RICE TRANSFORMATION AS A MEANS TO STUDY GENE EXPRESSION

A Dissertation

by

YIMING JIANG

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

August 2009

Major Subject: Genetics

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Approved by:

Chair of Committee,	Timothy C. Hall
Committee Members,	Clint Magill
	Jean Gould
	Keerti S. Rathore
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ABSTRACT

Rice Transformation as a Means to Study Gene Expression.

(August 2009)

Yiming Jiang, B.S., Guangxi Agricultural College;

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Chair of Advisory Committee: Dr. Timothy C. Hall

An exceptionally effective transformation procedure has been established by using class I embryo-derived rice callus. Every treated callus clump yielded multiple independently transformed plants (average 40 plantlets). Analysis of genomic DNA blots and pollen expressing green fluorescent protein (GFP) from T_0 plants revealed that 64% bore a single locus T-DNA insertion in which half had one T-DNA copy. Additive transgene expression was observed fromT_0 plants with GFP driven by mUbi1 promoter. Transgenic plants could be rapidly characterized by analyzing GFP pollen from T_0 plants without the need for further generations or genomic DNA blot analysis.

Agrobacterium tumefaciens-mediated transformation of microspore-derived callus for generating large numbers of T-DNA haploid and doubled haploid(DH) plants has also been investigated. The established transformation procedure resulted in 100% transformation frequency for class I microspore-derived rice callus. Each callus typically yields multiple independent transgenic plants. Genomic DNA blot analysis suggested 98% of the transgenic plants are independent events. About half of the transgenic plants were identified as haploid

plants, whereas half are DH hemizygous or homozygous transgenic plants. DH homozygous transgenic plants were obtained from T_0plants and confirmed by pollen GFP expression and genomic blot analysis in T_0transgenic DH plants. In this study, about 60% ofT_0transgenic DH plants had a single locus T-DNA insertion of which 45% bore one T-DNA copy. Furthermore, in a population of over 2,000 haploid and doubled haploid T-DNA plants , about 25% showed phenotypic differences from non-transformed haploid plants. Approximately 5% were seriously phenotypically abnormal including lethal or semi-lethal mutants. This highly efficient transformation procedure using microspore-derived callus could be valuable in speeding up plant breeding and in new gene discovery.

Diversification of the mUbi1 promoter led to a minimal promoter that has a similar function as the original mUbi1. Transient and stable transformation as measured from gene expression driven by the minimal promoter suggested that it has a similar function as the original wild type promoter.

DEDICATION

TO MY PARENTS

TO MY WIFE AND SON

TO MY SISTER AND BROTHERS

ACKNOWLEDGMENTS

I would like to thank the Genetics Program and Department of Biology for providing me the opportunity to study at Texas A&M University. I was fortunate enough to have tremendous support and help from many kind people during this time. It was impossible to finish my dissertation without their help and support.

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CHAPTER I

INTRODUCTION

Rice, the main food for about half of the world's population, is one of the most important crops in the world. Its high and stable production is crucial for feeding the 8.3 billion people that are predicted to be in existence by the year 2025 (Borlaug, 2000). Therefore, rice is a very attractive candidate for genetics and biology studies. Its small genome (400-430Mb) also makes it an excellent monocot model for genomic sequencing and many other studies (Shimamoto, 1995).

ORIGIN, DISTRIBUTION AND VARIATION OF RICE

Indica and Japonica rice are the most common and important varietal types in *Oryza sativa* that are distributed in the rice cultivation countries around the world. Due to their economic importance in the world, these two types of rice have been studied extensively and deeply at different levels, such as morphology, physiology, and genetics including classical genetics and molecular genetics. Various studies between the indica and japonica types show that indica rice is quite different from japonica rice, not only in origin and distribution, but also in both morphology and molecular composition.

This dissertation follows the format of Plant Molecular Biology.

Origin of rice

There are two hypotheses about the origin of indica and japonica rice; the diphyletic hypothesis and the monophyletic hypothesis (Oka, 1991). The first hypothesis assumed that indica and japonica originated independently, while the second hypothesis assumed that both indica and japonica was domesticated from same wild rice.

