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Transactivation Lines in Rice

Evaluation of insertional mutants and development of effective transactivator platform for FTO and co-ordinate gene expression

A report for the Rural Industries Research and Development Corporation

by Marie Connett Porceddu, Thach Tran, Richard Jefferson and Andrzej Kilian, CAMBIA.

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Foreword

This project aimed to help secure freedom to operate for the Australian rice industry in the biotechnology era. The initial focus of this project was in a gene discovery mechanism, hoped to lead to the discovery of rice promoters with useful gene expression patterns, and mutants that might have improved gene expression properties. However, the rice genomic sequencing efforts external to Australia, rapidly accelerated during the course of the project, changed the terms of reference. What was needed was no longer large numbers of mutations, but

- Ways to work around the large number of patent applications that followed the release of genomic sequence, to the extent that licenses to such patents may not be available or may block access to external markets.
- Ways to understand and direct gene expression now that we understand the genome to be more complex and studies of single gene insertions effects to be too simplistic.

"Freedom to co-operate" considerations are equally important with "Freedom to operate" and technical evaluation. The new transactivator is now available to the rice industry, but even more useful, it is presented as a capability to use it, together with the accumulated know-how, in a form that enables ready collaboration with other researchers and capture of the data they collect and the improvements they make, in the BioForge project (www.bioforge.net) being developed since 2005 by CAMBIA with US-sourced funding from the Rockefeller Foundation. Facilitating joint improvement and leverage, all technology presented on the BioForge is available to other researchers in the public and private sectors *only* if they agree to the collaborative terms of the BiOS license (www.bioslicense.net). This unique legal instrument developed by CAMBIA provides for norms of sharing, as well as legally binding covenants that protect those who have invested in a technology, such as RIRDC, from being subject to the situation of inability to use valuable improvements to the technology.
A summary of the BiOS License conditions follows:

In return for the benefits of the technologies, a licensee institution agrees to allow and encourage its employees and students to post on the website any improvements made to the technologies and safety information relevant to use of the technology and potential regulatory approval of products embodying it, and agrees not to assert any intellectual property rights to the improvements and information against other licensees.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 1500 research publications. It forms part of our Established Industries Rice R&D sub-program which aims to improve the profitability and sustainability of the Australian rice industry.

Most of our publications are available for viewing, downloading or purchasing online through our website:

- downloads at www.rirdc.gov.au/fullreports/index.html
- purchases at www.rirdc.gov.au/eshop

Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

Acknowledgments

The scientific work was carried out under the direction of Andrzej Kilian while employed at CAMBIA. The work of Ph.D. students was critical to the project: Dr. Xiqin Fu, now at the China National Hybrid Rice R&D Centre in Hunan, China, and Thach Tran, now at the Cuu Long Delta Rice Research Institute in Vietnam developed the majority of the DNA constructs that were used. Many of the crosses were analysed by Sujin Patarapuwadol, now at Kasetsart University in Thailand, whose tuition and accommodations were generously funded by the Rockefeller Foundation and whose PhD has been written under the supervision of Dr Peter Sharp of Sydney University. Upon completion of the student components of the laboratory work, further transformations were done and analysis of the expression patterns was continued at CAMBIA by Dr Brian Weir, Julie Bleeze, Margaret Irwin, and Lijun Tian. The cambiaDB LIMS database for entering the transformation history and expression data was created by Grzegorz Uszynski.

Rice germplasm for the transformations was provided by Dr Russell Reinke of the Yanco Agricultural Institute. The GUS gene was provided by Dr Richard Jefferson in plasmids constructed at CAMBIA. CAMBIA also provided the GUSPlus gene, and constructs containing it and the EGFP reporter gene, for this work. The two-construct strategy was invented by Dr Richard Jefferson, Dr Andrzej Kilian, Dr Carol Nottenburg, Dr Paul Keese, Dr Jorge Mayer, all of CAMBIA, and Dr Scott Stachel, on sabbatical at CAMBIA from the University of California.

Freedom to operate analysis for the minimal promoter used in the construct of Thach Tran and other promoters used in this study was carried out at CAMBIA by Carolina de la Roa-Rodrigues under the guidance of Dr Carol Nottenburg, J.D. This analysis was mounted on CAMBIA's www.patentlens.net website in commentable form at the conclusion of the RIRDC funded project, by CAMBIA's IT team under the direction of Dr. Marie Connett Porceddu and Dr Richard Jefferson with funding assistance from Business ACT. Updating of the the technology landscape is currently being carried out by Dr Yang Wei under the guidance of Dr Marie Connett Porceddu and Dr Dianne Rees, J.D., and the annotations interface has been improved by Dr Nick dos Remedios, with funding from the Ministry of Foreign Affairs of the Government of Norway via the International Rice Research Institute.

The constructs are being made available under the terms of the BiOS (Biological Open Source) license for Plant Enabling Technologies, developed by Dr. Marie Connett Porceddu of CAMBIA with major input from Dr Richard Jefferson of CAMBIA and Dr Michael Rabson J.D. of Maxygen, and reviewed by a number of attorneys and technology transfer specialists internationally. The license was mounted on CAMBIA's www.bios.net website in commentable form by Steve Irwin. To obtain continued improvements and details of use by licensees, the strategy is described on a project of CAMBIA's distributive collaboration website, www.bioforge.org, created by Dr Marie Connett Porceddu with graphics assistance from Rob Shafer, IT support from Steve Irwin and HTML assistance by Amanda Harms, all with the generous support of the Rockefeller Foundation.

Stocks of the constructs are now being maintained at CAMBIA by Leon Smith, and stocks of the seeds by Lijun Tian, whom CAMBIA also employs, currently with funding from the Lemelson Foundation, as the caretaker of the RIRDC-owned GM-containment glasshouse constructed on the ANU campus. Pesticide spraying and other technical assistance is provided by Steve Dempsey of ANU. The glasshouse was constructed and is maintained principally for the benefit of the Australian rice industry. The glasshouse is now administered by a committee consisting of Dr Russell Reinke and Dr Randall Williams representing the Australian rice industry, Dr. Marie Connett Porceddu of CAMBIA (replacing Dr Andrzej Kilian), Professor Murray Badger of ANU, and a representative from RIRDC (during the duration of the funded project, this was Jeff Davis).

Clerical assistance for the preparation of this report was provided by Stephanie Goodrick, and graphics assistance by Amanda Harms and Nick dos Remedios.

Abbreviations

Ac:	Activator transposase gene from *Zea mays*
AD:	activation domain
Ara: E. coli	gene encoding a protein involved in arabinose metabolism
AvrXa10:	avirulence gene from *Xanthomonas oryzae* pv *oryzae*, rice bacterial blight
CaMV 35S:	a promoter isolated from Cauliflower Mosaic Virus
Cro:	lambda phase repressor
DBD:	DNA-binding domain
DIG:	digoxigenin, a label used for DNA in hybridisations
dNTP:	dinucleotide triphosphates
EGFP:	enhanced green fluorescent protein, used as a reporter of gene expression
Gal4:	a portion of a yeast transcriptional activator gene
GFP:	green fluorescent protein, used as a reporter of gene expression
GUS:	beta-glucuronidase enzyme encoded by an *E.coli gusA* gene.
gusA:	gene encoding a beta-glucuronidase enzyme, used as a reporter of gene expression
GUSPlus:	beta-glucuronidase enzyme encoded by a *Staphylococcus gusA* gene.
gusR:	gene encoding a repressor of a beta-glucuronidase gene
Hpt:	a hygromycin resistance gene
Km(s):	kanamycin-sensitive
NaCl:	sodium chloride (salt)
NaOH:	sodium hydroxide (alkali)
OD:	optical density, a measurement for quantifying DNA and bacterial populations
PCR:	polymerase chain reaction, a method used for checking the presence of particular DNA
RT:	room temperature
SDS:	sodium dodecyl sulfate
SOC:	A medium for bacterial growth based on Tryptone/yeast extract
SSC:	a buffer used for DNA hybridization
TAE:	Tris-acetate EDTA buffer, used for DNA electrophoresis
TAFET:	Transcriptional activator-facilitated enhancer trap
UAS:	upstream activation sequence bound by a transcriptional activator
Ubi: *Zea mays*	ubiquitin promoter
VP16:	activation domain isolated from the human herpes simplex virus

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Executive Summary

This project aimed to help secure freedom to operate for the Australian rice industry in the biotechnology era. The initial focus of this project was in a gene discovery mechanism, hoped to lead to the discovery of rice promoters with useful gene expression patterns, and mutants that might have improved gene expression properties. However, the rice genomic sequencing efforts external to Australia, rapidly accelerated during the course of the project, changed the terms of reference. What was needed was no longer large numbers of mutations, but

- Ways to work around the large number of patent applications that followed the release of genomic sequence, to the extent that licenses to such patents may not be available or may block access to external markets.
- Ways to understand and direct gene expression now that we understand the genome to be more complex and studies of single gene insertions effects to be too simplistic.

At the end of the project, these concerns have both been addressed. A patent work-around was developed, a strategy to co-transform or cross the vector containing the open reading frame for the transcriptional activator, and the vector containing the DNA-binding activation site, so that the insertions would be unlinked. In patent claims over the use of promoters, often what is claimed is a construct that comprises the promoter "operably linked" to a gene of interest. In transactivation, the promoter of interest is not linked to a gene of interest, but to a transcriptional activator. We also constructed a set of new transactivator cassettes, through identification of an activation domain and a DNA binding domain that work better in plants and are less clearly dominated by IP rights.

Lines that express this transactivator in a defined pattern are determined by the enhancers or promoters near the site of insertion. Genes that it is desired to switch on can be placed into cassettes with a minimal promoter and an activation sequence for the transactivator. When these cassettes are transformed into plants containing or crossed with the pattern lines, the gene(s) of interest will be brought into the expression pattern shown by the reporter genes in a highly controlled useful manner without the need to define, analyse, and clone new promoters to drive the gene(s) of interest. Thus:

- the expression of multiple genes encoding different enzymes in a multi-step pathway, such as the starch biosynthesis pathway, can be controlled to occur at the same developmental stages and in the same amounts in the plant.
- The expression of down-regulation constructs for multiple steps in a pathway can similarly be coordinately regulated
- The expression of either type of construct can be monitored by reporter gene expression.

"Freedom to co-operate" considerations are equally important with "Freedom to operate" and technical evaluation. The new transactivator is now available to the rice industry, but even more useful, it is presented as a capability to use it, together with the accumulated know-how, in a form that enables ready collaboration with other researchers and capture of the data they collect and the improvements they make, in the BioForge project (www.bioforge.net) being developed since 2005 by CAMBIA with US-sourced funding from the Rockefeller Foundation. Facilitating joint improvement and leverage, all technology presented on the BioForge is available to other researchers in the public and private sectors *only* if they agree to the collaborative terms of the BiOS license (www.bioslicense.net). This unique legal instrument developed by CAMBIA provides for norms of sharing, as well as legally binding covenants that protect those who have invested in a technology, such as RIRDC, from being subject to the situation of inability to use valuable improvements to the technology.
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1. Introduction

Background

This project was developed to secure freedom to operate for the Australian rice industry in the biotechnology era. As it is becoming clear that rice is a preferred model system for gene discovery and biotechnology for all cereals, the research focus of many companies has turned towards rice. The initial focus of this project was in a gene discovery mechanism, enhancer traps, which it was hoped would lead to the discovery of many rice promoters with useful gene expression patterns, as well as the creation of gain of function mutants that might have improved gene expression properties.

Since the funding of this proposal, the magnitude of investment from international corporationsin rice genomics has been astounding. While the project proposal was being finalised, the sequencing of the rice genome was already underway, having been announced as initiated in 1998 with the plan for completion set at around 2008. However, in 2000 Monsanto completed a working draft of the sequence of over 90% of the complete genome and released it under restriction to the plant community for research purposes only (*Plant Physiol*, March 2001: The Use of the Monsanto Draft Rice Genome Sequence in Research). Further progress in genome sequencing has been achieved by other multinational corporations (notably Syngenta), by a Chinese program, and by the International Rice Genome Sequencing Consortium, substantially led by scientists from Japan (the Rice Genome Program in Tsukuba) and in the US. This rapid progress in rice genome sequencing had a profound effect on the project, as new strategies had to be developed to best respond to changing situation. Genomic sequence isolated in these large-scale sequencing projects was resulting in large-scale patent applications, accelerating the trend already seen in patenting of plant-effective promoters. With free trade agreements in place there is little doubt that intellectual property practices of multinational corporations based on intellectual property restrictions will extend into new markets, especially in Asia.

Additionally, although CAMBIA achieved very high transformation frequencies in the rice transformation protocol, the enhancer trap strategy originally envisioned as the basis of the project was not as successful as hoped, because the data upon thorough analysis showed an enhancer effect of the promoter used to drive the selectable marker in constructs that confounded the plant gene expression results of enhancer trap insertions. The original enhancer trap constructs used, and the rice transformation method, though improved at CAMBIA, were also significantly encumbered by patent rights of multinational companies.

Accordingly, the focus of this project shifted, though still within the original purpose of the CAMBIA proposal to secure freedom to operate for the Australian rice industry in the biotechnology era. A patent application was developed for a way of using the trans-activation constructs, originally developed as enhancer traps, for a work-around to many types of promoter patent claims. Additionally, new trans-activator constructs were developed and tested, which are free not only of some dominating patent claims but also of potential public concerns interacting with regulatory processes. Finally, a new method of licensing this intellectual property (maintaining capability to use improvements and access to biosafety data) and a new platform for distributive collaboration was developed, and both were tested for a rice transformation protocol different from that used in this project but which will be available together with the deliverables of this project under the new license.

2. Initial Objectives

The yeast transcriptional activator *Gal4* can activate transcription of any target genes bearing a *Gal4* binding site UAS (upstream activation sequence) by driving the expression of 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. This is useful in two major ways:

- to drive *Gal4* expression using characterized promoters (*e.g.* a tissue-specific or heat shock promoter).
- enhancer trapping, in which the *Gal4* expression pattern is directed by flanking genomic enhancer elements proximal to the insertion site of the transactivator construct, which are thereby discovered and described.

Exploiting a method in which a minimal promoter under the control of UAS elements was introduced into multiple sites in the *Drosophila* genome to drive ectopic expression of endogenous genes downstream of the inserted UAS-regulated promoter in a pattern directed by the *Gal4* activator, 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 seemed to be a potential approach to study functions of genes, for mis- or overexpression phenotypes are much less likely to arise by traditional loss-of-function mutagenesis.

A key feature of this *Gal4* system was that the *Gal4* gene and UAS-target gene are initially separated into two distinct transgenic lines. This ensured 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. Accordingly, in our project as it developed, the UAS-target gene lines were generated separately by transformation witha cassette of UAS-minimal promoter-target gene to generate target transgene lines. Usually several independent insertion lines would 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).

An important consideration of our data analysis platform was the selection of the pattern lines which would be used to transactivate the genes tagged with UAS element. Selection criteria should depend on the experimental goals; for example, if novel leaf phenotypes are sought, then the leaves should be examined for trans-activation, and this would allow discovery of a potential leaf pattern line identifying a leaf promoter that could be used. While judicious choice of the pattern line may facilitate experimental success, the novel patterns that would be generated with this approach could not be conceived *a priori*. The project focused on development of technology and building the capacity for isolation of agriculturally useful mutations and identification of genes responsible for the novel phenotypes. Therefore, during the course of the project the consultation process with the industry attempted development of phenotypic screens to target the current (or future) industry needs.

3. Materials and methods

Plant material

The Japanese japonica rice cultivar Nipponbare was the initial genetic material because it had been selected as the reference genotype by the Rice Genome Project in Tsukuba, Japan, and is the best characterised rice genome. At the same time Nipponbare has been a successful cultivar in Japan and a substantial plant physiology and genetics knowledge base has been developed for it. High resolution genetic and physical maps using Yeast Artificial Chromosome clones have been generated for Nipponbare, and the international rice genome sequence consortium used Nipponbare as the template DNA source.

As the focus of the project shifted from gene discovery to more specific industry outcomes there was a change to using the Australian japonica rice cultivar Millin, which performed better in our high throughput transgenics production facility. Millin is a commercial semi-dwarf medium grained variety bred and released in Australia in 1995. Any mutation developed in Millin background had a better chance of maintaining the pattern-specific effect when crossed to other Australian germplasm (Millin seed obtained from Yanco Rice Research Institute, NSW, Australia)

Reporter Gene Detection

GUS histochemical assay

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 catalytic action 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 X-gluc staining solution for 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.* (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.

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). 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.

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.

DNA manipulation and cloning

The techniques used for DNA manipulations, including quantitation, electrophoresis, purification, cloning and hybridisation, were generally as described by Sambrook *et al.* (1989). All plasmids were maintained and propagated in the E. coli strain DH5α following introduction by electroporation. Restriction endonucleases, polymerases, phosphatases and ligases were mainly purchased from New England Biolabs. Restriction 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 a miniprep protocol described by Sambrook *et al.* (1989) or a simple and fast method using the QIAPrep Miniprep-Kit (Qiagen). Southern blotting was done using the procedure described by Sambrook *et al.* (1989) except that the dNTPs were labelled with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals). Positively charged nylon membranes (Boehringer Mannheim) were used.

Polymerase chain reaction (PCR)

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).

Reaction conditions:
Denaturation 2 min at 94°C
3-step cycling (30-35 cycles):
Denaturation 30 sec at 94°C
Annealing 30 sec at 50-60°C
Extension 1 min/kbp at 72°C
Final extension 5 min at 72°C

Components in PCR reaction:
Template DNA 0.1-10 ng
PCR buffer 1x
MgCl2 1.5 mM
Primer 1 – 0.5 µM
Primer 2 - 0.5 µM
dNTP mix 200 µM of each dNTP
Tag DNA Polymerase 0.5 U

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, Brisbane, Queensland for gel separation.

Rice transformation and culture

Preparation of Agrobacterium containing constructs

Agrobacterium strain EHA105 was used in this study. For each transformation, 50 µl of electrocompetent cells were thawed on ice for 10 min, after which 10-50 ng of plasmid DNA were added into the cells and mixed by pipetting gently. The cells were then loaded into a 2-mm pre-chilled electroporation cuvette, and held on ice for 1 minute before applying a pulse to the cuvette on the Gene Pulser apparatus (Bio-Rad) with the following settings: 2.45 kv (voltage), 25 µF (capacitance), 200 ohms (resistance), 5 msec (calculated pulse time).

