STUDY OF GENE SILENCING IN RICE: A ROOT-PREFERENTIAL GENE *RCG*2

A Dissertation

by

XIANGYU SHI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Genetics

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Approved by:

Chair of Committee,	Timothy C. Hall
Committee Members,	Clint W. Magill
	Jean H. Gould
	Z. Jeffrey Chen
Intercollegiate Faculty Chair,	Craig J. Coates

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ABSTRACT

Study of Gene Silencing in Rice: A Root-Preferential Gene *RCg*2. (May 2009)

Xiangyu Shi, B.S., Shandong University

Chair of Advisory Committee: Dr. Timothy C. Hall

The *RCg*2 promoter was identified in a search for root-specific genes to combat the rice water weevil (RWW) but expressed at low frequency (~10%). Spatial expression of RCg2 was investigated using two reporter constructs YXA (*RCg2-gus-ocs*) and YXB (*RCg2-gus-RCg2*) that included 1.6 kb of the *RCg*2 5' sequence fused to the β-glucuronidase (*gus*) coding region. YXB plants were generated via *Agrobacterium*-mediated transformation but only 8 of 158 plants analyzed showed strong GUS activity despite the presence of an intact construct. Reactivation of *RCg*2 gene in rice was investigated by treatment of R0 and R1 of YXB transgenic plants with 5-azacytidine. Reactivation of *RCg2-gus* was observed in some transgenic plants indicating different mechanisms involved in the gene silencing of the YXB lines. DNA methylation analysis, northern blotting, RT-PCR and small RNA analysis supported the conclusion that PTGS and TGS are present in the silenced plants. Promoter analysis *in silico* and using promoter deletion assays predicted that the *RCg*2 promoter contains a complex region that includes miRNA homologs, *MITE*s and repetitive sequences. The high frequency of promoter-related silencing suggests functional interactions of these elements of the transgene and the homologous endogenous gene. To identify key elements contributing

to the root-preferential expression of *RCg*2 and the high frequency of silencing observed in transgenic (YXB) lines, several *RCg*2 promoter deletion constructs were designed. These include 5' deletions MC1, MC2, MC4, MC7 and MC8 and internal deletions MC5, MC11, MC12 and MC13. The frequency with which silencing was encountered in populations of the deletion mutants was used to characterize the effects of various promoter elements. Deletion of the region from -406 to -208 (compared MC11 to YXB, and MC13 to MC1) revealed that region contains a negative element. Among 36 independent transformants, 33% with MC11 expressed GUS and 85% with MC13 showed GUS expression. Comparing MC7 transgenic plants to MC1 revealed that the region –888 to –729 is another negative regulatory element, and comparing MC11 to MC12, the proportion of expression of transgenic plants indicated the region –729 to –406 is a positive regulatory element.

DEDICATION

To My Husband and Daughter

To My Parents

To My Sisters and Brothers

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CHAPTER I

INTRODUCTION

Rice (*Oryza. Sativa* L) is not only a very important food resource on a global basis, including both developed and developing countries, but also an excellent monocot crop plant for genomic sequence and analysis (Shimamoto, 1995). Rice is a monocot diploid plant (2n=24), which bears a haploid nuclear DNA content of about 415-463 x 10^6 bp (0.86-0.96 pg/2C) (Arumuganathan and Earle, 1991) (Xu, dissertation 1995). The size of the nuclear genome of *Arabidopsis thaliana* is 145 x 10^6 bp/C or 0.3 pg/2C, which let it become the smallest known nuclear DNA content among flowering plants. However, rice genome is about three times larger than the *Arabidopsis* genome. In 2002, the International Rice Genome Sequencing Project (IRGSP) announced completing the Japanese rice variety Nipponbare genome sequence which will accelerate efforts to feed the hungry by improving the world's most important food (International et al., 2005).

This dissertation follows the style of The Plant Cell.

Although Conventional breeding has successfully improved the rice development, mainly in a remarkable raise of production in the last decade (Khush, 1997), the genetic engineering technology has gained focus for a long time as a way to supplement these efforts. Many areas of research have been reported, which include resistance against disease and environment, such as, pests, pathogens, salinity and drought, and also improvement of the nutritional quality of rice (Tyagi et al., 1999). In fact, rice is the easiest cereal plant to transform genetically. This and the fact that it possesses the smallest genome among the major cereal crops (400-430 Mb) have identified rice as the model cereal (Shimamoto, 1995).

However, genetic transformation techniques met a strong obstacle in the unpredicted phenomenon of gene silencing, since variability or instability of transgene expression is not desirable for future commercialization of a genetically engineered crop. But it is irrefutable that genetic engineering techniques have opened a large range of opportunities to study various fundamental problems in plant biology and to elucidate various principles of gene regulation in monocots in general. Regulation of expression is modulated by factor interactions, epigenetic events, and chromatin structure and in many other ways and can determine when, where and how a specific gene is to be expressed in the plant. The study of gene silencing triggered by the introduction of alien genes to a given genome offers an excellent approach to survey the inner mechanisms of plant gene regulation.

Although early reports on gene silencing dealt with dicotyledonous plants, it is apparent that transgenes in monocots are equally susceptible to silencing processes. This

topic has been reviewed by our group (Iyer and Hall, 2000; Iyer et al., 2000) and several questions, such as the susceptibility of different genomes to silencing, the influence of the transformation strategy or specific transgenes in triggering silencing, and the stochastic nature of silencing and its inheritance in progeny, were addressed.

Understanding the molecular basis for tissue-specific gene expression is of fundamental importance to life science and also has broader applications such as crop improvement. The larval stages of the rice water weevil (RWW, an insect pest worldwide and the worst in Texas) develop over a period of 1 month, during which they devour the roots, typically causing severe loss in grain yield (Stout et al., 2001). Since the root system is an indispensable part of plant, understanding root development and regulation of root specific gene expression is of great significance in crop improvement through biotechnology approaches (Xu et al., 1995). To successfully use recombinant DNA technology for agronomic improvement of rice, molecular mechanisms for regulation of gene expression in normal as well as in transgenic rice plants need to be well understood.

The graminaceous monocots, which include such major food crops as wheat, maize, and rice, were not routinely suitable to gene transfer using *Agrobacterium*. It took a long time to overcome the difficulty of gene transfer using *Agrobacterium*. Molecular transformation for the delivery of foreign genes into rice calli induced from scutellar and mature embryo and immature embryo has become routine since 1995 (Hiei et al., 1994; Dong et al., 1996; Hiei and Komari, 2008).

The *RCg*2 gene was isolated in the Hall' laboratory in a search for promoters that would target insecticidal protein production to root tissues (Xu et al., 1995). In some rice lines transformed with construct YXB (*RCg2/GUS/RCg2*: Figure 3.1A), *GUS* reporter expression driven by *RCg*2 can be strong and has a characteristic pattern (Figure 3. 1A). However, expression in the vast majority of independent transformants (79/93) is absent or very weak. That the gene insert is capable of expression has been confirmed for many lines by exposing the roots to 5-azacytidine (5-azaC) (Figure 3. 6). This frequency of promoter silencing is, to our knowledge, unique and the purpose of this project is to elucidate the mechanisms or mechanisms involved.

Gene Silencing

Plant genetic engineering has been used as a research tool for regenerating transgenic plants, which held favorite characteristics for several decades. However, the unexpected and unpredicted gene silencing effects have slow down the crop improvement through transformation. Gene silencing is initially classified as two classes: transcriptional gene silencing (TGS) which has been often associated with cytosine methylation of promoter regions (Jones et al., 1998; Meyer, 2000; Habu et al., 2001; Kloti et al., 2002); Posttranscriptional gene silencing (PTGS) which was discovered in transgenic Petunia as loss of expression of both a transgene and its homologous endogenous genes (Napoli et al., 1990; Brodersen and Voinnet, 2006). It occurs through mRNA degradation and has been correlated with cytosine methylation in coding regions in some cases (Ingelbrecht et al., 1999; Kovarik et al., 2000; Morel et al.,

2000). Both transgenes and endogenous genes are subjected to these two processes. Despite these differences between TGS and PTGS, these two silencing pathways are mechanistically related. Recent studies revealed that there is an intriguing relationship between DNA methylation, histone methylation, chromatin remodeling and RNA interference (RNAi) (Tariq and Paszkowski, 2004). For example, double-stranded RNA (dsRNA), an intermediate product in PTGS processes, can be a trigger of TGS (Morel et al., 2000). A single transgene locus, *271*, can trigger both TGS and PTGS by simultaneously producing dsRNA corresponding to both promoter and transcribed sequences (Mourrain et al., 2007). Additionally, mutation of *Argonaute,* a gene that is involved in PTGS, can profoundly affect heterochromatin formation (Martienssen et al., 2005; Kim et al., 2006). Consequently, it is evident that both PTGS and TGS processes are intimately involved in the regulation of gene expression (Sijen et al., 2001).

Transcriptional Gene Silencing (TGS)

Transcriptional gene silencing is often related with DNA methylation and protein modifications such as histone deacetylation, methylation, and phosporylation (Li et al., 2002). DNA methylation is an epigenetic process, which has been discovered in both prokaryotes and eukaryotes. Methylation has been occurred in eigher CpG dinucleotides or CpNpG triplets in plants (Pradhan and Adams, 1995); however, in animals is usually confined to cytosines in CpG dinucleotides (Bestor, 2000). Cytosine methylation plays a great role in regulating gene expression (Chaudhury et al., 2001), genome defense

mechanisms (Matzke et al., 2001; Vaucheret et al., 2001), gene silencing (Kumpatla et al., 1997), genomic imprinting (Baroux et al., 2002), X-chromosome inactivation (Rakyan et al., 2001), vernalization (Sheldon et al., 2000b) (Sheldon et al., 2000a) and sex determination (Siroky et al., 1998). Cytosine methylation not only controls gene expression in a developmental stage and tissue dependent manner, but also it is involved in transgene repression as well as the regulation of the activity of the endogenous DNA, such as transposable elements and retrotransposons (Hirochika et al., 2000; Miura et al., 2001).

DNA methylation plays a major role in maintaining genes in a repressive state, and is likely carried out by the maintenance DNA methyltransferase, Dnmt1/MET1 (Vaucheret et al., 2001). The Dnmt1/MET1 enzyme has high affinity for hemimethylated DNA and functions during DNA replication (Bestor and Verdine, 1994; Vertino et al., 2002). The discovery of several MBPs, MeCP1, MeCP2, MBD1, MBD2, MBD3 and MBD4 (Ballestar and Wolffe, 2001; Wade, 2001), that interact with DNA MTase and are capable of recruiting repressive complexes and histone deacetylases, suggests the existence of additional mechanisms for gene suppression that may act through histone deacetylation (Brackertz et al., 2002; Feng et al., 2002). This, together with the finding that MBD proteins are associated with distinct histone deacetylase (HDAC) complexes, suggests that various MBP/HDAC interactions have different roles in gene silencing and may act at different stages of development (Hendrich and Bird, 1998; Jiang et al., 2002). In addition to histone deacetylation, histone methylation processes have been related to DNA methylation by several reports, including the recent

evidence demonstrating that MeCP2 can recruit a histone methyltransferase through its transcriptional repression domain and lead to a repressive chromatin state (Fuks et al., 2003). However, the finding in Arabidopsis that MOM1 can modulate silencing in the absence of changes in CG methylation status (Scheid et al., 2002), suggests that some forms of gene silencing are either methylation-independent or act downstream of cytosine methylation.

Posttranscriptional Gene Silencing (RNA Interference Silencing) (PTGS)

PTGS in plants was a mystery finding resulting from transformation that placed a sense orientation chalcone synthase (CHS) gene into wild type petunia in which an endogenous CHS already existed. The resulting shut down of enzyme activity from both copies was initially termed co-suppression (Napoli et al., 1990). Some flowers of co-suppressed plants are totally white whereas some contain only white sectors. RNA analysis revealed that transcription of CHS occurred, but the level of the CHS transcript in white sectors of co-suppression plants was much lower than in purples sectors of both co-suppressed and wild type plants (Napoli et al., 1990; van der Krol et al., 1990). This phenomenon later was defined as posttranscriptional gene silencing in plants, also called RNA silencing, and confirmed to share similar features to quelling in fungi (Cogoni and Macino, 1999; Pickford et al., 2002), and to RNA interference in *C. elegans* (Fire et al., 1998). Thoroughly research revealed that RNA silencing in plants as a mechanism when invading nucleic acid, such as transgenes and virus, are silenced through the action of

small (20-26nt) homologous RNA molecules (Brodersen and Voinnet, 2006), and suggest that PTGS can be divided into three steps: ignition, spreading and maintenance, and concluded that small RNA fragments created by enzymes named Dicers and Argonautes are the biochemical core of RNA silencing (Brodersen and Voinnet, 2006).

CHAPTER II

STABLE TRANSFORMATION AND MOLECULAR CHARACTERIZATION OF *RCg*2 GENE: A PUTATIVE RICE ROOT SPECIFIC GENE

Introduction

The isolation and study of root-specific genes fulfills a basic interest in understanding plant root development and the control mechanisms of root-specific expression, and a more applied goal, as it is the expression of agronomical important transgenes in a root-specific way. The production of insecticidal proteins in rice roots to combat the rice water weevil (RWW) in its early stages was one of the research objectives of Dr. Hall's laboratory. In 1995 Xu et al. (Xu et al., 1995) reported the isolation of two cDNA clones (RCc2 and RCc3) and one corresponding genomic sequence (RCg2) that were highly expressed in a root-specific manner in rice seedlings.

In order to test the expression pattern of a transgene regulated by the *RCg*2 promoter, two different chimeric gus gene constructs were designed, YXA (*RCg*2pro-*gus*-*OCS*ter) (Xu et al., 1995) (Figure 2.1C) and YXB (*RCg2*pro-*gus*-*RCg2*ter) (Hall et al., 2001). (Figure 2.1C)

Transgenic YXA plants were recovered following direct gene transfer (Battraw and Hall, 1990); transgenic YXB and YXA plants were recovered following *Agrobacterium*-mediated techniques (Dong et al., 1996). Although strong GUS

expression was observed in roots, in a very characteristic pattern, expression was also observed in leaves of the same transgenic plants. These findings suggested that some other important regions of the gene and not only the 5' and 3' sequences might additionally determine the *RCg*2 spatial regulation (Xu et al., 1995). A thorough characterization of the transformants and of the *RCg*2 promoter was subsequently conducted.

We focused our attention in two different objectives: (1) characterization of the YXB transformants and the *RCg*2 silencing phenomenon, and (2) diversification of *RCg*2 promoter to avoid silencing in the expression of transgenes.

Materials and Methods

Plant Materials

The model organism used in this research was rice (*Oryza sativa* subspecies japonica CV. Taipei 309, Gulfmount, Nipponbare, IR-72, and Texas 6). Rice seeds were surface sterilized in 25% commercial bleach for 50 minutes, then thoroughly rinsed with sterile distilled water until clean. Subsequently, the seeds were germinated on Murashige Skoog (MS) medium (Murashige and Skoog, 1962) at 26°C under an 18/6 (light/dark) regime for 15 days then transferred to soil and grown in greenhouse at 26ºC with a 14/10 (light/dark) photoperiod until mature. Leaf and root samples were taken at 15 days after germination. Inflorescence and immature embryo, and leaf sample were collected upon flowering. Callus was induced from immature embryo and mature seeds and maintained on N6 medium (Chu, 1978) for 3-5 weeks and then subjected to *Agrobacterium* mediated transformation, and biolistic transformation.

Plasmid Constructions, Plant Transformation and Regeneration

The T-DNA of plasmid pJD4YXB contains an herbicide resistance gene [Maize *ubiquitin* promoter *ubi-bar-nos*], an antibiotic resistance gene construct (5' *35S-hpt-35S*), as well as an *RCg*2 reporter gene construct (5' *RCg*2-*gus*-*RCg*2, YXB) (Figure 2.1A). Plasmid pJD4YXB was mobilized into *Agrobacterium tumefaciens* LBA4404 resulting in LBA4404 (pJD4YXB) as described (Bevan, 1984) using a modified tri-parental mating method (Dong et al., 1996). The bacterial strain was used to transform callus

induced from immature as described in Dong et al. (Dong et al., 1996) or mature embryos of Japonica cultivar Taipei 309 (T309).

Plasmid YXA used for transformed rice was provided by Xu (Xu et al., 1995). To make pJD4YXA 5' RCg2-gus-ocs-3' in YXA replaced 5' 35S-gus-ocs 3' in pJD4, the binary vector of stable transformation used for Agrobacterium-mediated bacterium made in our lab {Dong, 2001 #10222). Plasmid pJD4YXA was mobilized into *Agrobacterium tumefaciens* LBA4404 resulting in LBA4404 (pJD4YXA). Plasmid LBA4404 (pJD4) (Figure 2.1B), the binary vector for transformed rice served as control.

Genomic DNA Blot Analysis

Genomic DNA was prepared from rice tissue by a CTAB (hexadecyl trimethyl ammonium bromide) method (Taylor and Powell, 1982; Saghai-Maroof et al., 1984). Fresh leaf tissue or 10-14 days seedling was powdered with pestle and mortar in the presence of liquid nitrogen and immediately transferred to a centrifugation tube (30 mL). The extraction buffer (9mL) [Tris-HCl (100mM, pH 8.0), EDTA (50 mM, pH 8.0), NaCl (500 mM), mercaptoethanol (0.2 mL) and CTAB 0.2 g)] was added fine powdered tissues (600 mg), and incubated at 65°C for 3 h, with occasional mixing. Chloroform/octanol (24:1, 10 mL) was added to the tissue-homogenate to remove the chlorophyll, followed by inverting the tube 4-5 times and centrifugation at 8,000xg for 15 min at 4°C. The supernatant was then transferred to a new tube (50 mL) and the chlorophyll removal step with chloroform/octanol was repeated for one more time. An equal volume of isopropanol was added to the DNA extract, gently mixed, and DNA

was precipitated for 5 min. The white thread of DNA was then removed to a new microcentrifuge tube by glass hook or cut-end pipette tip (1 mL) and DNA was collected by centrifugation of the supernatant in centrifuge tube (50 mL) at 10,000xg for 10 min at 4°C. DNA pellets were washed by adding washing buffer I (1mL) [76% ethanol, NH_4OAC (10 mM)] and shaking vigorously for 20 min. Washing buffer I was removed by pipetting and the DNA pellet was further washed with washing buffer II (1 mL) [76% ethanol, NH_4OAC (10 mM)] for 5 min. The pellet was air-dried, and then dissolved in TE buffer (300 μL), depending upon the size of pellet. The DNA was dissolved at 4°C overnight and stored at -20°C.

Total genomic DNA was isolated from mature leaves and seedlings and genomic blot analysis was conducted as described in Buchholz et al. (Buchholz et al., 1998). Briefly, 2 μg genomic DNA was digested to completion with the indicated restriction enzyme, size fractionated by electrophoresis through 1% agarose gels. After electrophoretic separation of DNA on 1% agarose gel for 12-15 hours at constant 23 volts, the DNA was transferred to HybondTM-N+ nylon membrane (Amersham, Piscataway, NJ). Genomic DNA blot analysis was as described by Buchholz et al. (Buchholz et al., 1998). [32P]dCTP-labeled probes were made using a DECAprimeTM II DNA labeling kit (Ambion, Austin, TX). Membranes were washed with 2×SSC [1×SSC = 0.15 M sodium chloride /0.015 M sodium citrate (pH7)]/0.1% SDS at 65°C for 1 hr (low stringency), or with 0.3×SSC /0.1% SDS at 65°C for 1 hr (moderate stringency). The DNA immobilized on membrane was hybridized with random-primed 32P-labeled DNA probes.

Small Scale DNA Extraction from the Young Leaves and Seedling

The small-scale method for PCR analysis of rice plants is described in section 3.1.1 (p.401) of Buchholz et al. (Buchholz et al., 1998). This method is suitable for some plants, such as rice young leaves and seedlings and can give a sufficient yield to perform PCR analysis and DNA analysis. 2 sq. cm leaf or 14 days seedling were harvest into a 1.5 mL microfuge tube. Liquid nitrogen was added to the collected leaf and seedling samples that were then ground into a fine powder with a disposable plastic pellet pestle (#749521, Kontes Glass Co., Vineland, NJ. USA).

Bialaphos Dipping, 5'-azacytidene Treatment and GUS Staining

Assessment of bialaphos resistance by leaf dipping and reactivation of seedlings by 5'-Azacytidine (5-azaC) treatment were as described previously (Kumpatla and Hall, 1998a). For reactivation, calli or seedlings were grown on N6 medium for 14 days, and then transferred to N6 medium containing 5-azaC. GUS expression was examined by histochemical staining (Jefferson et al., 1987a). Plant tissues (rice root and leaf segments) were vacuum infiltrated in a phosphate-buffered solution containing 5-bromo-4-chloro-3 indolyl glucuronide (X-Gluc; 0.16 mg/mL) and incubated at 37^{o}C for 16 hours (Jefferson et al., 1987a). Chlorophyll in tissues was bleached out by soaking in ethanol (95%) overnight.

5-azaC Treatments

Reactivation of YXB using an inhibitor of DNA methylation (demethylating agent) 5-azacytidine (5-azaC) was conducted by growing rice tillers in nutrient solution containing 5-azaC. The tillers were placed in nutrient solution for 8 days to allow new roots to grow and then 5-azaC was added to the nutrient solution to a final concentration of 50 mg/L. Root samples were collected and assayed for GUS expression after 5 days of 5-azaC treatment. Seeds of self-crossed R0 plants were germinated in the presence of 5-azaC (50 mg/L) and one week or two-week-old seedlings were assayed for GUS expression.

Histochemical and Fluorometric Assays for GUS Activity

Histochemical GUS staining was performed for vegetative tissues (leaves, roots, inflorescences) with 5-bromo-4-chloro-3-indoxyl-â-D-glucuronic acid (Xgluc) as a substrate (Jefferson et al., 1987b). Samples were stained overnight (16 h) at 37 ^{o}C and chlorophyll was removed by 95% ethanol after staining (Chandrasekharan et al., 2003). Fluorometric assays of GUS activity of leaves and roots were performed as described by (Jefferson et al., 1987b). GUS activity was calculated as pmol 4-MU per hour per microgram protein and data were analyzed with SPSS 11.0 for Windows software. For each construct, three independent assays were performed unless otherwise specified.

Histochemical Assay (Jefferson et al., 1987a)

Sections were cut by hand from unfixed stems of plants grown in vitro, essentially as described (O'Brien and McCully, 1981), and fixed in 0.3% formaldehyde in 10 mM MES, pH 5.6, 0.3 M mannitol for 45 min at room temperature, followed by several washes in 50 mM NaH2PO4, pH 7.0. All fixatives and substrate solution were introduced into sections with a brief (1 min) vacuum infiltration. Histochemical reactions with the indigogenic substrate, X-Gluc were performed with 1 mM substrate in 50 mM NaH2PO4, pH 7.0 at 37°C for times from 20 min to several hours. After staining, sections were rinsed in 70% ethanol for 5 min, and then mounted for microscopy.

Fluorometric Assay (Jefferson et al., 1987a)

The fluorogenic reaction was carried out in 1 mM MUG extraction buffer with a reaction volume of 1 ml. The reaction was incubated at 37°C, and 200 ILI aliquots removed at zero time and at subsequent times and the reaction terminated with the addition of 0.8 ml 0.2 M Na2CO3. The addition of Na2CO3 serve the dual purposes of stopping the enzyme reaction and developing the fluorescence of MU, which is about seven times as intense at alkaline pH. Fluorescence was then measured with excitation at 365 nm, emission at 455 nm on a Kontron SFM 25 spectrofluorimeter, with slit widths set at 10 nm. The resulting slope of MU fluorescence versus time can addtherefore be measured independently of the intrinsic fluorescence of the extract. The fluorimeter was calibrated with freshly prepared MU standards of 100 nM and 1 ttM MU in the same

buffers. Fluorescence was linear from nearly as low as the machine can measure (usually 1 nM or less) up to 5-10ItM MU. A convenient and sensitive qualitative assay was done by placing the tubes on a long-wave UV light box and observing the blue fluorescence. This assay could be scaled down easily to assay very small volumes (reaction volume 50 d41, terminated with 25 l 1 M Na2CO3 in microtitre dishes or Eppendorf tubes).