The monophyletic hypothesis was supported by studies on intermediate wild-cultivated rice from the Jeypore Tract, India (Oka, 1991). The observations showed that japonica-like plants can be produced from typical indica-indica crosses and indica-like plants can be produced from typical japonica-japonica crosses (Oka, 1982). Therefore, it was believed that indica and japonica were derived from the same progenitor.

The diphyletic hypothesis was widely supported by studies at the biochemical and molecular level. Studies of isoenzymes indicated the indica and japonica most likely originated independently(Second, 1982; Morishima, 1986). The results from cpDNA comparisons of 93 cultivated rice, 32 indica and 61 japonica, also supported the diphyletic hypothesis and suggested that indica and japonica are from different centers of origin (Sun et al., 1998).

RAPD, RFLP, nuclear SSLP and chloroplast SSLP analyses on 4 japonica and 5 indica rices (Bautista et al., 2001) showed that indica rice is similar to *O. rufipogon* from tropical countries (India, Malaysia, Myanmar and Indonesia) and possibly originated from these areas, while the japonica rice is strongly similar to *O. rufipogon* from China where the japonica most likely originated. It is also supported by the archaeological evidence in which

that the oldest japonica grains were discovered along the Changjiang river in China, further suggesting that China is the origin center of japonica rice (Jiang et al., 1996).

Short interspersed nuclear elements (SINEs) was also used to study the origin of cultivated rice. It was found that the indica subspecies likely arose from one group of *O. rufipogon* lines while the japonica arose from another group of *O. rufipogon* lines. This result also supported the indica and japonica originated diphyletically (Cheng, 2003).

Geographical distribution of rice

Rice is widely distributed from tropical countries to temperate countries. However, indica rice is mainly grown in the tropical and subtropical areas while japonica rice is widely planted from the tropics to northern temperate zones. In Asia, the indica rice is mainly distributed on the continent (mainly in India, China, Thailand and Vietnam), so it was called "Continent" rice. The japonica is distributed both from Japan to Indonesia and the continent areas, but it was also called "Island" rice (Jiang et al., 1996).

The distribution of Indica and Japonica is also affected by altitude. The studies on the distribution of rice in Yunnan, China, found that the indicas are mainly cultivated in the mountainous areas below 1,200m while both indicas and japonicas are distributed between 1200-1700m. Only japonica was found in the mountainous regions above 1,800 (up to 2,370m). From those data, it is clear that the distribution of indica and japonica in Yunnan is mainly affected by temperature (Jiang et al., 1996).

Morphological variations of rice

The morphological characters of typical indica plants are quite different from typical japonica rice. The most conspicuous difference may be in grain form in which indica is slender and flattened while japonica is short and round. The typical indica leaf is broad and light green while japonica is narrow and dark green. The tillering ability of indica is stronger than that of japonica. There are many other characters that are different from each other (Jiang et al., 1996). Although typical indica and japonica plants differ in many characters, there are still many intermediate varieties that were difficult to group to either indica or japonica. Never is it possible to distinguish the two subspecies from each other by single character comparison. It was found that over 39% of rice cultivars were wrongly grouped when relying only on the length-width ratio of grains (Morishima and Oka, 1981). Jiang (1996) investigated the indica and japonica intermediates fromYunnan province, China. It was inferred that those intermediates are mixture of indica and japonica due to natural crosses between each other and natural selection (Jiang et al., 1996).

Isozymes variations of rice

Investigation of 1,688 native cultivars from different Asian countries for allelic frequencies at 15 isozymes loci using a multivariate technique (Glaszmann, 1987)showed that 95% of those rices can be grouped into six enzymatic groups and 5% fall into an intermediate type which can not be grouped into any other groups. In this study, the indica rices can be

found in five different isozyme groups and showed great variation while japonica (both tropical and temperate varieties) are relatively uniform and distributed in one isozyme group. If the allelic variability for isozyme loci is calculated by the average gene diversity using Nei's formula (Nei 1975), isozyme variation is observed more clearly between indica and japonica lines than within (Oka, 1991).

DNA variations of rice

Chloroplast DNA is very conservative in evolution. Rice cpDNA is 130kb (Hirai et al., 1985). The study of fragment length polymorphism of chloroplast DNA using three endonucleases found that nine types of chloroplast DNA are different between indica and japonica rices (Ishii et al., 1988). The cpDNA of indica and japonica subspecies were also different from each other (Dally and Second, 1990). A study on the large chloroplast DNA insertion on the long arm of chromosome 10 that the insertion is nearly identical in indica and japonica. It suggested that the cpDNA transfer from wild rice occurred before the divergence of indica and japonica rice (Yuan et al., 2002).