To the cells on ice was then added 500 µl of SOC medium to suspend the electroporated cells by gentle pipetting. Electroporated cells suspended in SOC medium were incubated at 28°C for 1-2 hours, then plated on LB. 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 (per liter)

- Bacto tryptone 20 g
- Bacto yeast extract 5 g
- NaCl 0.5 g
- $MgCl_2 \cdot 6H2O$ 2 g
- KCl 0.2 g
- Glucose 3.6 g
- pH 7.0

LB Medium (per liter)

- Bacto tryptone 10 g
- Bacto yeast extract 5 g
- NaCl 0.5 g
- Bacto agar 15 g (for gelled media)

Agrobacterium-mediated transformation of rice callus

The protocol for *Agrobacterium*-mediated transformation of rice was established in CAMBIA based on work by a Japanese group (Hiei *et al*., 1994). For all plant transformation experiments, the Agrobacterium strain EHA105 (Hood *et al.*, 1996) was used.

Callus Induction from rice seeds:

Seeds, usually 20-30 grams per experiment were dehusked and treated with 70% ethanol for 1 min, then washed thoroughly with distilled water and sterilized with a solution of 70% commercial bleach (4.5% active chlorine) and 1 drop of Tween 20, shaken gently for 30 min at room temperature. The seeds were then washed in a laminar flow cabinet with about 300 ml of distilled water and blotted dry on sterile filter paper.

Seeds were plated on 2N6 medium, approximately 15-20 seeds per plate, and cultured at 26°C in the dark for 3 weeks for callus induction.

Callus subculture

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

Transformation

Two days before the transformation, *Agrobacterium* containing plasmid of interest was streaked on solid AB medium with appropriate antibiotics (usually chloramphenicol at 100 μg/ml) and cultured at 28°C for two days.

Agrobacteria from the solid AB medium were scraped using an inoculation loop into AAM medium containing 100 μM acetosyringone (19.6 mg/l) and resuspended for growth at room temperature for 2-3 hours. Concentration of the suspension was adjusted to an OD reading at 600 nm of 0.7-1.0. In every experiment, the OD600 readings for different constructs were adjusted to more or less the same value. At this point the suspension was transferred to a larger container, to which the rice callus was added, swirled, and incubated 25-30 min at room temperature. Callus was then blotted dry on sterile filter paper and plated onto 2N6-AS medium for co-cultivation at 26°C in the dark for 3 days.

After co-cultivation the agrobacteria was removed from the callus through several washing steps using a solution of 100 mg/l timentin and 250 mg/l cefotaxime in autoclaved distilled water. Callus was placed in the wash solution, swirled and left for 30 min. (during this time most of the agrobacteria is released from the callus.) The wash solution was poured off and the callus was re-washed until the solution was clear. Washed callus was blotted dry on sterile filter paper and transferred to 2N6-TCH medium with timentin and cefotaxime for eliminating agrobacteria and hygromycin for selection of transformed cells, 25 calli to a 9-cm petri dish. The callus was cultured at 26°C in the dark and transferred to fresh 2N6-TCH medium every 2 weeks. Typically, tiny brownish translucent protuberances began to appear from the callus surface after two to three weeks on selection.

Lines were made with small proliferating globular calli from the initial callus after about 3-4 weeks on 2N6-TCH medium, by placing them on fresh 2N6-TCH medium. All 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.

Callus lines were allowed to continue proliferating for another 2 weeks on new 2N6-TCH plates under the same incubation conditions. Compact, opaque and yellowish callus was then transferred to RGH6 regeneration medium for plant generation in the dark for one week at 26°C, and then transferred to the light. Calli on RGH6 medium were 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, typically calli are found turning green and in about 14 days shoots will start differentiating. Shoots were 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.

Media used for transformation

2N6, callus induction medium (per liter)

- N6 salts and vitamins (Table 1)
- Casamino acids 1g
- 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)*

20x salts (per liter)

- NH_4Cl 20 g
- $MgSO_4 \cdot 7H_2O$ 6 g
- KCl 3 g
- $CaCl_2 \cdot 2H_2O$ 3 g
- $FeSO_4 \cdot 7H_2O$ 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 (per 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)

- $Na_2HPO_4 \cdot 2H_2O$ 1.696 g
- $MgSO_4 \cdot 7H_2O$ 5 g
- KCl 29.5 g
- $CaCl_2 \cdot 2H_2O$ 1.5 g

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

- $MnSO_4 \cdot 4H_2O$ 1 g
- $NaMoO_4 \cdot 2H_2O$ 25 mg
- H_3BO_3 300 mg
- $ZnSO_4 \cdot 7H_2O$ 200 mg
- $CuSO_4 \cdot 5H_2O$ 3.87 mg

- $CoCl_2 \cdot 6H_2O$ 2.5 mg
- KI 75 mg

AA Iron: (100x, 100 ml):
280 mg of $FeSO_4 \cdot 7H_2O$

AA Amino acids: (100x, 100 ml)
- Glycine 75 mg
- Arginine 1.74 mg
- Glutamine 8.76 mg

Preparation of AS:
19.6 mg acetosyringone is dissolved in 1 ml of dimethyl sulphoxide (DMSO) and added to the medium after autoclaving.

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.

2N6-AS, co-cultivation medium (per liter)
- N6 salts and vitamins (Table 1)
- 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

2N6-TCH, selection medium (per liter)
- N6 salts and vitamins (Table 1)
- 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 (per liter)
- N6 salts and vitamins (Table 1)
- Sucrose 30 g
- Glutamine 500 mg
- Proline 500 mg
- Casein enzymatic hydrolysate 300 mg

- 6-benzylaminopurine (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

½ MS-H, rooting medium (per liter)

- 0.5x MS salts and 1x vitamins (Table 2)
- Sucrose 10 g
- pH 5.8
- Phytagel 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 1: Components of N6 salts and vitamins (Chu *et al*., 1975)

Chemicals	Final conc.	Stock solutions
N6 Macro-elements	mg/l (1x)	g/l (20x)
$(NH4)_2SO_4$	463	9.3
KNO_3	2830	56.6
$CaCl_2{\cdot}2H_2O$	166	3.3
$MgSO_4{\cdot}7H_2O$	185	3.7
KH_2PO_4	400	8
N6 Micro-elements	mg/l (1x)	g/l (1000x)
$MnSO_4{\cdot}H_2O$	4.4	4.4
H_3BO_3	1.6	1.6
$ZnSO_4{\cdot}7H_2O$	1.5	1.5
KI	0.8	0.8
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.025
$CoCl_2{\cdot}6H_2O$	0.025	0.025
Fe_2EDTA solution	mg/l (1x)	g/l (200x)
$FeSO_4{\cdot}7H_2O$	27.85	5.57
Na_2EDTA	37.25	7.45
N6 Vitamins	mg/ml (1x)	mg/l (100x)
Nicotinic acid	500	50
Pyridoxine·HCl	500	50
Thiamine·HCl	1000	100
Glycine	2000	200
Myo-inositol	100000	10000

Table 2: Components of MS salts and vitamins (Murashige & Skoog 1962)		
Chemicals	Final conc.	Stock solutions
MS Macro-elements	mg/l (1x)	g/l (10x)
$(NH_4)NO_3$	1650	16.5
KNO_3	1900	19
$CaCl_2 \cdot 2H_2O$	440	4.4
$MgSO_4 \cdot 7H_2O$	370	3.7
KH_2PO_4	170	1.7
MS Micro-elements	mg/l (1x)	mg/l (10x)
$MnSO_4 \cdot H_2O$	16.9	169
H_3BO_3	6.2	62
$ZnSO_4 \cdot 7H_2O$	8.6	86
KI	0.83	8.3
$Na_2MoO_4 \cdot 2H_2O$	0.25	2.5
$CuSO_4 \cdot 5H_2O$	0.025	0.25
$CoCl_2 \cdot 6H_2O$	0.025	0.25
Fe_2EDTA solution	mg/l (1x)	g/l (200x)
$FeSO_4 \cdot 7H_2O$	27.85	5.57
Na_2EDTA	37.25	7.45
MS Vitamins	µg/l (1x)	mg/l (100x)
Nicotinic acid	500	50
Pyridoxine·HCl	500	50
Thiamine·HCl	500	50
Myo-inositol	100000	10000

Rice genomic DNA isolation

Plant leaves were collected on ice (the samples could be left in a -70°C freezer until extraction).
S buffer (see below) was preheated 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 (Hope, 1999)

- 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 up 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 up 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 up the volume to 250 ml by adding distilled water.

4. Results including statistical analysis

Testing of rice transformation at high production capacity

A series of binary vectors had been constructed for testing elements of enhancer traps. In this transformation experiment, four enhancer trap constructs were used, all of them harboring the enhancer trap cassette (minimal 35S-*Gal4*/VP16), the reporter cassette (UAS-minimal 35S-GUSPlus::EGFP) and the selection cassette (CaMV 35S-Hygromycin). The summary of results at key steps of the transformation process is shown in Table 3.1. 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. This efficiency was assessed as sufficient to fulfill the requirements of the project, in which large populations of transgenic lines were to be developed.

Table 3: Results of the test transformation experiment

Construct	No. of scutellar calli used	No. of hygromycin-resistant callus lines generated	No. of calli transferred to regeneration medium	No. of transgenic lines obtained
pFX-E24.1-4	200	1085	520	215
pFX-E24.1-12R	200	1330	656	230
pFX-E24.2-3	200	875	384	189
pFX-E24.2-15R	200	1400	656	287
Total	**800**	**4690**	**2216**	**921**

Testing the components of the TAFET system

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.

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. In other vectors there has been no detectable expression of *Gal4*/VP16 except for its integration into the vicinity of genomic enhancers. Therefore, this cassette is the sensor and transducer responding to enhancers and is usually located close to the right border of T-DNA. Through the sensing, and often amplifying, the enhancer signal, the 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 our project we used 6 copies of UAS, which had been shown to provide sufficient strength and specificity in a number of Gal4/VP16 applications. We used several reporter genes in our tests:

- β-glucuronidase gene from *E.coli* (GUS)
- GUSPlus
- the enhanced green fluorescent protein (EGFP)

The reporter cassette 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 theoretically 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 initial stages of the program.

The nomenclature and descriptions of the binary vectors produced to test the components of the TAFET system using GUS and GUSPlus reporters are shown in Table 4.

Table 4: Nomenclature and description of the binary vectors produced to test the components of the TAFET system using GUS and GUSPlus reporters

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

Table 4 outlines the TAFET constructs containing GUS and GUSPlus reporter genes. 35S: the CaMV35 promoter, HPTII: *hpt* II Hygromycin resistance gene, mGFP5ER: modified Green Fluorescent Protein, 5UAS, 6UAS: Upstream activation sequence of the GAL4, pBS: plasmid Blue-script, intron: the castor bean *catalase* intron, GAL4/VP16: transcriptional activator fusion.

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

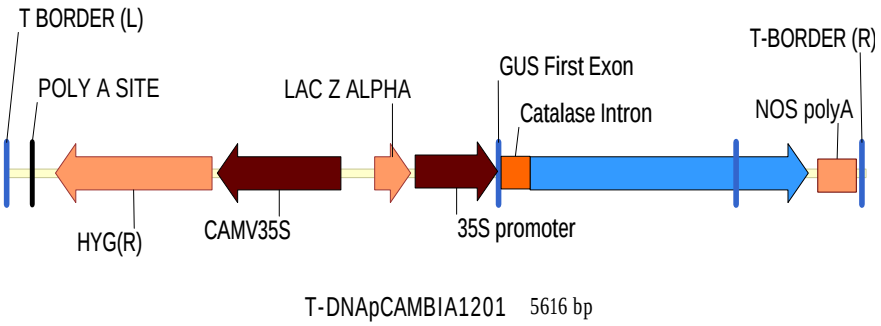


Figure 1: The pCAMBIA1201 plasmid as a positive control for the transactivator constructs.

Initial observation of reporter gene expression was performed on calli 3 days after co-cultivation with *Agrobacterium* carrying the various binary vectors described above. Both GFP and β-glucuronidase expression were analysed. However, the signal from mGFP5ER, used in the initial constructs, was very weak and difficult to discriminate from callus autofluorescence. Therefore, we focused our attention on the glucuronidase histochemical assay and did not analyse GFP expression in calli and plants obtained using these TAFET constructs. In later experiments, described below, EGFP was used.

The average percentage of calli exhibiting blue foci of GUS expression ranged from 14.4% to 54.3% for various GUS TAFET constructs, from 42% to 71.5% with GUSPlus TAFET constructs (Fig. 3.13), whereas calli with pCAMBIA 1201 (positive control, Figure 1) had 60.6% displaying blue foci of GUS expression. In contrast, calli transformed with both TAFET constructs that contained a deletion of the GAL4/VP16 DNA-binding domain (pSKD15.1 and pSKD15.2) displayed no blue foci of reporter gene expression. Transformation of these two deletion constructs were replicated three times (in each case observations were carried out on 3, 7 and 14 days after co-cultivation) with similar outcome: no GUS staining in negative controls. These results were interpreted as an indication of the ability of GAL4/VP16 to act as a transactivator in rice.

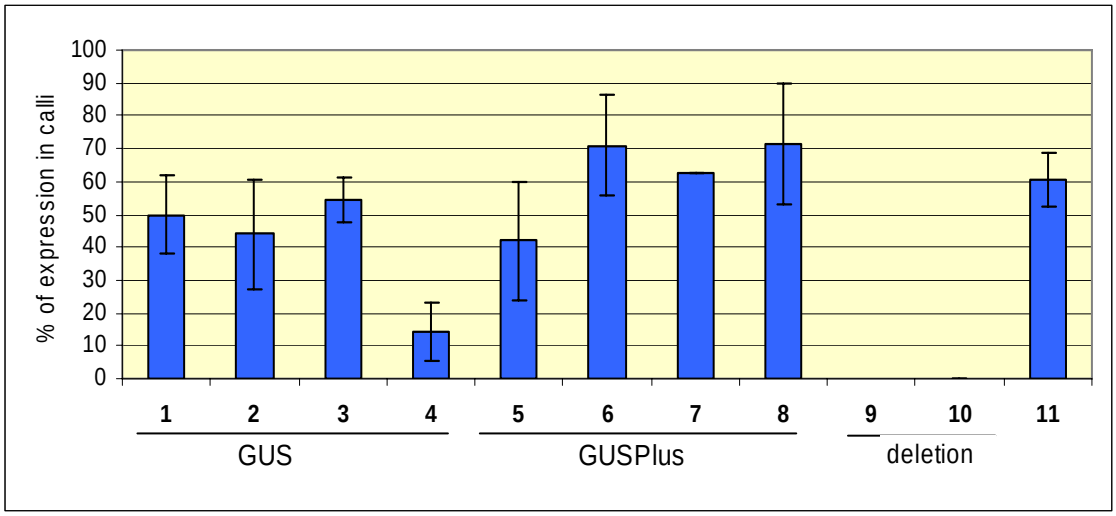
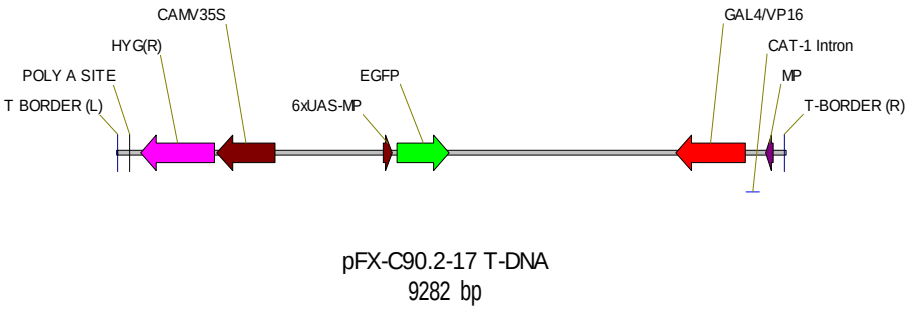


Figure 2: Expression of enhancer trap constructs in callus stage. 1-8: transactivator constructs as per Table 6.2; 9-10: GAL4/VP16 deletion, 11: Positive control pCAMBIA

Further tests of the system were performed in the later phase of the project using EGFP-based constructs. In one such transformation experiment the enhancer trap vectors were pFX-C90.1-12R and pFX-C90.2-17 and their corresponding *Gal4*-deletion vectors pFX-C103.1-2 and pFX-C103.2-3 (Fig. 3).



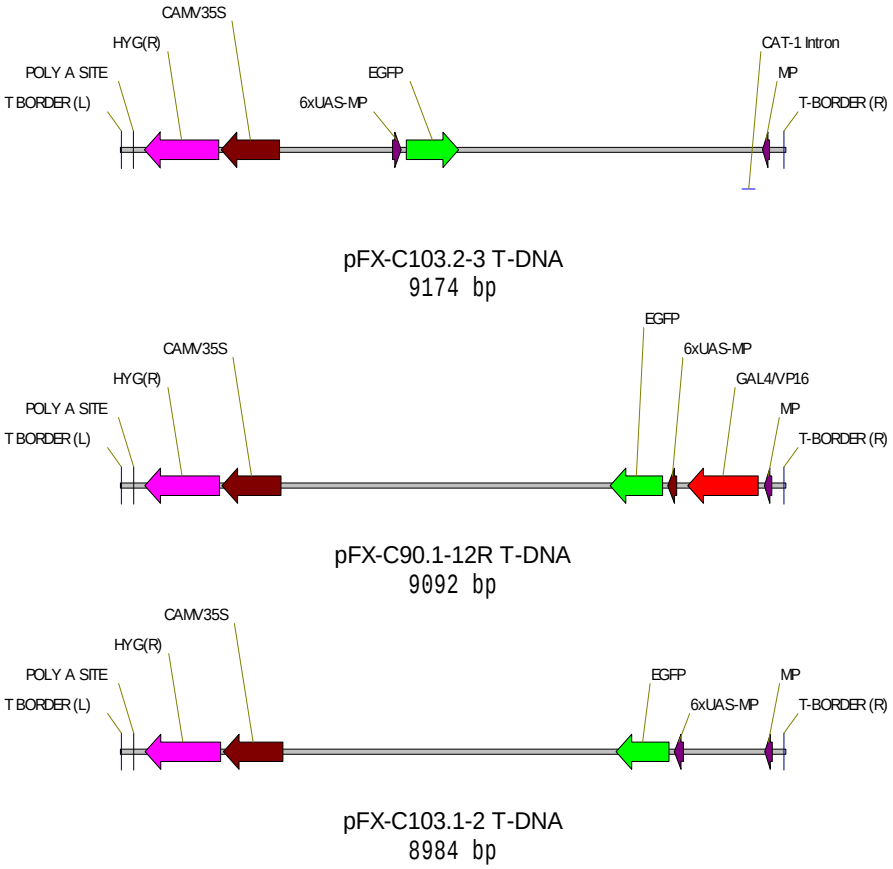


Fig. 3: T-DNA organisation of vectors with functional *Gal4* transactivator (pFX-C90.2-17 and pFX-C90.1-12R and EGFP as the reporter; and the corresponding *Gal4*-deletion vectors pFX-C103.2-3 and pFX-C103.1-2

In each experiment the number of calli was 200 for each of the constructs. GFP expression was observed once every week after transformation using the Leica MZ FLIII fluorescence stereomicroscope equipped with GFP3 filter set. Based on the results (data not shown) in repeated experiments, the transformation efficiency was found to be similar to the one described above.