Protein concentrations of plant extracts were determined by the dye-binding method of Bradford (1976) with a kit supplied by Bio-Rad Laboratories. DNA concentrations in extracts were determined by measuring the fluorescence enhancement of Hoechst 33258 dyes as described by Labarca and Paigen (1980), with the calibrations performed by addition of lambda DNA standards to the extract to eliminate quenching artefacts.

Bialaphos Leaf-Painting Bioassay

Transgenic plants were tested for herbicide resistance by dipping a portion of a leaf (the apical 8 to10 cm of mature leaves was used) into 0.5% (w/v) solution of a commercial herbicide (Kumpatla et al., 1997), containing 20% (w/v) bialaphos, for 4 min. Herbicide resistance (normal versus yellow and dried appearance) was scored 4 to 5 days after treatment.

Results

Stable Transformation and Regeneration of Transgenic Rice

To determine the spatial expression of an introduced *RCg*2 (a root specific gene from Xu. et al. (Xu et al., 1995) gene in transgenic plants, chimeric gene constructs LBA4404 (pJD4YXA) (Figure 2.1B) were made and transformed into rice immature embryo and mature embryo induced calli (*Oryza sativa* L. cv. Taipei 309) via *Agrobacterium*-mediated transformation. LBA4404 (pJD4) (Dong et al., 1996) and LBA4404 (pJD4YXA) were used as controls. As biolistics and other direct DNA transfer procedures frequently result in gene silencing in rice (Kumpatla et al., 1997; Kumpatla and Hall, 1998a, b), *Agrobacterium*-mediated transformation was used to deliver constructs into plants. Successful transformants were selected on medium containing 50mg/L Hygromycin. A total of 158 YXB, 45 YXA and 64 JD4 putative transgenic rice plants were regenerated and established in the greenhouse. All plants set seeds except YXB69a and YXB49a, and YXB69c produced only 13 seeds. High efficiency *Agrobacterium*-mediated transformation established the primary transformants population within few co-cultivation experiments.

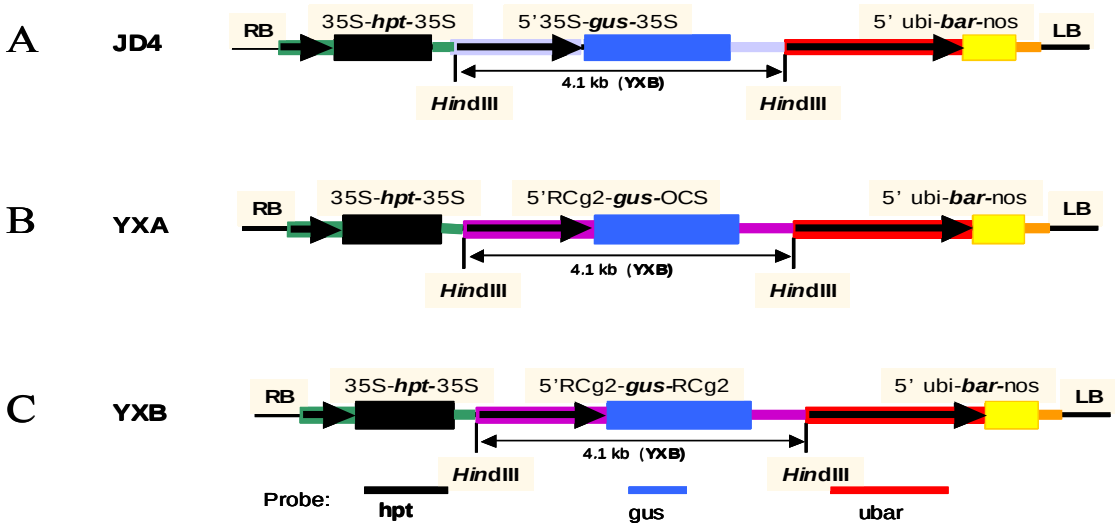


Figure 2.1. Organization of chimeric reporter gene constructs and genomic DNA analysis of transformants.

(A), (B), (C) The structures of Chimeric constructs pYXB, pJD4, pYXA are shown. 35S: Cauliflower mosaic virus 35S promoter; *hpt*: hygromycin phosphotransferase gene; *gus*: beta-glucoronidase gene; *bar*: bialaphos resistance gene. The bars under the construct were probes used for DNA analysis.

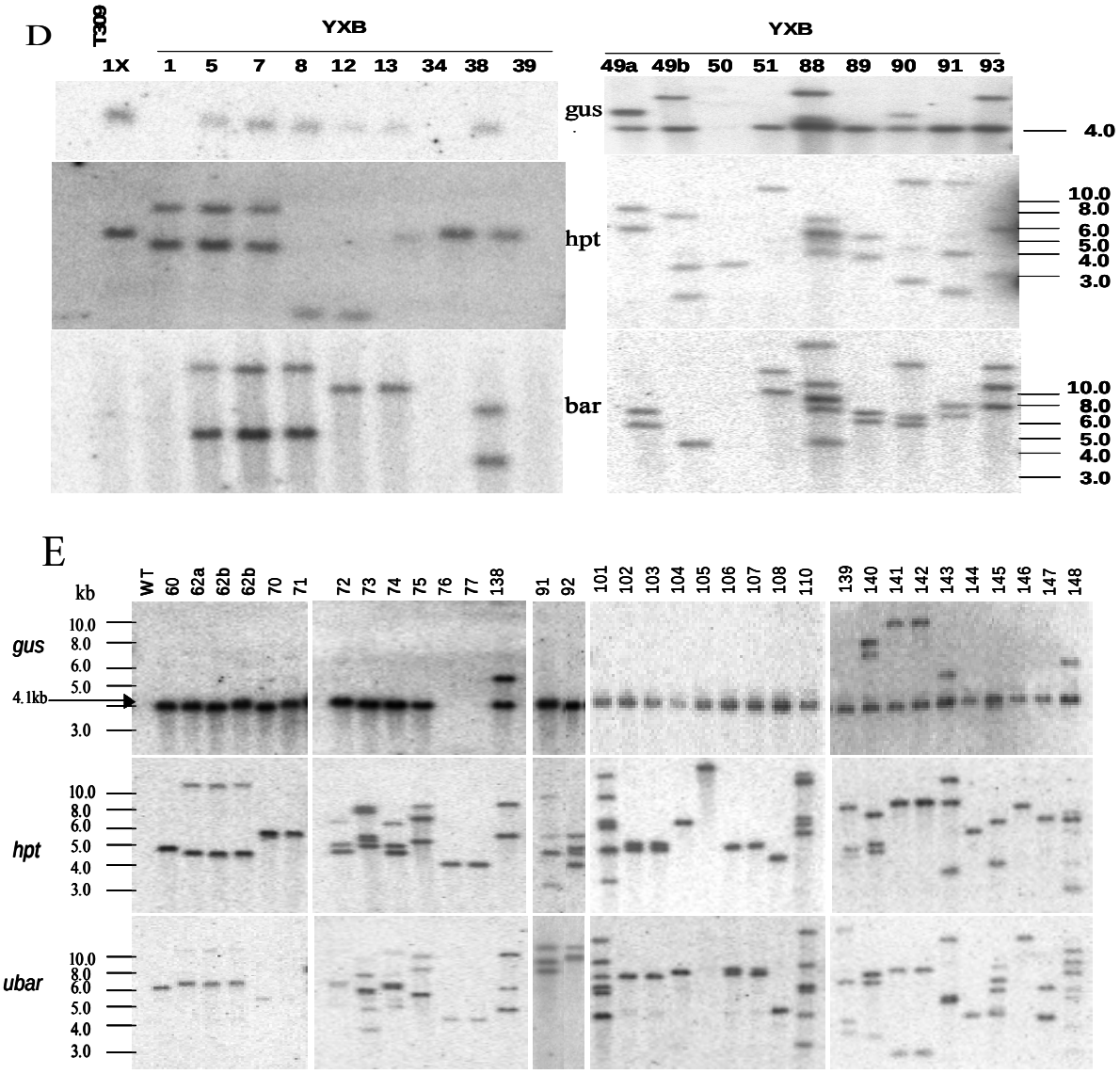


Figure 2.1. Continued.

(D), (E) Genomic DNA analysis of YXB transformants. Partial transformants genomic DNA digested with *Hind*III and electrophoresed in a 1% agarose gel. Expected *gus* fragment size is 4.1 kb.

Characterization of Transformants

Genomic DNA Blot Analysis

Genomic DNA blot analysis was conducted to determine the genomic organization of transgenes. Genomic DNA blots were probed with coding sequence of *gus* to determine the integrity of YXB. For the flanking sequences, *hpt* coding sequences or *maize Ubi* promoter sequences were used as probes to reveal right or left border sequences, which are highlight in Figure 2.1A. Of the158 transgenic plants analyzed, 93 independent lines were confirmed and 93 have at least one intact copy of RCg2-*gus* (4.1kb fragments such as in lines: 51, 89, and 91 (Figure 2.1D) and more lines showed in Figure 2.1E. We also observed rearrangements as shown in lanes: 49a, 49b, 88, 90, 93 (Figure 2.1D) and 138, 140, 142, and 143, and incomplete T-DNA transfer such as YXB1, 34, 39, 42 and 78 had only the hpt construct and lines YXB67, 105 (Figure 2.1D and 1E) and 129 (data did not show here), were found to have only gu*s* and hpt. High proportions of single copy and low copy transformants were obtained using *Agrobactriun*-mediated transformation methods. A summary of genomic DNA blot analysis of selected YXB plants is presented in Table 2.1. The putative number of copies of *hpt* construct (right border), intact and rearranged YXB construct and bar construct (left border), are included.

Table 2.1 Genomic blot data for number of copies inserted and organization of inserts in some of the YXB plants

	YXB 49-1	YXB 49-2	YXB 50	YXB 51	YXB 88	YXB 89	YXB 90	YXB 91	YXB 93	YXB 68	YXB 69-1	YXB 69-2	YXB 70	YXB 109
LB	2	1	-	2	5	2	3	2	3	2	4	1	1	-
C	1+1	1+1	-	1	1+1	1	1+1	1	1+1	1+1	1+1	1	1	1
RB	2	3	1	1	4	2	3	3	3	3	5	1	1	1

LB is left border - bar, RB is right border - hpt, and C is GUS band number on the southern blotting. 1+1 showed one intact GUS band and one rearranged copy of GUS

GUS and BAR Expression in YXB Plants

Histochemical staining for GUS expression in roots and leaves of the transformants showed GUS expressing lines in approximately 10% of the total independent transformants (Table 2.2.) and (Figure 2.2A). There are 21 single copy plants (gus/hpt/bar = 1), 2 of them are weak expressors (YXB127, 135) and 8 strong expressors (++) (YXB49a, 68, 69a, 91, 92, 93, 98, 138) but 6 of them carried 1 or more rearranged YXB sequences (T) (all except YXB91, 92) (Table 2.2). Seven silenced plants were recovere. For bar expression but only 1 of them is a single copy, plant (YXB19). Truncations on the YXB construct located in the promoter are apparently

related to GUS expression. Strong GUS expression was only detected in 8 R0 plants YXB15, YXB49a, 68, 69a, 91, 92, 93, 98 and 138. Weak GUS expression was detected in R0 YXB56-59, 61, 78-79, 126-128, 130, 135 and147-158 (Table 2.2) (Hall et al., 2001). Nineteen single copy plants (gus/hpt/bar = 1) are silenced and 2 are weak expressors, YXB127 and YXB135.

Table 2.2 A summary of transgene expression analysis of YXB plants

Expressing			Non-Expressing	
GUS	Strong [8]	Weak [19]	Gene* present [55]	Absent [11]
	49a, 68, 69a, 91-93, 98, 138	56-59, 61, 79, 122, 126-128, 130, 135, 147, 149, 151, 153, 156-158	2, 12, 19, 24, 25, 33, 37, 44, 49b, 51-52, 54, 55, 60, 62, 63, 65-66, 69b, 71-73, 75, 80, 82, 84, 88-90, 100-102, 104, 106, 108-109, 114-115, 119, 123-125, 131-134, 136-137, 139-141, 143-146	1, 32, 50, 64, 76, 78, 86, 96, 105, 121, 129
BAR	Resistant [74]		Gene* present [7]	Absent [12]
	2, 12, 24, 25, 33, 37, 44, 49a, 49b, 51, 52, 54-61, 63, 66, 68, 69a, 69b, 71-73, 75, 79, 80, 82, 84, 88-93, 98, 100, 102, 104, 106, 108, 114, 115, 119, 121-128, 130, 131, 133, 135, 137-141, 143-147, 151, 153, 156-158		19, 62, 76, 96, 101, 136, 149	1, 32, 50, 64, 65, 78, 86, 105, 109, 129, 132, 134

*intact transgene

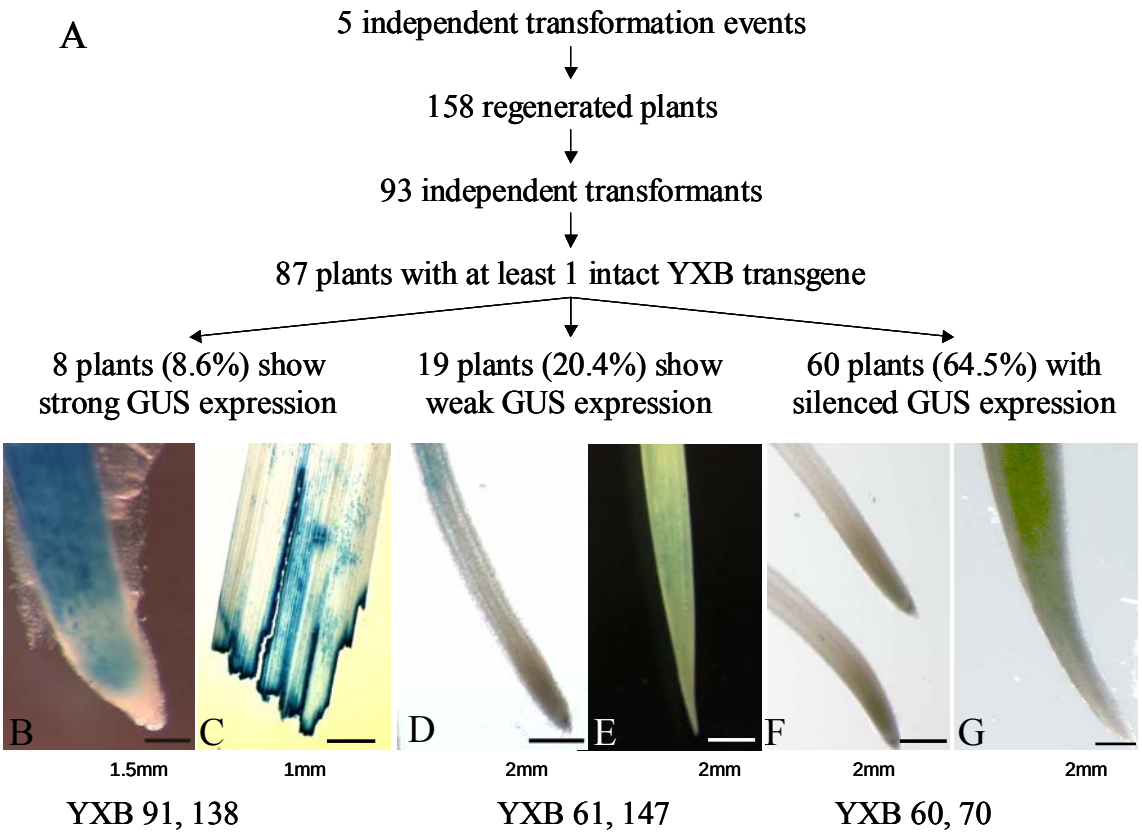


Figure 2.2. Independent transformants and GUS expression in transformants.

(A) Independent experiments and transformants.

(B)– (G) Histochemical GUS staining of roots and leaves from transgenic plants.

(B), (C) GUS staining of root and leaf show a strong GUS expression.

(D), (E) weak expression in the root and leaf.

(F), (G) GUS staining of root and leaf shows silenced expression.

A total of 158 plants were found to be resistant to bialaphos except: YXB1, 19, 32, 34, 39, 42, 50, 64, 65, 78, 86, 105, 109, 129, 132 and 134 were sensitive to bialaphos (table 2.1 and 2.2), and genomic DNA blot analysis showed that 1, 32, 50, 64, 65, 78, 86, 105, 109, 129, 132 and 134 lacked the Ubi-bar construct. In YXB19, 62a-62c, 76, 96, 101, 136 and 149, Ubi-bar construct was found to be present and silenced (Table 2.3) and (Figure 2.3).

Table 2.3 Bialaphos leaf-painting bioassay

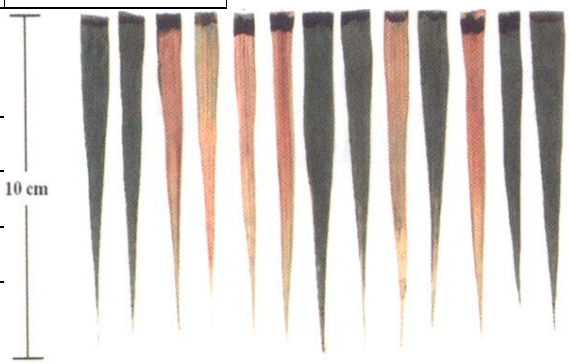
Sensitive Plants	Resistant Plants
19, 62a-62c, 76, 96, 101, 136, 149	2, 4, 5, 7, 8, 9, 10, 12, 13, 14, 18, 24, 25, 26, 28, 37, 38, 44,45

Plants transgenic for pYXB were tested for herbicide resistance by dipping an apical portion of a leaf into 0.5% (w/v) solution of a commercial herbicide (herbiace), containing 20% (w/v) bialaphos. Resistance to the herbicide (normal versus yellow and dried appearance) was scored 4-5 days after treatment.

A

Non-expressing (bar)		Expressing (bar)
16 (17.2%)		77 (82.8%)
With at least 1 bar gene copy (Silenced)		Without bar gene copy
6 (6.5%)		10
Single copy	Multiple copies	
1	5	

B



Bialaphos painting assay → sensitive (bar-silenced)
→ resistant (bar-expressing)

Figure 2.3. Bar expression: Bialaphos leaf-painting assay on leaves of rice plants.

(A) Bialaphos expression in YXB transgenic plants.

(B) Bioalaphos leaf painting assay on the leaves of YXB. Green color showed bar resistant (bar expressing) and brown color showed bar sensitive (bar-silenced).

Characterization of Progenies of the YXB Population

An initial population of 158 YXB transformants was characterized at the molecular level. Genomic DNA analysis were carried out and, after discarding the siblings, it was noted that only about 10% of the transgenic lines showed strong GUS expression, while about 90% were weak or non-expressors (silent lines) (Figure 2.2A.). Moreover, only 10% of these silent lines were sensitive to bialaphos (bar-silenced lines) (table 2.3.), indicating that the flanking genes are not silenced at the same rate as the

RCg2-regulated *gus* gene. All the lines with a single-copy integration pattern showed no GUS expression. Another curious observation from the YXB population was that 70% of the expressing plants contained a truncated copy of the YXB construct along with an intact T-DNA copy (Table 2.1.). All these data suggest that we are probably observing a phenomenon of gene silencing caused by a promoter especially prone to be suppressed.

GUS expression was maintained in R1 lines of YXB15, 61, 68, 91-95, 97, 138 (Figure 2.2), and not of YXB56-59, 78-79, 126-128, 130, 135. GUS expression in R1 seedlings of selected silenced lines: YXB53, 63, 66, 73, 84 and 120, was assayed histochemically, and GUS expression in the form of sporadically blue dot or faint blue was observed in lines: YXB53, 63, 73, and 120 between 12-72 hours after germination. Seeds from selfed R0 GUS expressing plants were germinated, and root and leaf samples were assayed for GUS activity. R1 progeny from R0 plants that have strong expression of GUS maintained the expression pattern, and R1 progeny from R0 plants that have low-level GUS expression lost the expression in the R1 generation. Histochemical GUS staining assay revealed similar YXA and YXB driven reporter expression pattern (Figure 2.2 B). GUS expression was also observed in leaves of transgenic plants (Figure 2.2C). High levels of GUS activity were seen in mature cells and in the elongation and maturation zones of roots, and in root caps, but no GUS activity was detected in the root meristematic region (Figure 2.2 B). The pattern of GUS expression is not uniform in leaves (Figure 2.2C).

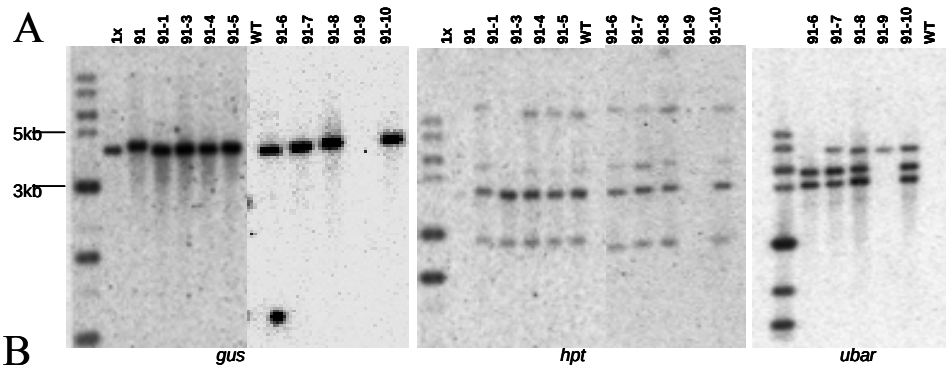
Accordingly, we selected some expressing lines (YXB91, 92, 138) from expressing lines: 49a, 68, 69a, 91-93, 98, 138 and some single-copy lines (YXB33, 60,

66, 70, and 100) from the transgenic population to perform further analyses (see genomic analysis of these lines). Different parameters were evaluated on the expressing lines: expression pattern in R1 progenies, possibility of recovering a single-copy expressing line after segregation of the T-DNA loci, recovery of a homozygous expressing line, and molecular analysis of the truncated T-DNA copy from line YXB138. Similarly, the transgene expression pattern in R1 progenies, recovery of homozygous R1 lines, and the type of silencing responsible for the lack of expression were some of the objectives addressed from the evaluation of single-copy lines.

Expressing Lines: YXB91, 92, 138

YXB91 originated from callus 1 in the 1st experiment with LBA4404 (faint GUS expression on callus) and showed strong expressing in the R0 line. Their integration patterns give a probable copy number after *Hind*III digestion of 1 bands of *gus*, 3 bands of *hpt* and one additional faint band indicated with an "f" (Figure 2.4A). 10 seedlings from YXB91 R0 plant after self- pollination were subjected to Southern blotting and histochemical GUS staining. The result showed that 90% of the siblings showed strong GUS expression.

A



B

	GUSexp	BARexp	hpt	gus	bar
91	+	+	3+1f	3	2
91-1	+	+	1+1f	3	na
91-2	+	+	3+1f	3	na
91-3	+	+	3+1f	3	na
91-4	+	+	3+1f	3	na
91-5	+	+	3+1f	3	na
91-6	+	+	3+1f	3	2
91-7	+	+	3+1f	3	2+1f
91-8	+	+	3+1f	3	2+1f
91-9	-	-	0	0	1f (?)
91-10	+	+	3+1f	3	2+1f

C

- 90% expressing R1 plants
- No cases of silencing
- The inheritance in the transgenic R1 progeny (same band pattern than R0 transformant) suggests integration in a single locus.

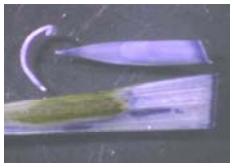


Figure 2.4. Genomic DNA analysis of R1 progeny from line YXB91 (*Hind*III-digested DNA).

(A) Southern blots of YXB91 progenies with gus, hpt and bar probes.

