.1</td>
<td>251</td>
<td>77</td>
<td>30.7</td>
</tr>
<tr>
<td>pFX-G85.2</td>
<td>142</td>
<td>52</td>
<td>36.6</td>
</tr>
<tr>
<td>Gal4-deletion</td>
<td>138</td>
<td>7</td>
<td>5.1</td>
</tr>
<tr>
<td>Positive control</td>
<td>130</td>
<td>107</td>
<td>82.3</td>
</tr>
</tbody>
</table>

GFP expression in the positive controls was quite normal, with a small amount of transgenic lines (18%) showing no GFP signal. In the enhancer trap construct pFX-G74.1 and pFX-G85.2, the percentages of transgenic lines showing GFP expression were 30.7% and 36.6%, respectively (Table 6.1). The frequencies of
GFP-positive lines in these enhancer trap constructs with Ubi-1-HPT cassette were apparently much lower than those with CaMV 35S-HPT cassette (70%-84%, Table 5.1). The result also suggested that there was no dramatic difference in the percentages of GFP-positive lines between the two enhancer trap constructs, implying that the ubi intron influenced only the survival of transgenic calli under hygromycin selection pressure, but not the function of enhancer trapping.

There were only 7 lines showing GFP expression in the Gal4-deletion constructs, much less than the enhancer trap constructs containing the functional transactivator (pFX-G74.1 and pFX-G85.2). The result indicated that (i) GFP expression in the enhancer traps was apparently triggered by the functional Gal4/VP16; and (2) though there was GFP expression in callus stage possibly imposed either by the cis-activation of enhancer elements from the Ubi-1 promoter or by some unknown regulatory proteins, the occurring frequency of such phenomenon was very low in transgenic plants.

6.6.2 Patterns of GFP expression in enhancer trap lines

Patterns of GFP expression were recorded based on tissue/organ or combinations of tissues/organs with GFP expression. The recorded categories and codes of tissues were root (A), root branch (B), stem base (C), leaf (D), stomata (E), vascular band (F), collar (G), auricle (H), ligule (I), trichome (J), mesophyll (K), stigma (L), ovary (M), anther (N), lodicule (O), glumes (P), sterile lemma (Q), pedicel (R), and panicle branch (S).

A total of 84 different patterns of GFP expression were detected from the 393 transgenic lines obtained with the enhancer trap construct pFX-G74.1 and pFX-G85.2. Of the 393 lines, only 129 lines showed GFP expression in 84 different patterns. The expression patterns were apparently very diverse (Appendix B).

Table 6.2 shows the categories and percentages of enhancer trap lines in which GFP expression was detected in one to several (up to 13) different tissues/organs
that included root, root branch, stem base, leaf, stomata, vascular band, collar, auricle, ligule, trichome, mesophyll, stigma, ovary, anther, lodicule, glumes, sterile lemma, pedicel and panicle branch. Over 60% of the GFP-positive pattern lines showed GFP expression in two to four tissues/organs (26.4%, 21.7%, 16.3% in two, three, four tissues/organs, respectively). Twelve lines showed specific GFP expression in only one tissue/organ, including 4 lines in root, 3 lines in stomata and one line each in vascular band, collar, trichome, stigma, and pedicel, respectively.

Table 6.2 Complexity of reporter gene expression patterns among the lines obtained with enhancer trap construct pFX-G74.1 and pFX-G85.2.

<table>
<thead>
<tr>
<th>Number of tissues/organs involved in a pattern</th>
<th>Number of lines</th>
<th>Percentage of pattern lines</th>
<th>Percentage of transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>9.3</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>26.4</td>
<td>8.7</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>21.7</td>
<td>7.1</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>16.3</td>
<td>5.3</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>6.2</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>10.1</td>
<td>3.3</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>4.7</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>0.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The frequencies of GFP expression in various tissues and organs of rice plants are listed in Table 6.3. Between the two enhancer trap constructs, there seems no significant difference in the distribution of tissue or organ-specific expression frequencies except for the expression in mesophyll. In both enhancer trap constructs, GFP expression was detected relatively frequent in root (15.9%-21.1%), stomata (16.7%-19.0%), leaf (13.5%-18.3%), and glumes (11.6%-13.4%). Besides, relatively high frequency (14.1%) of GFP expression was found in pFX-G85.2 plants in the form of irregular granules in mesophyll, while mesophyll with GFP expression were observed with lower frequency in pFX-
G74.1 plants (6.0%). However, the molecular mechanism of this difference was unclear.

To compare with the result obtained with 35S-HPT series enhancer traps described in Chapter 5, data from different constructs of the same series were pooled together and listed in Table 6.4. The overall frequency of GFP-positive lines in Ubiquitin-HPT series (32.8%) was much lower than that of 35S-HPT series (78.9%). Frequencies of GFP expression in collar (67.3%), stem base (52.4%), leaf (33.0%) and root (32.7%) were very high with 35S-HPT constructs, while those with Ubiquitin-HPT constructs were only 3.3%, 0.8%, 15.3% and 17.8%, respectively. Relatively frequent expression of GFP in stomata was observed with both 35S-HPT (16.5%) and Ubiquitin-HPT (17.6%) constructs. GFP expression in mesophyll was observed frequently with Ubiquitin-HPT constructs (8.9%), but not 35S-HPT constructs.

The Ubiquitin-HPT and the 35S-HPT enhancer trap constructs differ mainly in the promoters that drive the hygromycin resistance gene. Based on the comparison, highly frequent expression of GFP in collar, stem base, leaf and root of the 35S-HPT lines would be most probably imposed by the within T-DNA cis-activities from CaMV 35 promoter. In Ubiquitin-HPT series enhancer trap lines, such phenomenon was not apparent.
Table 6.3 The frequencies of GFP expression in various tissues and organs of rice plants transformed with enhancer trap constructs.