Two weeks after transformation, there was no GFP expression detected in the two *Gal4*-deletion constructs. This result was consistent with the results obtained using constructs with glucuronidase reporters, providing additional support for *Gal4*/VP16 functionality in the enhancer trap vectors (Fig. 6.2d).

However, three weeks after transformation, GFP expression was detected in the two *Gal4*-deletion constructs and the number of calli showing GFP expression increased dramatically at 4 weeks and stabilised thereafter.

A possible explanation of GFP expression in these *Gal4*-deletion constructs could be the *cis*-activity of enhancer elements from the CaMV 35 promoter. The difference of GFP expression levels between pFX-C103.1-2 and pFX-C103.2-3 further supports this hypothesis. Rice calli transformed with pFX-C103.2-3 showed a higher rate of GFP expression, expected if the 35S promoter has this enhancer effect, since the 6xUAS-MP-EGFP cassette was located very close to the CaMV 35S promoter (1609 bp), whereas in pFX-C103.1-2, showing a much lower level of GFP expression, the distance between the 6xUAS-MP-EGFP cassette and the CaMV 35S promoter was much larger (5644 bp).

Analysis of the initial TAFET line population: GUS reporters

In the initial stage of the project about 1,000 TAFET lines and control lines were generated using *Agrobacterium* transformation: 330 lines transformed with GUS reporter gene, 663 lines with GUSPlus reporter gene, 3 negative control lines (pSKD15.1) and 64 positive control lines generated with the pCAMBIA 1201 vector. About 5% of lines did not show any gene expression and of those expressing the reporter gene, about 34%, 36% and 25% of TAFET lines had weak, medium and strong intensity of expression, respectively.

GUS lines had a higher percentage of weak expression than GUSPlus (47% and 30%, respectively). GUSPlus constructs produced a higher percentage of lines with strong expression (36%) than GUS constructs (12%). These results may indicate GUSPlus is a more sensitive reporter gene than GUS.

Gene Expression Patterns in Vegetative Tissues

T_0 plants were observed for their expression patterns in vegetative tissues, during the initial growth phase before flowering. Of 1000 TAFET lines, 222 GUS TAFET lines and 321 GUSPlus TAFET lines showed gene expression in vegetative parts, in the root, shoot and leaf tissues. The percentage of GUS lines showing expression in vegetative tissues is lower than for GUSPlus lines. Percentages for each transactivator GUSPlus construct were similar. These results are probably due to the fact that GUSPlus constructs produced more lines with expression than GUS constructs, confirming the observation in the callus stage that GUSPlus is a more sensitive reporter gene than GUS.

Gene Expression Patterns in Floral Tissues

Four hundred and twenty lines were observed to show reporter gene expression in various floral tissues: lemma, palea, lodicules, filament, anther sac, pollen, stigma, style and ovule. More than 60% of lines showed expression in male parts (anther sacs and/or pollen), 21% in lodicules, more than 30% in stigmas and styles, but only 3% of lines showed expression in ovules.

Tissue specific expression patterns in floral parts were displayed by 17.3% of TAFET lines. The percentages of lines that had expression only in one, two or three tissues, were about 2.14%, 10.5% and 4.76%, respectively. Moreover, the percentage of lines that had expression in the ovule and another tissue was only about 0.5% (2 of 420 lines observed), while the lines that had gene expression only in the lodicule, and the lodicule and another floral tissue was about 6.4% (27 of 420 lines observed) (Table 5).

Table 5: Percentage of lines with tissue-specific expression patterns in TAFET population.	
Tissues	Percentage
Palea and lemma	4.00
filament	0.95
lodicules	6.42
stigma	2.86
style	1.20
ovule	0.48
anther sac	1.40
Total	17.31

Analysis of TAFET lines with EGFP reporter

A total of 590 transgenic lines (T0) with the EGFP reporter (including the positive control pFX-B114.1) were generated. Before transplanting into soil pots, all the plantlets were surveyed for GFP expression using the Leica MZ FLIII fluorescence stereomicroscope. The GFP signals were scored as strong, medium and weak, and patterns of GFP expression were recorded.

Table 6 Number and percentage of transgenic lines showing different strengths of GFP expression					
Construct	Total number of lines	Strong	Medium	Weak	Negative
pFX-C90.1-12R	109	38	26	26	19
(%)	100.00	34.86	23.85	23.85	17.43
pFX-C97.1-2	138	41	45	30	22
(%)	100.00	29.71	32.61	21.74	15.94
pFX-C90.2-17	123	22	22	42	37
(%)	100.00	17.89	17.89	34.15	30.08
Gal4-deletion	102	23	29	18	32
(%)	100.00	22.55	28.43	17.65	31.37

As seen in Table 6, 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. Like the situation in callus stage, pFX-C90.2-17 showed lower GFP expression, most probably due to the negative effect of the CAT-1 intron flanking *Gal4*/VP16.

Table 7: GFP expression patterns in enhancer trap and *Gal4*-deletion lines				
Construct	Tissue with GFP expression	Number of lines	Percentage of all GFP lines	Percentage of all transgenic lines
Enhancer trap	Root	121	41.4	32.7
	Root branch	13	4.5	3.5
	Stem base	194	66.4	52.4
	Leaf	122	41.8	33.0
	Stomata	61	20.9	16.5
	Vascular tissue	2	0.7	0.5
	Collar	249	85.3	67.3
	Auricle	31	10.6	8.4
	Ligule	8	2.7	2.2
	Trichome	1	0.3	0.3
Gal4-deletion	Root	24	34.3	23.5
	Root branch	4	5.7	3.9
	Stem base	49	70.0	48.0
	Leaf	12	17.1	11.8
	Stomata	2	2.9	2.0
	Vascular tissue	1	1.4	1.0
	Collar	55	78.6	53.9
	Auricle	6	8.6	5.9
	Ligule	2	2.9	2.0
	Trichome	0	0.0	0.0

The most important information in the experiment was GFP expression in the *Gal4*-deletion constructs. The percentage of transgenic lines with this negative control construct showing GFP expression was unexpectedly high (Table 6). The results obtained in callus stage both with glucuronidase reporters and EGFP indicated that *Gal4*/VP16 was not functional in the *Gal4*-deletion constructs, but it was apparent that the enhancer elements of the CaMV 35S promoter still allowed the reporter gene expression in TAFET constructs in regenerated plants.

Furthermore, 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.

It was apparent that GFP expression in *Gal4*-deletion constructs was not only similar in strength to that of enhancer traps, but also gave quite the same frequencies of expression patterns (table 7). The most frequent expression patterns in the *Gal4*-deletion transgenic lines were collar (78.6%), stem base (70.0%), root (34.3%) and leaf (17.1), whereas in the enhancer trap lines, they were also collar (85.3%), stem base (66.4%), root (41.4%) and leaf (41.8%), showing a similar tendency in the distribution of expression patterns.

Development of TAFET constructs with weaker *cis*-effects of the selection cassette.

The discovery of the 35S promoter enhancer effect on the expression of reporter genes in the TAFET system demanded development of another system, one which would allow creation of more diverse patterns of transactivator expression. The first attempt was through the replacement of 35S promoter driving hygromycin resistance gene with the ubiquitin promoter (*Ubi1*) from maize (Christensen, 1992).

Characteristics of the ubiquitin promoter

Several ubiquitin genes have now been isolated from higher plants. An intron commonly found in the 5'UTR of plant ubiquitin genes has been implicated as a quantitative determinant of expression in transient assays in *Arabidopsis* (Norris, 1993). The *Ubi1* promoter (with and without the first intron) was used to replace the 35S promoter in a series of TAFET and control constructs (Figures 4-5).

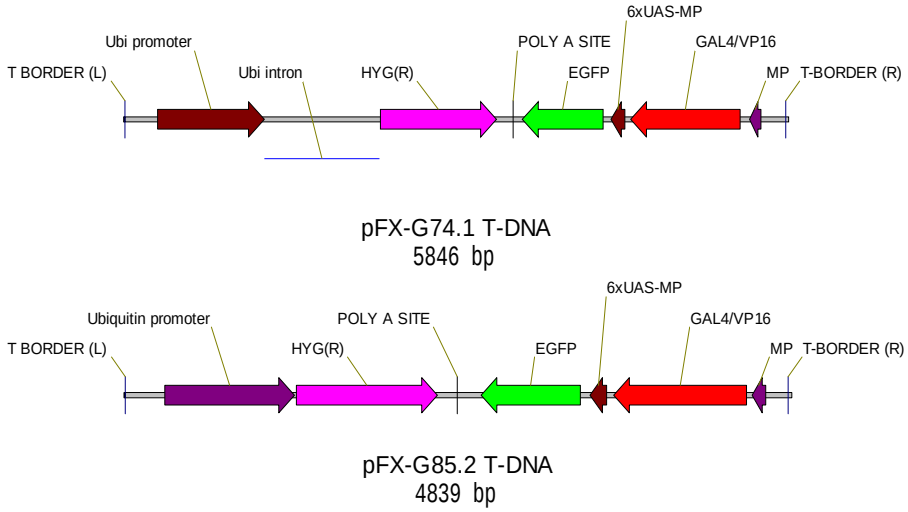


Figure 4: T-DNA components of the enhancer trap (TAFET) vectors.

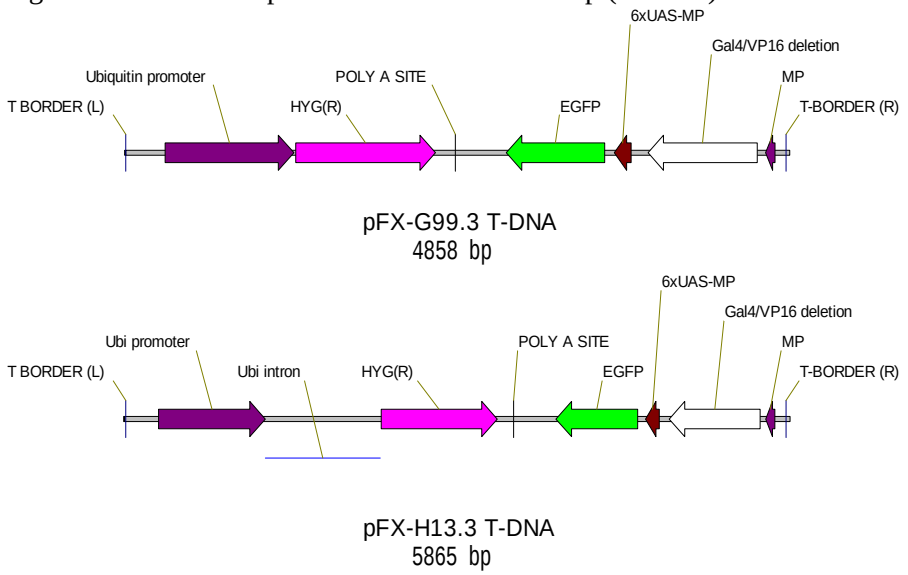


Figure 5: T-DNAs of the *Gal4*-deletion vectors pFX-G99.3 and pFX-H13.3.

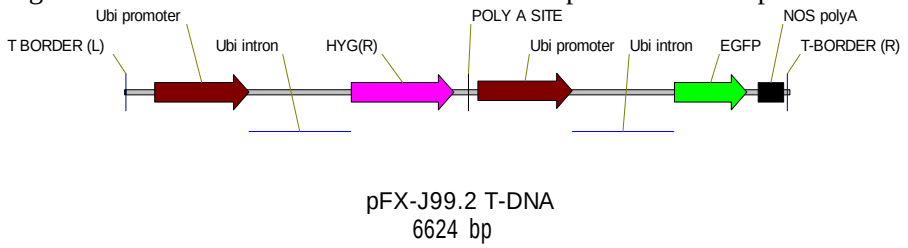


Figure 6: T-DNA components of the positive control binary vector pFX-J99.2.

Frequency of GFP expression in transgenic lines

A total of 661 transgenic lines were generated with the *ubi* promoter constructs, 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 these were transplanted into soil pots, plantlets were surveyed for GFP expression at the vegetative stage. At the generative stage, samples of young panicles were taken from each of the transgenic lines at the beginning of flowering, and from each transgenic line 15-20 spikelets were carefully dissected under a stereo microscope.

GFP expression in the positive controls was similar to described in the literature, with a relatively small number of transgenic lines (18%) showing no GFP signal. In the enhancer trap constructs pFX-G74.1 and pFX-G85.2, the percentages of transgenic lines showing GFP expression were 30.7% and 36.6%, respectively (Table 8), much lower than those with CaMV 35S-hygromycin cassette (70%-84%, Table 8). The result also showed that there was no dramatic difference in the percentages of GFP positive lines between the two enhancer trap constructs.

Table 8: Number and percentage of transgenic lines showing GFP expression

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

Only 7 lines showed GFP expression in the *Gal4*-deletion constructs, much fewer than the non-deleted enhancer traps. The result indicated that GFP expression in the enhancer traps was triggered by the functional *Gal4*/VP16.

Patterns of GFP expression in enhancer trap lines with Ubi1::hpt

Patterns of GFP expression were recorded based on tissues/organs or combinations of tissues/organs with GFP expression. 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. Specific description of these patterns is reported in Appendix 1. The expression patterns were very diverse, suggesting little influence from the *Ubi1* promoter.

Table 9 shows the categories and percentages of enhancer trap lines in which GFP expression was detected in up to 13 different tissues/organs that included root, root branch, stem base, leaf, stomata, vascular band, collar, auricle, ligule, trichome, leaf granules, stigma, ovary, anther, lodicule, glumes, sterile lemma, pedicel and panicle branch. Over 60% of the 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 tissue, collar, trichome, stigma, and pedicel, respectively. The phenomenon of GFP expression in multiple tissues/organs of a pattern line was also found in enhancer trap lines of *Drosophila* (Braun, 1997). This phenomenon could be most probably due to (i) multiple activities of an enhancer in rice genome, or (ii) multiple insertions of the enhancer trap T-DNA into different locations of the rice genome.

Table 9: Expression of EGFP in tissues/organs in pattern lines obtained with enhancer trap constructs pFX-G74.1 and pFX-G85.2

Number of tissues/organs involved in a pattern	Number of lines	Percentage of pattern lines	Percentage of transgenic lines
1	12	9.3	3.1
2	34	26.4	8.7
3	28	21.7	7.1
4	21	16.3	5.3
5	8	6.2	2.0
6	13	10.1	3.3
7	6	4.7	1.5
8	2	1.6	0.5
9	1	0.8	0.3
10	2	1.6	0.5
11	1	0.8	0.3
12	0	0.0	0.0
13	1	0.8	0.3

The frequencies of GFP expression in various tissues and organs of rice plants are listed in Table 10. 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 leaf granules. 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%). A relatively higher frequency (14.1%) of GFP expression was found in pFX-G85.2 plants in the form of irregular granules in leaves, while it was lower in pFX-G74.1 plants (6.0%).

Table 10: The frequencies of GFP expression in various tissues and organs of rice plants

Construct	Tissue with GFP expression	Number of lines	Percentage of all GFP lines	Percentage of all transgenic lines
pFX-G74.1	Root	40	51.9	15.9
	Root branch	3	3.9	1.2
	Stem base	3	3.9	1.2
	Leaf	34	44.2	13.5
	Stomata	42	54.5	16.7
	Vascular tissue	13	16.9	5.2
	Collar	8	10.4	3.2
	Auricle	1	1.3	0.4
	Ligule	1	1.3	0.4
	Trichome	4	5.2	1.6
	Granules	15	19.5	6.0
	Stigma	11	14.3	4.4
	Ovary	6	7.8	2.4
	Anther	18	23.4	7.2
	Lodicule	9	11.7	3.6
	Glumes	29	37.7	11.6
	Sterile lemma	11	14.3	4.4
	Pedicel	17	22.1	6.8
	Panicle branch	20	26.0	8.0
pFX-G85.2	Root	30	57.7	21.1
	Root branch	7	13.5	4.9
	Stem base	0	0.0	0.0
	Leaf	26	50.0	18.3
	Stomata	27	51.9	19.0
	Vascular tissue	8	15.4	5.6
	Collar	5	9.6	3.5
	Auricle	0	0.0	0.0
	Ligule	0	0.0	0.0
	Trichome	2	3.8	1.4
	Granules	20	38.5	14.1
	Stigma	7	13.5	4.9
	Ovary	3	5.8	2.1
	Anther	8	15.4	5.6
	Lodicule	4	7.7	2.8
	Glumes	19	36.5	13.4
	Sterile lemma	5	9.6	3.5
	Pedicel	10	19.2	7.0
	Panicle branch	10	19.2	7.0

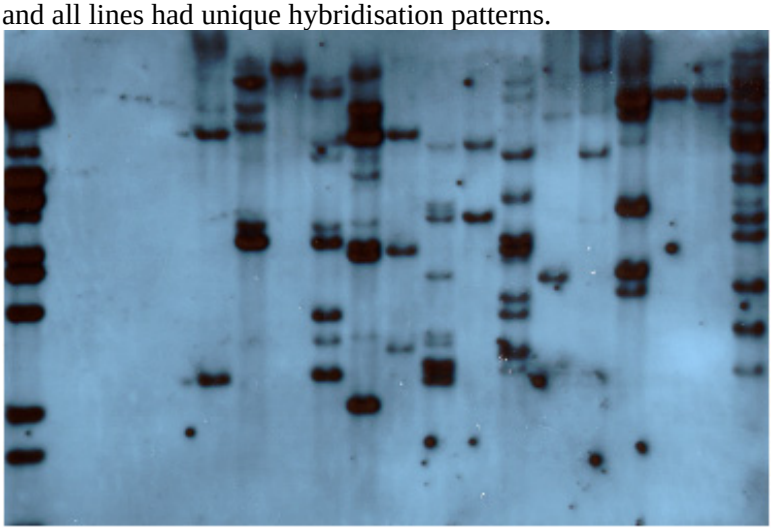
The observed diversity of reporter gene expression patterns provided us with confidence that *Ubi1* promoter did not have the problematic enhancer side effects of the 35S promoter in the TAFET constructs. However, some influence of the hygromycin resistance gene on the UAS-reporter gene remained even with *Ubi1* replacing 35S promoter. While the influence presented itself primarily at the tissue culture stage, it is possible that the high frequency of lines with certain expression patterns (stomata, for example) was a result of the *Ubi1* enhancer interaction with the USA-minimal promoter upstream of the reporter gene.