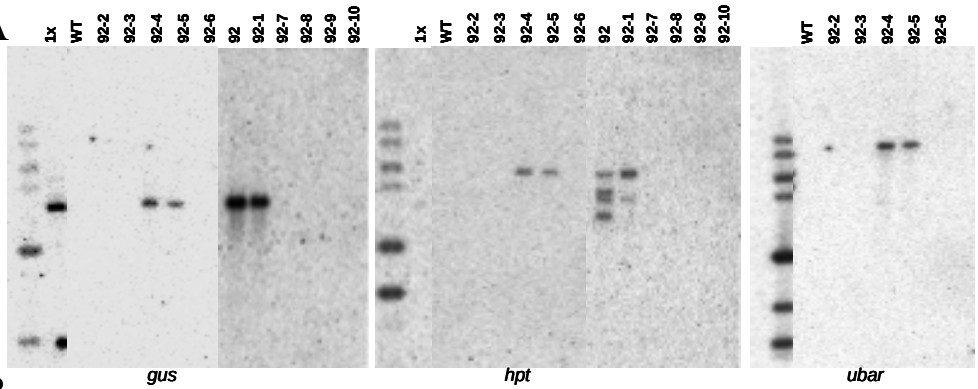
(B) Table of summary of Southern analysisand expression of YXB progenies.

(C) Figure showing root and leaf GUS expression in of progeny plants.

No cases of silencing were found in the R1 progeny. Transgene inheritance in the R1 progeny (transgenic R1 lines showing the same band pattern as the R0 transformant) suggested integration in a single locus. There is a faint band also appearing in some of the R1 lines in the hybridization with bar (Figure 2.4A).

Primary transformant YXB92 originated from callus 1 in 1st experiment with LBA4404 and is a strong expressing line. Its integration pattern (probable copy number) after *Hind*III digestion showed 3 bands of gus, 3-4 bands of hpt and 3 bands for bar. 10 seedlings taken from YXB92 R0 plant after self-crossing were subjected to Southern blotting and histochemical GUS staining. The result showed that 30% of the siblings showed weak GUS expression (only strong expression during the seedling stage). No cases of silencing were found in the R1 progeny. Inheritance pattern in the R1 progeny suggests integration in at least two separate loci. There are two single-copy lines, YXB92-4 and 92-5, that show weak expression in mature tissues. Line YXB92-4 is a homozygous single-copy line (Figure 2.5A). In 20 seedlings of R1 progeny from crossing 92 x WT (T309), 11 seedlings express only in the seed coat and 9 are non-expressing.

A



B

	GUSexp	BARexp	hpt	gus	bar
92	+	+	3-4	3	3
92-1	+	+	2	2	na
92-2	-	-	0	0	0
92-3	-	-	0	0	0
92-4	+	+	1	1	1
92-5	+	+	1	1	1
92-6	-	-	0	0	0
92-7	-	-	0	0	na
92-8	-	-	0	0	na
92-9	-	-	0	0	na
92-10	-	-	0	0	na

C

- 40% expressing R1 plants
- No cases of silencing
- The inheritance in the R1 progeny suggests integration in at least two separate loci.

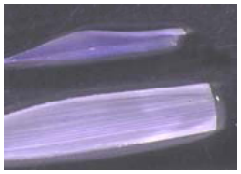


Figure 2.5. Genomic DNA analysis of R1 progeny from line YXB92 (*Hind*III-digested DNA).

(A) Southern blots of YXB92 progenies with gus, hpt and bar probes.

(B) Table of summary of southern and expression of YXB progenies.

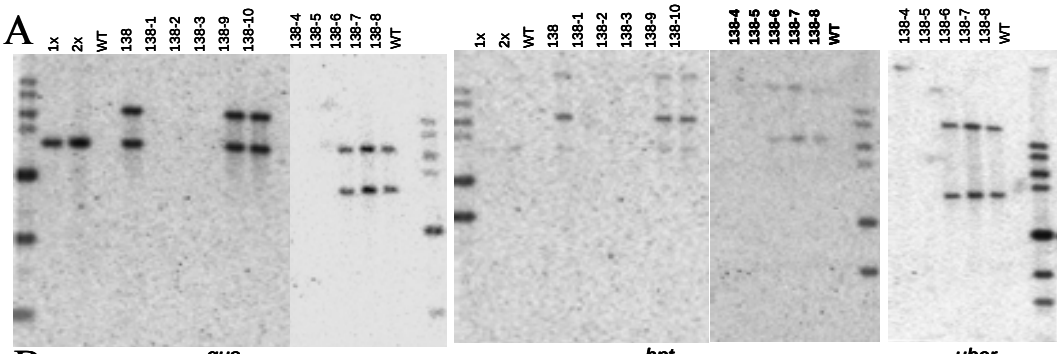
(C) Figure showing GUS expression in root and leaf of progeny plants.

YXB138 originated from callus 4 in second experiment with LBA4404 (faint GUS expression on callus) and is one of the strong expressing lines. Its integration pattern (probable copy number) after *Hind*III digestion shows a truncated copy of the YXB construct, indicated with a "T" (Figure 2.6B), along with an intact YXB copy, 2

hpt bands and 2 bands for bar. In further analysis it was confirmed that this "T" band hybridizes with both *RCg*2pro and *RCg*2ter (data was not show). In general, the truncated bands appearing in blots from expressing lines hybridized with *RCg*2ter, but not with *RCg*2pro (data not shown). On the contrary, in the case of some non-expressing lines also showing truncations of the YXB construct, the truncated band hybridizes with *RCg*2pro but not with *RCg*2ter. 10 seedlings taken from plant YXB138 R0 after self-crossing were subjected to Southern blotting and histochemical GUS staining. 50% of the siblings showed strong GUS expression. No cases of silencing were found in the R1 progeny. Transgene inheritance in the R1 progeny (transgenic R1 lines show the same band pattern as the R0 transformant) suggests integration in a single locus. Line YXB138-7 is a homozygous expressing line (Figure 2.6). In R1 progeny from crossing 138 x WT (T309), 8 out of 20 seedlings showed strong expression, 2 expressed only in the seed coat, and10 were non-expressing (data not shown).

Homozygous expressing lines YXB 92-4 (coming from an expressing line) and YXB138-7 were taken for further investigation. YXB 92-4 showed weaker GUS expression than the primary transformant, and totally lost GUS expression after the R2 generation. YXB138-7 was a homozygous expression line but the plant died after transferring to the greenhouse. 100% of the progeny of the 138-9-4, 138-9-2, 138-9-5 line are GUS expresser; SB was used to verify the homozygozity. The data are not shown.

A



B

	GUSexp	BARexp	hpt	gus	bar
138	+	+	2	1+1T	2
138-1	-	-	0	0	na
138-2	-	-	0	0	na
138-3	-	-	0	0	na
138-4	-	-	0	0	0
138-5	-	-	0	0	0
138-6	+	+	2	1+1T	2
138-7	+	+	2	1+1T	2
138-8	+	+	2	1+1T	2
138-9	+	+	2	1+1T	na
138-10	+	+	2	1+1T	na

- 50% expressing R1 plants
- No cases of silencing
- The inheritance in the transgenic R1 progeny (same band pattern than R0 transformant) suggests integration in a single locus.

C

Figure 2.6. Genomic DNA analysis of R1 progeny from line YXB138 (*Hind*III-digested DNA).

(A) Southern blots of YXB138 progenies with gus, hpt and bar probes.

(B) Table of summary of southern and expression of YXB progenies.

(C) Figure showing GUS expression in root and leaf of progeny plants.

Other expressing lines YXB68, 69, 88, 93, 98, 147 and148 SB are shown in figure 2.7. GUS histochemical staining of R1 seedlings without the truncated band after segregation showed weaker expression than the seedling with the truncated band after

segregation. This phenomenon indicated the truncated copy is related to an increase in the expression of GUS.

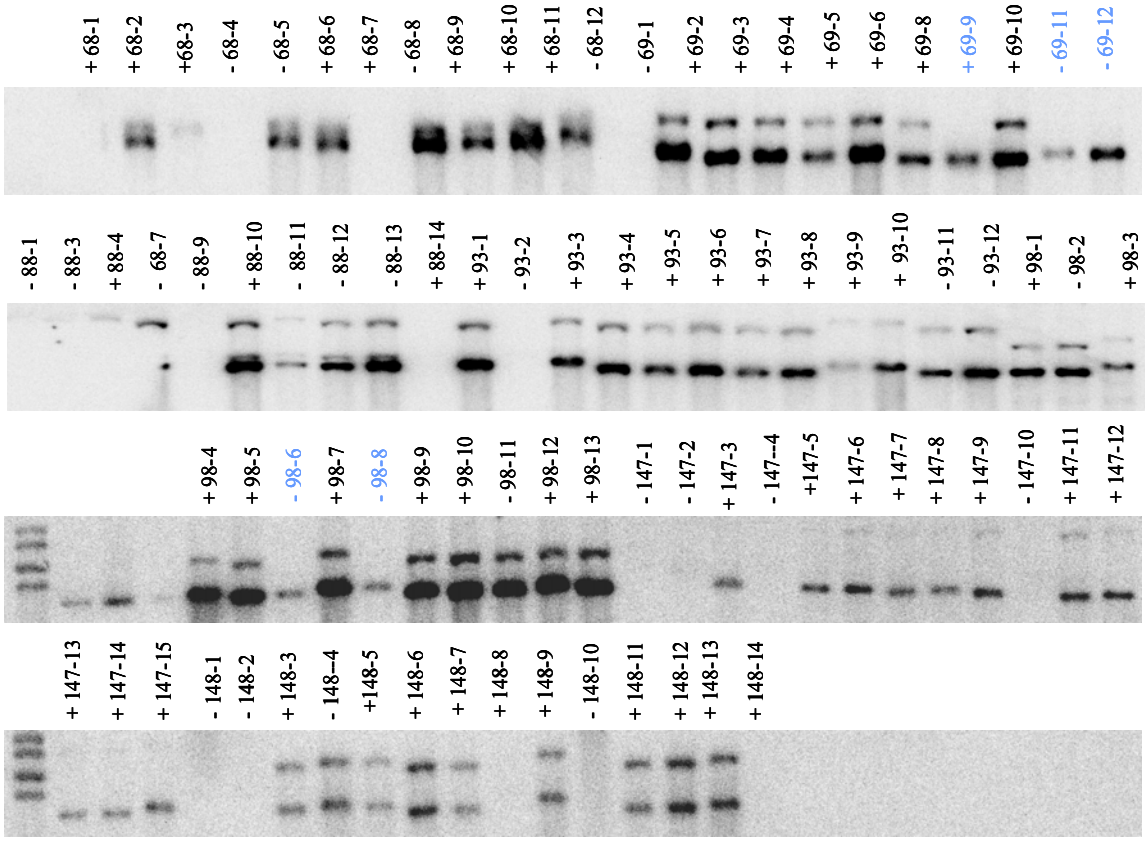


Figure 2.7. Genomic DNA analysis of R1 progeny from lines YXB68, 69, 88, 93, 98, 147, 148. *Hind*III digested DNA and using the gus probe.

Single-Copy Non-expressing Lines

YXB33, 60, 66, 70, and 100 from the transgenic population were used for further analyses. YXB 60, YXB 66, YXB 70 and YXB 100 originated from callus 5, 10, 12 and 10 respectively in the 1st experiment with LBA4404 (non-expressing). They are all non-expressing lines. YXB 66 is a sibling of YXB 67 and YXB 70 is a sibling of YXB 69b. YXB 33 originated from previous experiments. YXB 100 originated from callus 2 in the 1st experiments with LBA4404 (weak-expressing). The integration patterns (probable copy number) after *Hind*III digestion are given in the table below and all showed the same pattern.

hpt	gus	bar
1	1	1

For R1 progenies from self-crosses, YXB 60, YXB 66, YXB 70, YXB 33, YXB 100 and YXB 144, 70%, 80%, 70%, 90%, 85% and 75% respectively expressed gus in the young seedlings, but all lines were non expressing after maturity (6-7 leaves). About 90% showed high expression 90 hours after germination (Table 2.4 and Table 2.5). This loss of expression during plant growth is suggestive of PTGS or perhaps an indication of reversion of GUS expression after meiosis. Interestingly, there is a 2.8-kb band appearing in many blots from progeny of non-expressing plants. The size is regular in blots from different R1 progenies (Figure 2.8). Could it be a recombination event during meiosis? YXB 60-2, 70-3, 6 and 33-5 are homozygous single copy silencing lines identified for further investigation.

Table 2.4 Characterizations of progenies of single copy silenced lines

	gus (intensity)	zygosis		gus (intensity)	hpt (intensity)	zygosis
60-1	1+1*	hemi	66-1	0+1*	0+1*	-
60-2	2	homo	66-2	2+1*	2+1*	homo
60-3	1+1*	hemi	66-3	0+1*	0+1*	-
60-4	1+1*	hemi	66-4	0	0	-
60-5	1	hemi	66-5	1+1*	1+1*	Hemi
60-6	1	Hemi	66-6	1	1	Hemi

	gus (intensity)	Zygosis		gus (intensity)	hpt	Zygosis
70-1	1+1*	hemi	144-1	1+1	1+1	
70-2	0+1*	-	144-3	1+1	1+0	
70-3	2+1*	homo	144-4	2+1	2+1	homo for locus1?
70-4	1+1*	hemi	144-5	1	0+1	
70-5	1	hemi	144-6	1	1+1	
70-6	2	homo				

	gus (intensity)	zygosis
33-1	1	hemi
33-2	1	hemi
33-3	0	-
33-5	2+1*	homo
33-6	1	hemi

Table 2.5 Histochemical staining single copy seedlings

YXB60 GUSexp	42h	90h	3 leaves	6-8 leaves
+/-	28/12	16/1	17/7	0/21
%+	70	94	71	0

YXB66 GUSexp	42h	90h	3 leaves	6-8 leaves
+/-	32/10	16/0	0/16	na
%+	76	100	0	na

YXB70 GUSexp	42h	90h	3 leaves	6-8 leaves
+/-	28/12	16/1	2/18	0/20
%+	70	94	10	0

YXB33 GUSexp	42h	90h	3 leaves	6-8 leaves
+/-	34/6	13/1	0/17	na
%+	85	93	0	na

YXB100 GUSexp	42h	90h	3 leaves	5 leaves
+/-	16/22	15/2	11/5	0/18
%+	42	88	69	0

YXB144 GUSexp	42h	90h	3 leaves	5 leaves
+/-	30/10	16/1	15/3	0/17
%+	75	94	83	0

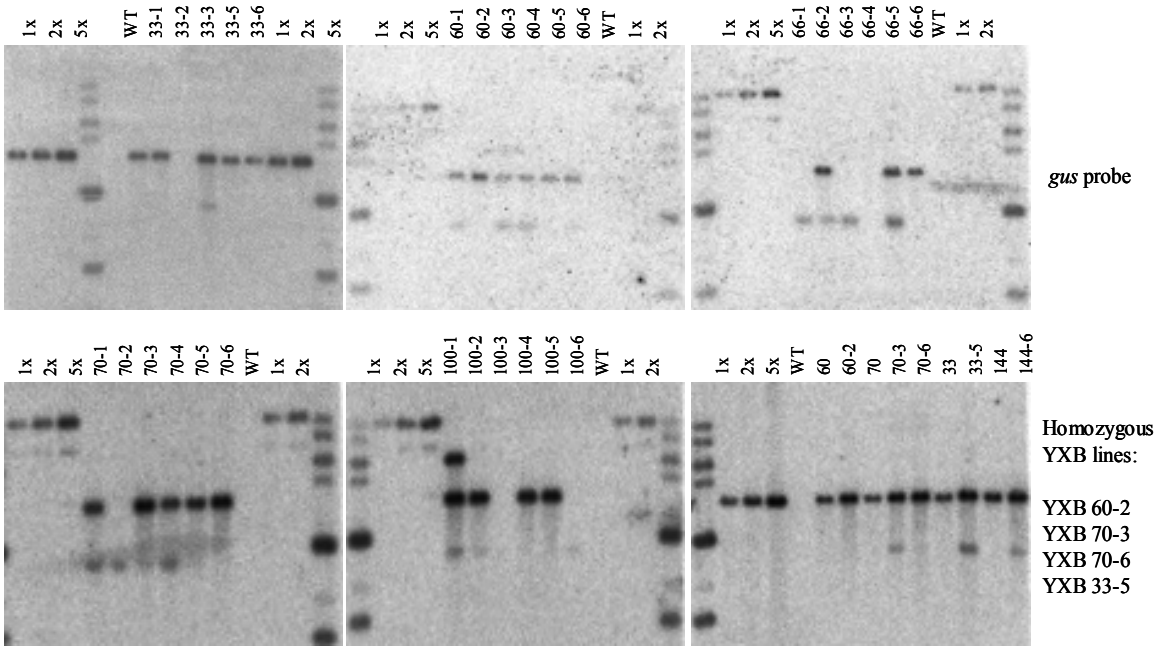


Figure 2.8. Genomic DNA analysis of R1 progeny from Single-copy lines YXB33, 60, 66, 70, 100. *Hind*III digested DNA and gus as a probe.

Inheritance and Integration of Transgenes in YXB Plants

Studies on progeny (self-crossing) (Table 2.6.) showed that the observed ratio in the segregating progeny doesn't correspond to the 3:1 Mendelian segregation ratio expected for a single dominant locus (or even higher for multiple loci), and also showed different rates of expression in different lines. Silencing phenomena in the progeny seedlings must be altering the ratio. The progenies from the strong expression lines, such as YXB138 and YXB91, showed strong expression and higher expression frequency in their progeny. The progeny from the weak expression lines, for example YXB 79 had the lowest proportion of GUS-expressing progeny.

Table 2.6 Inheritance of transgenes in YXB plants

Line (SE)	Number of seedlings	Number of GUS expressing seedlings	Line (WE)	Number of seedlings	Number of GUS expressing seedlings
68	19	10 (53%)	**58**	18	10 (56%)
69c	6	5 (83%)	**61**	19	10 (53%)
91	15	8 (53%)	**79**	14	1 (7%)
93	34	23 (68%)	**130**	10	9 (90%)

*Studies on progeny (self-crossing)

*The observed ratio in the segregating progeny doesn't correspond to the Mendelian segregation 3:1 expected for a single dominant locus (or even higher for multiple loci). Silencing phenomena in the progeny seedlings may be altering the ratio.

Table 2.7 Integration pattern

Single copy of T-DNA (YXB intact)			**Multiple copies of T-DNA (only intact copies of YXB)**			**Multiple copies of T-DNA (with truncated copies of YXB)**		
21			39			18		
Silenced		**Non-silenced**	**Silenced**		**Non-silenced**	**Silenced**		**Non-silenced**
21		0	37		2	12		6
NE	**WE**		**NE**	**WE**		**NE**	**WE**	
19	2		25	12		9	3	

*up to 6 copies of T-DNA fragments NE: no expression; WE: weak expression

*truncations and rearrangements of T-DNA

*all the plants with a single copy of T-DNA are silenced

*all expressing plants show multiple copies of T-DNA fragments and 6 of them carry a truncated copy of YXB construct

Interestingly every plant with a single copy of T-DNA is silenced (Table 2.7). Even the single copy lines of progeny from the strong expressing line YXB 92 became silent after two generations (Figure 2.9). This indicated that copy number also affected the expression of *gus* transgenes driven by the *RCg*2 promoter. All expressing plants show multiple copies of T-DNA fragments and 6 of them carry a truncated copy of the YXB construct. After segregation, the progeny lines with a truncated copy of GUS showed much stronger expression than the progeny lines without the truncated GUS copy.

Figure 2.9. GUS expression pattern in progeny of YXB lines. The order of the tube (L-R): root, leaf, inflorescence, and immature seed.

Cloning of a Truncated Copy of the YXB Cassette

Is a truncation of the YXB cassette, present in a 70% of the expressing lines, relevant for its expression? A truncated copy of the YXB cassette appears in *Hind*III-digested genomic DNA analysis as a band of more than 4.1kb in the pattern of some of the expressing lines and after segregation, the lines with this truncated copy of the YXB cassette showed stronger GUS expression than the lines that lost this truncated allele. These results led us to clone and analyze the function of the truncated copy. The lines chosen for this objective were 138 and 69a. The process was as follows: isolation of the DNA fragment after cutting the corresponding slice from the agarose gel (at the same migration level as the band appearing in the SB). (1) Cloning of the DNA fragments into the *Hind*III site of pPCRscript, or (2) Recircularization by ligation and PCR amplification of the whole fragment with primers known to be in the sequence. A clone was recovered from YXB138 in the *Hind*III site of pPCRscript. After sequencing, it was observed that unfortunately the cloned fragment was not the one expected, but a piece of rice genomic DNA. It did not include any sequence from the *gus* gene. [NOTE: The plasmid pPCRscript hybridizes with all the probes (*gus*, *hpt*, *ubar*) used to confirm the clone, which gave confusing results.] No results were obtained from the PCR method, since only small fragments could be amplified in very relaxed conditions.

Discussion

Transformed Rice with *RCg*2-gus

Agrobacterium-mediated transformation of plants generally yields transformation events with low copy number insertion of discrete T-DNA. Rearrangement and transfer of vector DNA that resides outside T-DNA border sequences are known to occur (Kumpatla and Hall, 1998b; Hall et al., 2001). Analysis of 158 YXB rice plants revealed 93 independent transformants with intact T-DNA insertion and/or partial transfer of the T-DNA. The copy number of T-DNA insertion ranged from 1-6 and independent events with single copy insertion of intact T-DNA have been found in 22/82. Four lines have only the *hpt* gene construct and 3 lines have YXB and *hpt*, but not the complete bar construct. Transfer of vector DNA outside the T-DNA border sequences into rice genome was not detected (Figure 2.10). The vector that we used in *Agrobacterium*-mediated transformation of rice is effective in delivering intact and discrete gene constructs at a low copy number into the rice genome. The efficiency of transformation is very high; co-cultivation experiments produced a large number of transformants and established a very effective transformation system.

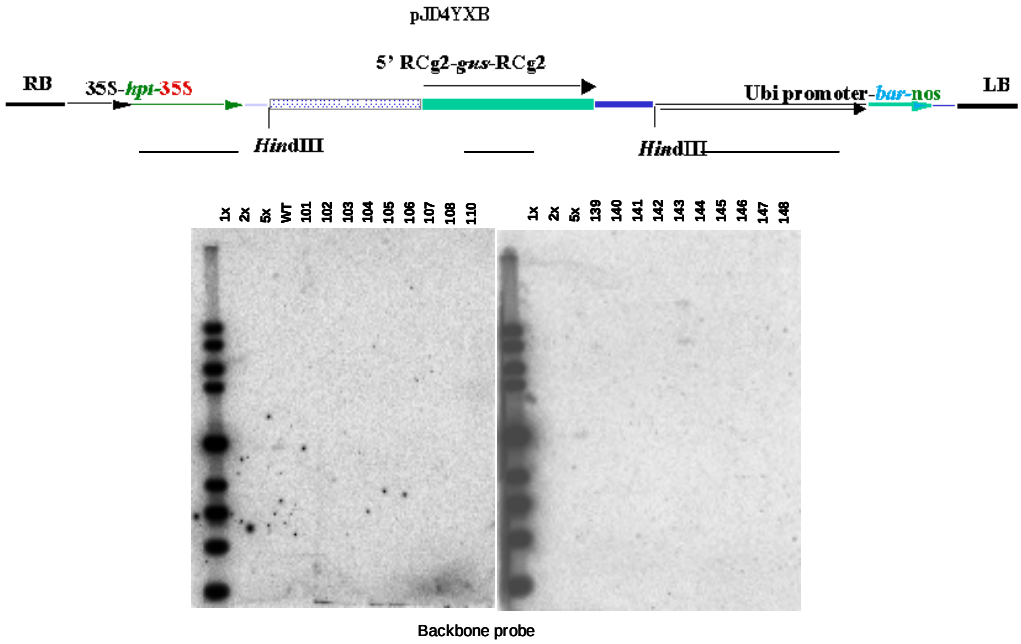


Figure 2.10. Analysis of genomic DNA digested with *Hind*III and electrophoresed in a 1% agarose gel. Plasmid backbone sequence as probe.