<table>
<thead>
<tr>
<th>Tissue with GFP expression</th>
<th>pFX-G74.1</th>
<th>pFX-G85.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of lines</td>
<td>Percentage of all GFP lines</td>
</tr>
<tr>
<td>Root</td>
<td>40</td>
<td>51.9</td>
</tr>
<tr>
<td>Root branch</td>
<td>3</td>
<td>3.9</td>
</tr>
<tr>
<td>Stem base</td>
<td>3</td>
<td>3.9</td>
</tr>
<tr>
<td>Leaf</td>
<td>34</td>
<td>44.2</td>
</tr>
<tr>
<td>Stomata</td>
<td>42</td>
<td>54.5</td>
</tr>
<tr>
<td>Vascular band</td>
<td>13</td>
<td>16.9</td>
</tr>
<tr>
<td>Collar</td>
<td>8</td>
<td>10.4</td>
</tr>
<tr>
<td>Auricle</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>Ligule</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>Trichome</td>
<td>4</td>
<td>5.2</td>
</tr>
<tr>
<td>Mesophyll</td>
<td>19.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Stigma</td>
<td>11</td>
<td>14.3</td>
</tr>
<tr>
<td>Ovary</td>
<td>6</td>
<td>7.8</td>
</tr>
<tr>
<td>Anther</td>
<td>18</td>
<td>23.4</td>
</tr>
<tr>
<td>Lodicule</td>
<td>9</td>
<td>11.7</td>
</tr>
<tr>
<td>Glumes</td>
<td>29</td>
<td>37.7</td>
</tr>
<tr>
<td>Sterile lemma</td>
<td>11</td>
<td>14.3</td>
</tr>
<tr>
<td>Pedicel</td>
<td>17</td>
<td>22.1</td>
</tr>
<tr>
<td>Panicle branch</td>
<td>20</td>
<td>26.0</td>
</tr>
</tbody>
</table>
Table 6.4 Frequencies of GFP expression in various tissues/organs of rice plants transformed with Ubiquitin-HPT and 35S-HPT series enhancer trap constructs.

<table>
<thead>
<tr>
<th>Tissue with GFP expression</th>
<th>Ubiquitin-HPT</th>
<th></th>
<th>35S-HPT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of lines</td>
<td>Percentage of all GFP lines</td>
<td>Percentage of all transgenic lines</td>
<td>Number of lines</td>
</tr>
<tr>
<td>Root</td>
<td>70</td>
<td>54.3</td>
<td>17.8</td>
<td>121</td>
</tr>
<tr>
<td>Root branch</td>
<td>10</td>
<td>7.8</td>
<td>2.5</td>
<td>13</td>
</tr>
<tr>
<td>Stem base</td>
<td>3</td>
<td>2.3</td>
<td>0.8</td>
<td>194</td>
</tr>
<tr>
<td>Leaf</td>
<td>60</td>
<td>46.5</td>
<td>15.3</td>
<td>122</td>
</tr>
<tr>
<td>Stomata</td>
<td>69</td>
<td>53.5</td>
<td>17.6</td>
<td>61</td>
</tr>
<tr>
<td>Vascular band</td>
<td>21</td>
<td>16.3</td>
<td>5.3</td>
<td>2</td>
</tr>
<tr>
<td>Collar</td>
<td>13</td>
<td>10.1</td>
<td>3.3</td>
<td>249</td>
</tr>
<tr>
<td>Auricle</td>
<td>1</td>
<td>0.8</td>
<td>0.3</td>
<td>31</td>
</tr>
<tr>
<td>Ligule</td>
<td>1</td>
<td>0.8</td>
<td>0.3</td>
<td>8</td>
</tr>
<tr>
<td>Trichome</td>
<td>6</td>
<td>4.7</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>Mesophyll</td>
<td>35</td>
<td>27.1</td>
<td>8.9</td>
<td>0</td>
</tr>
</tbody>
</table>
6.6.3 Patterns of GFP expression in Gal4-deletion lines

As the negative control in this experiment, 138 T₀ plants obtained with the Gal4-deletion constructs (pFX-G99.3 and pFX-H13.3) were evaluated for reporter gene expression in the same tissues and development stages as the plants obtained with enhancer trap constructs. Interestingly, only 5.1% (7 plants) showed any GFP expression (Table 6.1), implying that the present design of enhancer trap constructs imposed no significant background problem for screening genomic enhancers being trapped. However, there were still a few Gal4-deletion lines showed GFP expression. Expression patterns of these Gal4-deletion lines are listed in Table 6.5. GFP expression in all the 7 lines was found in single tissue/organ-specific patterns, three lines in anther and one line each in root, stomata, collar and glumes, respectively.

To further compare GFP expression between the Gal4-deletion and enhancer trap constructs, expression frequencies based on tissue/organ were pooled together for the enhancer trap constructs. The result is shown in Table 6.5. The distribution of expression frequencies in the Gal4-deletion lines was apparently not correlated to that in the enhancer trap lines. The most frequently detected GFP expression was in anther (42.9%) in the Gal4-deletion lines, whereas in the enhancer trap lines the most frequently observed GFP expression was in root (54.3%), stomata (53.5%), leaf (46.5%) and glumes (37.2%). There was no indication of cis-acting influences within the T-DNA components.
Table 6.5 The frequencies of GFP expression in various tissues/organs of transgenic plants obtained with enhancer trap and Gal4-deletion constructs.

<table>
<thead>
<tr>
<th>Tissue with GFP expression</th>
<th>Enhancer trap (non-deletion)</th>
<th>Gal4-deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of lines</td>
<td>Percentage of all GFP lines</td>
</tr>
<tr>
<td>Root</td>
<td>70</td>
<td>54.3</td>
</tr>
<tr>
<td>Root branch</td>
<td>10</td>
<td>7.8</td>
</tr>
<tr>
<td>Stem base</td>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>Leaf</td>
<td>60</td>
<td>46.5</td>
</tr>
<tr>
<td>Stomata</td>
<td>69</td>
<td>53.5</td>
</tr>
<tr>
<td>Vascular band</td>
<td>21</td>
<td>16.3</td>
</tr>
<tr>
<td>Collar</td>
<td>13</td>
<td>10.1</td>
</tr>
<tr>
<td>Auricle</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Ligule</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Trichome</td>
<td>6</td>
<td>4.7</td>
</tr>
<tr>
<td>Mesophyll</td>
<td>35</td>
<td>27.1</td>
</tr>
<tr>
<td>Stigma</td>
<td>18</td>
<td>14.0</td>
</tr>
<tr>
<td>Ovary</td>
<td>9</td>
<td>7.0</td>
</tr>
<tr>
<td>Anther</td>
<td>26</td>
<td>20.2</td>
</tr>
<tr>
<td>Lodicule</td>
<td>13</td>
<td>10.1</td>
</tr>
<tr>
<td>Glumes</td>
<td>48</td>
<td>37.2</td>
</tr>
<tr>
<td>Sterile lemma</td>
<td>16</td>
<td>12.4</td>
</tr>
<tr>
<td>Pedicel</td>
<td>27</td>
<td>20.9</td>
</tr>
<tr>
<td>Panicle branch</td>
<td>30</td>
<td>23.3</td>
</tr>
</tbody>
</table>
6.7 T-DNA copy numbers in T<sub>0</sub> lines

The copy numbers of integrated T-DNA in the T<sub>0</sub> enhancer trap lines were estimated by DNA Southern blot hybridization with randomly selected samples. Genomic DNA was digested with the EcoRI restriction enzyme and hybridized with the Gal4/VP16 fragment (660 bp) as the probe. The hybridization results showed that the numbers of T-DNA insertion ranged from 1 to 6 (Table 6.6). Of the 125 T<sub>0</sub> lines assayed, 59 had one copy of the transgene, 31 had two copies, and 35 had three or more copies. Thus, approximately 47.2% of the enhancer trap lines contained single-copy T-DNA insertion and the estimated average copy number was 1.96 per line.