Development of TARGET lines through biolistic and *Agrobacterium* transformation.

Our initial plan for Target line development was based on using the *Ac/Ds* transposon system. While our initial tests of the *Ac/Ds* system performance were encouraging, the challenge of high throughput crossing between the transposase line and Target lines was substantial. This prompted us to test a modified approach with delivery of UAS sequences through biolistic transformation (gene gun) and *Agrobacterium* methods.

We performed molecular analysis of several hundred Target lines and established that the average number of UAS elements inserted into rice genome using this procedure is above 4, with over 80% of lines containing at least a single UAS, outperforming *Ac/Ds*. In addition to eliminating the need to first cross and then segregate away the transgene coding for *Ac* transposase, our modified approach offers unambiguous identification of the causal factor(s) involved in novel phenotypes. If GAL4/VP16 from the TAFET construct present in one genomic location transactivates a gene linked with the UAS element in another location, segregation of both elements eliminates the new phenotype, while bringing them back together re-establishes it. In contrast, in a so called "cis" system, segregation of the molecular event responsible from the resulting phenotype is practically impossible.

Figure 7. Southern blot of randomly selected Target lines to determine the copy number of UAS insertions. DNA of Target lines was digested with *Eco*RI restriction enzyme and hybridized with a labeled fragment of the *hyg* gene. The first lane is the molecular size marker and the three empty lanes contain negative controls. The average number of UAS insertions per transgenic line was above four, and all lines had unique hybridisation patterns.

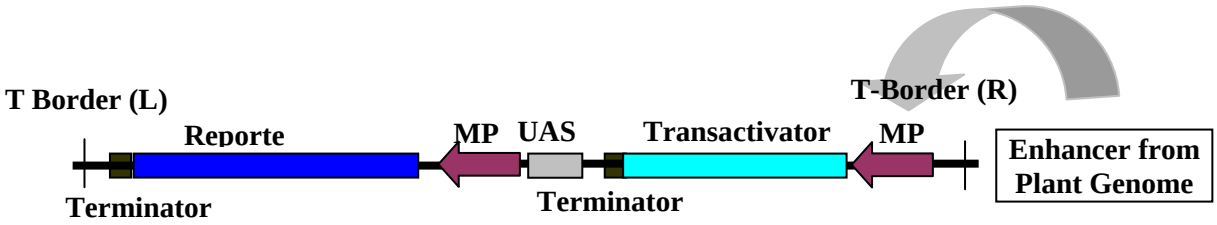


Co-transformation system for TAFET and Target

An alternative strategy was developed to deliver both transactivator and target into plant genome at the same time via *Agrobacterium*-mediated co-transformation of two separate plasmids. The "enhancer trap" plasmid contained in its T-DNA a transactivator gene under control of a minimal promoter, and GusPlus, a version of β-glucuronidase from *Staphylococcus* sp, under control of the 6xUAS and minimal promoter (Figure 8). The expression of the transactivator and the reporter gene remains at a very low level unless the insertion is in the vicinity of a strong enhancer in the plant genome. As they lack *hph*, tissues having only this T-DNA in the genome will not survive in the selection medium. In contrast, the T-DNA of the selection/activation plasmid carries the *hph* gene for selection and the transactivator-responsive promoter (UAS and a minimal promoter) for mis-expression of a random gene in the plant genome. Tissues having only this T-DNA in the genome can survive in the selection medium, but no GUS expression will be expected. Only tissues that both survive in the selection medium and show GUS expression contain both T-DNAs. These have succeeded in trapping a strong enhancer to express the target gene.

(A): “Enhancer Trap”

(pTNT15GAL4)



(B): Selection/activation

(pPSIA67.1)

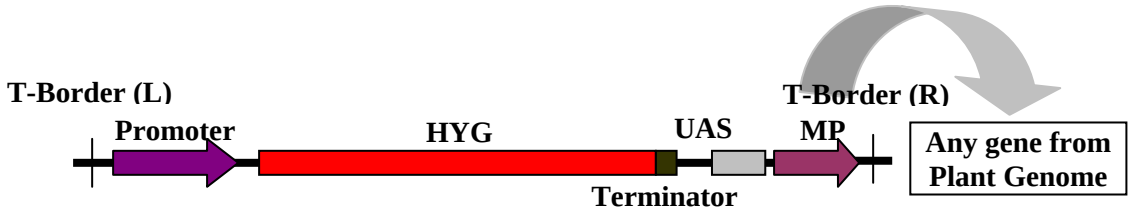


Figure 8. T-DNAs of plasmids for a two-cassette “enhancer trap” strategy. When inserted near a strong enhancer in the plant genome, the transactivator gene in T-DNA of the enhancer trap plasmid will be expressed, activating the expression of the reporter gene and any other gene near the right border of T-DNA of the selection/activation plasmid. The expression of the reporter gene helps identify patterns the “trapped” enhancer has imposed on the transactivator gene.

Table 11. Experiments performed using the combination of pTNT15GAL4 + pPSIA67.1 plasmids

Experiment barcode	Treatment	Number of calli	Cultivar	Number of plants	Cumulative plant number	Transformation efficiency
102003099001		840	NB	257	257	31
102003084001		660	ML	262	519	40
102003099002		1710	ML	198	717	12
102003099003		690	NB	176	893	26
102003099004		1470	NB	64	957	4
102003099005		1200	ML	48	1005	4
102003099006		600	ML	73	1078	12
102003099007		540	NB	143	1221	26
102003099008		600	ML	51	1272	9
102003099009		450	NB	54	1326	12
102003099010		150	ML	7	1333	5
102002330004		480	NB	203	1536	42
102002330001	1:1 ratio	250	NB	167	1703	67
102002330002	3:1 ratio	250	NB	26	1729	10
102002330003	10:1 ratio	250	NB	79	1808	32
102002330005		480	ML	197	2005	41
102002330006	1:1 ratio	250	NB	124	2129	50
102002330007	3:1 ratio	250	NB	23	2152	9
102002330008	10:1 ratio	250	NB	51	2203	20
102002179001		1000	NB	290	2493	29
102002336003		1000	NB	291	2784	29
102002352001		1000	NB	35	2819	4
102002352002		560	ML	72	2891	13
102503049001	10:1 ratio	240	ML	0	2891	0
102503049002	3:1 ratio	240	ML	0	2891	0
102503049003	1:1 ratio	240	ML	7	2898	3
102003061001	26C	400	ML	43	2941	11
102003061002	22C	400	ML	63	3004	16
102003085001		420	NB	13	3017	3
102003113001		650	ML	183	3200	28
102503136001		330	ML	0	(contaminated-discarded)	
102503136002		330	ML	0	(contaminated-discarded)	
102503206001	22C	210	ML	77	3277	37
102503206002	26C	210	ML	0	(contaminated-discarded)	
102503210001	1:1 ratio	260	ML	36	3313	14
102503227001	3:1 ratio	260	ML	20	3333	8
102503227002	10:1 ratio	260	ML	8	3341	3
102503234001	22C	250	ML	47	3388	19
102503234002	26C	250	ML	50	3438	20
102003248001	22C	350	ML	7	3445	2
102003248002	26C	350	ML	64	3509	18

Using this strategy over 3500 lines were generated using pTNT15GAL4 + pPSIA67.1 plasmids.
All the pTNT15GAL4 + pPSIA67.1 experiments were set up in a 1:1 ratio of the two constructs and were co-cultured at 26C. In the experiments listed above, a ratio of 1:1 is only noted for comparison to the 3:1 and 10:1 ratios set up on the same date. Similarly, where a co-culture temperature of 26C is given above, it is only to note the comparison of a 22C co-culture set up on the same date. ML = rice cultivar Millin, NB = rice cultivar Nipponbare.

In total 19830 calli were used in the above transformation experiments. In total, 3509 plantlets were transferred to the glasshouse. Based on these numbers, the transformation efficiency (no. plantlets/ total no. calli) is 19%. For the cultivar Nipponbare, the transformation efficiency was 21% (1996 plantlets/ 9390 calli) and for Millin, the efficiency was 16% (1513 planlets/ 9570 calli).

Testing transactivation system through genetic crosses

To verify the function of transactivators in rice transformed with the enhancer trap constructs described above, a series of genetic crosses were designed. Target lines were generated with the GUS reporter gene or the diphtheria toxin (DTA) gene cloned downstream of a minimal promoter that comprises a TATA-box and the binding sites for the transcription factor Gal4/VP16. Consequently, the GUS or DTA gene is unlikely to be expressed when introduced into wild-type plant cells. This allows "genetically 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 presented in Figure 9.

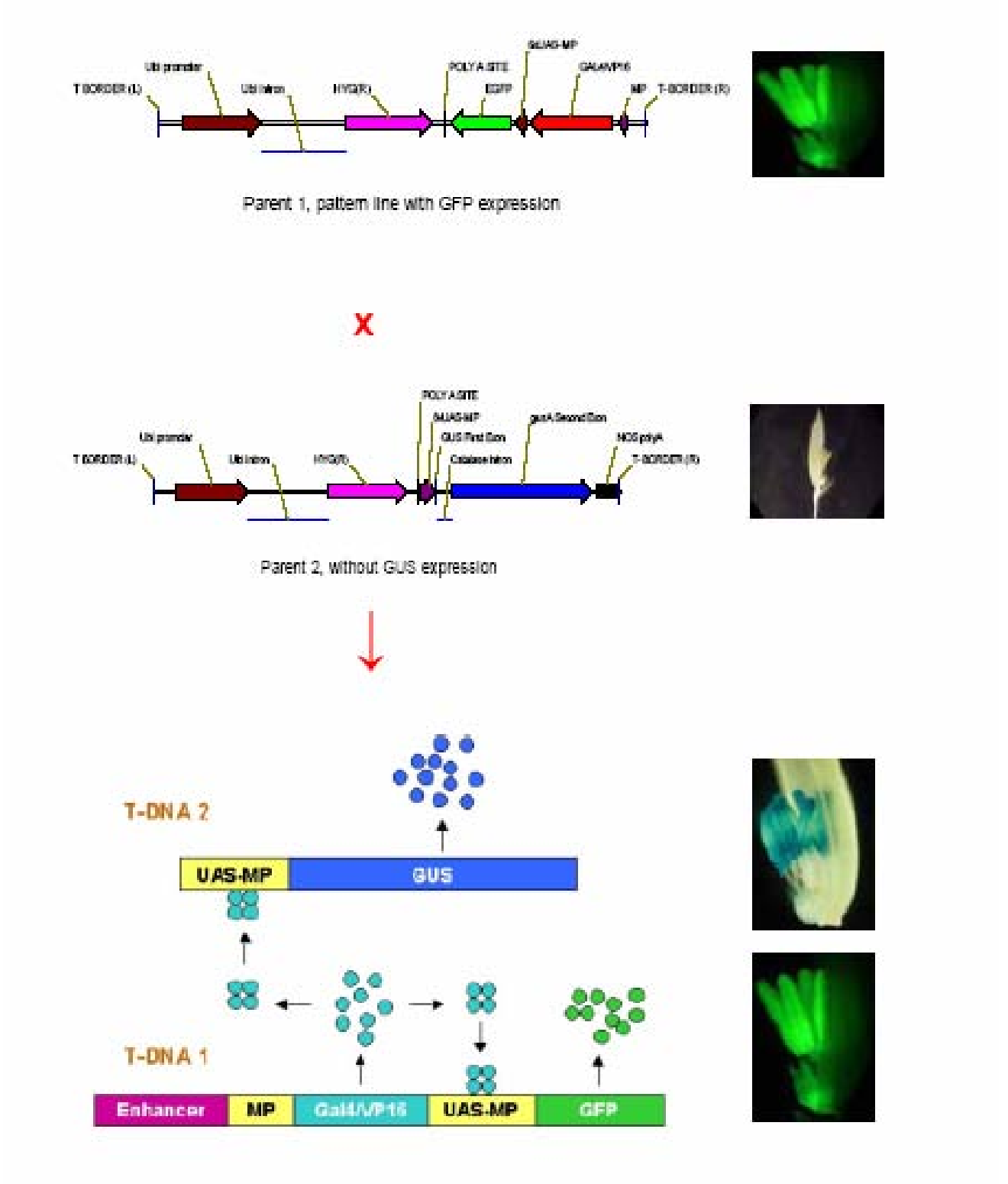


Figure 9: Construct design (top panel) and molecular mechanism underpinning the testing of transactivation through sexual cross (bottom panel).

Characteristics of parental lines used for genetic test and combinations of sexual crosses

Enhancer trap lines

Three enhancer trap lines obtained with the pFX-G74.1 construct 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 T_1 populations. The lines had the following expression patterns: FU01 131: stomata, anther, pedicel; FU01 171: root, stomata, lodicule; FU01 191: stomata, anther, glumes, pedicel.

Target gene lines

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

The major component of these constructs was a Gal4-responsive cassette (6xUAS-MP-GUS) that is identical in all four target gene constructs. Though the selection cassettes were somewhat different among the four constructs, no significant influence on expression of the neighboring Gal4-responsive cassette in plant level was expected based on the results obtained with negative controls.

In all four lines, no detectable target gene expression was 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 T_1 populations. To simplify analysis of the transactivator, only homozygous transgene individuals were used for making crosses.

Table 12: Code numbers of the target gene lines used for crossing.

Code number of lines in T1	Code number of plants used for crossing	Construct	Target gene
FU01 297	2, 3, 5, 8	pWSA 89.18	GUS, DTA
FU01 299	1, 2, 5	pWSA 50.2	GUS
FU01 301	1, 5	pWSA 60.1	GUS
FU01 302	1, 2	pWSA 59.27	GUS

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 (maternal) effects.

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 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. The target parental lines were also planted and used as the negative control. There was no GUS expression detected in any of the T_2 target gene lines used for the genetic crossing.

Co-expression of GFP and GUS in F plants.

Leaf samples from 16 plants of each F1 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 and observed under a Leica MZ FLIII fluorescence stereomicroscope. After GFP observation, the same set of samples was immediately put into GUS stain solution following the protocol. 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 F1 progenies. The expression patterns of GFP and GUS in a specific F1 population were very similar and were also consistent to the GFP pattern of its parental line used for crossing. The result indicated that in these seven F1 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.

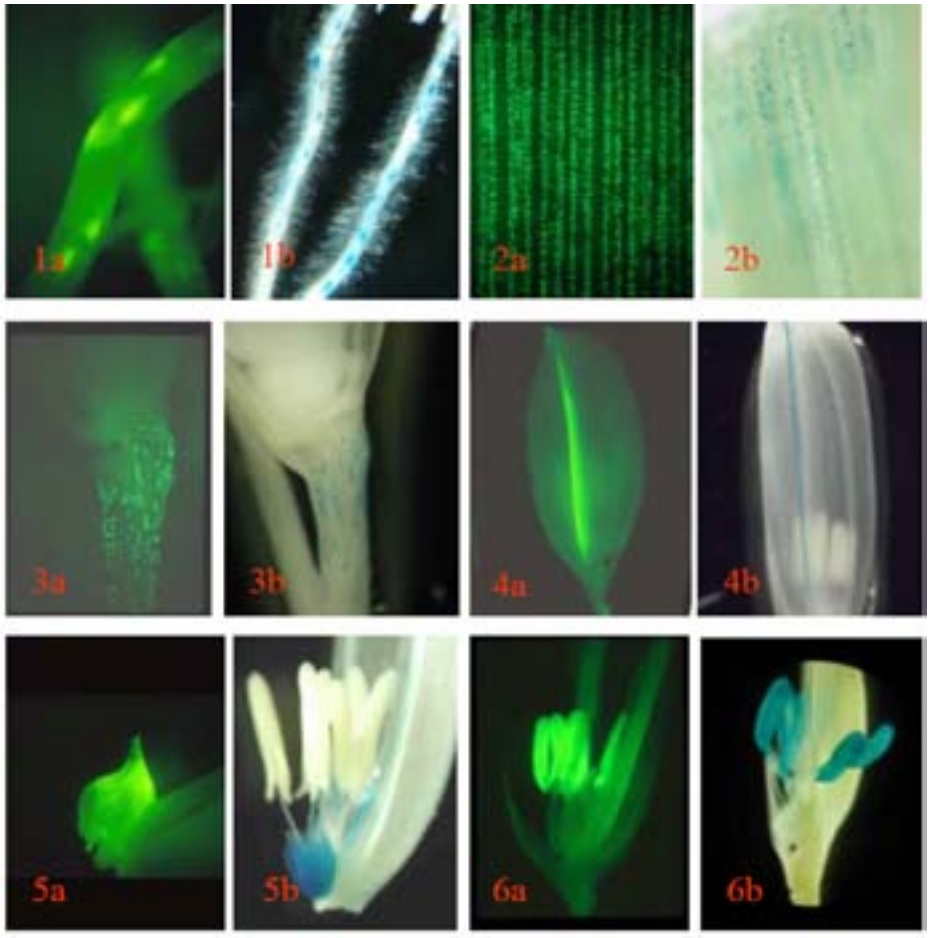


Figure 10: Co-expression of GFP and GUS in F1 progenies of enhancer trap lines crossed with target lines. 1-root, 2-leaf stomata, 3-pedicel, 4-glume (out edge of lemma), 5- lodicule, 6-anther; a-GFP expression, b-GUS expression.

Development of a new transactivator

The initial project was designed around using GAL4/VP16 as a transactivator of choice. GAL4/VP16 has been widely used as a transactivator for identifying novel genes and enhancers in *Drosophila* and other organisms, suggesting its universal applicability. Its application in plants, however, may be obstructed by problems such as the methylation of its 17 nucleotide long DNA recognition sequence, as suggested by research using this transactivator in tobacco (Galweiler *et al.*, 2000). In rice, we

observed about 3 % silencing in GAL4/VP16-induced GUS expression in the T_1 generation (CAMBIA Ph.D. thesis of Sri Koernati).

The activation domain of chimeric transactivator used in the above-described work is VP16-AD (411-490) from human Herpes Simplex Virus. VP16 is an abundant protein of the virus and acts in the early stage of the infection to stimulate transcription of the viral immediate-early genes, thereby facilitating the onset of the lytic program of viral gene expression. Although VP16 is not directly involved in the human disease process, using part of a human pathogen's genome in rice may be undesirable in the current climate where genetically modified organisms (GMO) use is being hotly debated.