Inheritance of Expression in Expressing and Silenced Lines

Extensive investigation on the inheritance and expression of the *gus* transgene in YXB transgenic plants identified some high expressing R0 lines where expression remain stable for many generations after segregation. Also, progeny of several strong expressing lines with rearranged *gus* copies showed stronger expression levels than the progeny without the rearranged *gus* copy. Once they were homozygous, the expression levels remained stable for many generations at the same level. This result revealed that the rearrangements affected the expression level of GUS in the transgenic *RCg*2 rice plants. However, the strong expressing YXB92 primary line gradually lost expression after the segregation into single copy homozygous progeny YXB92-4 and YXB92-5.

Expression was completely eliminated after the R2 generation in YXB92-4-7. The 19 contained a single copy *gus* inserted but silenced lines, continued to be silenced for many generations in their homozygous progeny. These single copy silenced lines together with YXB92-4-7 single copy silenced progeny line indicated that there are different silencing mechanisms underlying the silencing phenomena of *RCg*2 transgenic plants, and provided an excellent system to study the mechanisms of transgene silencing.

*RCg*2-*gus* Is Silenced at a High Frequency

Generally, silencing of an introduced gene or/and an endogenous gene is a result of genomic modification of the introduced gene via largely uncharacterized or partially characterized mechanisms (Kumpatla and Hall, 1998b; Kumpatla and Hall, 1999). The gene-silencing phenomenon is found to be more frequently associated with transformation events that carry multiple copy insertion or duplicated sequences, such as transgenic plant material obtained using direct DNA delivery methods (Kumpatla et al., 1997). Low copy insertion of T-DNA via an *Agrobacterium*-mediated DNA delivery method reduced the frequency of silencing of introduced genes. The T-DNA in pJD4YXB carries three gene constructs: 35S-*hpt*, *RC*g2-*gus*, and mubi-*bar*. Expression of the mubi-*bar* was assayed using bialaphos painting of leaf segments. Of 82 independent events, nine are bialaphos sensitive indicating that the frequency of silencing of ubi-*bar* is low, and this may be due to absence of homologous sequence between rice genome and mubi-*bar*. Expression of GUS from RCg2-*gus* in rice plants were found to be silenced in nearly 90%. This high frequency of silencing demonstrated

that the 5' *RCg*2 sequence used in *RCg*2-*gus* is prone to silencing and is highly valuable for studies on genome modification of introduced gene. Among the GUS-expressing lines, low level GUS expression was detected in lines YXB56-59, 61, 78-79, 126-128, 130, 135, 147-158, initially, and eventually silenced (Table 2.2.). This implies existence of different levels of gene silencing mechanisms that affect the silencing of YXB, or gradual onset of silencing of YXB might have occurred in the lines that had initial low level of GUS. For high-level GUS-expressing lines with a characteristic histochemical staining pattern in the root, GUS expression remained active in the R1 generation. The uniqueness of high frequency silencing is that it is highly targeted to the YXB DNA while the flanking *hpt* and *bar* genes are not silenced in most lines. Interestingly, a unique and dramatic silencing phenomenon was observed in the YXB population. This system could offer an excellent opportunity to study transgene silencing in detail.

CHAPTER III

*RCg*2 GENE IS A ROOT-*PREFERENTIAL* GENE, NOT ROOT SPECIFIC AND STUDY OF *RCg*2 GENE SILENCING IN RICE

Introduction

To understand root development, genes that preferentially or specifically express in roots have been isolated from crop plants (Miao et al., 1991; Tsay et al., 1993). Detailed studies involving root-specific gene expression have been conducted in transgenic tobacco (Keller et al., 1989). These studies have shown that several hundred base pairs of immediate 5'-flanking sequence are important and sufficient for correct spatial expression of heterologous reporter genes (Xu et al., 1995). A few highly expressed root-specific genes have been identified in monocots such as barley (Lerner and Raikhel, 1989), maize (Held et al., 1993) and rice (Xu et al., 1995).

The expression pattern directed by the *RCg*2 promoter was characterized using a *gus* chimeric gene construct (5' RCg2-*gus*-OCS, YXA) in transgenic rice (Xu et al., 1995). Transgenic YXA plants were recovered following direct gene transfer (Battraw and Hall, 1990) and *Agrobacterium*-mediated transformation respectively. Transgenic YXB rice plants carrying YXB (5' RCg2-*gus*-RCg2-3', YXB) (Hall et al., 2001) were recovered following *Agrobacterium*-mediated techniques (Dong et al., 1996). Although strong GUS expression was observed in a very characteristic pattern in roots, expression was also observed in leaves of the same transgenic plants. These findings suggested that

some other important regions of the gene and not only the 5' and 3' sequences might additionally determine *RCg*2 spatial regulation. Also there is a possibility that *RCg*2 gene is not root specific in its expression.

Interestingly, a unique and dramatic silencing phenomenon was observed in the YXB population. This system could offer an excellent opportunity to study transgene silencing in detail. A thorough characterization of the transformants and of the *RCg*2 promoter was subsequently conducted.

A variety of silencing effects have been described in the literature, involving single transgene loci (Meyer et al., 1992; Elmayan, 1996; Iglesias et al., 1997), interactions between unlinked loci (Hobbs et al., 1993; Meyer and Saedler, 1996), or even interactions with or through an endogenous homologous gene (Jorgensen et al., 1996; Stam et al., 1998). (Trans)gene silencing can occur in a transcriptional (TGS) or a posttranscriptional (PTGS) manner, and it seems dependent on a large number of factors. Aspects such as insert location (Hobbs et al., 1990), rearrangements, organization (Yang et al., 2005a), multiple-copy loci (Waterhouse et al., 1998; Hamilton and Baulcombe, 1999; Lechtenberg et al., 2003; Mishiba et al., 2005); (Tang et al., 2007), homology to an endogenous sequence (reviewed by Meyer P, (Meyer and Saedler, 1996)), excessive level of transcription and others cause have been claimed to be the apparent triggers of silencing. Transformants containing a single transgene copy are also known to undergo silencing (Elmayan, 1996) and the presence of a homologous endogenous sequence may explain such gene silencing. Recent studies make it evident that at least some silencing mechanisms are part of the gene regulation system during plant growth and development

and are also involved in plant defense systems against invasive DNA or RNA sequences (virus, transposons) and surveillance processes that check the genome integrity (Kumpatla, 1997; Jorgensen et al., 1998; Kumpatla and Hall, 1998b; Matzke and Matzke, 1998; Iyer et al., 2000).

Previous studies in our laboratory focused attention on transgene silencing. Biolistics and other direct DNA transfer procedures frequently resulted in gene silencing in rice. These studies have been reported in detail (Kumpatla, 1997; Kumpatla and Hall, 1998a, b). Generally, *Agrobacterium*-mediated transformation give rise to lower transgene copy numbers compared to direct transformation methods. Consequently, the *Agrobacterium*-transformed YXB population may represent an excellent system to study silencing events derived from the presence of an endogenous promoter regulating a chimeric transgene. We focused our attention on two different objectives: (1) characterization of the YXB transformants and the *RCg*2 silencing phenomenon, and (2) diversification of the *RCg*2 promoter to avoid silencing the expression of transgenes. The finding that GUS was also expressed in leaves of YXB transgenic plants also made us rethink the expression profile of the endogenous *RCg*2 gene profile. RT-PCR and RNA gel blot were used to detect the expression of *RCg*2 gene in various wild type varieties of rice: Taipei309, Nipponbare, Gulfmont, IR-72 and TX-6 and the YXB transgenic rice.

Materials and Methods

RNA Isolation and Analysis

For leaf tissues, 14 day seedling (leaves) growing in a Magenta box (PHYTATRAY II, SIGMA CHEMICAL CO, ST. LOUIS, USA) was collected into 1.7 ml microtubes, and root tissues were collected and the medium washed away with distilled water. Roots and leaves were dried with Kimwipes and put into microtubes. The collected tissues were frozen with liquid nitrogen; ground with pellet pestles (KONTES Glass Company). Two methods were used to isolate total RNA in this research, TRIZOL® reagent (Invitrogen Inc., Carlsbad, CA) and RNeasy Plant for Mini kit (QIAGEN). RNA was extracted according to the manufacturer's instructions.

TRIZOL reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, was an improvement to the single-step RNA isolation method developed by Chomczynski (Chomczynski and Sacchi, 1987)**.** The tissue samples were homogenized in TRIZOL reagent (0.5 – 0.8 ml, 1 ml TRIZOL per 100 mg of tissue). After incubation of the homogenized samples at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes, 0.2 ml of chloroform was added; the tubes were then mixed vigorously and incubated at room temperature for 3 min. The mixture was centrifuged at 12,000xg for 15 min at 4°C. Centrifugation separated the biphasic mixtures into the lower red, phenol-chloroform phase and the upper colorless, aqueous phase. The upper phase was transferred to a new microtube and mixed with an equal volume isopropanol. The sample was incubated at room temperature for 10 min

and centrifuged at 12,000xg for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed once with cold 75% ethanol (1 ml) and centrifuged at 12,000xg for 10 min at 4°C. The washed RNA pellet was air-dried and dissolved in RNase-free water. The RNA was quantified by spectroscopic measurement of absorbance at wavelength 260nm. The total RNA was precipitated and aliquots kept in -20°C for short-term use or -80°C for long-term storage.

RNeasy Plant Mini Kit

The ground plant tissue (about 100 mg) was homogenized in RLC buffer (450 μL), containing guanidine hydrochloride (GITC), which immediately inactivated RNases. RNA was extracted according to the manufacturer's instructions. Each sample was mixed vigorously and then applied to a QIA shredder spin column, sitting in a collection tube (2 ml). The sample was centrifuged at 12,000xg for 2 min, which allowed the supernatant to pass through the column. The supernatant was transferred to a new tube, mixed with 0.5 volume of absolute ethanol, and applied to an RNeasy mini column, followed by centrifugation at 12,000xg for 1 min. The RNA sample on the column was washed once with RW1 buffer (700 μL) and twice with RPE buffer (500μL). The RNeasy column was transferred into a new collection tube and total RNA was eluted from column with RNase-free water (20 μL). Total RNA was extracted from various generations of YXB transgenic plants and various varieties of wild types of rice using Trizol.

RNA Blots Analysis

RNA concentrations were determined using Ribogreen as recommended by the supplier (Molecular Probes, Eugene, OR, USA) or by spectrophotometer. RNA blot analysis was conducted as described in (Yang et al., 2005a; Yang et al., 2005b). The total RNA was electrophoretically separated (5 V/cm) under denaturing conditions in a formaldehyde-MOPS, 1% agarose gel supplied with a NorthernMax kit (Ambion), then transferred (40 V/cm) in chilled 1 × MOPS buffer for 1 h to a Hybond nylon membrane (Amersham Biosciences, Piscataway, NJ, USA) using a BioRad Trans-blot Cell. The resulting RNA blot was cross-linked using a UV Stratalinker (Stratagene, La Jolla, CA) and labeled DNA probes (the gus coding sequence, *RCg*2 coding sequence and 3'UTR of *RCg*2, or the EF1-a coding sequence) were prepared using a Decaprime II kit (Ambion) and hybridized overnight at 42°C in ULTRAhyb ultrasensitive hybridization buffer (Ambion). The membrane was washed twice with 2 × SSC, 0.1% SDS for 5 min then twice with 0.1 ×SSC, 0.1% SDS for 15 min. Hybridization signal densities were measured using a Fujix 2000 phosphorimager and MacBAS v2.5 software (Fuji Machine Manufacturing, Chiryu, Aichi, Japan) (Yang et al., 2005a; Yang et al., 2005b).

For RNA gel blot analyses, samples loaded on 1% formaldehyde denaturing agarose gels were run at 5 V/cm for 2 to 3 h in 13 3-(N-morpholino)- propanesulfonic acid buffer. RNA was transferred using a Trans-Blot Cell (Bio-Rad, Hercules, CA) for; 1 h at 40 V. Probes were made from DNA templates using the DECAprimeII system (Ambion, Austin, TX), and hybridization was performed with Ultrahyb buffer (Ambion).

Densities of hybridization signals were measured with MacBas software (Fujifilm, Tokyo, Japan), and background signals were subtracted from the reading.

Primers and Probes Made by PCR

Genomic DNA was extracted from seedlings of WT T309 using a CTAB (hexadecyl trimethyl ammonium bromide) method (Taylor and Powell, 1982; Saghai-Maroof et al., 1984). PCR was performed using PCR master mix from Promega (Madison, WI, USA). PCR primers sequence for the internal control Rice EF1-α:

Forward: 5'-GCCCATGGTTGTGGAGACCTTCTC-3'

Reverse: 5'-TCATTTCTTCTTGGCGGCAGCCTTG-3'

Primers sequence for RCg2 coding region:

Forward: 5' –ATGGCTGCTTCCAAGGTCGCTC-3' Tm 58°C

Reverse: 5' TTAGCAGGTGAAGTCGGAGGGG 3' Tm 59°C

Primer sequence for RCg2 3' untranslated region (3' UTR) primers:

Forward: RCg2 3'UTR F: TAATTGAGCATGGAAAGAGCTCAAAC Tm 57.8°C

Reverse: RCg2 3'UTR R: CTAACACAAGGGGGGATAATAGCTTATC Tm 58.4°C

Primer sequence for Gus coding region:

Forward: 5' GGT GGG AAA GCG CGT TAC AAG-3' Tm 56°C

Reverse: 5'-GTTTACGCGTTG CTT CCG CCA-3' Tm 56°C

Thermocycling conditions were: 94°C for 5 min, followed by 38 cycles of 94°C for 1 min, various annealing temperature (53°C for EF1-α, 55°C for RCg2 coding region,

53°C for 3' UTR and 51°C for *gus*) for 1 min and 72°C for 2 min, with a final polymerization step of 72°C for 10 min. The products of the amplification were subjected to electrophoresis through 1% agarose gel, followed by staining with ethidium bromide (100ng/L). The gel was then digitally imaged and was analyzed using semi-quantitative method Image J.

*RCg*2 Coding Region, 3'-*UTR* Region Probes and *EF*1-α Were Amplified Using PCR and Purified with a PCR Purification Kit

Gus probes were made either from PCR or isolated from plasmid pJD4 digested with *BamH*I and *EcoR*V. Hpt probe were made from pJD7XbaI plasmid digested with *BamH*I.

Quantitation and Normalization of PCR Signals with Semi-quantitative Method: Image J

Densitometry of data obtained from Southern blot and ethidium bromide staining of PCR products for at least two completely independent experiments was performed using ImageJ software (National Institutes of Health). Normalized *RCg*2 signals were obtained by subtraction of the background signal from the experimentally obtained value. Normalized *EF-1*α control signals were determined similarly and, for each variety, the signal enrichment for the normalized *RCg*2 signal was calculated relative to the normalized EF-1 α signal. Normalization of PCR product signals and relative enrichment (RE) values were derived as described in Ng et al. (Ng et al., 2006).

RT-PCR Analysis

Total RNA samples were treated with DNase I before RT-PCR amplification using the QIAGEN One-Step RT-PCR kit (Qiagen, Valencia, CA). Total RNA (1 mg) was combined with 10X DNase buffer (1μL) [Tris-HCl (200 mM, pH 8.4), $MgCl_2$ (20 mM), KCl (500 mM)], and DNase I (1 unit, 1 μL, Invitrogen) in the final volume of 10-μl. The reaction was carried out at 25^{o}C for 10 min and the enzyme was inactivated at 65^{o}C for 15 min in the presence of EDTA (2.5 mM). The RNA samples were used directly for RT-PCR reaction.

Total RNA (0.5μg) was isolated from rice leaves and root using Trizol and digested with RNase-free DNase I, was subjected to RT-PCR analysis employing the Qiagen one-step RT-PCR kit. The DNA free RNA was mixed with dNTP (100 μM each), 1X buffer (supplied with the kit), enzyme mix (Omniscript and Sensiscrip reverse transcriptase, 2 μL), and forward and reverse gene specific primers (0.6 μM each) in the total volume of 50 μL. Before the reaction started, a thermal cycler was heated to 50^{o}C then the tube was placed in the machines and reverse transcription reaction was performed at 50^{o}C for 30 min, followed by incubating at 95^{o}C for 15 min to inactivated reverse transcriptase, denature the cDNA template, and activate HotStar Taq DNA polymerase. The PCR amplification was performed for different number of cycles of denaturation at 95^{o}C for 1 min, annealing for 1 min and extension at 72^{o}C for 1 min, followed by extension at 72^{o}C for 10 min. The annealing temperature (Ta) was the melting temperature ™-5^{o}C. The RT-PCR reactions included 0.6μM gene-specific primers and 0.08 μM EF1 -specific primer (as an internal control). Primer sequences

used in assessing transcript abundance and reaction conditions are provided as supplemental material.

Following RT-PCR, the products were resolved using agarose (1%) gel electrophoresis and stained with ethidium bromide (1 g/L). Images of the stained gels were captured using a digital camera, and relative densitometry intensity (pixels/mm2), normalized relative to EF-1α (internal control), was obtained using MacBAS v2.5 software (Fuji, Tokyo, Japan). Values shown are for RT-PCR analyses of two entirely independent experiments and were within the linear amplification range. For DNA contamination controls in RT-PCR, RT enzyme mix was replaced by Taq DNA polymerase from Invitrogen.

5-azaC Treatments

Reactivation of YXB using an inhibitor of DNA methylation (demethylating agent) 5-azacytidine (5-azaC) was conducted by growing rice tillers in nutrient solution containing 5-azaC. Methods were as described in Chapter II.

Histochemical and Fluorometric Assays for GUS Activity

Histochemical GUS staining was performed for vegetative tissues (leaves, roots, inflorescences) with 5-bromo-4-chloro-3-indoxyl-â-D-glucuronic acid (Xgluc) as a substrate (Jefferson et al., 1987b). Samples were stained overnight (16 h) at 37 ^{o}C and chlorophyll was removed by 95% ethanol after staining (Chandrasekharan et al., 2003). Fluorometric assays of GUS activity of leaves and roots were performed as described by

(Jefferson et al., 1987b). GUS activity was calculated as pmol 4-MU per hour per microgram protein and data were analyzed with SPSS 11.0 for Windows software. For each construct, three independent assays were performed unless otherwise specified.

High Molecular Weight RNA Was Separated from Low Molecular Weight RNA by Precipitation with 10% PEG8000 in 0.5 M NaCl (Ambion, Austin, TX)

For smRNA detection (Reinhart and Bartel, 2002), low molecular weight RNA present in the supernatant after treatment of the total RNA with 10% PEG8000 in 0.5 M NaCl was precipitated by being made to 75% ethanol, dissolved in RNase-free water, and subjected to denaturing PAGE (16% acrylamide, 7 M urea: Hamilton and Baulcombe, (Hamilton and Baulcombe, 1999). The separated RNAs were electrotransferred to Zeta Probe GT membrane (BioRad, Hercules, CA), UV cross-linked and hybridized with DNA probes prepared using a Decaprime II kit (Ambion) and purified on a Biospin 6 column (BioRad). Both digested and undigested probes prepared from the gus transgene and *RCg*2 coding region and 5' UTR and 3' UTR were used for hybridization. The membrane was hybridized in ULTRAhyb –Oligo buffer (Ambion) at 42^{o}C overnight. The blot was then washed twice with 2 × SSC, 0.5% SDS at 42^{o}C for 30 min.

Two-week-old rice seedlings growing on MS medium with and without 5-azaC (25 mg/L) were harvested and ground in liquid nitrogen. Trizol reagent (Invitrogen, Carlsbad, CA) (20 mL) was added to 2 g of ground tissue, and RNA was extracted according to the manufacturer's instructions.

Low molecular weight RNAs and high molecular weight RNAs were fractionated from total RNAs using the method of Hamilton and Baulcombe (Hamilton and Baulcombe, 1999).

For smRNA detection, 60 mg of low molecular weight RNAs was separated on a 17% polyacrylamide gel containing 7 M urea. Gels were transferred onto Hybond^{+} nylon membranes (Amersham, Piscataway, NJ), and hybridization was performed using Ultrahyb-Oligo buffer (Ambion) at 42 and 408C. Full-length *RCg*2 probe and gus, as well as synthesized DNA oligonucleotides corresponding to their double-stranded regions, were used as probes.

Total RNA from silenced and expressing YXB lines was extracted with Trizol, dissolved in TE buffer, and made to a final conc. of 10% PEG, 1M NaCl, for 2 h. High mol. wt RNA was sedimented by centrifugation and small RNAs in the supernatant precipitated with ethanol. Northern analysis was used to detect miRNA. RNA was separated by 7M urea – 17% PAGE and transferred to a nylon membrane. Six miRNA homologs predicted for Oryza from an Arabidopsis miRNA cloning study were used as queries against rice EST databases and, based on a maximum number of hits; mir160a was selected as a probe for detection of miRNA. The same blot was stripped and re-probed for detection of siRNA. Analyses were repeated at least twice. Hybridizations and washes were carried out at 40ºC. Probes for detection of *RCg*2-related siRNA were based on the *RCg*2 promoter and/or coding sequence.

Genomic DNA Methylation Analysis

Genomic DNAs extracted from rice lines T309, expression line YXB138 homologous progeny, YXB92 silenced progeny, silenced lines YXB70 progeny and YXB60 were digested using methylation-sensitive restriction enzyme *Pvu*I (CGAT|CG). DNA was treated and after electrophoretic separation of DNA on 1% agarose gel for 12-15 hours at constant 23 volts, the DNA was transferred to HybondTM-N+ nylon membrane (Amersham, Piscataway, NJ). Genomic DNA blot analysis was as described by Buchholz et al. (Buchholz, 1998 #7800). [32P]dCTP-labeled probes were made using a DECAprimeTM II DNA labeling kit (Ambion, Austin, TX). Membranes were washed with 2×SSC [1×SSC = 0.15 M sodium chloride /0.015 M sodium citrate (pH7)]/0.1% SDS at 65°C for 1 hr (low stringency), or with 0.3×SSC /0.1% SDS at 65°C for 1 hr (moderate stringency).

Bisulfite Genomic DNA Methylation Sequencing

Genomic DNAs extracted from rice lines T309, expression line YXB138 homologous progeny, YXB92 silenced progeny, and silenced lines YXB72 progeny and YXB60 were digested by *Hind*III and purified with phenol chloroform. DNA was treated with bisulfite, which selectively deaminates cytosine but not 5-methylcytosine to uracil. This leads to a primary sequence change, as unmethylated cytosines are converted to uracil and then thymidine after PCR; however, 5-methylcytosine is not converted by bisulfite and remains as a cytosine after PCR. This primary sequence change can be quantified using direct sequencing, restriction digestion, or pyrosequencing. In brief, a

total of 500 to 750 ng of purified genomic DNA was used for bisulfite treatment for 16 h using the EZ DNA methylation kit from Zymo Research (Orange, CA). After elution of the treated samples, 2 to 4 mL of elute was used for subsequent PCR amplification with AmpliTaq Gold DNA polymerase (Foster City, CA). PCR analyses were performed according to the manufacturer's recipe with an optimized $MgCl_2$ concentration of 4 mM. The cycling parameters were as follows: 94ºC for 2 min; four cycles of 94ºC for 1 min, 53ºC for 2 min, and 72ºC for 4 min; 39 cycles of 94ºC for 45 s, 53ºC for 2 min, and 72ºC for 2 min; and 72ºC for 10 min. The primers to amplify the *RCg*2 promoter top strand DNA were 5'-AGAAGGGGYGAGYTAGAGGATTG-3', the reverse primer of endogenous *RCg*2 promoter was 5'-ARCRACCTTRRAARCARCCAT-3'. Reverse primer of transgenic *RCg*2 promoter was 5' -CRATTCARACTRAATRCCCACA -3'. PCR products were cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and plasmids containing inserts of the correct size were sequenced using T7 primer. This method was as described in Yang et al. (Yang et al., 2005b).