Rice mtDNA is very conserved in evolution as well. A study on mtDNA in rice showed that the mtDNA arrangement commonly found in wild rice mtDNA is mainly found in indica, rarely in japonica. Further study showed that the common wild rice mtDNA found in indica is very conservative. But the mtDNA in japonica is quite different from that in indica. It is very interesting that some japonicas also have the common wild rice mtDNA. Studies on

restriction fragment length polymorphism (RFLP) of mitochondrial DNA from rice showed that the RFLP patterns in Indica rice were different from Japonica rice (Abe et al., 1999).

Restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and simple sequence length polymorphisms (SSLP) were used to study the genetic variations of indica and japonica rice. (Sun et al., 2000; Bautista, 2001; Zhu et al., 2001). RFLP results showed that the genetic variation of indica is larger when comparing with japonica in *O. sativa* using proportion of polymorphic loci, average number of alleles per loci and average gene diversity as the parameters. The RFLP data was used to calculate the genetic distance indica and japonica accessions. It was found that genetic distance within indica rice is larger than that within japonica rice.

The RAPD, RFLP, nuclear SSLP and chloroplast SSLP analyses on rice also showed that the patterns of both RAPD and SSLP variants of indica rice is different from those of japonica, while no difference in RFLP variants was detected between these two subspecies (Bautista, 2001).

Genome of rice

Rice (*Oryza Sativa*. L.)has a chromosome number of 2N=24. A draft sequence of both indica and japonica rices was published (Goff et al., 2002; Yu et al., 2002). The complete sequence of the japonica cultivar Nipponbare is available from TIGR database. From the published data, the genome size of indica is 466 megabase, while that of japonica is 420 Mb. The estimated number of genes in the indica genome ranges from 46,022 to 55,615 genes,

while that in japonica the range is from 32,000 to 50,000 genes. The difference between indica and japonica on chromosome 4 is 16% estimated at the gross level(Yu et al., 2002). When compared at the nucleotide level, although the alignment is only partial, the single nucleotide polymorphisms (SNP) rate is 0.43% and the rate of divergences in insertion/deletion polymorphisms (InDels) is 0.23%.

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF RICE

The first green revolution was based on the introduction of high-yielding semi-dwarf varieties of wheat and rice, in combination with applications of large amounts of nitrogen fertilizer to increase grain yield. It was crucial to feed the almost doubled world population that occurred in the 1960's. Sixty years later, it was estimated the world population will reach 8.3 billion by 2025. A second green revolution for plant breeding and cultivation is needed to feed the increasing world population. Genetic engineering of food crops has turned out to play an important role for the expected second revolution. Rice is a staple food for almost half the world's population. It is crucial to improve rice production to achieve the projected food productivity to feed the growing world population.

For almost a decade, the development of methods for genetic transformation of rice and other cereals lagged in comparison with the progress for dicotyledonous crops. The major cause of this delay was the fact that transformation mediated by the soil bacterium *Agrobacterium tumefaciens* was initially thought to be inapplicable to cereal plants, necessitating the use of direct transformation techniques such as bombardment (Cao et al., 1992). However, Hiei et

al.(Hiei et al., 1994)demonstrated efficient transformation of rice by *Agrobacterium* and similar procedures (Dong et al., 1996)are now widely used. Nevertheless, particle bombardment remains a powerful tool for analysis of transient expression of foreign genes in plant cells (Komari et al., 1998).

The soil phytopathogen *Agrobacterium tumefaciens* has been utilized routinely for transformation of dicotyledons. Advantages include the transfer of pieces of T-DNA with defined ends and minimal rearrangement, the transfer of relatively large and intact segments of DNA, the integration of small numbers of copies of genes into chromosomes, and the high fertility of resultant transgenic plants in contrast to direct DNA uptake methods (Sheng and Citovsky, 1996; Hiei et al., 1997)

In the past few decades, although various attempts to infect monocotyledons with *Agrobacterium* were made, no conclusive evidence of integrative transformation was obtained until in the middle of1990s. Successful transformation ofcereal crops *via Agrobacterium* has been achieved in wheat (Cheng et al., 1997), maize (Ishida et al., 1996; Shen et al., 1999) and rice. Efficient transformation of several subspecies of rice via *Agrobacterium* has been reported (Chan et al., 1993; Hiei et al., 1994; Aldemita and Hodges, 1996; Dong et al., 1996; Komari et al., 1996) and genes of interest have been transferred into rice for commercial purposes (Burkhardt et al., 1997; Cheng et al., 1998; Ye et al., 2000).