There are also patent rights granted on the use of GAL4 as a transactivator to E. I. du Pont de Nemours and Company, which limits the use of GAL4 in transgenic plants in certain jurisdictions. While the US patent has lapsed, it appears to be still in force in Australia at time of writing, and continuations and other patent applications may be pending in multiple jurisdictions.

All of the above pointed to the necessity of developing an alternative transactivator in order to deal effectively with technical problems, public acceptance issues and Freedom to Operate challenges for the project outcomes.

Transactivators are normally modular in structure and consist of at least two domains; a DNA binding domain and an activation domain. In this study, the strategy for developing new transactivators relied on the replacement of these two domains using proteins (or subdomains of proteins) for which a substantial amount of scientific information available.

Candidates for DNA-binding domain (DBD)

A total of five candidates were selected for testing as new DNA-binding domains. These candidates represent different classes of DNA-binding proteins and contain different numbers of potentially methylated sites in their DNA recognition sequences.

Transposase Ac from maize-Zea mays

The transposase of the maize transposon Activator (*Ac*) is an 807 amino acid protein, which catalyzes the "cut" and paste" of Ac transposon in the maize genome (Kunze *et al.*, 1987). The protein can bind specifically to a hexamer motif AAACGG, which is present many times in direct or inverted orientation at the terminal repeats of Ac (Kunze and Starlinger, 1989). Although the activity of Ac depends on the state of methylation of its DNA recognition sequence (Ki *et al.*, 2002), the transposon has been used quite intensively for transposon-tagging in many plant species such as *Arabidopsis thaliana* (Dean *et al*., 1991) suggesting that it may work for a large number of species.

In this project, the 427 amino acids region (amino acid 103 to 530) covering the NLS and DNA-binding domain (Feldmar and Kunze, 1991) was used as a DNA-binding domain candidate.

Regulatory protein AraC from Escherichia coli

AraC is a regulatory protein, which binds the inducer *L*-arabinose and activates the transcription of three widely separated *ara* operons involved in arabinose metabolism in *Escherichia coli* (Hirsh and Schleif, 1977; Lee *et al.*, 1981; Hendrickson and Schleif, 1984; Lobell and Schleif, 1990). The 292-amino acid protein consists of an N-terminal half (amino acids 1-170) responsible for *L*-arabinose binding and dimerization, a linker of five amino acids and C-terminal half (amino acids 178-292) for specific DNA-binding (Bustos and Schleif, 1993).

AraC binds to different regulatory sequences in *ara* operons depending on the availability of *L*-arabinose such as $araO_1$, $araO_2$, and *araI* (with two half sites $araI_1$ and $araI_2$) (Hendrickson and Schleif, 1984; Lee *et al.*, 1987; Hamilton and Lee, 1988; Bustos and Schleif, 1993). The binding of AraC to the reconstructed binding sites $araI_1$-$araI_1$ (or I_1-I_1) which contains no potential site for

methylation ($araI_1$: TATGGATAAAAATGCTA), does not require the presence of *L*-arabinose (Bustos and Schleif, 1993).

Furthermore the structure of this protein has been studied in detail (Schleif, 1996), which may open the opportunities for its further engineering as a DNA binding domain for new transactivators.

In this project, the whole AraC protein was used as a DNA-binding domain candidate with its reconstructed binding (I_1-I_1) site.

Regulatory protein cro from Escherichia coli

The λ phage cro repressor is involved in the regulatory switch that determines the lytic or lysogenic, development pathways, of an infecting λ phage in E. coli (Johnson et al., 1981). This small protein of 66 amino acids binds to its 17bp operator as a dimer with different affinity, with the highest being to the consensus (TATCACCGCCAGTGGTA) and OR_3 sequence (TATCACCGCAAGGGATA) (Kim et al., 1987). The structure of the protein with or without its operator has been well studied (Anderson *et al.*, 1981; Brennan *et al.*, 1990). The fusion between cro and an activation domain from λ repressor has been demonstrated as a transactivator (Bushman and Ptashne, 1988).

In this project, the cro repressor was used with its consensus and OR3 sequence.

Regulatory protein GusR from Escherichia coli

GusR is one of the proteins that negatively controls the expression of the β-glucuronidase gene in the gusRABC operon of *E coli* (Blanco *et al*. 1986; Blanco, 1987). GusR consists of 197 amino acids (Jefferson *et al.*, 1997) and binds specifically to two DNA sequences in the *gusA* gene with a greater affinity for one of them with the sequence CGAACGAACGTTCGGTTGC (Blanco, 1987).

In this study, *GusR* was used together with its best DNA recognition sequence.

Intron-encoded homing endonuclease I-PpoI

I-PpoI protein is an intron-encoded homing endonuclease from *Physarum polycephalum.* This endonuclease is encoded by the third intron located within the extra-chromosomal nuclear DNA encoding the large ribosomal RNA subunit (Muscarella *et al.*, 1990). I-PpoI is a small protein with two alternative translation products of 163 and 183 amino acids. Both are active, recognize a sequence of 13-15 nucleotides and induce a double-strand break within this sequence for insertion of the intron (Ellison and Vogt, 1993). The protein can bind to its target in the absence of Mg^{2+}, but requires a divalent cation as its only cofactor for cleavage (Wittmayer and Raines, 1996). Although highly specific, the protein tolerates some level of degeneracy within its recognition sites (Wittmayer *et al.*, 1998).

The structure as well as the chemical mechanism for DNA cleavage of this protein has been studied in detail. Replacement of histidine at position 98 by alanine abolishes the catalytic activity of the protein, but it retains its DNA binding ability (Friedhoff *et al*., 1999; Galburt *et al.*, 1999; Mannino *et al*., 1999).

This variant of *I-PpoI* (variant H98A) was therefore considered as a good candidate for the DNA binding domain of a new transactivator and was used for testing as DNA binding domain with its original and three modified DNA recognition sequences.

Candidates for Activation Domain (AD)

Four activation domain candidates were selected among plant or plant related bacterial proteins. In contrast to DBD candidates, the plant origin of AD candidates may be more critical for the interactions of new transactivators with other proteins involved in the transactivation process and the basal transcriptional machinery.

AvrXa10 from Xanthomonas oryzae pv. oryzae

AvrXa10 from *Xanthomonas oryzae* pv. *oryzae*, the causal agent for rice bacterial blight, is a member of the *avrBs3* avirulence gene family (Bonas *et al.*, 1989). These *avr* genes interact with the corresponding resistance genes from plant cultivars to trigger several reactions which lead to suppression of the growth and ingress of the pathogen (Scheel 1998). AvrXa10 protein contains an activation region of 38 amino acids at the C-terminus, which is required for avirulence activity. Thirty one amino acids from this region when fused to GAL4-DBD was able to activate transcription in yeast and *Arabidopsis thaliana* (Zhu *et al.* 1998). One mutant, with replacements at three hydrophobic amino acid residues in the region, was defective in transcriptional activation in yeast and avirulence activity in rice. When it was replaced by the activation region from VP16, the avirulence activity of AvrXa10 was restored. Thus, this region is clearly not involved in the interaction with other components in the host organism to determine the specificity of avirulence, but purely in transactivation. This region therefore was used as an AD candidate for new transactivators in this study.

Maize-Dof1

Maize *Dof1* (or *MNB1a*, Yanagisawa and Izui 1993) is a member of the *Dof* plant transcriptional factor family which are involved in tissue-specific and light regulated gene expression. The protein contains two separable domains, a DNA-binding domain in the N-terminal region and an activation domain in the C-terminal region. The C-terminus (136-238) was shown to be sufficient as an activation domain when fused to the GAL4-DBD (Yanagisawa and Sheen, 1998). The activation domain of this protein is functional not only in plants, but also in yeast and human cells (Yanagisawa 2001).

In this study, the region of 63 amino acids between positions 175-238 was used as an AD candidate for new transactivators.

Viviparous1 from maize-Zea mays

Viviparous 1 (VP1) from maize is a transcriptional activator involved in the process of seed maturation (McCarty *et al*., 1991). The N-terminal region of VP1 (amino acids 1-121) contains an activation sequence, which was independently functional when fused with the GAL4-DBD in maize protoplasts. In addition, VP1 functioned normally when its 121-amino acid activation region was replaced by VP16 AD (McCarty *et al*., 1991).

In this study, this 121-amino acid region was used as an activation domain candidate for new transactivators.

RisBz1 from rice-Oryza sativa

RisBz1 is one of the five rice basic leucine zipper transcription factors of maize Opaque 2-like proteins (Onodera *et al*., 2001). They are endosperm-specific and are involved in the regulation of seed storage protein synthesis (Dierks-Ventling, 1981; Kodrzycki *et al.*, 1989). RisBz1 consists of 436 amino acids with a proline-rich activation domain located in the first 27 amino acids from the N-terminus. This region, when fused with the GAL4-DBD, was able to activate the expression of UAS(GAL4)-MP-GUS in rice protoplasts (Onodera *et al*., 2001). This region of 27 amino acids was used as an activation domain candidate for new transactivators.

Establishment of stably transformed rice callus lines with DNA binding domain candidates fused to VP16 activation domain

The stably transformed callus lines were generated by co-transforming transactivator plasmids containing different DBD candidates fused to VP16-AD and their corresponding GUS reporter plasmids. As the negative control, pFX.J.99.2 carrying the *hph* gene and EGFP in its T-DNA was used in the place of transactivator plasmids.

The plasmids were co-transformed into rice callus of Millin cultivar using the *Agrobacterium*-mediated DNA transformation method. The transformation was repeated three times, each with about 150 pieces of callus per combination. Independent hygromycin-resistant lines were established in the

3-4 weeks after transferring onto the selection medium by separating different proliferating secondary callus from each initial callus. This early separation minimized the chance of taking multiple proliferations from the same transformation event within a callus because of overgrowth at later stages.

Lines surviving selection were transferred onto the fresh selection medium every two weeks and were grown for a period of 3-4 weeks before harvesting for GUS staining, extracting protein for GUS assay, extracting RNA for RT-PCR or regenerating plants.

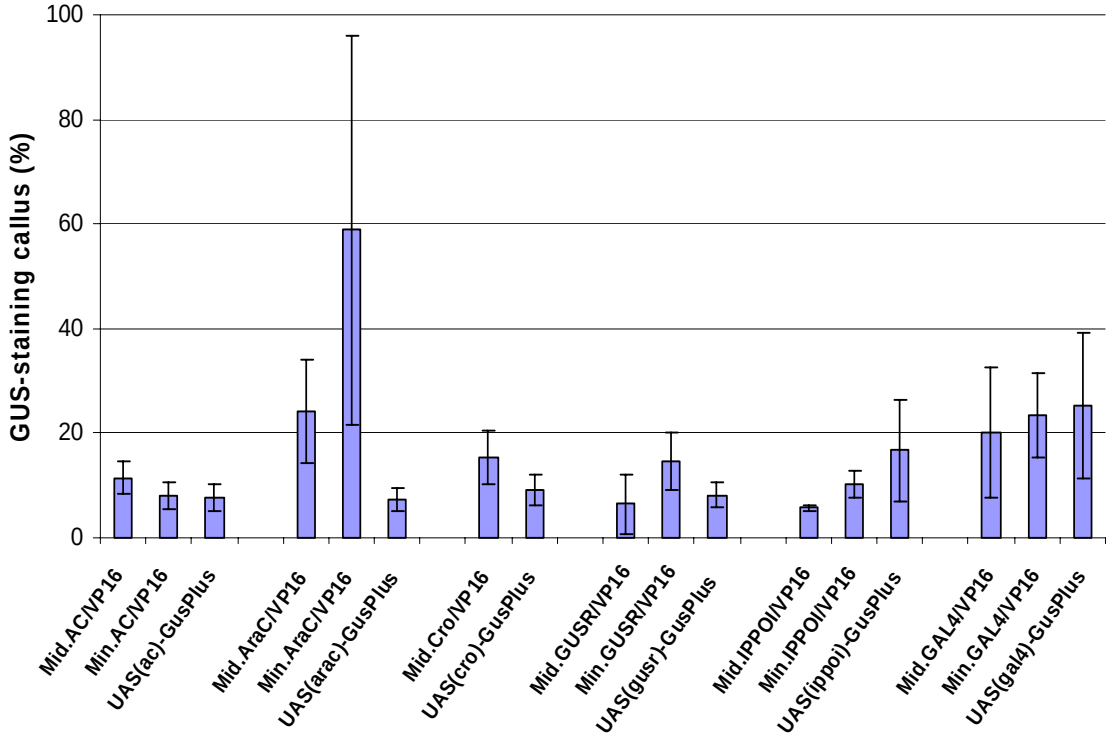


Figure 11. GUS staining of callus from hygromycin-resistant lines with different DBD/VP16 candidates and their negative controls ($UAS_{(DBDs)}$-GusPlus). Callus was stained by incubating at $37^{O}C$ in GUS staining solution. Callus with any GUS-stained spot was considered as GUS stained callus. The average percentage of GUS-stained callus was calculated for each transformation.

Activity of DNA binding domain candidates fused to VP16 activation domain: Results of GUS staining

After about 6-8 weeks in the selection medium, callus from each hygromycin-resistant line was harvested into individual wells in 96-well plates. The materials were incubated at 37 C in GUS staining solution after vacuum-infiltration for 5 min. The staining of callus was observed and recorded after 5 min, 1 hr and 12 hrs. Callus with any GUS-stained spots was considered as expressing GUS. GUS staining in the callus was considered "strong" if it turned blue after 5 min, "medium" after 1 hr and "weak" after 12 hrs in the staining solution at 37 C.

On average, the percentage of GUS-stained callus ranged between 5.7-58.9 % (Figure 11). The percentage of GUS stained callus was generally low in the negative controls, but higher with the presence of DBD/VP16 candidates. Among the negative controls, the highest value of GUS-stained calli was from *I-PpoI*/VP16 and GAL4/VP16. A significant increase in GUS staining was observed in the calli transformed with *AraC*/VP16 in both versions compared to the respective negative control. The introduction of extra DNA fragments at 5' end of the DBD candidates led to a decrease in the activity of DBD/VP16 candidates, although not significantly in most cases.

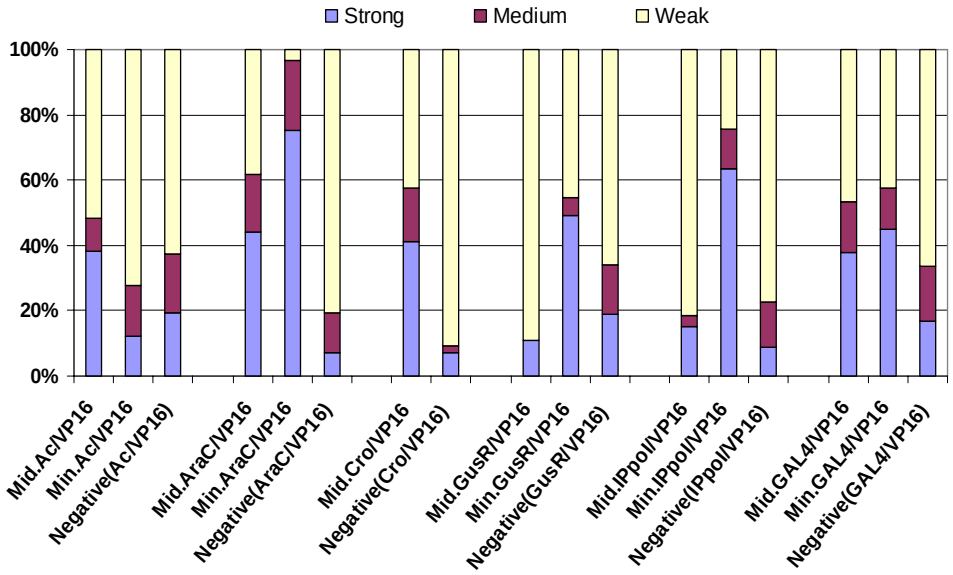


Figure 12. Intensity of GUS staining expressed as percentage of the overall GUS-stained callus. GUS staining was considered to be strong, medium and weak if the callus stained blue after 5 min, 1 hr and 12 hrs, respectively at 37^{O}C in the staining solution.

More than 63% of GUS-positive callus in the negative controls stained weakly. A significant increase in the percentage of strongly GUS-stained callus was observed for both versions of Arac/VP16 and GAL4/VP16, the Mini version of IPpoI/VP16 and the Midi Version of Cro/VP16 (Figure 12). AraC/VP16 (Mini Version) had not only more callus with GUS staining, but also the staining of these callus was stronger than that of GAL4/VP16. The negative control of AraC/VP16 had fewer and weaker GUS staining calli compared to the negative control of GAL4/VP16. *AraC* was therefore selected as the best performing candidate DBD for the fusion with activation domain candidates.

RT-PCR analysis of DNA binding domain candidates fused to VP16 activation domain

To confirm proper expression and processing of the transactivator genes (DBD/VP16) at the RNA level, reverse transcription of these genes from RNA samples isolated from the transformed materials described above was carried out. Total RNA was extracted from eight different GUS-stained lines using Triazol Reagent (Life Technologies). About 1 µg of total RNA was treated with RNA-free DNase (Promega) to remove any trace of genomic DNA contamination. A half of µg each of these DNAse-treated RNA samples was reverse-transcribed and amplified by specific primers for each DBD/VP16 candidate or in combination with primers for GUSPlus (Table 13) and One-step RT-PCR kit (QIAGEN). For the negative controls, the other half of each DNase-treated RNA sample was amplified using RedTaq DNA Polymerase (Sigma) and the same sets of specific primers, but without reverse transcription. These negative controls were used to confirm the absence of DNA in the DNase-treated RNA samples. The amplified products were separated on 1.2% agarose gels.