Table 6.6 T-DNA copy numbers in T<sub>0</sub> sample lines.

<table>
<thead>
<tr>
<th>Copy number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of lines</td>
<td>59</td>
<td>31</td>
<td>20</td>
<td>12</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>% of total</td>
<td>47.2</td>
<td>24.8</td>
<td>16.0</td>
<td>9.6</td>
<td>1.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

6.8 GFP expression in T<sub>1</sub> generation

Based on the data obtained in T<sub>0</sub> generation, a sample of 103 lines showing different strengths and patterns of GFP expression was taken for growing T<sub>1</sub> populations. For 94 lines of the sample, 25 seeds were germinated and planted in pots. Sixteen plants were finally established for each line. For the purpose of segregation analysis, 40 plants were planted for each of the other 9 lines with relatively strong GFP expression.

Leaf samples including the collar from 8 plants of each line (40 plants for some selected lines) were taken at the tillering stage and GFP expression in leaf, stomata, vascular band, collar, auricle, ligule, trichome and mesophyll was investigated. For surveying GFP expression in stigma, ovary, anther, lodicule,
glumes, sterile lemma, pedicel and panicle branch at the generative stage, samples of young panicles were taken at the beginning of flowering stage. For each plant, 10-15 spikelets were carefully dissected under a stereomicroscope and GFP observations were done using the Leica MZ FLIII fluorescent stereomicroscope equipped with GFP3 filter set. GFP expression in root, root branch and stem base was not investigated.

In general, the GFP expression in T₁ generation of all the 103 lines was fairly consistent with the expression of T₀ generation plants. Among the 103 lines, 7 negative control lines (Gal4-deletion) and 46 enhancer trap lines showed no GFP expression in both T₀ and T₁ generations; the other 50 lines with GFP expression in T₀ showed similar patterns of GFP expression in T₁ population. In more than 50% of the GFP-positive lines (27 lines), however, not all the individual T₁ plants within an enhancer trap line showed perfectly the same pattern and strength as observed in T₀. The segregation in terms of GFP expression strength and pattern in T₁ generation could be due to multiple-copy T-DNA insertion at different chromosome loci.

6.9 Discussion

The GFP expression patterns in various tissues/organs of rice have been detected for all 531 lines obtained with the Ubiquitin-HPT series constructs. A wide variety of different expression patterns of GFP were observed. Some lines exhibited broad expression patterns (i.e. most cells, tissues/organs) while other patterns were much more restricted (i.e. a small number of cells or a single tissue/organ). Even in a single tissue/organ (for example, root, leaf, stomata), GFP expression patterns were found apparently different with lines. The GFP expression patterns of the enhancer trap lines obtained were very diverse, providing a rich genetic stock useful for researchers studying various aspects of development. Another important use of these lines is the ability to identify and clone genes of interest based on expression pattern. Since these lines are tagged with T-DNA, cloning of flanking DNA is easy and straightforward.
The phenomenon of reporter gene expression in multiple tissues/organs of a pattern line was also found in enhancer trap lines of *Arabidopsis* (Campisi et al., 1999) and *Drosophila* (Braun et al., 1997). This phenomenon could be most probably due to (i) multiple activities of an enhancer trapped, or (ii) multiple insertions of the enhancer trap T-DNA into different locations of the genome. Further studies, especially the copy numbers of all GFP-positive lines, would lead to a better understanding of enhancer activities. Final characteristics of an enhancer trap line should be determined based on single copy or single site T-DNA insertion.

The present study showed that frequency of GFP-positive enhancer trap lines was 32.8% obtained with the improved Ubiquitin-HPT series constructs. There was only one report on enhancer trapping in rice presented by Wu et al. (2003). In their study, GUS expression was detected in 25-59% of the enhancer trap lines in the various tissues assayed and 68.5% of transgenic plants showed GUS-positive leaves at seeding stage (Wu et al., 2003), therefore, the overall frequency of GUS-positive lines was at least 68.5%. Such high frequency of enhancer trapping was most probably due to the within T-DNA cis-activities imposed by the CaMV 35S promoter in the construct (pFX-E24.2-15R, Chapters 3 and 5) they used to establish the enhancer trap lines. In *Arabidopsis*, it was reported that 31-48% of the enhancer trap lines exhibited reporter gene expression in the inflorescence or seedling (Sundaresan et al., 1995; Campisi et al., 1999).

Galweiler et al. (Galweiler et al., 2000) reported that Gal4-mediated expression of the reporter gene in transgenic tobacco was extremely variable, became lost as plants matured, and was almost undetectable in most of their progeny because of the extensive methylation of UAS that prevented Gal4-binding activity. The results from the present study with rice showed that there was no indication for apparently reduced or changed expression patterns of GFP in the T₁ populations compared with those in the corresponding T₀ plants, implying that the UAS elements used in rice experiments were not significantly affected by such methylation.
Significant improvements have been made in greatly reducing background problem imposed by the within T-DNA cis-activities at plant level. With the improved Ubi-HPT series enhancer trap constructs, there were only a few (5.2%) Gal4-deletion lines that showed GFP-positive in anther and some other tissues. However, results of GFP expression at the callus stage indicated strong cis-activities caused by the Ubi-1 promoter. It seems that Ubi-1 imposes different extents of cis-activities depending on tissues or organs the promoter is expressed. Base on histochemical localization of GUS activity, Cornejo et al. (1993) reported that Ubi-1 was expressed in many, but not all, rice tissues and was most active in rapidly dividing cells. To completely remove unwanted within T-DNA cis-activities, a co-transformation system should be considered so that the whole selection cassette is dismissed from the final transgenic plants.

Selectable markers are commonly used to distinguish between plant cells with an integrated transgene and the bulk of non-transformed cells. These selectable markers are mostly based on genes conferring antibiotic or herbicide resistance. In recent years, concerns have been raised regarding the spread of antibiotic resistance genes in nature or the escape of herbicide resistance genes to wild weedy species. To remove the cause of concern, scientists have developed several methods to generate marker-free transgenic plants. These plants are initially selected for resistance to an antibiotic or herbicide, but the selection marker is removed on subsequent manipulation. Several methods have been proposed to eliminate the selection cassette from the primary transformant, including:

(i) Use of a site-specific recombination system, such as Cre-lox or Flp-Frt (Dale and Ow, 1991; Bayley et al., 1992; Albert et al., 1995; Lyznik et al., 1996; Vergunst et al., 1998) to remove the selection cassette.