Primers of methylation sequence PCR:

RcgMS (F): 5’ -AGA AGG GGY GAG YTA GAG GAT TG -3’

RcgCMS (Rev): 5’ -ARC RAC CTT RRA ARC ARC CAT- 3’

RcgGMS (rev): 5’ - CRA TCC ARA CTR AAT RCC CAC A - 3’

Nuclear Run-on Transcription Assay

Isolation of leaf nuclei and run-on transcription were performed essentially as described by Ingelbrecht and de Carvalho (Ingelbrecht and de Carvalho, 1992). Slot blots were prepared using nitrocellulose membranes (Schleicher and Schuell, Keene, NH) containing 1 μg of linearized plasmid DNA (or the fragment isolated from 1 μg of plasmid DNA). Hybridization was performed for 2 d and the filters were washed to a final stringency of 0.3XSSC and analyzed on a Fujix BAS 2000 Bio-Imaging Analyzer.

Results

RNA Gel Blot Analyses Confirmed *RCg*2 Is a Root-Preferential Gene Rather Than Root- Specific Gene

As histochemical analysis of GUS expression of YXB (in this research) and YXA (Xu et al., 1995) showed expression in both roots and leaves, involvement of the 3' -downstream sequence of *RCg*2 gene would not explain the unexpected expression in leaf tissue seen in the transgenic plants. These results made us rethink the expression profile of the endogenous *RCg*2 gene.

RNA gel blots were used to profile the expression of the *RCg*2 gene in various wild type varieties of rice: Taipei309, Nipponbare, Gulfmont, IR-72 and TX-6. The result showed that the expression of *RCg*2 genes did not vary greatly in the same organ between varieties, Comparisons among T309 leaf (T309L), Nipponbare leaf (NPL), IR-72L, Gulfmont leaf (GUFL) and TX-6, showed similar expression, and their root samples also showed similar expression level for either the *RCg*2 coding region as probe or the 3'-UTR used as a probe. However, there was a significant difference between roots and leaves (Figure 3.1A) with expression in roots 2X as high as in leaves using semi-quantitative method Image J (Figure 3.1 B and 3.1C). T309 showed the highest expression 137.63 in root, and TX-6 showed the highest expression 85.5 in leaf (no root sample here). However, the expression of NIP *RCg*2 in leaf showed the lowest expression. The expression ratio of root to leaf samples differed slightly among the varieties. T309 showed the highest ratio, about 2.05, the ratio of Nipponbare was 1.84,

but the expression ratio of Gulfmont and IR-72 was almost same (Figure 3.1C). The RNA samples in the blot labeled with *RCg*2 coding sequence showed much stronger signal than those labeled with *RCg2* 3'-UTR (Figure 3. 1A), so could explain the contradiction results with Xu et al. (Xu et al., 1995) where it was stated that *RCg*2 is root-specific gene.

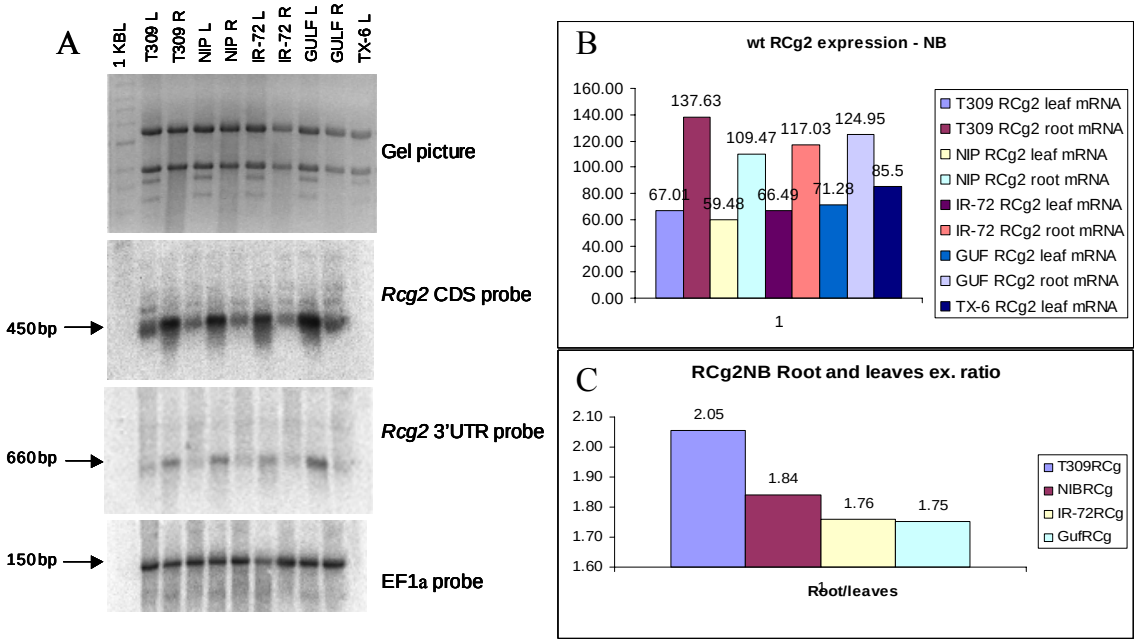


Figure 3.1. Northern blotting analysis of *RCg*2 expression in different varieties.

(A) 5ug of total RNA was used for the Northern blot using *RCg*2 coding sequence 450bp, 3'-UTR 660bp and EF1a 100bp as probes.

(B) Image J data shows relative expression level of *RCg*2 in different tissues and varieties.

(C) The ratio of *RCg*2 expression in root and leaves.

RT-PCR Analysis of *RCg*2 Expression in Various Varieties and Tissues also Confirmed that *RCg*2 Is a Root-Preferential Gene

More precisely RT-PCR analysis of *RCg*2 expression was performed to confirm the expression profile of *RCg*2 in various varieties and tissues. Total RNA (1 g) was isolated from rice leaves and roots using Trizol and digested with RNase-free DNase I, was subjected to RT-PCR analysis employing the Qiagen one-step RT-PCR kit. The results shown in figure 3.2 reveal that *RCg*2 expression in T309, NIP and GULF roots was stronger than the expression in leaf samples. However the *RCg*2 expression in IR-72 and TX-6 root samples was not higher than the expression in the leas samples, and actually was a little bit lower than expression in leaf. This probably is due to the RNA sample quality and degradation. The ratio of expression in root samples to leaves samples in T309 was about 2, consistent with northern blotting data. The ratio of expression in root samples to leaf samples in NIP and GULF was 1.3 and 1.7. Although the expression of IR-72 and TX-6 in root is not higher than the expression in leaf, we still know the expression of GUS in root is 1.5 – 2 times higher than in leaf. The expression in the leaf from the RT-PCR also confirmed that *RCg*2 not only expressed in roots but also in leaf, and the expression level is significantly different between root and leaf.

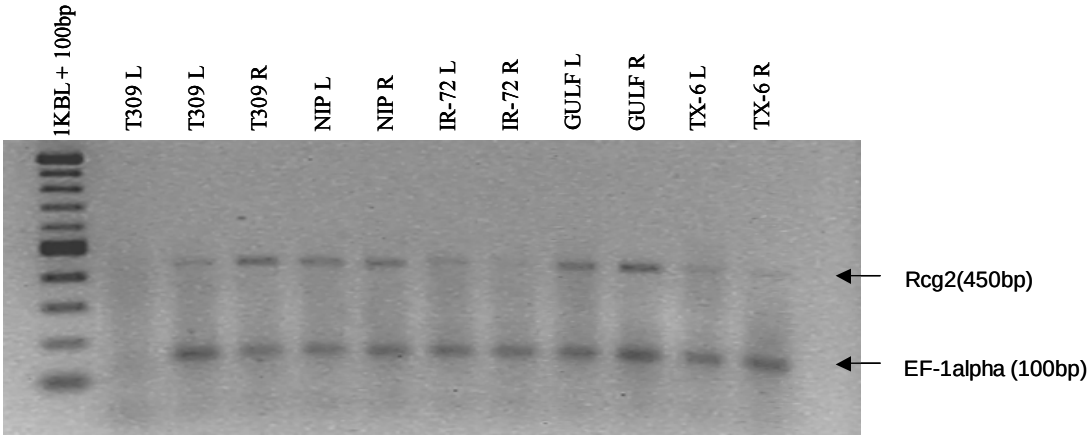


Figure 3.2. RT-PCR analysis of *RCg*2 expression in different varieties and tissues. RT-PCR using 0.5 ug total RNA as template, 6 μM (50 ng) of each RCg2 primer and 0.08 μM (30 ng) EF-1 alpha as internal control. PCR run 26 cycles.

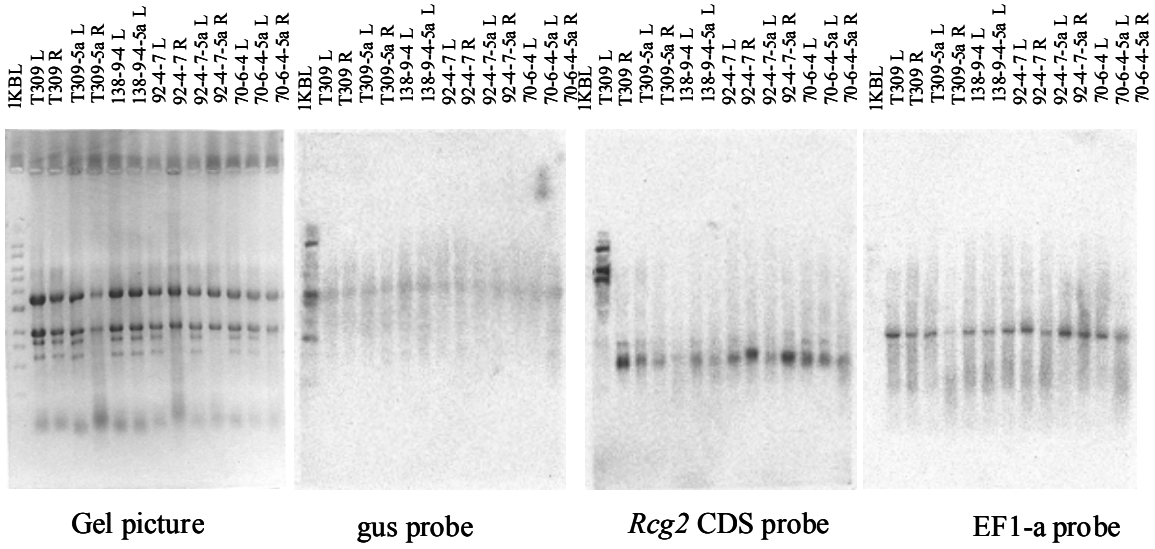


Figure 3.3. Northern blotting analysis of transgenic YXB. 4 ug of total RNA was used for the northern blot using *RCg*2 coding sequence, gus and EF1-α as probe.

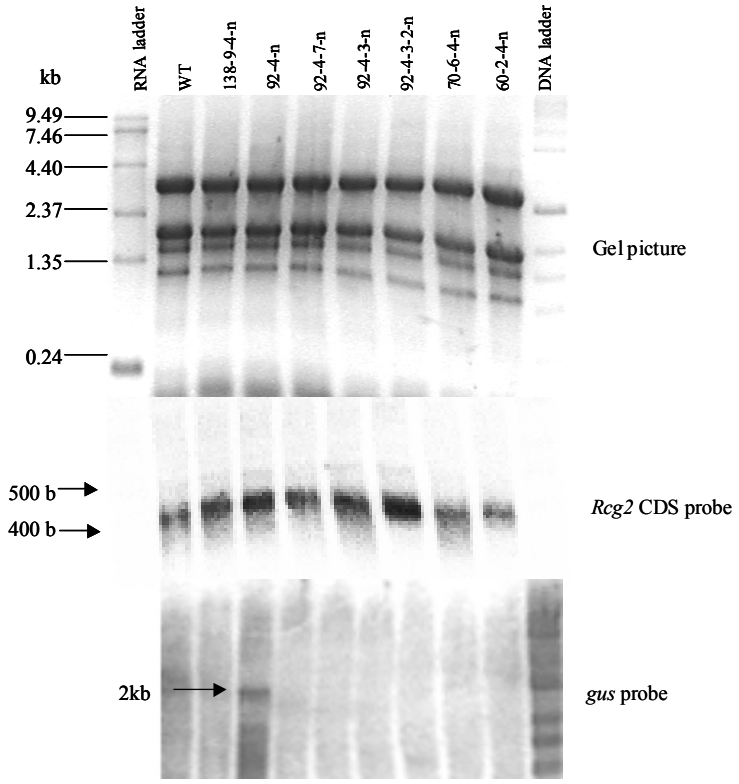


Figure 3.4. Northern blot analysis of YXB. *RCg*2 coding sequence as probe. RNA from leaf.

RT-PCR and Northern Blot Analysis of *RCg*2 Expression and Transgene *gus* Expression in Transgenic Homozygous Lines Progeny also Confirmed that *RCg*2 is a Root-Preferential Gene

Total RNA samples from the YXB homozygous lines progeny were subjected to northern blotting analysis. The results showed the endogenous *RCg*2 existent in all the progeny from both homozygous expressing lines YXB 138-9, YXB 92-4 and silenced lines YXB70-6 and YXB60-2 (Figure 3.3 and Figure 3.4). The transgene *gus* only

appeared in strong expression line YXB138 and its progeny (Figure 3-4). GUS expression was lost after the R2 generation in YXB92-4, which is a segregate from the strong expression line, YXB 92. Rice elongation factor *EF-1α* was used as internal control. RT-PCR results also showed similar result for either expressing lines or silenced lines for RCg2 endogenous gene (Figure 3.5A).

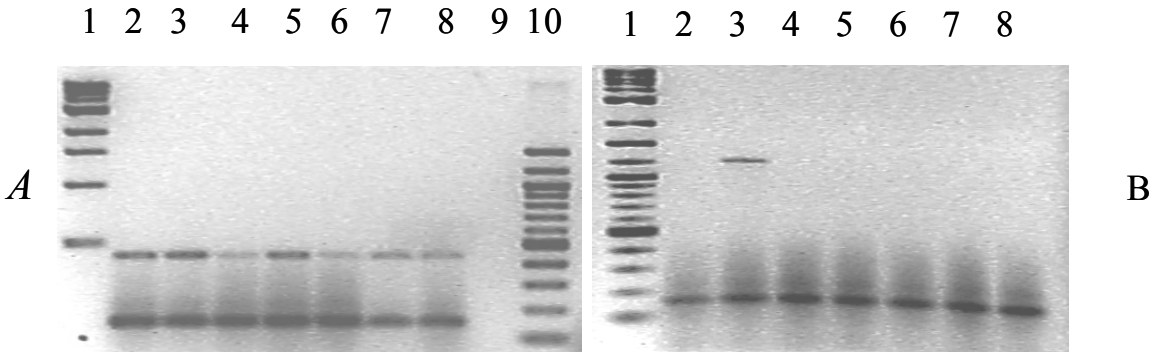


Figure 3.5. RT-PCR analysis of endogenous *RCg*2 and transgene *RCg*-GUS.

(A) RNA was used for reverse transcription using RCg2 primers and amplified by PCR in a one step reaction using 28 (optimum) cycles. Silenced lines progeny of YXB 70, 60 and show a reduction in expression, and YXB 92 is variable over three generations. The upper band is the endogenous gene-specific band; the lower band is an internal control (EF1 alpha).

(B) Similar experiment to that shown in A, but with a GUS-based internal primer. Expression of the transgene is only seen in line YXB 138. Lane 2: T309; lane 3: 138-9-4-n (expressing); lane 4: 92-4-n (silenced); lane 5: 92-4-7-n (silenced); lane 6: 92-4-3-2-n (silenced); lane 7: 70-6-4-n (silenced); lane 8: 60-2-4 -n (silenced); Lanes 1 and 10 are 1 kb and 100 bp DNA ladders, respectively; lane 9 is a negative control (no RNA).

These findings suggest that the 1.65kb of 5' -flanking sequence and 3' -flanking sequence of the *RCg*2 gene probably do not provide sufficient information for precise spatial regulation in transgenic rice plants.

5- Azacytidine (azaC) Treatments Reactivated the GUS Expression of Silenced YXB Lines

The data shown in Table 3.1 and additional studies about of pYXA and pJD4 transgenic rice plants (not shown) reveal that the *RCg*2 promoter is silenced at a high frequency in transgenic rice. An important control was to show that the integrated transgene was capable of expression. This can be accomplished in many cases by reactivation with 5-azacytidine or trichostatin A (Kumpatla et al., 1997; Kumpatla and Hall, 1998a, b).

Methylation is one of the most frequently documented genome modifications of introduced and endogenous genes. Methylation inhibitors, such as 5-azaC, can change methylation status (Weber et al., 1990). Fresh grown root samples were collected from tillers of primary transformants: YXB1, 5, 7, 8, 12 and 38 that had been grown in the presence of 5-azaC (50 mg/L) for 5 days. X-gluc staining of the root samples revealed patchy blue staining in areas other than root tips of YXB5, 7, 8, 12, 38, 60 and 70. Seeds from YXB5, 13, 25, 28, 33, 37, 38, 44, 60, and 70 were germinated in the presence of 50 mg/l of 5-azaC. Root samples from these plants were taken after one and two weeks and stained for GUS activity. Reactivation of GUS was observed in these lines (Table 3.1)

(Figure 3.6 and Figure 3.7) indicating that methylation very likely is involved in silencing of YXB in these lines.

Table 3.1 Reactivation of GUS expression of YXB plants after 5-azaC treatments

	Lines	GUS expression	
		No treatment	+5- azaC (50mg/l)
R0 Root	2	-	+
	12	-	+
	37	-	+
	60	-	+
	70	-	+
R1 seedling	2	-	-
	12	-	-
	24	-	-
	25	-	-
	37	-	+
	60-2	-	+
	70-4	-	+
	92-4	+	+
	92-5	+	+
	33-5	-	+
R2 seedling	60-2n	-	+
	70-4n	-	+
	92-4n	-	+
	92-5n	-	+
	33-5n	-	+

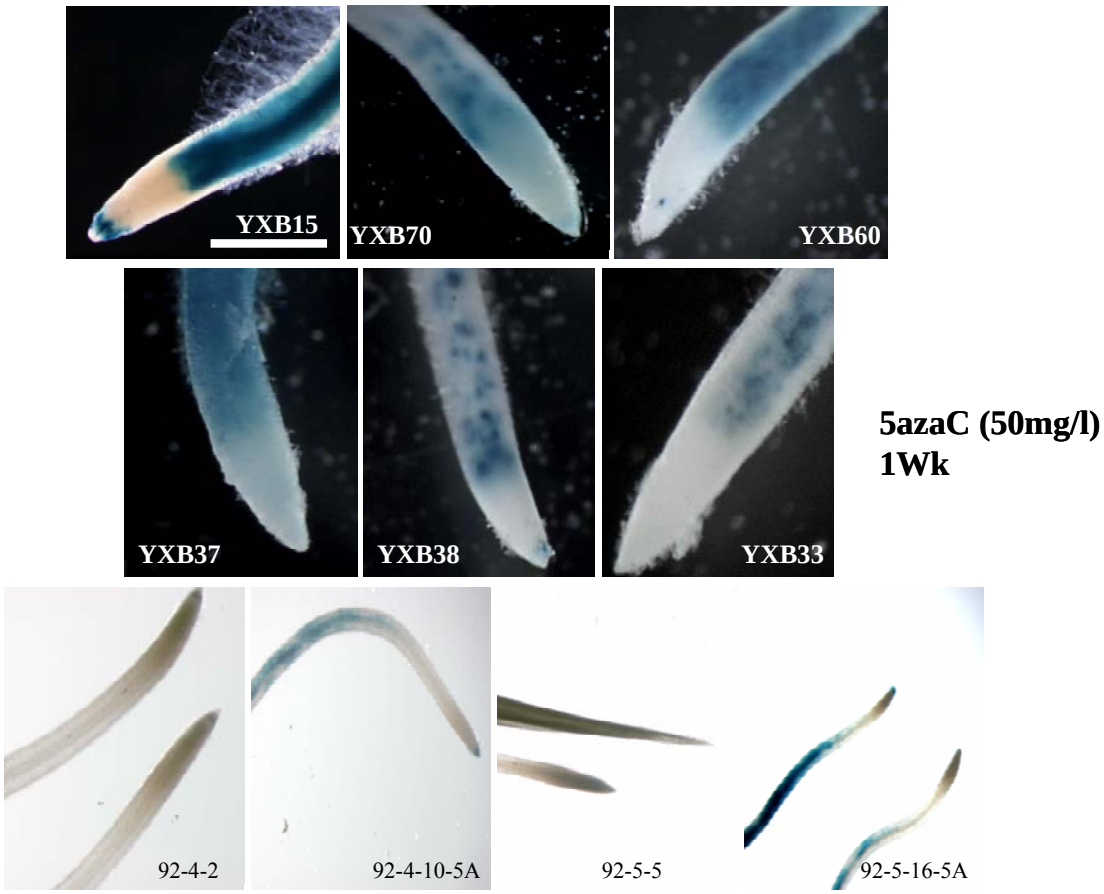


Figure 3.6. YXB GUS staining pattern after 5- azaC treatment.

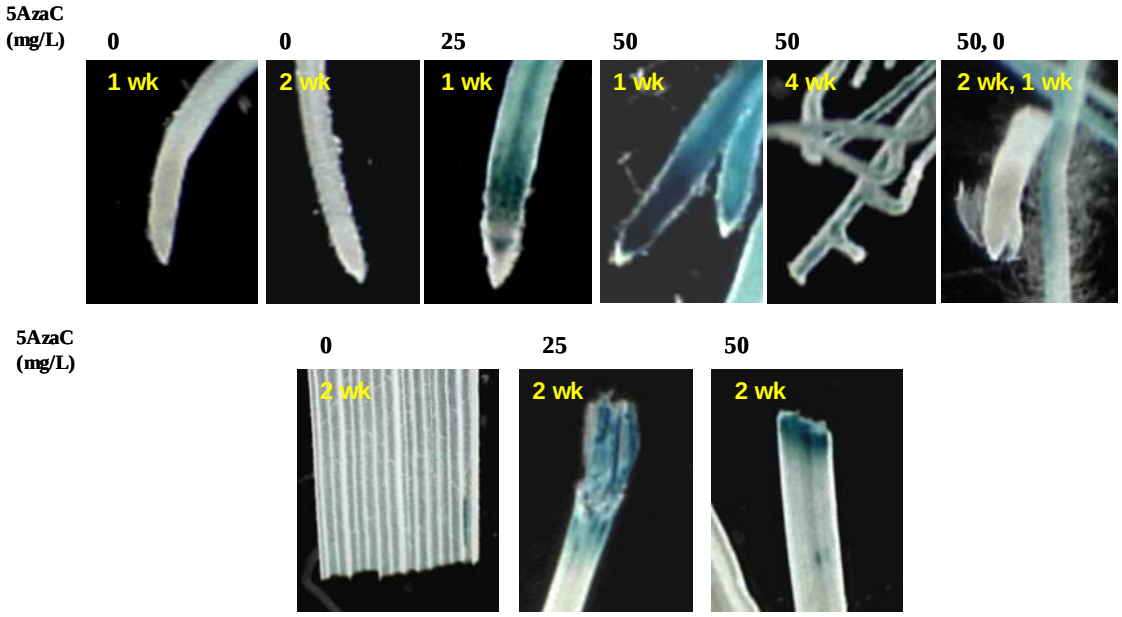


Figure 3.7. X-Gluc staining of root and leaf tissues of YXB10 R1 seedlings germinated in the presence of 5-azaC.

Seeds from self pollination silenced (GUS) lines: YXB13, 28, 33, 37, 38 and 44 were germinated in the presence of 5-azaC (50 mg/L) and hygromycin B (50 mg/L) for two weeks and transferred to soil. Herbicide resistance was determined 45 days after transplanting and bialaphos resistance was found to be maintained for 8/10 (YXB13), 7/7 (YXB28), 2/3 (YXB33), 9/10 (YXB37), 4/6 (YXB38) and 3/3 (YXB44).

Reactivation of GUS expression confirms silencing and DNA methylation related silencing. The *Agrobacterium*-transformed YXB population appears to represent an excellent system to study silencing events derived from the presence of an endogenous promoter regulating a chimeric transgene.