Table 13: Specific primers used for RT-PCR analysis

DBD/VP16	Primers	Primer sequence	Product (bp)
Ac/VP16	TNT33 VP16-R	ACCATGGCTATTGTTCATGAACCAC CCACCGTACTCGTCAATTCCA	1526
AraC/VP16	TNT41N VP16-R	ATGGCTGAAGCGCAAAATGA (above)	1118
Cro/VP16	TNT12N VP16-R	ACCATGGAACAACGCATAACC (above)	446
GusR/VP16	TNT61 VP16-R	CCACCATGGATAACATGCAGACTGAAG (above)	811
IPpoI/VP16	TNT16N VP16-R	ATGGCGAAATCCAACCAAGC (above)	797
GAL4/VP16	TNT6N VP16-R	ATGGGCAAGCTCCTGTCCTC (above)	686
GUSPlus	306-F 306-R	AGCGAGCAATGTGATGGATTTC GTTGGCGATGCTCCACATCA	306

No product was recovered from the negative controls for all DBD/VP16 samples, indicating that DNA digestion during RNA sample preparation was complete. The RT-PCR products from both versions of DBD/VP16 candidates were single bands and with the expected size, except for both versions of *Ac*/VP16 (Figure 5.5 and 5.6). Co-amplification of transactivator genes and GUSPlus from Midi Version RNA samples was successful. The RNA product of the GUSPlus gene was present in all GUS-stained samples. The situation was not the same for the products of the transactivator genes, especially in the case of *Ac*/VP16. In this case, there were two RT-PCR products (500 and 750 bp) amplified from some of the tested samples in both versions. These products clearly were not full-length RT-PCR products of *Ac*/VP16 (the expected size of this would be approximately 1500 bp), but could be the products of alternative splicing of *Ac*/VP16 primary transcript or non-specific amplification product(s) from the rice genome.

The expression of GUS in the samples without the products of the transactivator genes may be explained by the influence of a strong enhancer from the genomic DNA region near the insertion site of the T-DNA fragment of the transactivator plasmid. The low frequency of GUS staining in the hygromycin-resistant callus and the absence of the products of transactivator genes in GUS-stained callus may explain the poor performance of these DBD candidates, especially *Ac*/VP16.

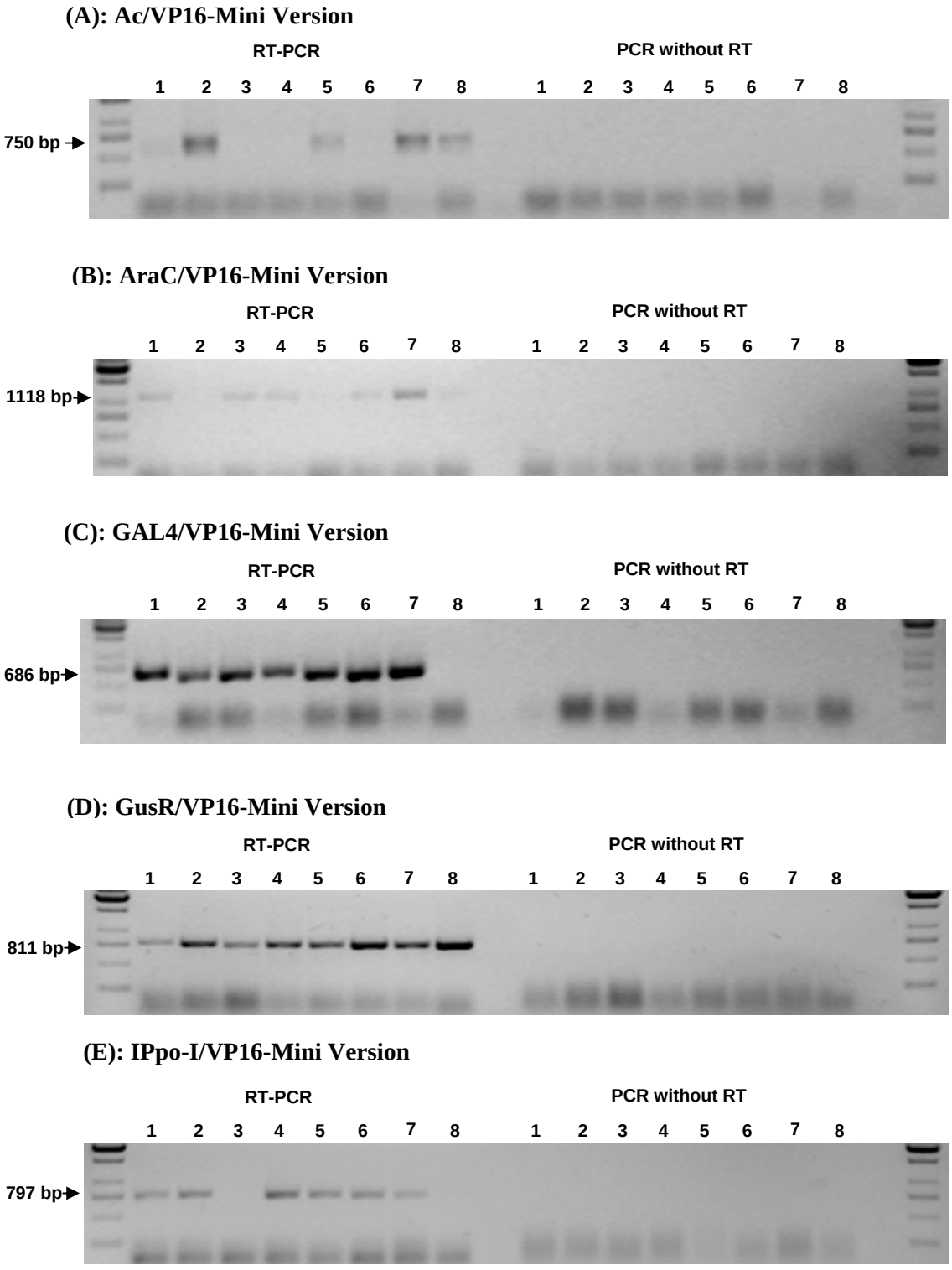
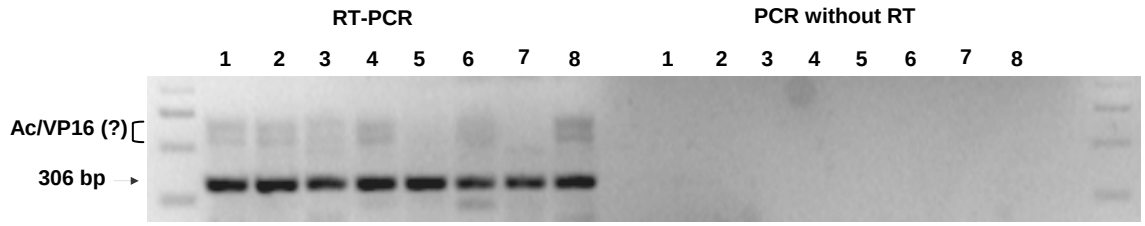
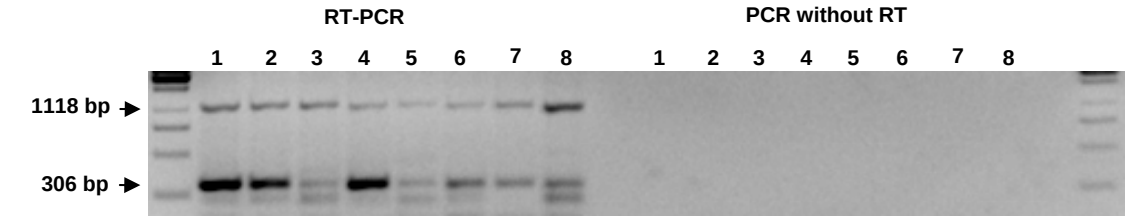


Figure 13. RT-PCR products of samples extracted from GUS-stained secondary callus. These calli were transformed with different DBD/VP16 (Mini Version) and their reporter-UAS-MP-GUSPlus. RNA samples were reverse-transcribed and amplified (eight samples in the left) or directly amplified without reverse transcription (eight samples in the right) using a pair of specific primers for each DBD/VP16 candidate. The numbered lanes within each gel represent, in each gel, the same corresponding samples.

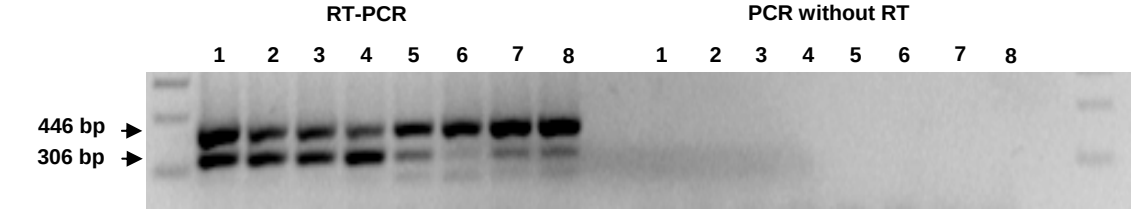
(A): Ac/VP16-Midi Version



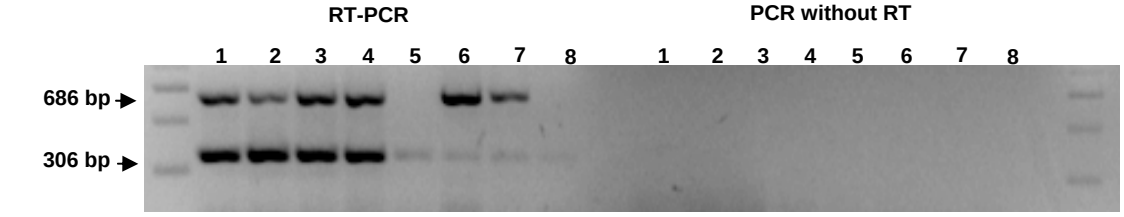
(B): AraC/VP16-Midi Version



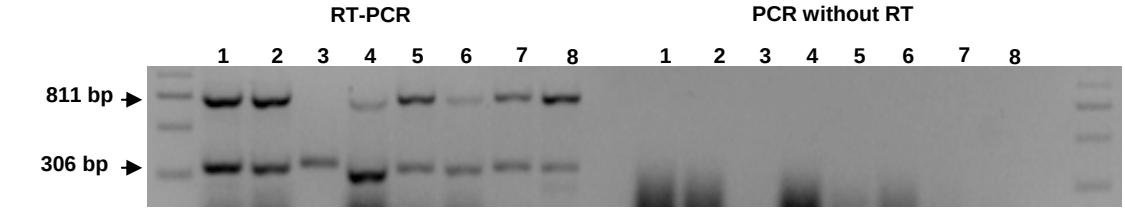
(C): Cro/VP16-Midi Version



(D): GAL4/VP16-Midi Version



(E): GusR/VP16-MiDi Version



(F): IPpoI/VP16-Mini Version

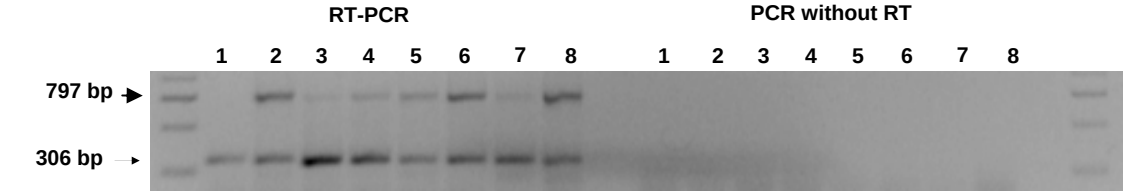


Figure 14. RT-PCR products of samples extracted from GUS-stained secondary callus. These calli were transformed with different DBD/VP16 (Midi Version) and their reporter -UAS-MP-GUSPlus. RNA samples were reversely transcribed and amplified (eight samples in the left) or directly amplified without reverse transcription (eight samples in the right) using a pair of specific primers for each DBD/VP16 candidate. The numbered lanes within each gel represent, in each gel, the same corresponding samples.

Testing activation domain candidates

The second and final step for assembling new transactivators is the identification of the best activation domain (AD) among the four candidates described above. These candidates are tested in the fusion with GAL4-DBD and AraC- the best DBD candidate identified. The final testing is carried out in rice and tobacco, the representatives of mono- and dicotyledonous plants.

Performance of activation domain candidates in tobacco

Transactivator plasmids carrying AD candidates fused to GAL4-DBD or AraC were co-transformed into tobacco leaf disks together with their respective GusPlus reporter plasmids. For the negative controls, the reporter plasmids for GAL4-DBD and AraC were used for transformation together with plasmid pFX.J.99.2 carrying no transactivator gene (Table 14). The transformation was carried out by the *Agrobacterium*-mediated method.

In total fifteen leaf disks of the same size were used for each co-transformation which was repeated three times. These leaves were co-cultivated with the bacteria on RMOP medium for 48 hrs in darkness at 25-26^{O}C before transferring to the selection medium RMOP-TCH for selection and plant regeneration. The regenerated shoots were rooted in MST-TCH before transferring to the glass house.

For each repeat, ten disks from each co-transformation were taken for GUS staining after seven days on the selection medium. The leaves were washed briefly in sterile distilled water before transferring into GUS staining solution. After 5 min vacuum infiltration, the samples were kept at 37^{O}C for overnight. GUS staining was observed and counted after de-staining in 70% ethanol for a few days. The average number of GUS-stained spots per disk was calculated.

Table 14: Plasmid combinations used for testing of AD candidates in Tobacco

Candidates	**Plasmid Combination**	**Plasmid's T-DNA Characteristics**
GAL4-DBD/AD candidates		
Negative Control (GAL4/VP16)	pFX.J.99.2 pTNT.F.68	Ubi Pro-*hph*, Ubi Pro-EGFP $UAS_{(GAL4)}$-MP-GusPlus
GAL4/VP16 (Positive Control)	pTNT.G.42.GAL4 pTNT.F.68	Ubi Pro-*hph*, 35S Pro-GAL4/VP16 $UAS_{(GAL4)}$-MP-GusPlus
GAL4/RisBZ1	pTNT.Q.23.1 pTNT.F.68	Ubi Pro-*hph*, 35S Pro-GAL4/RisBZ1 $UAS_{(GAL4)}$-MP-GusPlus
GAL4/Dof1	pTNT.Q.23.2 pTNT.F.68	Ubi Pro-*hph*, 35S Pro-GAL4/Dof1 $UAS_{(GAL4)}$-MP-GusPlus
GAL4/VP1	pTNT.Q.23.3 pTNT.F.68	Ubi Pro-*hph*, 35S Pro-GAL4/VP1 $UAS_{(GAL4)}$-MP-GusPlus
GAL4/AvrXa10	pTNT.Q.23.4 pTNT.F.68	Ubi Pro-*hph*, 35S Pro-GAL4/AvrXa10 $UAS_{(GAL4)}$-MP-GusPlus
AraC/AD candidates		
Negative Control (AraC/VP16)	pFX.J.99.2 pTNT.I.65	Ubi Pro-*hph*, Ubi Pro-EGFP $UAS_{(AraC)}$-MP-GusPlus
AraC/VP16 (Positive Control)	pTNT.G.42.AraC pTNT.I.65	Ubi Pro-*hph*, 35S Pro-AraC/VP16 $UAS_{(AraC)}$-MP-GusPlus
AraC/RisBZ1	pTNT.Q.22.1 pTNT.I.65	Ubi Pro-*hph*, 35S Pro-AraC/RisBZ1 $UAS_{(AraC)}$-MP-GusPlus
AraC/Dof1	pTNT.Q.22.2 pTNT.I.65	Ubi Pro-*hph*, 35S Pro-AraC/Dof1 $UAS_{(AraC)}$-MP-GusPlus
AraC/VP1	pTNT.Q.22.3 pTNT.I.65	Ubi Pro-*hph*, 35S Pro-AraC/VP1 $UAS_{(AraC)}$-MP-GusPlus
AraC/AvrXa10	pTNT.Q.22.4 pTNT.I.65	Ubi Pro-*hph*, 35S Pro-AraC/AvrXa10 $UAS_{(AraC)}$-MP-GusPlus

In the GAL4-DBD fusion, the highest number of GUS-stained spots per leaf disk was from *AvrXa10* with 34 spots/disk, but it was not statistically significant different at 1% level of significance to those from the fusion with *Dof1* and VP16, which both produced similar number of GUS-stained spots of 30 (Table 15).

Table 15: Average number of GUS-stained spots induced by co-transformation of two plasmids, which independently carry an activation domain candidate fused to GAL4-DBD or *AraC* and a GUS gene under GAL4- or *AraC*-responsive promoter

Candidates	**GUS-stained spots per leaf disk [(a)]**	**DMRT [(b)]**
GAL4-DBD/AD candidates		
Negative Control (GAL4/VP16)	0	d
GAL4/VP16 (Positive Control)	30	ab
GAL4/RisBZ1	0	d
GAL4/Dof1	30	ab
GAL4/VP1	9	bc
GAL4/AvrXa10	34	a
AraC/AD candidates		
Negative Control (AraC/VP16)	0	d
AraC/VP16 (Positive Control)	39	a
AraC/RisBZ1	0	d
AraC/Dof1	8	c
AraC/VP1	20	ab
AraC/AvrXa10	51	a

(a) Average of three replications, each with ten leaf disks
(b) Any two means having a common letter are not significantly different at the 1% level of significance

In an *AraC* fusion, AvrXa10 was the best AD candidate with the average number GUS-stained spots/disk of 51 and was followed by VP16 (Positive control) and VP1 with 39 and 20 spots/disk, respectively.

There was not a single GUS-stained spot observed in the negative controls and in the RisBZ1 fusion sample.

Although values for the top three AD candidates in fusion with GAL4 DBD or *AraC* were not significantly different due to the big variation among the repeats, *AvrXA10* was always the best candidates especially when fused to *AraC*. These three candidates were used for further testing in rice.

Performance of activation domain candidates in rice callus

Further testing of AD candidates was carried out in rice in both fusions- with GAL4-DBD (for control) and *AraC* (as the best DBD candidate). Plasmids carrying these candidates were co-transformed into callus of Millin cultivar with their corresponding EGFP reporter plasmids. GAL4/VP16 and its deleted version were used for the positive and negative controls, respectively. To identify the possible “direct”

interaction of these AD candidates with UAS of GAL4-DBD or *AraC*, the deleted version of GAL4-DBD was fused to these candidates and used in the transformation together with the reporter plasmid for GAL4-DBD or for *AraC* (Table 16).

The experiment was repeated three times, with approximately 200 pieces of callus for each co-transformation. The calli were washed after three days co-cultivation and transferred to the selection medium. Transfer of the callus to the fresh selection medium was every two weeks. EGFP was observed under Leica Fluorescent Microscope with filter sets for EGFP at 1, 7, 14, 21 and 28 days after transferring to the selection medium.

In the fusion with GAL4-DBD (Figure 15), the percentage of callus with EGFP expression was generally highest at the first day, then gradually decreased and stabilized after two weeks after transferring onto the selection medium. There was insignificant EGFP expression in the negative control and in the transformations where the fusions with the deleted version of GAL4-DBD were used. *AvrXa10* and *Dof1* performed better than the positive control –VP16-AD.