(ii) Co-transformation of multiple T-DNAs which can insert into unlinked sites for future segregation (Yoder and Goldsbrough, 1994; Hohn et al., 2001). Three approaches are adopted for co-transformation: (1) the introduction of two T-DNAs, each from a different bacterium; (2) the introduction of two T-DNAs
carried by different replicons within the same bacterium (An, 1985; Daley et al., 1998; Jacob and Veluthambi, 2002); and (3) the introduction of two T-DNAs located on the same replicon within a bacterium (Depicker et al., 1985; Komari et al., 1996; Xing et al., 2000; Lu et al., 2001; Matthews et al., 2001). In all cases, a fraction of transformants will carry the two transgenes unlinked. Co-transformation frequencies of up to 47% were reported with a high proportion of both tobacco and rice transformants carrying unlinked transgenes (Komari et al., 1996). This procedure requires fertile plants for genetic separation of the two transgene loci.

6.10 Conclusion

New enhancer trap constructs with the Ubi-1 promoter instead of the CaMV 35S were constructed and tested through transformation of rice calli. GFP expression of the enhancer traps, along with corresponding Gal4-deletion constructs, was studied during the callus stage. Comparisons in dynamics of GFP expression between enhancer traps and Gal4-deletion constructs confirmed the functionality of the Gal4/VP16. However, cis-activities probably due to enhancer elements of the Ubi-1 promoter were also detected in these constructs during the callus stage.

A total of 661 transgenic lines were generated and patterns of GFP expression in vegetative and generative stages were studied. From the 393 lines obtained with enhancer trap constructs pFX-G74.1 and pFX-G85.2, 129 enhancer trap lines (32.8%) were found GFP-positive. In these enhancer trap lines, expression of GFP was detected in various tissues/organs including root, root branch, stem base, leaf, stomata, vascular band, collar, auricle, ligule, trichome, mesophyll, stigma, ovary, anther, lodicule, glumes, sterile lemma, pedicel, and panicle branch. The expression patterns were found very diverse. Among the 129 GFP-positive enhancer trap lines, 84 different patterns of GFP expression were categorized. Over 60% of the pattern lines showed GFP expression in two to four tissues/organs. Twelve lines showed specific GFP expression in only one tissue/organ.
The present design of enhancer trap constructs imposed no significant background problem for screening genomic enhancers being trapped. At the plant level, cis-activities from the Ubi-1 promoter, if any, were not apparent. Among the 138 extensively studied Gal4-deletion lines, only 7 lines (5.2%) were found GFP-positive, much lower than the frequency (68.6%) in the case of CaMV 35S promoter. Ways to completely overcome such background problem are discussed.
Chapter 7 Genetic test and confirmation of the transactivator’s functionality

7.1 Introduction and experimental design

Brand and Perrimon (Brand and Perrimon, 1993) used a P element-based enhancer trap strategy to generate Drosophila enhancer trap lines that express the yeast transcription activator, Gal4, in different patterns. To generate enhancer trap lines expressing Gal4 in numerous cell and tissue-specific patterns, the Gal4 gene is inserted randomly into the genome, driving Gal4 expression from numerous different genomic enhancers. A chosen target gene could then be placed under the control of the Gal4 upstream activation sequences (UAS), transformed, and maintained silently in the absence of Gal4. Genetic crosses between this target line and any of the library of Gal4-containing lines could specifically activate the target gene in a particular tissue or cell type. The phenotypic consequences of mis-expression, including those lethal to the organism, could then be conveniently studied (Figure 7.1).


Figure 7.1 Directed gene expression in Drosophila.
One method of achieving clear phenotypic effects is through transactivation of the diphtheria toxin A chain (DTA) gene in order to selectively kill cell lineages. DTA is known to kill cells by ribosylating the EF2 translation initiation factor and inhibiting protein synthesis (Bellen et al., 1992; Tsugeki and Fedoroff, 1999). DTA has been used to selectively ablate tissues and cells in *Arabidopsis* (Day et al., 1995; Tsugeki and Fedoroff, 1999; Bougourd et al., 2000).

Bougourd et al. (Bougourd et al., 2000) targeted the expression of toxic and regulatory proteins to particular cells of the root meristem in *Arabidopsis*. DTA was transactivated in particular cells by crossing a Gal4-dependent DTA transgenic line to a selected enhancer trap line with GFP expression in columella and root cap. Ablation of cells in and around the area of the columella and root cap was observed, corresponding to the expression pattern of Gal4 and GFP in the enhancer trap line used.

To verify the function of transactivator in rice transformed with the enhancer trap constructs described in Chapter 6, a series of genetic crosses were designed. In essence, target lines are generated with the GUS reporter gene or the DTA gene cloned downstream of a minimal promoter that comprises a TATA-box and the binding sites for the transcription factor Gal4NP16. Consequently, the GUS or DTA gene is unlikely to be expressed when introduced into wild-type plant cells. This allows silent transgenic lines to be generated and propagated without interference from the target gene or selection against its expression. Expression of the target gene is achieved by crossing these target lines with an enhancer trap line that expresses Gal4/VP16 and the GFP reporter gene in a tissue or organ-specific pattern since the Gal4/VP16 protein specifically recognizes the binding sites upstream both the GFP reporter and the target gene. In this way the target gene will be expressed only in those cells of the progeny in which the transcription factor is expressed, and expression pattern of the target gene corresponds to the expression pattern of Gal4/VP16 and GFP in the enhancer trap line used. An illustration of the experiment design using GUS reporter as the target gene is shown in Figure 7.2.
Figure 7.2 Experiment design to verify functions of the transactivator.
In Figure 7.2, an enhancer trap line with GFP expression pattern in anther is crossed to a target line harboring the 6xUAS-MP-GUS cassette but showing no GUS expression. In the F1 progeny, there exists not only expression of GFP in anther following the pattern of its parent, but also co-expression of GUS in anther with the same pattern as GFP. This happens because the regulatory proteins of Gal4/VP16 recognize and bind to UAS sites sitting wherever in the genome, and direct expression of the gene downstream of the UAS sites.

![Diagram of T-DNA components](image)

Figure 7.3 T-DNA components of the enhancer trap pFX-G74.1.

Table 7.1 Characters of enhancer trap lines used for crossing.