Transgene Silencing in YXB Transgenic Plants

RT-PCR Analysis of RCg2 Expression and Transgene gus *Gxpression Showing Reactivation of GUS Expression in Homozygous Progeny of Transgenic Lines Confirmed that Transcription is Silenced by DNA Methylation*

Total RNA samples from roots and leaves from the YXB homozygous lines YXB 138-9-5 (the expressing line), YXB 92-4-n single copy silenced line (primary transformant with strong expression) and YXB 70-6, the single copy silenced line, and their seedlings after 5-azac treatment were subjected to RT-PCR analysis (26 cycles). The results verified the presence of the endogenous *RCg*2 in all the progeny from both homozygous expressing lines YXB 138-9, YXB 92-4 and silenced line YXB70-6 for both non-treated and 5-azac treated samples (Figure 3.8). However, mRNA from the transgene *gus* only appeared in samples from the strong expression line YXB138 and its progeny (Figure 3.8). Another primary strong expression line, YXB 92, and its homozygous single copy progeny YXB92-4 lost GUS expression after the R2 generation. The lanes of YXB92-4-n L and YXB92-4-n R showed no transcripts. The lanes of YXB YXB92-4-n La and YXB92-4-n Ra (a represents 5 azaC treatment) showed amplification of *gus* mRNA, indicating that the silenced transgene was reactivated. The same results were seen in the single copy silenced line YXB 70-6-n (Figure 3.8) and YXB 60-2 (data not shown). Rice elongation factor EF-1α was used as an internal control. The stronger expression in most root samples than in leaf was consistent with the wild type rice varieties and revealed the *RCg*2 is root preferential gene.

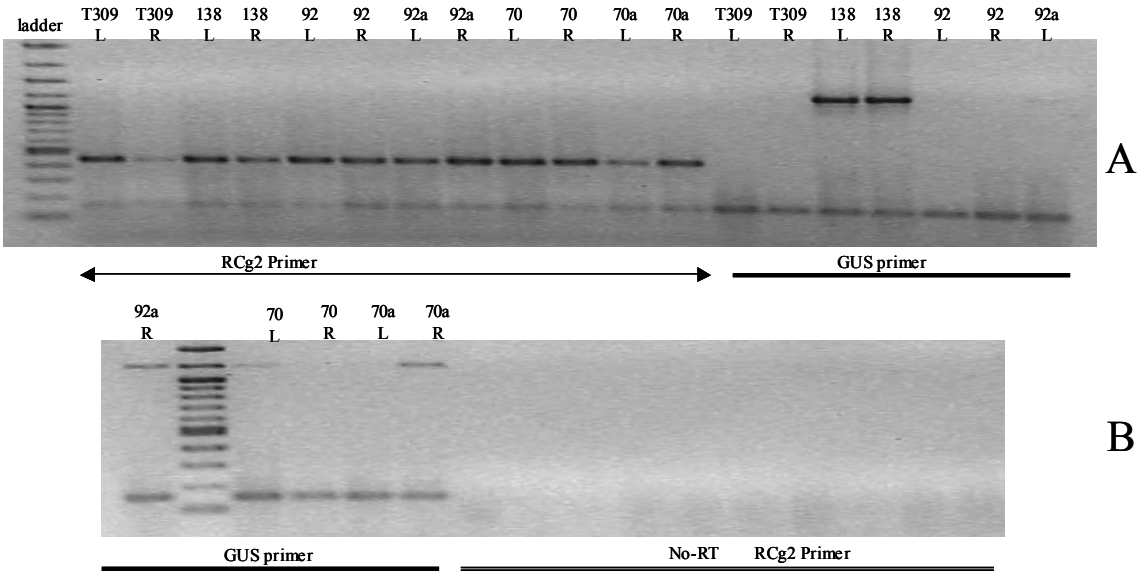


Figure 3.8. RT-PCR analysis of *RCg*2 expression and 5-azaC treatment in homozygous lines of transgenic YXB plants.

Genomic DNA Methylation Analysis Shows the YXB Transgenic Silence Is Methylation Related

Genomic DNAs extracted from rice lines T309, expression line YXB138 homozygous progeny, YXB92 progeny, silenced lines YXB70 progeny and YXB60 were digested with methylation-sensitive restriction enzyme *Pvu*I (CGAT|CG) and the intensity and its restriction fragments for expressing and silenced lines was compared (Figure 3.9). Wt T309 was digested with *Pvu*I (CGAT|CG) as a control. The expected size of the *RCg*2 promoter is 2600 bp and reconstructed samples to provide 5X, 2X, and 1X copy are shown in the right lanes of the blot (Figure 3.9C). All the samples, either expressing lines 138, 92 and 91 or silenced lines 60, 70, 144, and wt T309 showed

endogenous *RCg*2 gene promoter fragment bands of 770 bp and 712 bp. This indicated that the endogenous *RCg*2 promoter showed no methylation at any *Pvu*I site, and that most promoter sequences in the sample can be cut by *PvuI* enzyme to form smaller bands. Lighter intensity of bands at 2600 bp and 1747bp from expressing lines YXB138 and YXB91 provides evidence that methylation is associated with silencing of the *RCg*2 promoter. Expressing lines YXB138 and YXB91 lacked the 2600 bp band and had very faint 1700 bp bands indicating no methylation at the 6895 bp, 6797 bp and 6086 bp sites (Figure 3.9A and 3.9B) and less methylation at site 6042 bp or 6086 bp compared with silenced lines. Silenced line showed some methylation at all the *Pvu*I sites and strong methylation at site 6042 bp or 6086 bp. For the line YXB92 a very different pattern revealed that this line transgene locus is different than other expressing lines, and might explain the lost of expression after 2 generations with self-crossing (Figure 3.6). *RCg*2 transgene silence in rice transgenic plants is associated with methylation of the *RCg*2 promoter indicating that it is a transcriptional gene silencing is the cause.

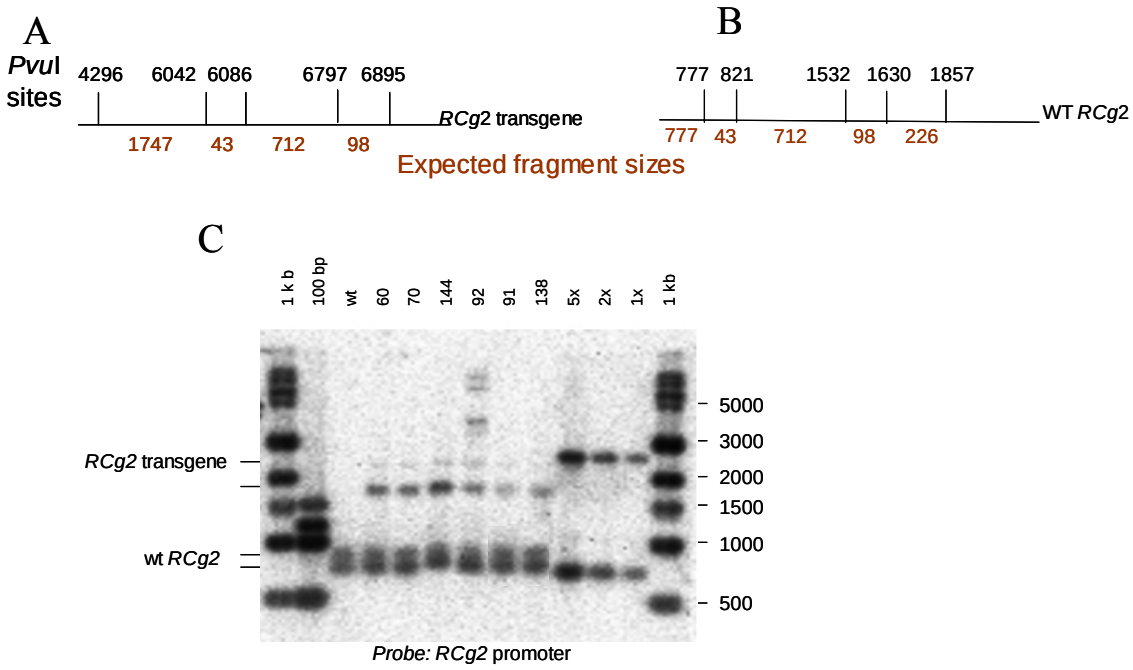


Figure 3. 9. Methylation analysis of silenced and expressing lines by Southern Blotting.

(A) Expected fragments sizes after digestion by *Pvu*I in trasgenic *RCg*2 promoter.

(B) Expected fragments sizes after digestion by *Pvu*I in endogenous *RCg*2 proomoter.

(C) Southern blot result shows methylation is related to gene silencing in transgenic plants.

Methylation Sequence and Silencing of the *RCg*2 Promoter

Genomic DNAs extracted from rice lines T309, expression line YXB138 homozygous progeny, YXB92 silenced progeny, and silenced lines YXB72 progeny and YXB60 were digested by *Hind*III and purified with phenol chloroform. DNA was treated with bisulfite, which selectively deaminates cytosine but not 5-methylcytosine to uracil. This leads to a primary sequence change, as unmethylated cytosines are converted to uracil and then thymidine after PCR; however, 5-methylcytosine is not converted by bisulfite and remains as a cytosine after PCR (Grunau et al., 2001) (Yang et al., 2004).

Figure 3.10. *RCg*2 promoter sequence and highlight CpG and CNG. CpG highlights with green color and CNG highlights with red color.

This primary sequence change can be quantitated using direct sequencing, restriction digestion, or pyrosequencing. The relationship between silencing, reactivation and methylation status was studied in collaborative work with Dr. Yeon-Hee Lee during Dr. Lee's visit to Texas A&M in May-July, 2004. Because there are no classical *Hpa*II - *Msp*I (CCGG) or *Sma*I - *Xma*I (CCCGGG) methylation sensitive restriction enzyme pair sites, methylation sequencing (Clark et al., 1994) was undertaken. 11 clones were obtained from line YXB70-6-4, 2 clones from 60-2-4, and 6 clones from YXB138-9-4 and 7 clones from YXB92-4-7.

There are 43 CNG sites and 37 CpG sites in the promoter sequence (Figure 3.10). The size of the PCR fragment of *RCg*2 promoter generated with the primers used is about 1100 bp. Within this part of the *RCg*2 promoter, there are 30 CpG sites dinuceotide and 27 CpNG sites. Of all cytosine positions, 49% were methylated for YXB138 progeny (138-9-4), 21% for YXB92 progeny (92-4-7), and 93.6 % for YXB70 (70-4-3-11) within the promoter fragment of the primer amplicon region (1030 bp. -950 to +78). Symmetric cytosine positions (CpG and CNP) of these three lines were 54%, 30% and 94% methylated, respectively. Two amplicon clones were obtained from YXB60 progeny, and the sequences are the same as the *RCg*2 promoter sequence, suggesting that the bisulfite treatment of genomic DNA did not work. Improved techniques for methylation sequencing are planned for future experiments. The result showed the cytosine methylation frequency is much different in transformant lines. It showed about 95% methylated cytosine in the single copy silenced line YXB70-2 and lower frequency in transgenic expressing lines. However, the switched line YXB90-4-7

showed very low methylation. This result also revealed that different mechanisms are involved in the *RCg*2 promoter transgenic silencing in addition to methylation related silencing.

Time Course of 5-azacytidine (5-azaC) Treatments Reactivation of the GUS Expression of Silenced YXB Lines

To optimize the reactivation conditions for GUS expression in transgenic YXB lines, the histochemical staining experiment was performed about one week after the seeds had germinated on MS medium without 5-azacytidine. Then the seedlings were transferred onto medium containing 25 mg/l and 50 mg/l 5-azacytidine respectively. GUS expression was initially detected via histochemical staining after 22 h, and 40% of seedlings stained in the leaves and roots after 24 h (Figure 3.11). 80% of the seedlings gave reactivated GUS expression with 25 mg/L concentration of 5-azaC and 60% of the seedlings reactivated with 50 mg/L concentration of 5-azaC. After 42 h, 100 % of seedlings were reactivated for GUS expression. This finding indicated that high 5-azaC concentration was not essential to reactivation in seedlings.

In another set of experiments, callus derived from mature embryos of silenced lines was exposed to 5-azaC to investigate the reactivation of GUS expression. Histochemical staining of callus revealed that GUS started to be reactivated on callus following 38 h exposure to 50 mg/L 5-azaC. Uniform GUS expression was observed for all treatments after 96 h of exposure to 50 mg/L 5-azaC (Figure 3.11A). No callus was reactivated with 25mg/L concentration of 5-azaC. This finding suggests that high 5-azaC

concentration and a longer exposure required for reactivation of GUS expression on callus.

A

	seedling (10 seedling)		Calli (10 pieces)	
Conc. of Aza-C (mg/l)	25	50	25	50
24 hr (% reactivated)	40	40	0	0
38 hr (% reactivated)	80	60	0	10
42 hr (% reactivated)	100	80	0	50
96 hr (% reactivated)	100	80	0	90

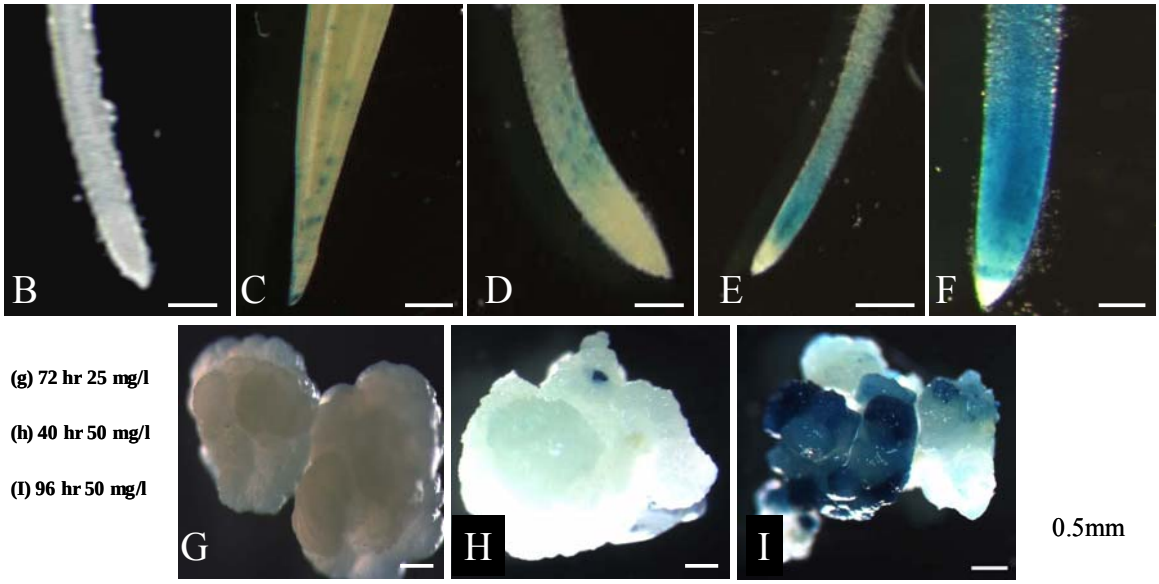


Figure 3.11. Reactivation of GUS expression in seedlings and calli of silenced YXB70-6.

(A) Reactivation of GUS expression by 5-azacytidine (5-azaC).

(B) GUS expression was not visible in roots before 24 hr exposure to 25 mg/L 5-azaC.

(C) And (D) GUS expression was visible in roots or leaves following 24 hr exposure to 25 mg/L 5-azaC.

(C) And (F) GUS expression was increased in roots or leaves following 38 hr exposure to 25 mg/L 5-azaC.

(G) GUS expression was not visible in calli after 72 hr exposure to 25 mg/l 5-azaC, and even after a long exposure.

(H) GUS expression started to show on calli following 40hr exposure to 50 mg/L 5-azaC.

(I) GUS expression started to show increased on calli following 90 hr exposure to 50 mg/L azaC.

Possible Functional Interactions of miRNA, siRNA and MITE Elements of the *RCg*2 Promoter

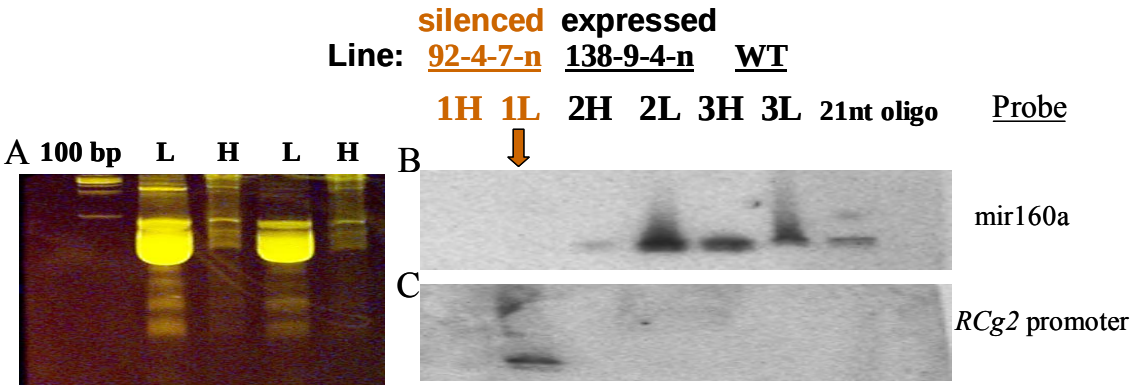


Figure 3.12. Appearance of a 21 nt siRNA is associated with the disappearance of miRNA in a silenced line.

(A) Total RNA for northern analysis was size-fractionated on a 17% polyacrylamide, 7M urea gel and separated into low (L) and high (H) mol. wt. RNAs by PEG treatment.

(B) When hybridized with a mir160a-based probe, the presence of a mir160a homolog was evident except in the 92-4-7-n line.

(C) When the blot shown in panel A was stripped and re-probed with the RCg2 promoter probe, 21-25 nt siRNAs were detected exclusively in the silenced progeny line 1L (YXB 92-4-7-n).

Initial experiments were undertaken to detect the presence of siRNAs and miRNAs in various expressing and silenced lines of YXB at different growth stages. Total RNA for northern analysis was size-fractionated on a 17% polyacrylamide, 7M urea gel and separated into low (L) and high (H) mol. wt. RNAs by PEG treatment. When hybridized with a mir160a-based probe, the presence of a mir160a homolog was

evident except in the 92-4-7-n line (Figure 3.12B). When the blot shown in panel B (Figure 3.12B) was stripped and re-probed with the *RCg*2 promoter probe, 21-25 nt siRNAs were detected exclusively in the silenced progeny line 1L (YXB 92-4-7-n) (Figure 3.12C). Mir160a – microRNA was originally cloned from Arabidopsis Reinhart. The sequence of the processed mir160a is UGCCUGGCUCCCUGUAUGCCA. On the basis of computational analysis, 3 microRNA homologs 160 a, b and c were predicted to be present in rice. Appearance of a 21 nt siRNA is associated with the disappearance of miRNA in progeny of silenced line YXB 92-4-7-n. To confirm this result, different silenced lines 92-4-7-3-mature plant (silenced line), 70-6-4-n mature plant (silenced line), and 92-4-7–n seedling stage (silenced line), 70-6-4-n seedling stage (silenced line), 60-2-4-n seedling stage (silenced line), and 60-2-4-n mature plant (silenced line) were subjected to northern blot analysis (Figure 3.13). SiRNAs only appeared in 92-4-7-n seedling stage RNA extracts hybridized with *RCg*2 promoter probes (Figure 3.13B). Other homozygous silenced progeny of lines 70-3 and YXB60-2 showed no hybridization with *RCg*2 promoter probe either in mature plants or in seedlings stage (Figure 3.13B). This result indicated that the silenced line YXB 92-4-7 from the primary expressing line has a different silencing mechanism than the silenced line YXB 70 and YXB 60. Interestingly in mature plants, all lines showed hybridization with mir160a, so further experiments were performed (Figure 3.13C and D). The result provided the first experimental evidence for the presence of a miRNA in rice. The mir160a homolog is present in seedlings and leaves of mature rice (*Oryza sativa* T 309) plants (Figure 3.13).

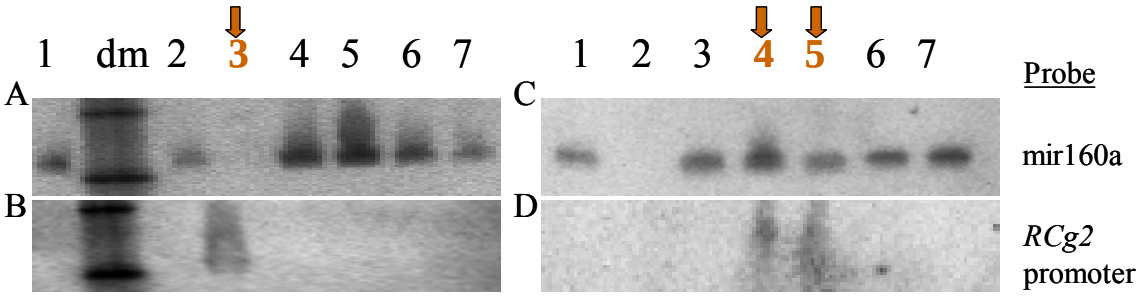


Figure 3.13. Northern analysis of total RNA from YXB lines and total RNA from mature YXB lines.

(A) Hybridization with a mir160a-based probe revealed the presence of a mir160a homolog except in the 92-4-7-n line.

(B) Stripping and re-probing with the full-length RCg2 probe (promoter+coding) showed 21-25 nt siRNA exclusively in the silenced progeny line YXB 92-4-7-n. Lane 1: 92-4-7-3-mature plant (silenced line); lane 2: 70-6-4-n mature plant (silenced line); lane 3: 92-4-7–n seedling stage (silenced line); lane 4: 70-6-4-n seedling stage (silenced line); lane 5: 60-2-4-n seedling stage (silenced line); lane 6: 60-2-4-n mature plant (silenced line); lane 7: T 309 Seedling stage (wild type); dm: 10 bp decade marker (20 and 30 bp shown).

(C) A mir160a-based probe showed the presence of mir160a homolog in all lines.

(D) The same blot as A, stripped and re-probed with the *RCg*2 promoter probe detected 21-25 siRNA exclusively in YXB 92-4 progeny. Low levels of siRNA, spread over a 21-30 bp range, were detected. Lane 1: 21 nt oligo (size marker); lane 2: - (empty lane); lane 3: 70-6-3-n (silenced line); lane 4: 92-4-7-n (silenced line); lane 5: 92-4-3-n (silenced line); lane 6: 138- 9-4-n (expressing line); lane 7: T309 (wild type).

Discussion

*RCg*2 Is a Root - Preferential Gene

Expression of *RCg*2-gus in leaves of YXA and YXB transgenic plants indicated that *RCg*2 is expression is not limited to roots. This is contrary to the finding reported by Xu et al. (Xu et al., 1995) that *RCg*2 is a root specific gene and in agreement with the observation with results discussed earlier for the YXA transformants. Northern blot analysis and RT-PCR results for two-week-old wt varieties rice and YXB showed existence of *RCg*2 transcripts in both of the plants and both in leaves and roots. Nuclear run-on results for the mature plant also revealed transcripts in YXB transgenic plants leaves (Figure 3.14). The conclusion is that the *RCg*2 is not root specific in rice but is a root preferential gene.