Table 16: Plasmid combinations used for testing of AD candidates in rice

Candidates	**Plasmids**	**T-DNA Characteristics**
GAL4-DBD/AD candidates		
GAL4/*RisBz1*	pTNT.P.13	*Ubi* Pro-GAL4/*RisBz1*
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
GAL4/*Dof1*	pTNT.P.14	*Ubi* Pro-GAL4/*Dof1*
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
GAL4/VP1	pTNT.P.15	*Ubi* Pro-GAL4/VP1
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
GAL4/*AvrXa10*	pTNT.P.16	*Ubi* Pro-GAL4/*AvrXa10*
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
AraC/AD candidates		
AraC/Dof1	pTNT.P.46	*Ubi* Pro-*AraC/Dof1*
	pTNT.Q.18	$UAS_{(AraC)}$-MP-EGFP, $UAS_{(AraC)}$-MP-*hph*
AraC/VP1	pTNT.P.47	*Ubi* Pro-*AraC*/VP1
	pTNT.Q.18	$UAS_{(AraC)}$-MP-EGFP, $UAS_{(AraC)}$-MP-*hph*
AraC/AvrXa10	pTNT.P.48	*Ubi* Pro-*AraC/AvrXa10*
	pTNT.Q.18	$UAS_{(AraC)}$-MP-EGFP, $UAS_{(AraC)}$-MP-*hph*
The Controls		
Negative Control (GAL4/VP16)	pTNT.M.47	*Ubi* Pro-deleted GAL4/VP16
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
Positive Control (GAL4/VP16)	pTNT.L.89	*Ubi* Pro-GAL4/VP16
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
Negative Control (*RisBz1*/GAL4)	pTNT.P.26	*Ubi* Pro-deleted GAL4/*RisBz*1
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
Negative Control (*Dof1*/GAL4)	pTNT.P.27	*Ubi* Pro-deleted GAL4/*Dof1*
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
Negative Control (VP1/GAL4)	pTNT.P.28	*Ubi* Pro-deleted GAL4/VP1
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
Negative Control (*AvrXa10*/GAL4)	pTNT.P.29	*Ubi* Pro-deleted GAL4/*AvrXa10*
	pTNT.L.114.1	$UAS_{(GAL4)}$-MP-EGFP, $UAS_{(GAL4)}$-MP-*hph*
Negative Control (*Dof1/AraC*)	pTNT.P.27	*Ubi* Pro-deleted GAL4/*Dof1*
	pTNT.Q.18	$UAS_{(AraC)}$-MP-EGFP, $UAS_{(AraC)}$-MP-*hph*
Negative Control (VP1/*AraC*)	pTNT.P.28	*Ubi* Pro-deleted GAL4/VP1
	pTNT.Q.18	$UAS_{(AraC)}$-MP-EGFP, $UAS_{(AraC)}$-MP-*hph*
Negative Control (*AvrXa10/AraC*)	pTNT.P.29	*Ubi* Pro-deleted GAL4/*AvrXa10*
	pTNT.Q.18	$UAS_{(AraC)}$-MP-EGFP, $UAS_{(AraC)}$-MP-*hph*

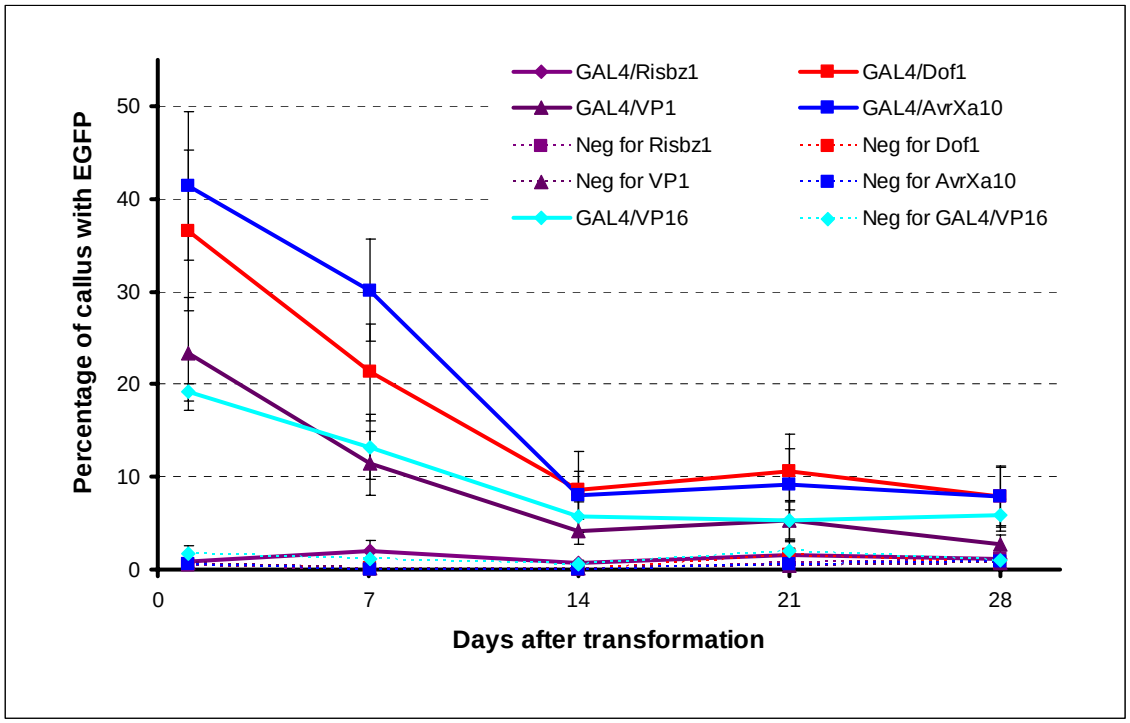


Figure 15. Activity of transactivator candidates in the fusion with GAL4-DBD in rice callus. Transactivator candidate genes and the reporter $UAS_{(GAL4)}$-MP-EGFP were co-transformed into rice callus. The experiment was repeated three times, each with approx. 200 calli per co-transformation. For negative controls, AD candidates fused to the deleted version of GAL4-DBD were used for transformation. The expression of EGFP was observed at different time points and the percentage of callus with EGFP expression was calculated.

The percentage of callus with EGFP for GAL4/*Dof1* and GAL4/*AvrXa10* was 36.6% and 41.4%, respectively, compared to 19.2% of GAL4/VP16 at the first day and 8.5% and 8.1%, respectively, compared to 5.7% of GAL4/VP16 after two weeks. GAL4/*RisBz1* could not induce any significant level of EGFP in the callus as shown before in the test in tobacco.

In the fusion with *AraC*, the percentage of callus with EGFP expression was more or less similar to that in the fusion with GAL4-DBD, but with a little delay. As a result the highest EGFP expression was observed between 1-7 days after transformation and stabilized between the second and the third week (Figure 16). Again, *AvrXa10* was the best AD candidate with EGFP expression about 52% compared to that of about 39-45% for GAL4/VP16 at the first week and stabilized at the level of about 20% compared to that of about 18% for GAL4/VP16 in the third week. Both *VP1* and *Dof1* in the fusion with *AraC* were weaker than GAL4/VP16. All the negative controls had insignificant number of callus with EGFP expression.

In all transformations, the only callus survived and proliferating were those with EGFP expression. Calli without EGFP turned brown and died slowly in the selection medium. However, none of EGFP callus in the negative controls survived. Plants were also regenerated from EGFP expressing callus. These plants grew and developed normally.

The results of these two experiments in rice callus clearly showed that *AvrXa10* is the best candidate in both fusions: with GAL4-DBD and *AraC*.

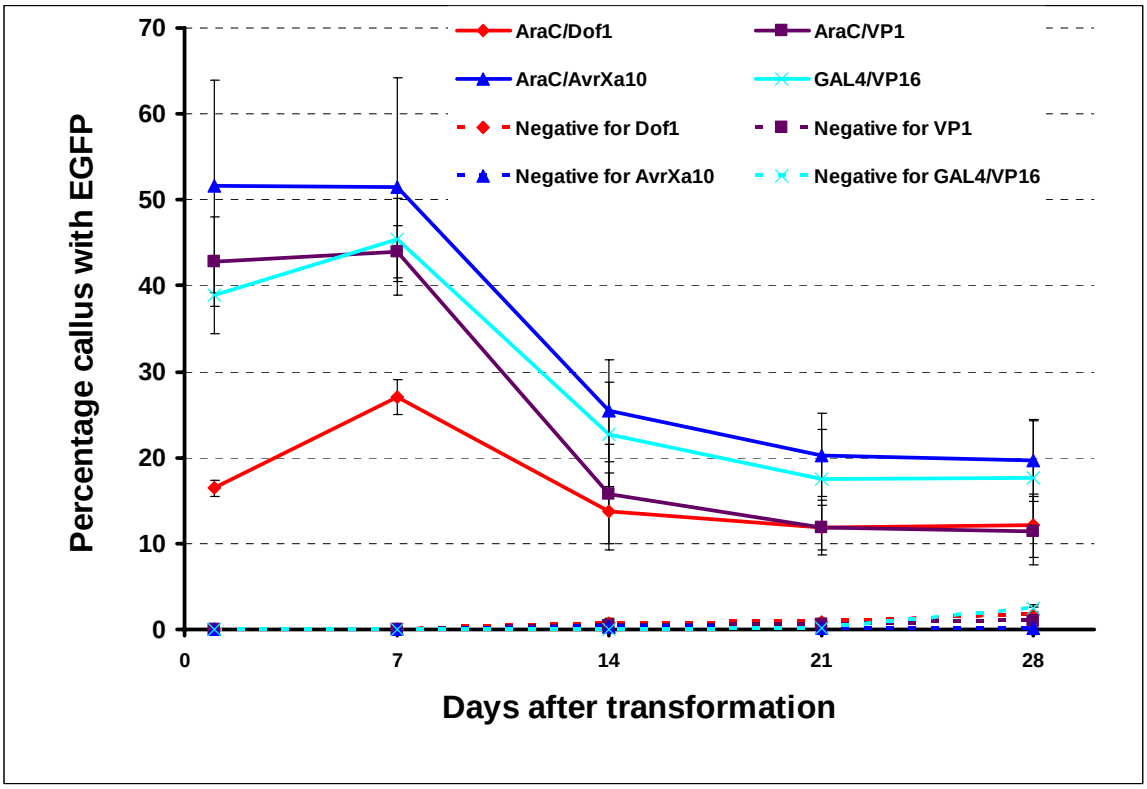


Figure 16. Activity of transactivator candidates in the fusion with *AraC* in rice callus. Transactivator candidates' genes and the reporter $UAS_{(AraC)}$-MP-EGFP were co-transformed into rice callus. The experiment was repeated three times, each with approx. 200 calli per co-transformation. For negative controls, AD candidates fused to the deleted version of GAL4-DBD were used for transformation. The expression of EGFP was observed at different time points and the percentage of callus with EGFP expression was calculated.

Development of Enhancer Trap population using novel transactivator

Construction of plasmids for new "Enhancer Trap" strategy with AraC/AvrXa10 transactivator

Two plasmids were constructed based on the newly developed transactivator: the *AraC/AvrXa10* "Enhancer Trap" plasmid and the target (or reporter) plasmid.

Transactivator plasmid- pTNT.Q.61-3

First, the DNA fragment containing the GAL4/VP16, the $UAS_{(GAL4)}$-MP and a part of the GusPlus gene (*NcoI* and *BglII*) in pTNT.K.15.GAL4 was replaced by the DNA fragment of the *AraC/AvrXa10* (*NcoI* and *BglII*) from pTNT.Q.22-4 (pTNT.Q.61-1). At the same time, the $UAS_{(GAL4)}$-MP (*HindIII* and *MfeI*) in pTNT.K.15.GAL4 was replaced by the $UAS_{(AraC)}$-MP (*HindIII* and *MfeI*) (pTNT.Q.61-2). Finally, the DNA fragment between two *BglII* sites in the second intermediate plasmid pTNT.Q.61-2 was inserted into the *BglII* site in the first intermediate plasmid pTNT.Q.61-1 to generate a new Enhancer Trap plasmid- pTNT.Q.61-3.

Target (Reporter) plasmid: pTNT.Q.60

This is a reporter plasmid for mis-expression of a gene which is located downstream of the UAS-MP by random insertion of this plasmid in the plant genome.

The plasmid was created by replacing the $UAS_{(GAL4)}$ from pPSI.A.67-1 (CAMBIA plasmid) by the $UAS_{(AraC)}$ from pTNT.P.42 using the *HindIII* and *SpeI* sites. PCR was used to verify the presence of

this DNA fragment between the *hph* and the $UAS_{(AraC)}$ using the specific primers of TNT82 and Hyg510F.

Co-transformation-based enhancer trap system

Rice populations (Millin) were established from the newly developed constructs (Table 17) co-transformed into rice callus using *Agrobacterium*-mediated DNA transformation method.

Table 17: Constructs used and lines regenerated from the transformation

Transformation	Plasmids used	Characteristics of the plasmids	Regenerated lines
Enhancer Trap	pTNT.Q.61-3	MP-*AraC*/*AvrXa10*, $UAS_{(AraC)}$-MP-GusPlus	200
	pTNT.Q.60	35S-*hph*, $UAS_{(AraC)}$-MP	
Negative Control	pTNT.Q.103	MP-del.*AraC*/*AvrXa10*, $UAS_{(AraC)}$-MP-GusPlus	Not regenerated plants
	pTNT.Q.60	35S-*hph*, $UAS_{(AraC)}$-MP	
Positive Control	pCAMBIA1305.1	35S-*hph*, 35S-GusPlus	Not regenerated plants

Callus was harvested for GUS staining at different time points. Callus from "enhancer trap lines" were used to regenerate plants after two months in the selection medium. Further GUS staining was carried out before plantlets being transferred into the glasshouse.

GUS expression at the callus stage

Callus from all transformations was harvested for GUS staining (overnight at $37^{O}C$) at three time points: one day, one week and three weeks after the removal of *Agrobacteria.* The sampling was carried out from three independent transformation experiments. The average percentage of GUS stained callus from the positive control (*i.e.* with pCAMBIA1305.1) was used as the denominator for normalizing data obtained from the transformations among the repeats.

The average number of calli found to be transformed with only one plasmid pCAMBIA1305.1 (the positive control) was 57.7, 48.1 and 81.8 % at one day, one week and three weeks, respectively (Figure 17). GUS staining of callus in these transformations after normalization were 27.5, 19.5 and 14.2 % in one day, one week and three weeks, respectively. Values for the negative control were 4.9, 0.8 and 1.3 % correspondingly.

Functionality of the enhancer trap system was apparent not only with the significantly lower number of staining calli in negative controls, but also with the typically weaker staining of callus in the negative control compared to that from an enhancer trap population

GUS expression of "Enhancer Trap" population at plantlet stage

From the total of 1115 calli used for transformation, 208 independent lines were regenerated, a transformation efficiency of 18.7 %. Before transfer into the glasshouse, callus from the base of the shoots and one shoot with roots from each line were harvested for GUS staining as described before.

The percentage of lines positive for GUS only in the callus and the shoot was 6.4 and 2.7 % respectively. No line expressed GUS only in the root (Table 18). About 1.8 % of the lines expressed GUS in the callus and either in the roots or the shoots or in both of them. Only one out of 110 lines

tested had GUS expressed only in the shoot and the root, but not in the callus. The percentage of lines expressing GUS either in the callus, the shoot or the root was about 15.5 %.

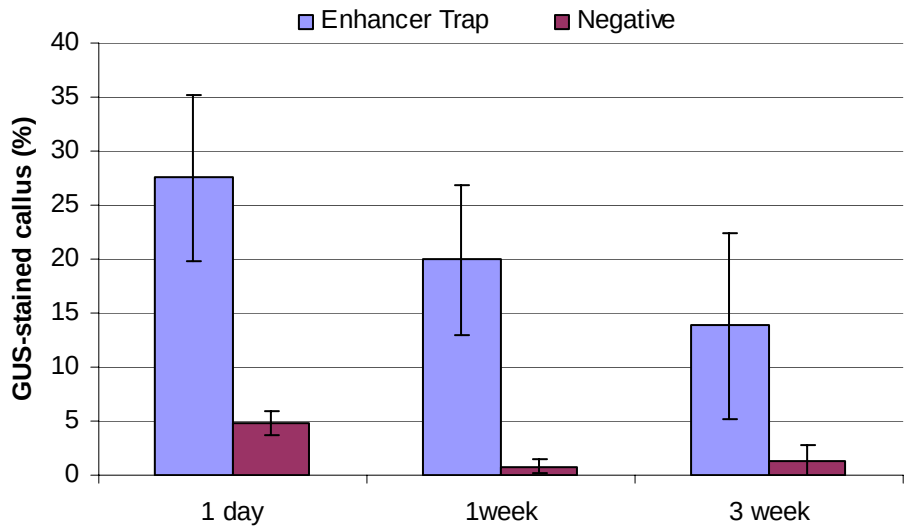


Figure 17: GUS staining of rice transgenic populations at callus stage. Rice callus from Millin were co-transformed with pTNT.Q.61-3 and its UAS pTNT.Q.60 using *Agrobacterium*-mediated DNA transformation method. For the negative control, pTNT.Q.103 with the deleted version of *AraC/AvrXa10* was used in the place of the "enhancer trap" plasmid for co-transformation. The experiment was repeated three times. About 80-90 calli were stained for GUS at each time point. pCAMBIA1305.1 was used as the positive control of the transformation and as the denominator for normalizing the data.

Table 18: Results of GUS staining of enhancer trap population at the plantlet stage

Tissues	Lines tested (No)	GUS positive lines	
		(No)	(%)
Callus only	110	7	6.4
Shoot only	110	3	2.7
Root only	110	0	0
Callus and shoot only	110	2	1.8
Callus and root only	110	2	1.8
Shoot and root only	110	1	0.9
Callus, shoot and root	110	2	1.8
Any of the tissues	110	17	15.5

Analysis of the enhancer trap lines developed with new transactivator

Thirty-two co-transformation experiments were conducted using a total of 21495 calli. Two of these experiments were found to be contaminated and were discarded. A total of 796 plantlets were transferred to the glasshouse. Transformation efficiency for these co-transformation experiments was 3.9% (total plants to glasshouse/ total number of calli less the contaminated experiments).

Most of the plantlets (at the *in vitro* stage) were assayed for GUS activity in the callus from the base of the shoot, and in the root and shoot (if possible). GUS activity was observed in 8% of the calli (62 out of 772 assayed), 2.8% of the roots (20 out of 702 assayed) and 3.2% of the shoots (22 out of 695 assayed).