<table>
<thead>
<tr>
<th>Code number of lines in T1</th>
<th>Code number of plants used for crossing</th>
<th>GFP expression pattern</th>
<th>Pattern code</th>
</tr>
</thead>
<tbody>
<tr>
<td>FU01 131</td>
<td>24, 32, 40</td>
<td>stomata, anther, pedicel</td>
<td>ENR</td>
</tr>
<tr>
<td>FU01 171</td>
<td>21, 25, 31, 33</td>
<td>root, stomata, lodicule</td>
<td>AEO</td>
</tr>
<tr>
<td>FU01 191</td>
<td>7, 8, 22, 35</td>
<td>stomata, anther, glumes, pedicel</td>
<td>ENPR</td>
</tr>
</tbody>
</table>

Notes to the code: A = root, E = stomata, N = anther, O = lodicule, P = glumes, R = pedicel.
7.2 Characteristics of parental lines used for genetic test and combinations of sexual crosses

7.2.1 Enhancer trap lines

Three enhancer trap lines obtained with the pFX-G74.1 construct (Figure 7.3) were selected for making crosses. GFP signal was relatively strong in all selected lines so that observations were easy to perform. These lines were phenotypically normal and uniform within T₁ populations. GFP expression patterns and code numbers of these enhancer trap lines are listed in Table 7.1.

7.2.2 Target gene lines

Four target gene lines obtained with pWAS89.18, pWSA50.2, pWSA60.1 and pWSA59.27 (Figure 7.4) were selected for making crosses.

All these constructs and their respective transgenic lines were generated in CAMBIA (Canberra, Australia). The major component of these constructs is a Gal4-responsive cassette (6xUAS-MP-GUS) that is identical in all the 4 target gene constructs. There is an extra Gal4-responsive cassette (6xUAS-MP-DTA) in pWAS89.18 to test the effects of expression of the DTA gene. Though the selection cassettes are somewhat different among the 4 constructs, no considerable influence on expression of the neighboring Gal4-responsive cassette in plant level was expected based on the results obtained with negative controls as described in Chapter 6.

In all 4 lines, there was no detectable target gene expression noticed. Neither GUS gene nor DTA gene expression was detected through GUS staining and observations under a stereomicroscope. Like the enhancer trap lines, all these target gene lines were phenotypically normal and uniform within T₁ populations. To simplify analysis of the transactivator, only homozygous transgene individuals were used for making crosses. Code numbers of the target gene lines are listed in Table 7.2.
Figure 7.4 T-DNA components of the target gene constructs.
Table 7.2 Code numbers of the target gene lines used for crossing.

<table>
<thead>
<tr>
<th>Code number of lines in T1</th>
<th>Code number of plants used for crossing</th>
<th>Construct</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>FU01 297</td>
<td>2, 3, 5, 8</td>
<td>pWSA 89.18</td>
<td>GUS, DTA</td>
</tr>
<tr>
<td>FU01 299</td>
<td>1, 2, 5</td>
<td>pWSA 50.2</td>
<td>GUS</td>
</tr>
<tr>
<td>FU01 301</td>
<td>1, 5</td>
<td>pWSA 60.1</td>
<td>GUS</td>
</tr>
<tr>
<td>FU01 302</td>
<td>1, 2</td>
<td>pWSA 59.27</td>
<td>GUS</td>
</tr>
</tbody>
</table>

Table 7.3 Combinations and code numbers of genetic crosses.

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>GFP pattern of enhancer trap parent</th>
<th>Code number of F1 population</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>FU01 297-2</td>
<td>FU01 171-33</td>
<td>AEO</td>
<td>FU02 105</td>
<td>Single enhancer trap line crossed to 3 different target lines</td>
</tr>
<tr>
<td>FU01 299-1</td>
<td>FU01 171-25</td>
<td>AEO</td>
<td>FU02 101</td>
<td></td>
</tr>
<tr>
<td>FU01 302-1</td>
<td>FU01 171-21</td>
<td>AEO</td>
<td>FU02 107</td>
<td></td>
</tr>
<tr>
<td>FU01 297-3</td>
<td>FU01 191-8</td>
<td>ENPR</td>
<td>FU02 106</td>
<td>Single enhancer trap line crossed to 3 different target lines</td>
</tr>
<tr>
<td>FU01 299-2</td>
<td>FU01 191-35</td>
<td>ENPR</td>
<td>FU02 102</td>
<td></td>
</tr>
<tr>
<td>FU01 302-2</td>
<td>FU01 191-7</td>
<td>ENPR</td>
<td>FU02 108</td>
<td></td>
</tr>
<tr>
<td>FU01 297-2</td>
<td>FU01 171-33</td>
<td>AEO</td>
<td>FU02 105</td>
<td>Single target line crossed to 3 different enhancer trap lines</td>
</tr>
<tr>
<td>FU01 297-3</td>
<td>FU01 191-8</td>
<td>ENPR</td>
<td>FU02 106</td>
<td></td>
</tr>
<tr>
<td>FU01 297-5</td>
<td>FU01 131-24</td>
<td>ENR</td>
<td>FU02 110</td>
<td></td>
</tr>
<tr>
<td>FU01 299-2</td>
<td>FU01 191-35</td>
<td>ENPR</td>
<td>FU02 102</td>
<td>Reciprocal crosses</td>
</tr>
<tr>
<td>FU01 191-22</td>
<td>FU01 299-5</td>
<td>ENPR</td>
<td>FU02 120</td>
<td></td>
</tr>
<tr>
<td>FU01 301-1</td>
<td>FU01 131-40</td>
<td>ENR</td>
<td>FU02 111</td>
<td>Reciprocal crosses</td>
</tr>
<tr>
<td>FU01 131-32</td>
<td>FU01 301-5</td>
<td>ENR</td>
<td>FU02 118</td>
<td></td>
</tr>
<tr>
<td>FU01 297-2</td>
<td>FU01 171-33</td>
<td>AEO</td>
<td>FU02 105</td>
<td>Reciprocal crosses</td>
</tr>
<tr>
<td>FU01 171-31</td>
<td>FU01 297-8</td>
<td>AEO</td>
<td>FU02 119</td>
<td></td>
</tr>
</tbody>
</table>

Numbers after dash indicate individual T₁ plants within a line; GFP expression code: A = root, E = stomata, N = anther, O = lodicule, P = glumes, R = pedicel.
7.2.3 Parental combinations of the genetic crosses

Using the 7 parental lines, 11 genetic crosses were made between an enhancer trap line and a target line. Different combinations were designed, including a single enhancer trap line crossed to 3 different target lines, a single target line crossed to 3 different enhancer trap lines and pairs of reciprocal crosses to study cytoplasmic effects. Details of the cross combinations are listed in Table 7.3.