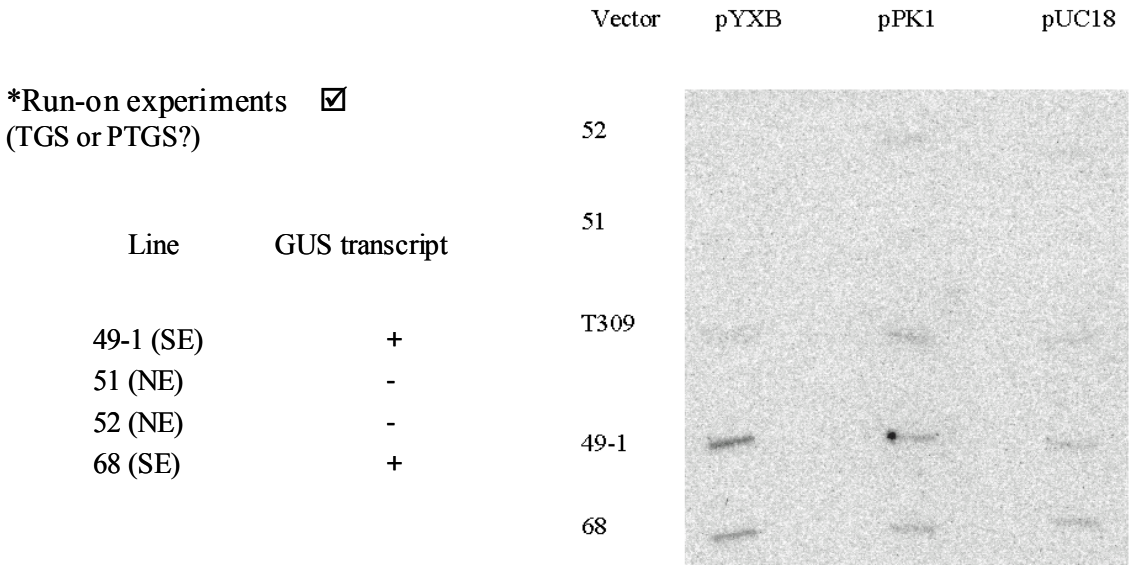


Figure 3.14. Nuclei run-on assay on transgenic YXB expressing and silenced lines. a-32P-UTP-labeled run-on transcripts prepared from wild type (T309), silenced YXB plants (51, 52) and expressing (49-1, 68) plants were hybridized to pYXB (gus coding fragment), pPK1 (RCg2 coding fragment) and pUC18 (plasmid vector).

Multiple Silencing Mechanisms Silenced the RCg2-*gus*

Methylation of an introduced gene and the corresponding endogenous gene is often associated with gene silencing phenomena. To study the unique and high frequency gene silencing in the YXB plants, we selected and demethylated 7 silenced YXB lines that have different T-DNA copy numbers (1 or 2) using 5-azaC. Contrary to the uniform and high-level reactivation of GUS expression in a 35S-gus transformed silenced line (JDV105) (Hall et al., 2001); we only observed partial reactivation in YXB38 (Figure 3.6 on page 69) that has 2 copies of the T-DNA (Figure 2.1B on page 19). This observation suggested existence of more than one gene silencing mechanisms towards YXB in that some plants were responsive to the demethylating agent and some were not. Demethylation not only reactivates the silenced gene but also affects the functional gene. We selected seeds from several self-crossed and bialaphos resistant YXB lines; they were germinated in the presence of hygromycin and 5-azaC and transferred to soil. After 45 days, herbicide resistence was assayed and some lines became sensitive to bialaphos. This finding indicates that demethylation using 5-azaC may have positive or negative effect on the introduced gene depending on different transformation events. To determine if silencing resulted from transcriptional or posttranscriptional inactivation, nuclear run-on transcription assays were carried out on nuclei isolated from both YXB expressing (YXB 49-1 and 68) and silenced (51 and 52) lines (Figure 3.14). The finding suggested that the silencing in lines 51 and 52 (no transcript was detected on run on blot) is transcriptional (Figure 3.14) but is post-transcriptional in line 92-4-7 progeny (Figure 3.12 on page 79). Nuclear run-on in the

mature plant also revealed transcriptional silencing (TGS) in YXB transgenic plants (Figure 3.14). This result suggests that the silencing in these plants is occurring at the transcriptional level. This is consistent to the results of 5-azaC reactivation experiments and DNA genomic analysis with restriction enzymes sensitive-to-methylation [*SnaB*I (TAC|GTA) and *Pvu*I (CGAT|CG)]. Although we do not have any good control plant to check the efficiency of these enzymes to cut in (un)methylated recognition sites, we can draw from the results that apparent methylation in *SnaB*I (data not shown) or *Pvu*I sites (Figure 3.8 on page 72) within the *RCg*2 promoter or *gus* coding sequence (different band intensity for expressing and non-expressing lines) is observed. This could be an indication that what we are observing in the *RCg*2 silencing is a TGS phenomenon.

The silencing observed in the YXB population could be related as well to another kind of regulation, in which the chromatin conformation of the promoter sequence is involved. If this is the case, the reversion of the expression after meiosis could be explained by a temporary loss of this silent conformation state during the first stages of plant life (Table 2.5 on page 37). Methylation is apparently involved. The transient expression after bombardment to silenced calli could be explained if it is necessary for the transgene (or promoter) to be integrated in the genome to adopt the silent configuration. The hypothesis that a truncation of the RCg2pro-*gus*-RCg2ter might be implicated in the efficient GUS expression of some YXB lines could also be supported by the involvement of a preferential conformation of the RCg2pro-regulated gene. The YXB lines would not be able to express the chimeric gene unless the promoter is broken and thus not capable of adopting the required configuration. Designing and preparing

constructs that contain different truncations of the *RCg*2 promoter would be useful to test this hypothesis.

Possible Functional Interactions of miRNA, siRNA and MITE Elements of the *RCg*2 Promoter

The *RCg*2 promoter contains a complex region that includes miRNA homologs, MITEs and repetitive sequences (Chapter IV). The high frequency of promoter-related silencing suggests functional interactions of these elements of the transgene and the homologous endogenous gene. Our experiments revealed that appearance of a 21 nt siRNA is associated with the disappearance of miRNA in progeny of silenced line YXB 92-4-7-n (Figure 3.11B and 3.11C on page 78). SiRNAs only appeared in 92-4-7-n at the seedling stage with *RCg*2 promoter probes. Other homozygous silenced lines progenies of 70-3 and YXB60-2 showed no RNA hybridization with the *RCg*2 promoter probe either in mature plants or in seedlings stage. This result indicated that the silenced line YXB 92-4-7 from the primary expressing line has a different silencing mechanism than the silenced line YXB 70 and YXB 60. Interestingly in mature plants, all lines showed hybridization with mir160a. So, further experiments were performed (Figure 3.12 on page 79). The results provided the first experimental evidence for the presence of a miRNA in rice. The mir160a homolog is present in seedlings and leaves of mature rice (*Oryza sativa* T 309) plants (Figure 3.12 on page 79).

CHAPTER IV

DISSECTION OF A COMPLEX PROMOTER *RCG*2 TO AVOID TRANSGENE SILENCING IN RICE

Introduction

In the previous chapters, it was shown that a high frequency of silencing occurred in the initial population of YXB transformants. Less than 10% of the transgenic lines showed strong GUS expression, while about 90% were weak or non-expressors (silent lines). Moreover, only 10% of these silent lines were sensitive to bialaphos (bar-silented lines), indicating that the flanking genes are not silenced at the same rate as the *RCg*2-regulated *gus* gene. The root expression pattern and high frequency silencing phenomena attracted us to examine the promoter structure and mechanisms underlying regulation of expression.

Knowledge of regulatory elements in plant promoters is of major interest in biotechnology and will provide ability to control gene expression in many research areas. PlantCARE is a database of plant *cis*-acting regulatory elements, enhancers and repressors (Rombauts et al., 1999; Lescot et al., 2002), and PLACE is a database of plant *cis*-acting regulatory DNA elements covered only vascular plants (Higo et al., 1997; Higo et al., 1999).

These sites gather and provide information on many *cis*-acting regulatory elements from both monocotyledonous and dicotyledonous species and some from conifers, describing many individual promoters from higher plant genes. Regulatory elements are represented by positional matrices, consensus sequence and individual site on particular promoter sequences. They also provide links to the EMBL, TRANSFAC and MEDLINE databases. These two databases are extracted mainly from previously published reports, supplemented with an increasing number of *in silico* predicted sites. They provide an excellent resource for *in silico* analysis of prospective promoter regulatory sequences.

Given the burgeoning information concerning the involvement of small interfering RNAs (siRNAs), micro RNAs (miRNAs) and transposons elements in gene silencing events, a reappraisal of the *RCg*2 promoter sequence was undertaken. *In silico* promoter analysis and promoter deletion analyses were used to provide insight for changes that could be made in order to avoid silencing in the expression of transgenes.

Materials and Methods

In Silico Analysis of the *RCg*2 Promoter

Using BLAST together with MAK, a computer search program developed in this laboratory (Yang and Hall, 2003b), we have obtained preliminary evidence for the potential presence of many small RNA-encoding elements in the *RCg*2 promoter. Database searches (against Rice genome and EST) also revealed the likely presence of many elements in *RCg*2 promoter region, including miRNAs; miniature inverted repeat transposable elements (MITEs) and other repetitive regions (Figure 4.1on page 97). This figure eventually formed the basis for subsequent promoter deletion analysis.

To identify putative regulatory elements, *in silico* analysis of the full-length the *RCg*2 promoter (-1656) was undertaken using PlantCARE (Rombauts et al., 1999; Lescot et al., 2002) and PLACE (Higo et al., 1999) databases. Taking 1656 bp of the upstream sequence (promoter) of *RCg*2 as a query sequence for *cis*-elements, searches were conducted against both the PlantCARE and PLACE databases. The resulting reports provide the site names, motif and location, as well as a link to a page with more detailed information on the particular element. Each potential motif or regulatory elements, identified was addressed by reading the relevant literature identified though Pubmed link and GeneBank accession numbers, and then the results of two databases compared to gather more information and predict the regulatory elements of *RCg*2. Examples of motifs identified include root-specific elements and positive and negative regulatory elements.

Deletion and Truncation of Segments of the *RCg*2 Promoter

Different constructs were designed from the original promoter sequence, which included mainly rough truncations of the whole *RCg*2 promoter sequence (MC7, MC1, MC2, MC42, MC8) and replacement of the 5' untranslated region or UTR with the UTR from brome mosaic virus (MC3, MC6, MC9, MC10). The constructs were derived from the pYXB plasmid and were named MC# (or pMC#). MC1, MC2, MC4, MC7, MC8, MC10 were made by digesting fragments from pYXB with appropriate restriction enzyme or after using PCR primers to create appropriate restriction sites. MC1 and MC7 removed the fragments from -1578 to -729bp, and -1578 to -888 respectively, which include a low repetitive sequence (LRS) –1137/-1088 and the OsMir –979/-958 sequence of the *RCg*2 promoter. MC2, MC4 and MC8 had segments removed from -1578/-268, -1578/-328 and -1578/-88 respectively. Plasmids pMC1, pMC7 and pMC10 were modified via PCR to add a *Hind*III site at the end of the *RCg*2 terminator. These plasmids were then inserted into a binary vector: the JD4-*Hind*III fragment without *gus*. And plasmids pJD4-MC1, pJD4-MC7 and pJD4-MC10 in binary vector pJD4 were mobilized into *Agrobacteria* LBA4404 via triparental-mating method.

To make internal deletion constructs pMC5, pMC11, pMC12 and pMC13; we first modified the pYXB to make pYXB-*Hind*III to serve as an intermediate plasmid suitable for site-directed mutagenesis (up to 8kb). Primers were designed to add two *Afl*II sites in the pYXB-*Hind*III plasmid sequence while changing as few nucleotides as possible The site-directed mutated plasmids were digested with *Afl*II to delete the sequences between two *Afl*II sites, the smaller plasmid was self-ligated and transformed

back into *E. coli* to create pMC5, pMC11, pMC12 and pMC13. Purified HindIII digestion products of pMC5, 11 and 12 were then ligated into JD4-*Hind*III vector to make pJD4-MC5, pJD4-MC11, pJD4-MC12 and pJD4-MC13. Triparental matings were used mobilize pJD4-MC5, pJD4-MC11, pJD4-MC12 and pJD4-MC13 into *Agrobacterium* strain LBA4404 in order to transform rice calli derived from mature and immature embryos and thus create the MJ transformants containing deleted *RCg*2 promoters. Transformants resistant to 50 mg/L hygromycin were analyzed by genomic DNA blots and histochemical GUS staining. Another set of constructs (MC5, MC4) was designed after the finding of a putative ORF of more than 100 aminoacids inside the promoter sequence (named RCgX).

pMC5: primer for deleting pYXB-HINDIII to make pMC5: 711-1544bp=833bp

Primer1: 5'- GCT CGA TCG AGA TTA TAT TAC CCT CTT AAG CAT GCA TGC ATG GGG GAA TAA TTC -3'

Primer 2 (2341b mut.): 5'- CAC TGT TCG ATC GAT CAC TAG CTC TTA AGC TGC ATC CAT GGT CCG TCC TG -3'

Primer for pMC11

Primer 3: for pMC11: deletion (mut. 1883) 1172- 5'- C AAG CAT TTC TCC ATA TTC ATC CC**C TTA AG**T TGG TTT TTA TAG GAC GAG AGG AG -3'

Primer 4: for pMC11: 5'- CTG AAG TAG AGT TAT ATG AAG CTG **CTT AAG** CCC AAC TTT GCA TGT CTA GTT C -3'

Primer for pMC12

Primer1: 5'- GCT CGA TCG AGA TTA TAT TAC CCT CTT AAG CAT GCA TGC ATG GGG GAA TAA TTC -3'

Primer 5 :(mut: 2187/2188)

5'- CT GTG CCA AAC AGG GCC TCC GTC TTA AGA GAA AGT AGC AAC GCA TCC ATG -3'

Primer design for MC13:

Forward: 5' –CGC**GGATCCAAGCT**TGGGGGAATAATTCAACAACGTCTG- 3'

Reverse: 5' – ACG GAC CAT GGA TGC AGC TAG - 3'

Transient Expression Using *Biolistics*-mediated Plant Transformation

Dehusked mature seeds from rice line T309 were rinsed with 70% ethanol for 1 min, and then incubated in 50% (v/v) bleach for 45 min with shaking at 120 rpm. The seeds were then washed five times with sterile distilled water prior to plating on N6 medium (Chu et al., 1975), embryo face-up, for two weeks at 28 °C. Induced calli were subcultured on N6 medium. After 10 to 14 days culture, actively growing calli were selected and precultured on high osmolarity N6 medium supplemented with mannitol and sorbitol (0.3 M each) for 4 h prior to bombardment using a Biolistic Particle Delivery System model PDS-1000 (E. I. du Pont de Nemours & Co., Wilmington, DE). For each experiment, Rice (*Oryza sativa* L., ssp. Japonica, cv. Taipei 309) (T309) immature or mature embryo-derived calli were subjected to particle bombardment (Bio-Rad PDS 1000/He biolistics system), and the calli were bombarded twice with gold particles (1 mg; 1.0 m diameter.) coated with different deletion and truncation constructs

at a 5:1 (w/w) ratio on 1 µm (o.d) gold particles (4 µg plasmid/2.4 mg particles) DNA (1 µg). The day after bombardment, some calli was subjected to GUS staining (Yang et al. 2005).

Stable Transformation by *Agrobacterium*-mediated Transformation Was as Described as in Chapter II

Tri-parental Mating to Transfer Deletion Constructs into *Agrobacteria* LBA4404

Tri-parental mating for transfer of plasmids pTVK291 (containing virogenes) was grown on 2XYT agar with 60 µg/mL nalidixic acid and 50 µg/mL kanamycin selections for overnight at 37 °C. The *E. coli* strain, harboring the plasmid of each deletion construct, was grown overnight on 2XYT agar containing 10 µg/mL gentamycin. *E. Coli* pRK2013 (helper strain) was grown on 2XYT agar with 50 µg/mL kanamycin overnight. The bacteria were scraped off their respective plates and mixed well on the medium with no antibiotics. PTVK291, the *E. coli* strain with the plasmid, and *E. coli* pRK2013 were mixed in the ratio 3:1:1 (v/v).

The mixture of three strains was then placed at the center of a 2X YT agar plate with no antibiotic selection. Mating was allowed to occur by leaving the plate upside down for 6-12 h at 37 °C. The bacteria were scraped off and streaked on a 2X YT plate containing 10 μg/mL gentamycin, 100 μg/mL kanamycin and 60 μg/mL nalidixic acid for isolation of single colonies. Start culture LBA4404 was grown on AB medium and pRK2013 (helper strain) was grown overnight on 2XYT agar with 50 μg/mL kanamycin. A single colony from the first mating, LBA4404 and *E. coli* pRK2013 were mixed in the ratio 3:1:1 (v/v). The mixture of three strains was then placed at the center of a 2X YT agar plate with no antibiotic selection. Mating was allowed to occur by leaving the plate upside down for 10-12 h at 28 °C. The three mixtures were streaked on an AB plate plus kanamycin (100 μg/mL) and gentamycin (50 μg/mL). For assurance of isolating single colonies, a second AB sucrose plate (kan + gent) was streaked for isolated colonies from an isolated colony on the first plate, and then an isolated colony from the second plate was checked to be sure the bacteria are Benedict's positive. Genomic DNA analysis Was as described as chapter II.

Results

Small RNA Encoding Elements Are Present in the *RCg*2 Promoter

Given the current interest in the involvement of small interfering RNAs (siRNAs), micro RNAs (miRNAs), miniature inverted repeat transposable elements (MITEs) and transposable elements in gene silencing events, we were interested in determining if match sequences for any of these elements detected in the *RCg*2 promoter are present in the expressed sequence tags (ESTs) of rice. An initial BLAST search using the two EST sequences which are ORF within the *RCg*2 promoter, as queries resulted in >206 hits in the genomic database. Using BLAST together with MAK, a computer search program (Yang and Hall, 2003b), we have obtained preliminary evidence for the presence of some small RNA-encoding elements in the *RCg*2 promoter. A comparison of GUS spatial distribution and expression levels provided insight to the contributions of these elements to promoter activity. Related studies have shown that MITEs can positively affect transcription, but also increase the incidence of silencing (Yang and Hall, 2003a).

Miniature Inverted Repeats Transposable Elements (MITEs) Are Present in the *RCg*2 Promoter

In a search for the genomic origin for the two EST sequences, AC123516 from 68330 to 69726 was found to be the gene for AU075832 and AL731624 from 18552 to19960 was found to be the gene for EST AA751445. After splicing of the introns from

the transcripts, both *mJanus* elements were retained within the mRNAs. It is widely known that Dicer or its homologs can degrade dsRNA or dsRNA regions in a transcript and produce small RNAs (siRNA or miRNA) that are able to act as a signal for the silencing of homologous regions. Coincidently, MITEs usually assume a stem loop structure. A folding of the transcribed *mJanus* on EST AU075832 indeed showed a stem loop structure and the ~30 bp stem region contains two small bulges thus giving it potential be the substrate for Dicer homologs in rice. Even more interestingly, the homologous region of the *RCg*2 promoter corresponds to the stem region of the hairpin structure of *mJanus*. Identifying the origin of the region of the *RCg*2 promoter that is homologous to the *mJanus* stem structure was the next project. It may be a relic of an ancient *mJanus* that had inserted into the locus. When the *RCg*2 promoter sequence was used to align with the *mJanus* family, the evolutionarily closest member of *mJanus* (AC136447) was identified. When this element and *RCg*2 promoter were aligned using low stringency parameters, a decayed copy of *mJanus* was identified within the *RCg*2 promoter from -276 to -108 with over >70% sequence similarity (7e-21) to the *mJanus* sequence from AC136447. Surprisingly, the ancient *mJanus* inserted 21 bp upstream of the TATA box. Except for the terminal invert repeat (TIR) region on the 5' end, which retained 93% identity in 40 bp, the rest of the element is significantly decayed. The biological significance of the retention of the 5' TIR region in silencing of the promoter remains unknown.

Another region on the *RCg*2 promoter (from -337 to -316) is also repetitive, and we were able to identify this region to be the relic sequence of a MITE belonging to

another MITE family, we named *Helios*. This MITE family has a TIR sequence of CTCCCTCTTGGTTGATAATACTTGTCGTTTTGG and a TSD sequence of TA, making it a member of the "Stowaway" group. Using MAK, we were unable to retrieve an anchor element from the current rice genome sequences.

Remarkably, database searches (against Rice genome and EST) revealed the likely presence of many elements in the *RCg*2 promoter region, including miRNAs; miniature inverted repeat transponsable elements (MITEs) and other repetitive regions (Figure 4.1A and 1B). These and other features of the *RCg*2 promoter are shown in Figure 4.1.

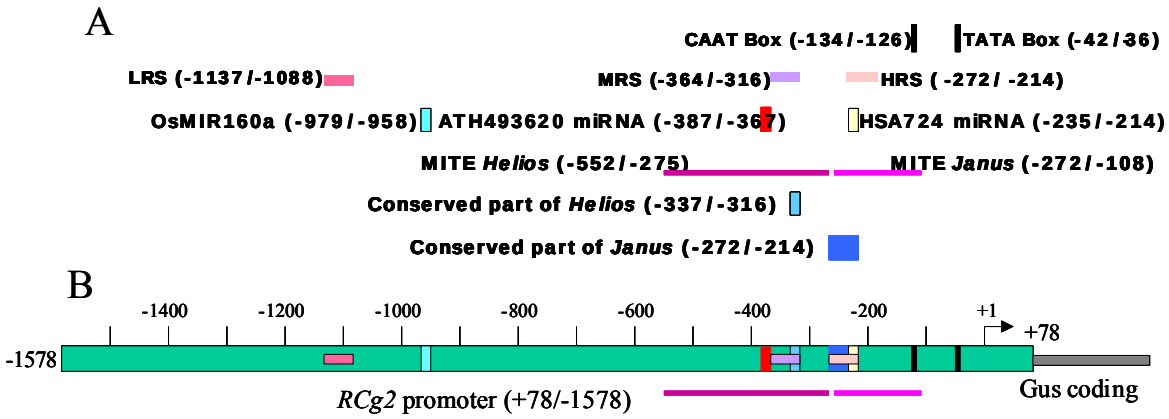


Figure 4.1. Scale diagram of the *RCg*2 promoter, conserved motifs and colored keys to their location within the *RCg*2 promoter.

(A) Conserved motifs and colored keys to their location within the *RCg*2 promoter.

(B) Scale diagram of the *RCg*2 promoter.

Diversification of the *RCg*2 Promoter to Avoid Silencing in the Expression of Transgenes

RCg2 Promoter Deletion Analysis

Promoter deletion analysis is a useful tool for identifying important regulatory regions involved in transcriptional control of gene expression (Buzeli et al., 2002; Chang and Sun, 2002; Kluth et al., 2002). To identify key elements contributing to the root-preferential expression of *RCg*2 and the high frequency of silencing observed in transgenic (YXB) lines, several *RCg*2 promoter deletion constructs were designed. These include 5' deletions MC1, MC2, MC4, MC7 and MC8 and internal deletions MC5, MC11, MC12 and MC13. The promoter constructs were fused to the *gus* reporter gene and used for *Agrobacterium*-mediated transformation of rice. Transformants resistant to 50 mg/L hygromycin were analyzed by genomic DNA blots and histochemical GUS staining. The frequency with which silencing was encountered in populations of the deletion mutants was used to characterize the effects of the various promoter elements. The large number (363) of independent transformants studied gave credibility to deduce both positive and negative regulatory elements (Figure 4.3C on page 103).

5' Deletion Analysis of RCg2 Promoter

*RCg*2 promoter deletion constructs have been made. These include 5' deletions MC1, MC2, MC4, MC7 and MC8. The constructs (Figure 4.2) were derived from pYXB and are designed to examine the effects of deleting the Low, Medium and High

repetitive sequences (LRS, MRS and HRS). MC7 (-888) and MC1 (-729) include a putative ORF (-713/-360) within the *RCg*2 promoter and 2 MITE families, miRNA and moderate and high repetitive sequence (Figure 4.1), so their negative regulatory effects are not expressed. MC2 (-268) contains the conserved part of *Janus* (-272/-214) and HSA724 miRNA (-272/-214) and one OsMIR 160a sequence. It also contains the highly repetitive sequence (-272/-214). MC4 (-328) contains part of Helios (-337/-316), the whole MITE *Janus* (-272/-108) sequence, HSA724 miRNA (-272/-214) and one OsMIR 160a sequence without ATH493620 miRNA (-387/-367). It contains a highly repetitive sequence (-272/-214). MC8 (-88) contains a basal promoter, without a CAAT box. These constructs were inserted in the *Hind*III site (located between the 35S-*hpt*-35S and mUbi-*bar*-Nos gene cassettes) of the transformation binary vector pJD4 (Figure 2.1C on page 19). The resulting plasmids were introduced into *Agrobacterium* strain LBA4404 by tri-parental mating.