Mature plants (glasshouse grown) were assayed for GUS activity in the mature leaves, inflorescence, and immature seed. GUS activity was observed in 2% of the leaves (4 out of 197 assayed), 36% of the inflorescences (36 out of 100 assayed) and 16.7% of the immature seeds (9 out of 54 assayed).

In general, analysis of the new population provides strong support for the improved performance of the new transactivator compared to Gal4/VP16. The wide diversity of reporter gene expression pattern and the results obtained for the negative controls suggest higher specificity of the *AraC* UAS. Also, the intensity of reporter gene expression is somewhat higher under control of *AraC/AvrXa10* than under control of the Gal4/Vp16 transactivator, both in rice and tobacco. Finally, the presence of reporter gene expression in various organs/ tissues indicates that at least in rice, the new transactivator does not require tissue-specific co-factors for its activity.

“Freedom to operate” considerations are equally important to technical evaluation, when considering biotechnological tools like tranactivator. The new transactivator will be available to rice industry without restrictions as an outcome of the project, together with the accumulated know-how.

Development of CAMBIAdb – Laboratory Information Management System for Transgenomics

CAMBIAdb was created in this project as a database to primary store plant transformation data and keep track of laboratory work. It was designed to follow standard lab procedures during the course of transformation experiment. For easy identification of each created physical entity during experiment, a bar coding system was introduced and all plates, pots and seed bags generated during each experiment were labeled and if necessary identified through barcode search utility.

CAMBIAdb was designed as typical client-server system, with multi-user secure access and user management utility. Users have various levels of access to functionality, so control of various procedures happening in the system is possible.

Additional features integrated into this system were:
Constructs management – closely related to each transformation experiment

Oligonucleotide management – related to PCR analysis, with automatic ordering system
Lab books management – record of users laboratory books

The main part of the system is designed in a "tree" approach, where user can define first transformation experiment and than track all plants generated during this experiment – stepping down from T0 generation to next generations of plants of the same line. Various attributes are attached to the whole experiment itself and also to each plant. Each plant can also be described through predefined phenotypic and DNA analysis parameters.

The system is a web-based application entirely made using open source components, i.e. LAMP architecture (Linux, Apache, MySQL, Perl). All a user needs to access it is a web browser and access to the server on which data and software components are located. The working system was also set up on a Windows based machine in the lab to establish platform independence.

Screen shots of CAMBIAdb are provided in electronic files appended to this report. Although most of the database aspects other than the oligo management were tailored to this particular transactivation project, as an open source software product it is available for further development if it is desired to adapt it to any other project.

Containment glasshouse operations

A GM containment glasshouse comprising two temperature-controlled rooms set at temperatures and daylength suitable for growing japonica rice was constructed and is maintained principally for the benefit of the Australian rice industry. The original site was to be ANU land in Weston Creek, but the Stromlo fires of 2003 resulted in siting it at the main ANU campus one block away from CAMBIA's leased premises on the CSIRO Black Mountain campus.

The GM containment glasshouse was administered during the term of the project by a committee consisting of Dr Russell Reinke and Dr Randall Williams representing the Australian rice industry, Dr Andrzej Kilian representing CAMBIA, Professor Murray Badger of ANU, and Jeff Davis representing RIRDC.

CAMBIA employed and continues to employ a full-time technician who maintains the plants in the GM containment glasshouse. ANU provides utilities, routine maintenance, and pesticide spraying, paid by RIRDC during the course of this project.

The largest portion of glasshouse space during the term of this project was occupied by rice plants grown for this project. The plants were grown from shoots regenerated from transformed and control material generated by CAMBIA. Transgenic rice plants were rooted onto soil trays and grown to maturity for collection of seed, which were assayed for reporter gene expression, counted, and weighed. Rice plants were also grown from these seed and controlled crosses were carried out in the glasshouse.

The glasshouse has also been used during the term of the project by two other rice culture projects. One is a project of ANU Fellow Dr Josette Masle to extend studies of DNA sequences that may be involved in gene expression related to root impedance from the model plant *Arabidopsis* to rice, and was facilitated by CAMBIA providing transformation services for a period of time and continuing to provide plant care. (CAMBIA has also provided plant care for a Charles Sturt University Ph.D. student working on the genetics of pigeonpea, who has been provided space in the glasshouse with the permission of the committee at the time she commenced the work). Experimental plans have not been made available to CAMBIA but may be available to RIRDC. This academic work is still ongoing, is of unknown duration, and has not resulted in any publications.

The other, a project of CAMBIA partially funded by the Rockefeller Foundation and partially by Horticulture Australia to develop a transformation system bypassing many patents surrounding *Agrobacterium* transformation (see a thorough analysis of the technology landscape at

www.bios.net/daisy/patentlens/tech_landscapes/78.html) has resulted in several publications and technology available royalty-free for improvement and commercialization by the Australian rice industry and all others willing to agree to open source terms of sharing technology and biosafety data.

The key publication is Broothaerts W, Mitchell H J, Weir B, Kaines S, Smith L M A, Yang W, Mayer J E, Roa-Rodriguez C, Jefferson R A (2005) Gene transfer to plants by diverse species of bacteria, *Nature* 433:629-633 . This was one of *Nature*'s most widely cited publications in 2005, according to the *Nature* website. This work was also analysed in detail for its technical value and ability to provide freedom to operate, in reviews in *Nature* and *Nature Biotechnology* in 2005 and in *Trends in Plant Sciences* in 2006.

Three patent applications are pending as a result of this project (published in the United States as US 2005/0289672 and US 2005/0289667, and as PCT Publication WO 2006/004914). The "Transbacter™" technology, which used the non-pathogenic soil bacterium *Sinorhizobium meliloti* to replace the pathogen *Agrobacterium* in transformation of rice and other species, is currently being further improved by CAMBIA with U.S.-sourced funding. All protocols used for the rice transformation by *Sinorhizobium* have been made available on the website www.bioforge.org maintained by CAMBIA, and the technology is available for license at www.bioslicense.net under the terms of sharing described above. To date over forty institutions have obtained strains on request.

5. Discussion of outcomes

In the course of generating large numbers of transactivation lines, information of use for rice tranformation was generated, such as the greater effectiveness of GUSPlus and EGFP over GUS and GFP as reporter genes.

Information of use for plant biotechnology in general was also derived, including demonstration of the enhancer effect of the 35S promoter driving the selectable marker, and that less of an enhancer effect is demonstrated with the *ubi1* promoter, which is quite effective in rice.

It was also demonstrated that it is possible to obtain interactions without operably linking the vector containing the open reading frame for the transcriptional activator, and the vector containing the DNA-binding activation site, by co-transformation, super-transformation, or in a cross.

A new transactivator construct was created, through identification of an activation domain from among multiple candidates, and a DNA binding domain from among multiple candidates, each of which work better in plants, and then recombining the best of the two domains into a single open reading frame.

This new transactivation gene was shown to be functional, and transformation using it gave rise to some pattern lines that could be useful later because the patterns in which reporter gene expression is enhanced are known and can be exploited for other genes, again without operably linking them to the transactivator constructs.

6. Recommendations to Industry

The transactivator constructs used in the early part of the work were not useful for locating large numbers of gain-of-function mutations in rice and characterising promoters, the initial goal of the project. The enhancer effect of the constructs was a problem that confounded phenotypic results. However, the rice genomic sequencing efforts external to Australia, which rapidly accelerated during the course of the project, changed the terms of reference. What was needed was no longer large numbers of mutations, but

- ways to work around the large number of patent applications that followed the release of genomic sequence, to the extent that licenses to such patents may not be widely available or that they may block access to markets external to Australia.
- Ways to understand and direct gene expression now that we understand the genome to be more complex and studies of single gene insertions effects to be too simplistic

One patent work-around was developed and a PCT application was filed in order to prevent its patenting by any other party that could exclude others from using it. This was a strategy to co-transform or cross the vector containing the open reading frame for the transcriptional activator, and the vector containing the DNA-binding activation site, so that the insertions would be unlinked.

The construction of a new transactivator construct, through identification of an activation domain and a DNA binding domain that not only work better in plants but are less clearly dominated by intellectual property rights, also provided a unique opportunity to address the second bullet point above.

The way that this can be used is that once lines have been established that express the transactivator in a defined pattern determined by the enhancers or promoters near the site of insertion (the case for the rice lines described in this study), genes that it is desired to switch on can be placed into cassettes with a minimal promoter and an activation sequence for the transactivator. When these cassettes are transformed into plants containing or crossed with the pattern lines, the gene(s) of interest will be brought into the expression pattern shown by the reporter genes. Thus it is possible to achieve highly controlled useful expression of transgenes without the need to define, analyse, and clone new promoters to drive the gene(s) of interest.

Thus, effective transactivation is not just an intellectual property workaround for promoter patents, but an opportunity for coordinate expression of genes. The genome may comprise multiple insertions of the corresponding upstream activation sequence linked to a minimal promoter and gene of interest, and the insertions may include multiple genes.

Thus, for example:

- the expression of multiple genes encoding different enzymes in a multi-step pathway, such as the starch biosynthesis pathway, can be controlled to occur at the same developmental stages and in the same amounts in the plant.
- The expression of down-regulation constructs for multiple steps in a pathway can similarly be coordinately regulated

The expression of either type of construct can be monitored by the expression of a reporter gene construct that is coordinately regulated in the same genome.

Intellectual Property generated

One patent work-around was developed and a PCT application was filed in order to prevent its patenting by any other party that could exclude others from using it. This was a strategy to co-transform or cross the vector containing the open reading frame for the transcriptional activator, and the vector containing the DNA-binding activation site, so that the insertions would be unlinked.

The value of this strategy as a work-around stems from wording, common, although not universal, in patent claims over the use of promoters (see CAMBIA's technology landscape on promoter patents at www.patentlens.net). Often, the promoter is not itself claimed as a composition of matter (or if it is, it is easy to work around such claims by slight modifications of the sequence). Instead, the composition of matter claimed is for use of the promoter within a construct that comprises the promoter "operatively linked" or "operably linked" to a gene of interest. In transactivator use, the promoter of interest is not linked to the gene of interest, but to a transcriptional activator. Upon transcription and translation of the transcriptional activator, the activation domain activates all copies in the genome, whether linked or unlinked to the transcriptional activator, of the corresponding upstream activation sequence linked to a minimal promoter and gene of interest. The latter gene would not otherwise be transcribed.

Accordingly, beyond the scope of the RIRDC funded project and upon recruitment of staff with IP analysis skills (see www.bios.net/daisy/patentlens/1521.html) and funding sourced through the International Rice Research Institute (see the press release and FAQ at www.bios.net/daisy/bios/1375), CAMBIA began a project in 2005 to update a technology landscape on promoters, covering freedom to operate with the minimal promoter used with the upstream activation sequence, (www.bios.net/daisy/promoters/768.html), and is developing a new technology landscape on transcriptional activators, which will be made available in 2006 within CAMBIA's Patent Lens (www.patentlens.org).

"Freedom to co-operate" considerations are equally important with "Freedom to operate" and technical evaluation, when considering biotechnological tools such as the transactivator constructs and the lines created using them. The new transactivator is now available to the rice industry, but will be available in an even more useful way when it is presented as a capability to use it, together with the accumulated know-how, in a form that enables ready collaboration with other researchers and capture of the data they collect and the improvements they make. This is the intent of the BioForge project (www.bioforge.net) being developed since 2005 by CAMBIA with US-sourced funding from the Rockefeller Foundation. All technology presented on the BioForge is available to other researchers in the public and private sectors *only* if they agree to the collaborative terms of the BiOS license (www.bioslicense.net). A summary of the BiOS License conditions follows:

In return for the benefits of the technologies, a licensee institution agrees to allow and encourage its employees and students to post on the website, for the benefit of all other licensees, any improvements made to the technologies and safety information relevant to use of the technology and potential regulatory approval of products embodying it, and agrees not to assert any intellectual property rights to the improvements and information against other licensees.

This unique legal instrument developed by CAMBIA provides for norms of sharing, as well as legally binding covenants that protect those who have invested in a technology, such as RIRDC, from being subject to the situation of inability to use valuable improvements to the technology.

The license is available to RIRDC without any upfront costs or royalties, of course, and provides for access to the BioForge collaborative platform and to all the Transbacter technology and GUSPlus technology, even though the BioForge was developed after the conclusion of RIRDC funding, and the other technologies listed were supported by other funding. RIRDC has only to execute the license available for download at www.bioslicense.net

Communications plan

One peer-reviewed paper arose from this work:
Wu C, Li X, Yua W, Chen G, Kilian A, Li J, Xu C, Li X, Zhou D-X, Wang S, Zhang Q (2003) Development of enhancer trap lines for functional analysis of the rice genome. *Plant J*. 35: 418-427 **.** The lines developed using the transactivator constructs in the earlier portion of the work described in this report are characterised to a limited degree in this paper, in the context of their use in field tests arranged by Andrzej Kilian in Wuhan, China. As described in this report, however, these lines were of limited utility because of the presence of the 35S enhancer.

Two Ph.D. theses now being reviewed by the university and external thesis examiners are expected to result in the submission of peer-reviewed publications relevant to the latter portion of the work described in this report. This portion has a greater significance for its capability to be used practically, both on technical grounds and freedom-to-operate grounds:

1. Thach Tran's thesis on development of the more effective transactivator constructs
2. Sujin Patarapuwadol's thesis partially covering the analysis of controlled crosses involving transactivator lines and the stability of transactivation effects in subsequent generations, particularly in lines where expression was seen in mature rice seed.

These will be mounted on the www.bioforge.net website as soon as the theses are accepted and/or the peer-reviewed papers are in press.

One international published patent application arose from this work:
Jefferson, Kilian, Nottenburg, Keese, Mayer and Stachel, WO 01/21781, which covers the two-construct strategy achieved by co-transformation, subsequent transformation, or genetic crosses.

As described above, the intellectual property described in the patent application and the Ph.D. theses, even though the latter are unpublished as yet, is available for license under the terms of a BiOS license, which allows both research and commercial exploitation by any public institution or public or private individual, subject to covenants that improvements to the technology and any biosafety data will be shared with all others agreeing to the terms of the license. The transactivator constructs and rice lines developed in this project are available under the materials transfer provisions of the related Technology Support Subscription agreement. For the terms of the license and subscription agreement, see www.bioslicense.net.

BiOS (Biological Open Source) licenses are designed to enable the sharing of the capability to use patented and non-patented technology, which may include materials and methods, within a "protected commons". The license can support both freedom to operate, and freedom to co-operate. "Biological Open Source" is not a new way to patent, but a new cooperative way to license the capability to use patented technology. Under the license, both products and improvements can be patented, and products and services can be developed for profit or for public good - but licensees may not assert rights to exclude from use of improvements, even patented improvements, against the licensor and other licensees that are contributors within the protected commons.

The essence of the open source concept: what is provided with wide access is not necessarily the product solution, but the enabling technology that allows products to be developed by innovative people. All those who agree to the terms of sharing have protected access to the capacity to use the enabling technology, and can make and commercialise products without the need to renegotiate a commercial license.

Collaboration between licensees to share improvements is further facilitated by licensee access to protected discussion spaces on the BioForge. The BioForge website, www.bioforge.org, is a portal for protocol-sharing, comments on patents, and discussion tools in both public and secure (confidential) environments. As it develops it will provide further resources for cooperative development of technologies and maintaining capabilities to use them. A BioForge project on using and improving transactivators has been developed by CAMBIA and will be made live by the release of this report.

Appendix 1. Number of different pattern lines obtained with enhancer trap and *Gal4*-deletion constructs

The recorded categories and codes of tissues were root (A), root branch (B), stem base (C), leaf (D), stomata (E), vascular tissue (F), collar (G), auricle (H), ligule (I), trichome (J), granules (K) stigma (L), ovary (M), anther (N), lodicule (O), glumes (P), sterile lemma (Q), pedicel (R), and panicle branch (S).

Patterns	pFX-G74.1	pFX-G85.2	*Gal4*-deletion
A	1	3	1
AB	0	1	0
ABDS	1	0	0
ACD	1	0	0
ACGIOP	1	0	0
AD	1	1	0
ADE	1	1	0
ADEFKLMNOPQRS	1	0	0
ADEFLKPQRS	1	0	0
ADEFLNOP	0	1	0
ADEGHLQ	1	0	0
ADEGJKNPR	1	0	0
ADEGKLMNQRS	0	1	0
ADEK	2	4	0
ADEKLN	0	1	0
ADEKPQ	0	2	0
ADEKPR	0	2	0
ADEKPRS	0	1	0
ADELNPQ	1	0	0
ADEMN	1	0	0
ADEPQR	1	0	0
ADEPRS	1	0	0
ADEPS	0	1	0
ADF	2	1	0
ADGK	1	0	0
ADGNPQ	1	0	0
ADGNPR	1	0	0
ADK	2	1	0
ADKLNOPQRS	0	1	0
ADLNOPQ	1	0	0
ADNPQR	1	0	0
AE	6	5	0
AEG	1	0	0
AEGPR	1	0	0
AEK	1	0	0
AEOPRS	1	0	0
AEPQ	1	0	0
AEPR	0	1	0
AF	1	0	0

AFLOPQS	1	0	0
AGNS	0	1	0
AJ	2	0	0
AK	0	1	0
BCDEK	1	0	0
BDFLOPQS	0	1	0
BDFP	0	3	0

BDK	0	1	0
BE	1	0	0
BEFG	0	1	0
DE	1	0	0
DEJNS	1	0	0
DEK	0	1	0
DEKPR	2	0	0
DEKPS	0	1	0
DENPRS	1	0	0
DF	1	0	0
DFJ	0	1	0
DFQR	1	0	0
DK	3	0	0
E	3	0	1
EFP	2	0	0
EFS	2	0	0
EK	0	2	0
ELMOPRS	0	1	0
ENPR	1	0	0
ENPS	1	0	0
ENS	2	0	0
EPR	0	2	0
EPS	2	0	0
F	1	0	0
G	0	1	1
GK	0	1	0
J	0	1	0
L	1	0	0
LMNO	3	0	0
LNO	1	0	0
LNS	0	1	0
MN	0	1	0
MPR	1	0	0
N	0	0	3
NP	0	1	0
P	0	0	1
PRS	1	0	0
PS	3	1	0
R	0	1	0
RS	1	0	0

Notes to the code: A = root, B = root branch, C = stem base, D = leaf, E = stomata, F = vascular band, G = collar, H = auricle, I = ligule, J = trichome, K = granules, L = stigma, M = ovary, N = anther, O = lodicule, P = glumes, Q = sterile lemma, R = pedicel, S = panicle branch.

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