7.3 Making crosses using hot water emasculation method

7.3.1 Selection and early growth of T-DNA harboring plants

T₁ seeds of the target lines were used for establishing plant population. To prevent establishment of segregated plants without T-DNA insertion, the T₁ seeds of target lines were treated with hygromycin at a concentration of 50 µg/ml in water. Seeds containing no T-DNA insertion (or with HPT gene silencing) did not generate normal shoot and root and were discarded (Figure 7.5). Only the well germinated transgenic individuals were planted in pots for crossing.

![Figure 7.5 Seeds treated in water containing hygromycin (50 mg/ml). The non-transgenic negative control (Millin) and some seeds of the target line FU01 297 did not generate normal shoot and root, and were discarded accordingly.](image)

For convenient operation of hot water emasculation, the mother plants were grown in a relatively small pot so that it could be handled with ease. To prevent self-fertilization or cross-fertilization by unwanted parents, emasculation was
done either early in the morning when rice plants in the glasshouse had not yet started flowering, or in the afternoon when rice plants had finished flowering.

### 7.3.2 Panicle selection and trimming

For successful crossing, it is necessary to choose panicles mature enough as mother parents. When the panicle has exerted from the flag leaf sheath for one or two days and a few spikelets on the top have started flowering, the panicle is a good candidate for crossing.

Before emasculation, the selected panicles were carefully trimmed using a scissors so that all the spikelets already flowered were removed. Younger spikelets at the lower portion of the panicle were also removed. After trimming, the top one thirds of all spikelets remaining on the panicle was cut off using a sharp scissors. The panicle was covered with a paper bag after trimming.

### 7.3.3 Emasculation

Emasculation was done by dipping the rice panicle for 5 minutes into the hot water maintained at 45 °C. Through this treatment the male organ in all spikelets on the panicle was killed whereas the female organ remained alive. After emasculation, the panicle was bagged immediately to avoid pollination with unwanted pollen.

### 7.3.4 Pollination

Panicles of father plants were cut before anthesis in the morning, and kept in a flask with water to make ready for crossing. Pollination was done when a lot of spikelets on the cut panicle became open, by sprinkling the pollen of opening father flowers on to the cut spikelets of mother parent. Pollination was also successfully done by simply covering the emasculated mother panicle and the father panicle together in a single paper bag.
Negative controls were designed to confirm effectiveness of the hot water emasculation. For each of the 7 parental lines, one of the emasculated panicles was simply bagged without pollination. No single seed was found on all these negative controls, indicating that the male organ in the mother parent was effectively killed and seeds obtained from mother parents were true hybrids.

7.3.5 Harvesting hybrid seeds

The mother panicles were kept being bagged for 10 days after pollination. Hybrid seeds set on the mother panicles were harvested after 40 to 45 days from pollination. The harvested hybrid seeds were stored under dry and low temperature conditions before planting.

7.4 Growing F₁ population

To promote germination, the F₁ hybrid seeds were pre-germinated on medium before planted in pots. F₁ seeds were treated with 70% ethanol for one minute, sterilized for 30 minutes with a solution of 70% commercial bleach and 0.2% Tween 20, washed thoroughly with distilled water, and germinated on ½ MS medium (without any hormone) at 26 °C. In this way, nearly 100% germination was achieved and 20-40 F₁ plants were established for each cross combination. T₂ populations of the parental lines were also planted as positive/negative controls for GFP/GUS expression.

7.5 Reporter gene expression in T₂ parental lines

For all the T₂ populations of enhancer trap parental lines used for crossing, 4 plants were taken at random to further check their GFP expression and to use as positive control in the analysis of F₁ plants. GFP expression patterns in T₂ generation of all the enhancer trap parental lines were consistent with T₁ generation patterns shown in Table 7.1.
The target parental lines were also planted and used as the negative control. There was no GUS expression detected in any of the T\textsubscript{2} target gene lines used for the genetic crossing.

**7.6 Validation of transactivator functionality through analysis of the F\textsubscript{1} populations**

**7.6.1 Co-expression of GFP and GUS in F\textsubscript{1} plants**

Leaf samples from 16 plants of each F\textsubscript{1} populations and root samples from those populations with the parental line FU01 171 involved were taken at the tillering stage and GFP expression in leaf stomata and root (for FU01 171) was investigated. For surveying GFP expression in anther, lodicule, glumes, and pedicel at the generative stage, samples of young panicles were taken from the same 16 plants at the beginning of flowering stage. For each plant, 10-15 spikelets were carefully dissected under a stereomicroscope. GFP observations were done using the Leica MZ FLIII fluorescence stereomicroscope equipped with GFP3 filter set.

After GFP observation, the same set of samples was immediately put into GUS stain solution following the protocol described in Chapter 2. GUS expression was investigated under a Leica stereomicroscope.

All the seven cross combinations with the 6xUAS-MP-GUS target involved showed clear co-expression of GFP and GUS in their F\textsubscript{1} progenies (Table 7.4). The expression patterns of GFP and GUS in a specific F\textsubscript{1} population were very similar and were also consistent to the GFP pattern of its parental line used for crossing (Table 7.3, Figure 7.6). The result indicated that in these seven F\textsubscript{1} progenies, the transactivator from an enhancer trap parent did perform its function *in trans*. The Gal4/VP16 proteins recognized and bound to its target (6xUAS-MP-GUS) brought by another parent and triggered the target gene expression in the same pattern as observed in the pattern line.
Figure 7.6 Co-expression of GFP and GUS in F1 progenies of enhancer trap lines crossed with target lines. 1-root, 2-leaf stomata, 3-pedicel, 4-glume (out edge of lemma), 5- lodicule, 6-anther; a-GFP expression, b-GUS expression.

In the F1 populations FU02 101, 102, 107, 108, 111, 118, and 120, almost perfect co-expression of GFP and GUS was detected, implying normal function of the whole system (Table 7.4). For example, in the FU02 118 population, all the 8 F1 plants with GFP expression showed also GUS expression in the same ENR (stomata, anther and pedicel) pattern. The same situation was observed in FU02 107, 111 and 120 populations.
However, there were still some cases showing one of the GFP-positive individuals with missing GUS signal. In the FU02 101 population, for example, 7 plants showed GFP expression but only 6 of them exhibited co-expression of GUS. Similar cases occurred in FU02 102 and 108. Further studies are needed to understand the molecular mechanisms of such phenomenon.

Table 7.4 Co-expression of GFP and GUS in F1 populations.