Internal Deletion Analysis of RCg2 Promoter

In silico promoter analyses revealed the presence of many elements in the *RCg*2 promoter region that are likely to have positive or negative effects on transcription. For example, our lab has shown that miniature inverted repeat transposable elements (MITEs) can positively affect transcription, but also increase the incidence of silencing (Yang et al., 2005b). The locations of MITEs *Janus* and *Helios* are indicated in (Figure 4.1), as are those of other repetitive regions. Internal deletion constructs including MC5, MC10, MC11, MC12 and MC13 (Figure 4.3A) were made and transferred into rice so

that GUS reporter activity could be assessed histochemically. Quantitative measurements of GUS activity were also undertaken using fluorometric MUG assays (data not shown). MC5 (-1578/-745) and MC4 were designed after the finding of a putative ORF (-713/745)(RCgx) encoding more than 100 amino acids inside the *RCg*2 promoter sequence. MC11 (-1578 Δ-406/-208) deletion conserved part (-337/-316, -272/-214) of the two MITE families, MRS, HRS, ATH493620 miRNA (-387/-367) and HSA724 miRNA (-272/-214). MC12 (-1578 Δ-745/-88) deletes two MITE families, ORF, MRS, HRS and ATH493620 miRNA (-387/-367), HSA724 miRNA (-272/-214), OsMIR160a (-706/-687; -196/-178) and the CAAT box. It was made via fusing MC5 (-1578/-745) and MC8 (-88/+78). MC13 (-729 Δ-406/-208) deleted the low repetitive sequence (LRS) (-1137/-1088) and rice MIR160a (OsMIR160a) (-979/-958). It was made either from MC1 (-729) by deleting (-729 Δ-406/-208) or from MC11 (-1578 Δ-406/-208) by deleting (-1578/-729). In MC10 (-88/-3 + BMV-UTR), the corresponding UTR is replaced by MMV-UTR (Figure 4.3).

Transient Expression Using *Biolistic*-mediated Plant Transformation

All the constructs were bombarded into wild-type rice callus (T309) to check their capability and efficiency to direct GUS expression. The bombardments were done generally in the same way, bombarding the plate at least twice with rupture disks at 1,300 psi, 26 mm Hg vacuum, and room a distance of 6 cm. MC1, MC2, MC7, MC12, MC13, MC11 and MC5, MC10 showed GUS expression, MC3, MC4 and MC9 did not show expression and the reason is unclear. However, MC5 and MC10 have positive

GUS expression. We finally concluded that a basal promoter could be obtained from the *RCg*2 5' sequence (construct MC8) and that the corresponding UTR could be replaced with the BMV-UTR (construct MC10) (see Table 4.1 and Figure 4.2). Plasmid pJD4 showed strong expression and was used as control. Corresponding MJ transgenic plants are shown in the right column in Table 4.1.

Table 4.1 Deletion constructs' transient expression and their correspondent *Agrobacteria* plasmids

Construct	Bombardment Transient GUS expression	Corresponding construct for *Agrobacterium* transformation
MC1	+	MJ1
MC2	+	MJ2
MC3	-	N/A
MC4	-	MJ4
MC11	+	MJ11
MC5	-	MJ5
MC12	+	MJ12
MC7	+	MJ7
MC8	+	MJ8
MC9	-	N/A
MC10	+	MJ10
MC13	+	MJ13

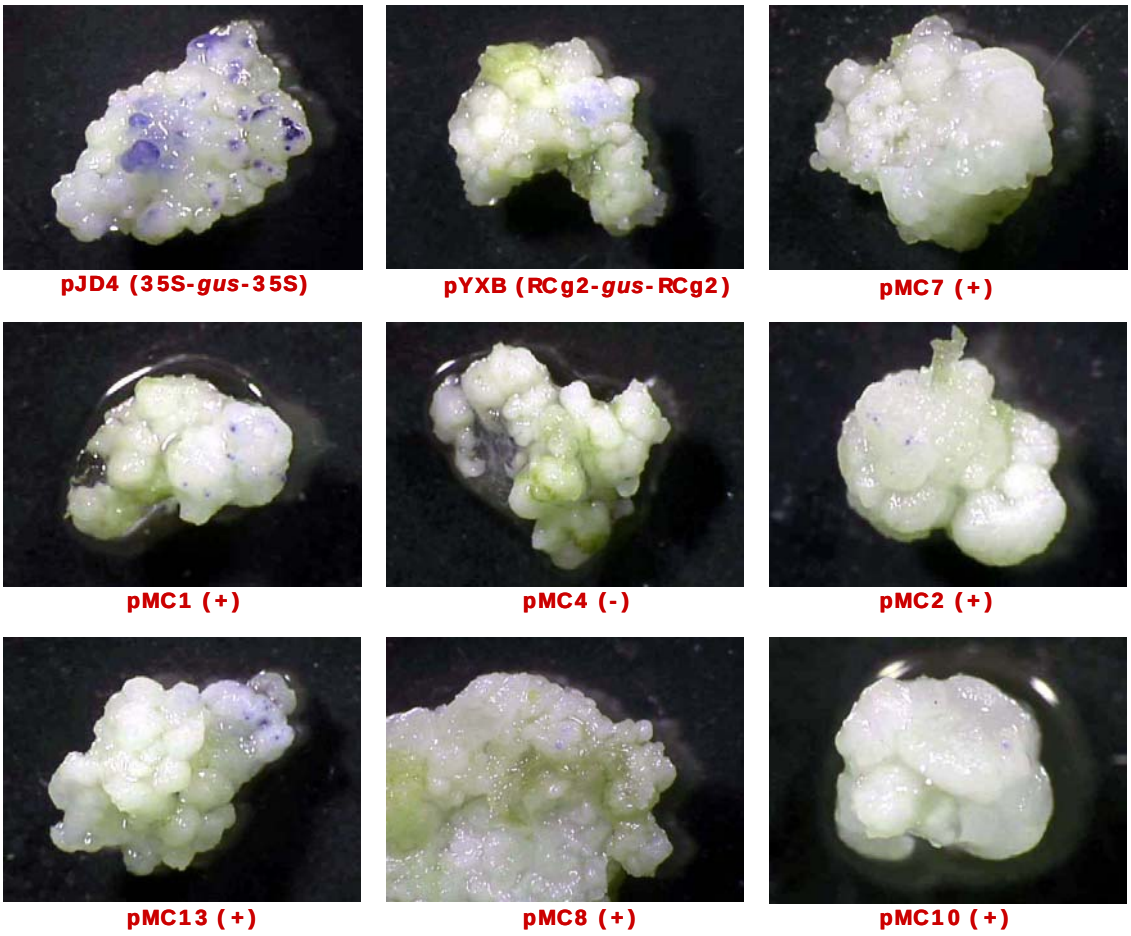


Figure 4.2. Bombardment and transient expression assays with deletion constructs and other modifications on the RCg2 promoter.

Characterization of MJ Transgenic Plants

Some of the constructs were transferred to the transformation vector pJD4, in the *Hind*III site located between the 35S-hpt-35S and mUbi-*bar*-NOS gene cassettes, after removing the 35S-*gus*-35S cassette. The resulting plasmids, named pMJ# (Table 4.1), were transferred by triparental mating to the *Agrobacterium* strain LBA4404. The new bacteria will be used to obtain stable rice transformants. Experiments will introduce MJ2, MJ4, MJ8, MJ10, among others using pJD4 and pJD4-YXB as controls. Another set of bacteria was obtained by incorporating the plasmids into *Agrobacterium* strain GV3101 by electroporation. These bacteria will be used for the recovery of stable *Arabidopsis* transformants to investigate the *RCg*2 transgene in *Arabidopsis*. No results are available at this time.

A summary of the extensive functional analysis that has been conducted of the truncations is shown in Figure 4.2 panel C. A total of 363 independent transformants were produced and analyzed by Southern blots and histochemical staining. The large number of independent transformants studied gives credibility to the positive and negative regulatory elements summarized in panel C of Figure 4.2. The region -272 to -214 includes elements marked on Figure 4.1B as: highly repetitive sequence (HRS: -272/-214), Homo sapiens (HSA) miRNA (-235/-214) and the conserved part of the MITE *mJanus* (-272/-214). While the relationship of these elements needs further evaluation, the initial results obtained for the MC11 construct and MC13 construct compared with YXB confirmed our prediction that this may be a negative regulatory region.

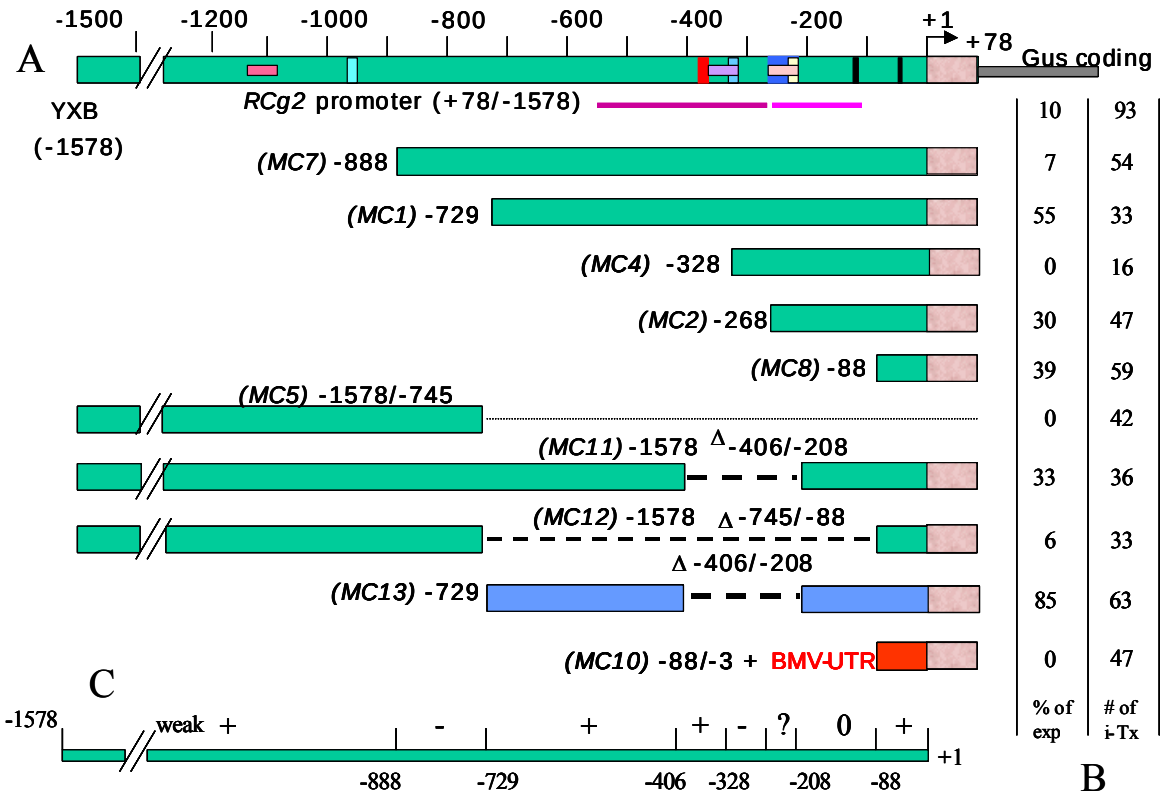


Figure 4.3. Scale diagram of the *RCg*2 promoter, deletion constructs and analysis of relative gus expression for each construct.

(A) Various deletion and truncation constructs used in these studies.

(B) Summary of transgenic plants analysis.

(C) Negative and positive regulation elements in the RCg2 promoter.

As shown in Figure 4.1B, the region from -406 to -208 is deleted in the MC11 promoter and, of 36 independent transformants, 33% expressed GUS. In the MC13 construct, of 63 independent transformants, 85% expressed GUS, and this result showed that the region of –406 to 208 is a strong negative regulatory region.

The difference in proportion of expressing transformants for MC4 (-328; no expressors) and MC2 (-268; 30% expressors) reveals that the region between -328 and -268, which corresponds with a medium repetitive sequence (G. Yang, personal observation), contains a major negative feature. As for MC11, construct MC12 lacks the -406 to -208 regions, but only 6% of 33 independent transformants were expressors (Figure 4.2B). This suggests that MC12 lacks positive regulatory regions present in MC11. These can be predicted to be present in the -729 to -406 and/or -208 to -88 regions that are present in MC11 but not in MC12.

Evidence for strong positive element(s) between -729 and -406 is derived from the finding for MC1 that 55% (of 33 independent transformants) express (Figure 4.4), whereas no expressors (of 16 independent transformants) were recovered for MC4. Together with the data for MC11 and MC12, this confirms the positive regulatory function of the region between -729 and -406. It remains to be determined if regions -406 to -328 and -268 to -208 have positive, negative or neutral effects and are, therefore, marked "?" in Figure 4. 3C.

As MC7 has only 7% expressing lines, it appears that another negative regulatory element exists between -888 and -729. Interestingly, MJ8 (-88), the shortest promoter fragment tested yielded 39% expressing plants (Figure 4.3B). A summary of characterization of deletion constructs showed in table 4.2. And expression of GUS showed in Figure 4.4.

Table 4.2 Characterization of the transgenic plants of *RCg*2 promoter deletion construct.

plants	C P from +1	length bp	# of transgenic plant	# of independer	SB (gus) 1 copy line	#. mutiple C	#.T + full l	% of Expression leaf	root	overall
MJ1	-729	807	33	33	EX. 2, 14, 3 S. 3	EX. 7 S.9	EX. 9 S. 3	60.6	48.6	60.6
MJ2	-268	346	49	47	EX. 53 S. 47, 77	Ex. 1 s. 0	Ex. 23 S. 13	38.7	48.9	55.1
MJ4	-328	406	17	16	Ex. 0 S. 55,65, 7	EX. 0 S.7 + 2	EX. 0 S. 3 + 2(	0	0	0
MJ5	-1578-754	824	42	42	Ex. 0 S. 3, 5, 31,	Ex. 0 S.19	Ex. 0 S.16	0	0	0
MJ8	-88	166	74	59	EX. 72®, 8 S.11 (60, 6	Ex. 12 S. 5	Ex. 21 S. 5	41.9	43.2	58.1
MJ11	-1578 D-406/-208	1460	36	36	Ex. 0 S.2, 18	Ex. 6 S.8	Ex. 9 S.10	30.6	30.3	38.9
MJ7	-888	966	54	48	Ex. 0 S. 29	Ex. 5 S. 18	Ex. 1(23) S. 16(10	5/48=10.4	5/48=10.4	5/48=10.4
MJ10	-88	94	43(40)	29	Ex.0 S.1(7, 14)	Ex.0 S.	Ex.0 S.	0	0	0
MJ12	-1578 D-745/-88	1014	48(47)	33	Ex. 0 S. 8	EX. 2 (27, 40 S. 11	EX. 0 S. 7	4	4	4
YXA	-1578	1656	48(46)	28	Ex.0 S.4(13,19,2	Ex.0 S. 14	Ex. 6(9,2 S.2(22,3	24.4	24.4	24.4
JD4	35S	714	52(18)	12	Ex.0 S.0	Ex. 4(21,28, S. 1(29)	Ex. 2(12, S. 0	7/12=58.3	7/12=58.3	7/12=58.3
MJ13	-729 D-406/-208	609	63	53	Ex. 17 S. 2	EX. 24 S.3	EX. 7 S. 0	85	85	85

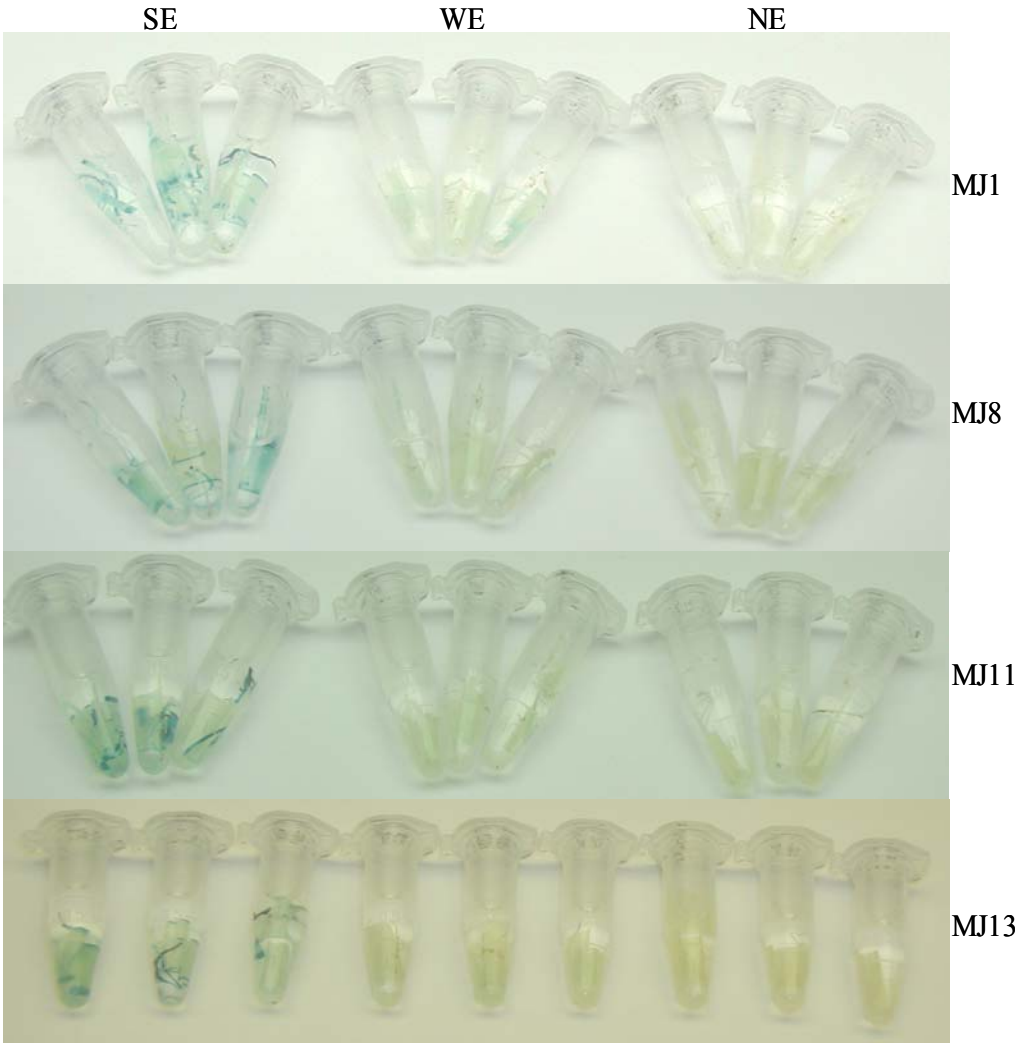


Figure 4.4. GUS expressions of various RCg2 promoter deletion constructs. SE: strong expression; WE: weak expression; and NE: no-expression. MJ# show transgenic plants from MC# constructs.

Discussion

The *RCg*2 Promoter Contains Regulatory Elements Related to Gene Silencing

The *RCg*2 promoter contains a complex region that includes miRNA homologs, MITEs and repetitive sequences. Miniature inverted repeat transposable elements (MITEs) can positively affect transcription, but also increase the incidence of silencing (Yang et al., 2005b). The locations of MITEs *Janus* and *Helios* are indicated in (Figure 4.1), as are those of other repetitive regions. Deletion constructs that deleted high repetitive sequence (HRS: -272/-214), Homo sapiens (HSA) miRNA (-235/-214) and the conserved part of the MITE *mJanus* (-272/-214) showed high proportional expression in transgenic rice. The high frequency of promoter-related silencing suggests functional interactions of these transgene elements and the homologous endogenous gene.

Effect of MITEs within Plant Promoters

The results summarized in Figure 4.1 and 4.2 support the prediction that the regions in the *RCg*2 promoter that have sequence similarity to MITEs *Helios* and *Janus* can negatively regulate expression shown by comparison of expression from YXB plants with that from MC11 plants (that contain and lack these MITE sequences, respectively). Quantitative MUG assays will be done on four expressing independent transformants of similar copy number for each construct. In separate studies, we have shown that the presence of a MITE sequence (Kiddo) in the rice *rubi*2 promoter has two opposing effects (Yang et al., 2005b). One effect is to enhance expression, but the other is to

increase the likelihood of silencing. If we can obtain rigorous evidence that MITE sequences in the *RCg*2 promoter also have specific up, down (or both) effects on expression, this will be an important addition to knowledge in the field.

The region -272 to -214 includes elements with features similar to those of highly repetitive sequences, to HSA miRNA from *Homo* sapiens and the conserved region of the MITE *mJanus*, and was found to be a negative regulatory region. Deletion of the region from -406 to -208 (the MC11 promoter) revealed that it also includes a negative element, as of 36 independent transformants, 33% expressed GUS. A third, major, negative regulatory region was identified: that between -328 and -268, which corresponds with a medium repetitive sequence. Evidence for strong positive element(s) between -729 and -328 is derived from the finding for MC1 that 55% (of 33 independent transformants) express, whereas no expressors (of 16 independent transformants) were recovered for MC4.

CHAPTER V

CONCLUSION

GUS expression was observed in leaves and roots for both YXA and YXB transformants. A very low percentage of YXB transformants showed stable GUS expression over several generations and the expression is stable and inheritable in the R1 generation and R2, R3 etc. There is a possibility of obtaining a single-copy expressing R1 plant by segregation of T-DNA loci, for example YXB92, 92-4 and 92-5. YXB92-4 is homozygous line. A fragment of the YXB cassette, present in 70% of the expressing lines, is relevant for its expression. The lines containing this fragment show stronger expression levels than do the lines lacking a fragment. This also applies to their progeny after segregation. Nineteen single copy lines can be reactivated by 5-azacytidine treatment. Expression can be restored after mitosis. We recovered homozygous R1 lines from single copy (SC) transformants for further experiments. They are YXB60-2, YXB70-3, 70-6, and YXB33-5 and also have one line from YXB92, YXB92-4. Expression of the transgene is only seen in expressing lines YXB138, YXB91, etc. and their progeny. But the expressing line YXB92 progeny (single copy) were silenced. RT-PCR and northern blotting (NB) analysis of the endogenous sequences revealed presence on both root and leaf.

*RCg*2 promoter is not a root specific gene. Northern blot and RT-PCR analysis of *RCg*2 expression confirmed that *RCg*2 gene is a root preferential gene. *RCg*2 can show expression in different tissues (leaf and root) of transgenic rice plants. Reactivation

confirmed that the transgene insert was capable of expression and was therefore silenced in the primary transformants and their progeny. *RCg*2 transgene silencing is DNA methylation related. Reactivation and run on experiment showed that *RCg*2 transgene silencing is transcriptional gene silencing in some lines, and posttranscriptional silencing in other. *RCg*2 promoter was silenced at a high frequency. Expression of GUS from the *RCg*2 promoter was silenced in more than 90% of the lines while the flanking hpt and bar genes are not silenced in most lines. This uniqueness of high frequency silencing indicated that the *RCg*2 promoter is valuable for studies on the cause of gene silencing. Multiple silencing mechanisms are related to the RCg2-*gu*s silencing. Contrary to the uniform and high level reactivation of GUS expression of 35s-gus in JDV92-8 line, there are different reactivation frequency in the YXB silenced lines that some plants responsive to the demethylating agent and some not. There is another YXB line, YXB92, primary transformant showed strong expression, R1 became weak, R2 was silenced, especially YXB92-4-7 line, its silencing show related to small RNA. Appearance of a 21 nt siRNA is associated with the disappearance of miRNA in a silenced line seedling stage of YXB92-4-7, and mir160 and *RCg*2 siRNA produce in mature plants.

The *RCg*2 promoter contains a complex region that includes miRNA homologs, MITEs and repetitive sequences. The high frequency of promoter-related silencing suggests functional interactions of these elements of the transgene and the homologous endogenous gene. The frequency of the expression of the transgenes related to different regions of the *RCg*2 promoter. Deletion and truncation of the *RCg*2 promoter revealed

that were useful methods to study the regulatory roles of MIETs, miRNAs and repetitive elements individually and in combinations, and also deleted the repress elements can restore the high expression frequency of the transgenes in rice. The possibility exists that the endogenous *RCg*2 promoter competes effectively with entering transgenes driven by the same promoter. The region from -406 to –208, that includes multiple elements, showed very strong negative regulation of *RCg*2.

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