<table>
<thead>
<tr>
<th>Line number</th>
<th>Number of plants investigated</th>
<th>GFP expression</th>
<th>GUS expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pattern</td>
<td>Number of positive plants</td>
</tr>
<tr>
<td>FU02 101</td>
<td>16</td>
<td>AEO</td>
<td>7</td>
</tr>
<tr>
<td>FU02 102</td>
<td>16</td>
<td>ENPR</td>
<td>8</td>
</tr>
<tr>
<td>FU02 107</td>
<td>16</td>
<td>AEO</td>
<td>7</td>
</tr>
<tr>
<td>FU02 108</td>
<td>16</td>
<td>ENPR</td>
<td>13</td>
</tr>
<tr>
<td>FU02 111</td>
<td>16</td>
<td>ENR</td>
<td>5</td>
</tr>
<tr>
<td>FU02 118</td>
<td>16</td>
<td>ENR</td>
<td>8</td>
</tr>
<tr>
<td>FU02 120</td>
<td>16</td>
<td>ENPR</td>
<td>16</td>
</tr>
</tbody>
</table>

Notes to the code: A = root, E = stomata, N = anther, O = lodicule, P = glumes, R = pedicel, na = not applicable.

7.6.2 Results of genetic crosses involving the DTA target gene

All the F1 populations with DTA as the target gene parent (FU02 105, 106, 110 and 119) showed no GUS expression (Table 7.5). These F1 plants were phenotypically normal and no detectable abnormalities were observed under the stereomicroscope with 40x magnification. The enhancer trap constructs involved for these crosses performed normal when crossed with other target lines, as shown in the first and second groups of crossing in Table 7.5. For example, in the first group, both FU02 101 and 107 exhibited co-expression of GFP and GUS in F1 individuals, but not in the FU02 105 population.

In these populations with the DTA target gene involved, GFP expression was normal. Consistent GFP expression patterns were still detected in their F1
individuals, implying that the target tissue was not killed by expression of the DTA gene.

Table 7.5 Results of GFP and GUS co-expression in F1 populations arranged in groups of crossing.

<table>
<thead>
<tr>
<th>Code number of F1 population</th>
<th>Female</th>
<th>Male</th>
<th>Target gene</th>
<th>No. of GFP positive plants in F1</th>
<th>No. of GUS positive plants in F1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FU02 105</td>
<td>FU01 297</td>
<td>FU01 171</td>
<td>GUS, DTA</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>FU02 101</td>
<td>FU01 299</td>
<td>FU01 171</td>
<td>GUS</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>FU02 107</td>
<td>FU01 302</td>
<td>FU01 171</td>
<td>GUS</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>FU02 106</td>
<td>FU01 297</td>
<td>FU01 191</td>
<td>GUS, DTA</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>FU02 102</td>
<td>FU01 299</td>
<td>FU01 191</td>
<td>GUS</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>FU02 108</td>
<td>FU01 302</td>
<td>FU01 191</td>
<td>GUS</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>FU02 105</td>
<td>FU01 297</td>
<td>FU01 171</td>
<td>GUS, DTA</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>FU02 106</td>
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<td>GUS, DTA</td>
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<td>GUS, DTA</td>
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<td>FU02 118</td>
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<td>8</td>
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<td>FU01 171</td>
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<tr>
<td>FU02 119</td>
<td>FU01 171</td>
<td>FU01 297</td>
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<td>9</td>
<td>0</td>
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</tbody>
</table>

Note: 16 plants were observed for each of the F1 populations.

7.6.3 Cytoplasmic effects of the transactivator activities

Three pairs of reciprocal crosses were made to investigate the cytoplasmic effects of the transactivator activities. The results listed in Table 7.6 indicated no apparent cytoplasmic effects on the activities of the transactivator, for F1 populations from every pairs of reciprocal crosses showed similar extent of co-expression of the two reporter genes.

For each of the F1 populations, 16 plants were observed. There was difference in number of GFP/GUS-positive plants between the reciprocal crosses. This
phenomenon is not considered to be cytoplasmic effects as discussed in next section.

Table 7.6 Co-expression of GFP and GUS in F1 populations generated through reciprocal crosses.

<table>
<thead>
<tr>
<th>Female</th>
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<th>GFP expression</th>
<th>GUS expression</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Pattern</td>
<td>Number of positive plants</td>
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<tr>
<td>FU01 299-2</td>
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<td>FU01 297-8</td>
<td>AEO</td>
<td>9</td>
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</tbody>
</table>

Notes to the code: A = root, E = stomata, N = anther, O = lodicule, P = glumes, R = pedicel, na = not applicable

7.7 Discussion

An important feature of the transacting system is that the Gal4 gene and UAS-target gene are initially separated into two distinct transgenic lines, which ensures that the parental lines are viable. In the Gal4 line, the activator protein is present, but has no gene-of-interest to activate. In the UAS-target gene line, the target gene is silent in the absence of transactivator. When the Gal4 and UAS lines are crossed, the target gene is turned on only in the progeny of the cross, allowing dominant phenotypes (including lethality) to be conveniently studied. Thus a library of Gal4-based enhancer trap lines can be built up, each line expressing Gal4 in a different tissue or cell-specific pattern. The UAS-target gene can then be ectopically expressed in a wide variety of patterns merely by crossing the UAS line to a library of Gal4 lines. In a complementary fashion, a library of UAS-target genes can also be constructed and a large number of
different genes, or combinations thereof, can be mis-expressed in precisely the same domain by crossing the appropriate Gal4 line to a UAS line library.

Since the Gal4-UAS system was described by Brand et al. (1993), the technique has been used to study almost every aspect of the nervous system in *Drosophila*, from development to behaviour (Brand and Dormand, 1995). In recent year, this system has also been adapted for targeted misexpression studies in other organisms, including zebrafish (Scheer and Camnós-Ortega, 1999; Koster and Fraser, 2001; Scheer et al., 2001), mouse (Rowitch et al., 1999), *Xenopus* (Hartley et al., 2002) and *Arabidopsis* (Kiegle et al., 2000). Kiegle et al. (2000) used the Gal4 transactivation strategy to target aequorin to specific cell types in the root to monitor the responses of epidermis, elongation zone epidermis and cortex, endodermis and pericycle to cold, osmotic and salt stress. The present study is the first successful utilization of the Gal4-UAS system to target gene (GUS) expression in various tissues/organs of rice.

The genetic crossing has provided solid evidence that the transactivator (Gal4/VP16) plays normal transacting functions in rice enhancer trap system established through the present study. Enhancer trap lines in which the transcriptional activator is expressed in a tissue or cell-specific fashion are useful for many types of experimental studies because any gene-of-interest can be subjected to Gal4 regulation by fusing the coding region of this gene to the Gal4 upstream activating sequence.

The technique is also potentially useful in rice breeding. An elite rice variety can be transformed with the enhancer trap construct and a library of enhancer trap lines with various Gal4 expression patterns can be established and used for a broad range of breeding purposes. For example, an endosperm-specific pattern line can be crossed to a target line harboring a UAS-lysine-rich gene cassette. Then, a new quality variety with high lysine content in rice grain can be bred, which is extremely difficult or impossible with conventional breeding methods because of the lack of lysine-rich genetic resources in rice species. Besides, the system can be used to drive multiple genes expressing in the same pattern or to
direct a single gene expressing in multiple tissues/organs. The present study, however, is not enough to suggest immediate use of the system for breeding practices, for there are still unclear aspects and rooms to improve.

In the crosses between FU01 299 and FU01 191, there was dramatic difference in number of positive plants detected between reciprocal crosses (the first pair, Table 7.6). All the 16 F1 plants (100%) showed GFP expression in the combination FU01 191-22/FU01 299-5, but only 8 GFP-positive plants (50%) in the combination FU01 299-2/FU01 191-35. This was not considered an indication of cytoplasmic effects because different individual enhancer trap plants (T1) were used for crossing. FU01 191-22 could be a homozygote while FU01 191-35 a heterozygote in terms of the transgene. This was not avoided because of a limitation in available panicles from the paired parental plants for making reciprocal crosses. Molecular analysis of T-DNA status in the parental plants and their F1 progenies was planned to verify this hypothesis but the Southern blot was not completed.

The competitive abilities of gametes for fertilization may be different among gametes with different copies of transgenes. Such hypothesis could not be verified without detailed data of T-DNA copy number.

The DTA target gene has been successfully used for genetic ablation in mammalian (Palmiter et al., 1987), *Drosophila* (Bellen et al., 1992) and plants (Day et al., 1995; Tsugeki and Fedoroff, 1999; Bougourd et al., 2000). Yang (CAMBIA, unpublished data) used a similar genetic crossing system (Gal4/VP16 and UAS-DTA) and has successfully targeted expression of the DTA gene in *Arabidopsis*. In the present experiment, the results of genetic crosses involving the DTA target gene were unfortunately unable to clearly confirm a functional transactivator in the system. All the F1 populations with DTA as the target gene parent showed complete missing of GUS expression. This might be caused by targeted expression of the DTA gene: the target cells would be destroyed by the DTA expression and the destroyed cells would not express GUS reporter gene. However, the GFP reporter which contained the same UAS-MP element as the
GUS and DTA gene, showed clear expression in these target cells where the GUS and DTA were expected to express. Moreover, all F₁ plants were phenotypically normal and no detectable abnormalities were observed under the stereomicroscope with 40x magnification. It was apparently not due to missing of the T-DNA because the seeds were treated with hygromycin during germination process and the plants established must be harboring the T-DNA. Therefore, there could be problem in the target gene cassettes of the construct (pWSA 89.18) used for generating the target gene line. Further studies on the DTA gene construct are needed.

7.8 Conclusion

To verify the function of transactivator in rice transformed with the enhancer trap constructs, a series of genetic crosses were designed. Three enhancer trap lines obtained with the pFX-G74.1 construct, and four target gene lines harboring 6xUAS-MP-GUS or 6xUAS-MP-DTA were selected for sexual crosses. Using these parental lines, 11 genetic crosses were made between an enhancer trap line and a target line. Different combinations were designed, including a single enhancer trap line crossed to 3 different target lines, a single target line crossed to 3 different enhancer trap lines and pairs of reciprocal crosses to study cytoplasmic effects.

Among the 11 F₁ populations, all the 7 crosses involving the 6xUAS-MP-GUS target (with no DTA!) showed clear co-expression of GFP and GUS in F₁ progenies. The expression patterns of GFP and GUS in a specific F₁ population were quite the same and consistent to the GFP pattern of its parental line used for crossing. The experiment results showed clearly that the transactivator from an enhancer trap parent did find its target brought by the target gene parent and tricked the target gene expression following acting pattern of the enhancer. The transactivator performed its functions as theoretically expected. Results from the reciprocal crosses showed no cytoplasmic effects on functions of the transactivator.
The other 4 crosses involving the DTA target gene parental line failed to be able to either confirm or reject a normal function of the transactivator. Further studies on the DTA gene construct are needed.
References


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Goff et al. (2002). A draft sequence of the rice genome (Oryza sativa L. ssp. japonica). Science 296, 92-100.


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a binary vector T-DNA in one Agrobacterium tumefaciens strain. Plant Science 163, 801-806.


# Appendix A. List of abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
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<tr>
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<td>adenine</td>
</tr>
<tr>
<td>Ac/Ds</td>
<td>activator/dissociation transposons</td>
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<td>adenosine-5'-triphosphate</td>
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<td>6-benzyladenine</td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
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<td>bar</td>
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</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
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<td>cytidine</td>
</tr>
<tr>
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<td>cauliflower mosaic virus</td>
</tr>
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<td>CTAB</td>
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</tr>
<tr>
<td>DNA</td>
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</tr>
<tr>
<td>dNTPs</td>
<td>mixture of dATP, dTTP, dCTP and dGTP</td>
</tr>
<tr>
<td>DsE</td>
<td>enhancer trap Ds element</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraactic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
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<tr>
<td>g</td>
<td>gram or gravity</td>
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<td>green fluorescent protein</td>
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<td>β-glucuronidase</td>
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<td>β-glucuronidase gene</td>
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<tr>
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<td>-------------</td>
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<tr>
<td>MS</td>
<td>medium of Murashige and Skoog</td>
</tr>
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<td>nanogram</td>
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<td>nanometer</td>
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<td>neomycin phosphotransferase II gene</td>
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<td>OD</td>
<td>optic density</td>
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<td>open reading frame</td>
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<td>polymerase chain reaction</td>
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<td>picogram</td>
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<tr>
<td>rpm</td>
<td>rotation per minute</td>
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<td>room temperature</td>
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<td>shrimp alkaline phosphatase</td>
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<td>sodium chloride/sodium citrate</td>
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<td>T</td>
<td>thymine</td>
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<td>transfer DNA</td>
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<td>melting temperature</td>
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<tr>
<td>U</td>
<td>unit, enzyme activity</td>
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<td>UAS</td>
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<td>reporter gene encoding β-glucuronidase</td>
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</tr>
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<td>X-Gluc</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
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**Appendix B.** List of different expression patterns of GFP and the number of transgenic rice lines representing these patterns.

<table>
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