An *Agrobacterium*-mediated rice transformation system using binary vectors has been established in our lab. It is similar to previously published procedures (Aldemita and Hodges, 1996; Chan et al., 1993; Dong et al., 1996; Hiei et al., 1994; Zhang et al. 1997; Cheng et al., 1998; Vijayachandra et al., 1995). An *Agrobacterium*-mediated transformation procedure

using inflorescence as the explant source was also established (Dong et al., 2001). The *Agrobacterium*-mediated transformation system has been used in generating a large number (more than1500) independent transformants from different gene constructs.(Vijayachandra et al., 1995; Cheng et al., 1998) Recently, a dual T-DNA transformation system for the production of marker-free transformants of rice was established in our lab.

TRANSGENE EXPRESSION AND SILENCING

Although efficient protocols for transformation of some cereal crops are now available, there are still many hindrances to the successful application of this biotechnology to crop improvement. Transgene silencing is one important constraint. A variety of silencing effects has been described in the literature, involving single transgene loci, interactions between unlinked loci, or even interactions with or through an endogenous homologous gene (Meyer et al., 1992; Iglesias et al., 1997; Iyer et al., 2000). Epigenetic silencing of transgenes and endogenous genes can occur at the transcriptional level (TGS) or at the posttranscriptional level (PTGS) (Fagard and Vaucheret, 2000). Aspects such as insert location, rearrangements, multiple-copy loci, homology to an endogenous sequence, excessive level of transcription and others have been claimed to be apparent triggers of silencing. Several studies make it evident that at least some silencing mechanisms are part of normal gene regulation systems that operate during plant growth and development. Additionally, silencing is now known to be involved in plant defense systems against invasive DNA or RNA sequences (virus, transposons) and surveillance processes that check the genome integrity to suppress the expression of abnormal or alien

transcription units (Jorgensen et al., 1998; Kumpatla et al., 1998; Iyer et al., 2000). Recent studies have shown that small RNA, such as small interfering RNAs (siRNAs) and micro-RNAs (miRNAs) play important roles as regulators of gene expression in eukaryotes (Hutvagner and Zamore, 2002; Lai, 2002; Rhoades et al., 2002).

CHAPTER II

HIGHLY EFFICIENT PRODUCTION AND CHARACTERIZATION OF DIPLOID TRANSGENIC RICE VIA *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION

INTRODUCTION

Transformation of rice (*Oryza Sativa* L.) was conducted via naked DNA uptake (Battraw and Hall, 1990; Battraw and Hall, 1992)or by biolistics (Christou, 1992). However, multiple and rearranged copies of the desired gene together with the plasmid vector was often integrated into the genome, which often results in gene silencing (Christou, 1992; Jorgensen et al., 1998; Kumpatla et al., 1998; Iyer et al., 2000). *Agrobacterium tumefaciens*-mediated transformation has been utilized routinely for transformation of dicotyledons where it has been shown that transfer of pieces of T-DNA with defined ends with minimal rearrangement. Also, the method allows transfer of relatively large and intact segments of DNA, leads to integration of small numbers of copies of genes into chromosomes, and the resultant transgenic plants have a high fertility in contrast to direct DNA uptake methods (Shen and Ho, 1995; Hiei et al., 1997). For almost a decade, the development of methods for genetic transformation of rice and other cereals was not very successful until Hiei et al.(Hiei et al., 1994)demonstrated efficient transformation of rice by Agrobacterium and Dong et al extended it to Javanica rice (Dong et al., 1996). The established procedures of *Agrobacterium tumefaciens*-mediated transformation of rice are now widely used.

With the complete genome sequence as well as abundant genetical and physiological data, rice is a well established model for cereal crops. Now the challenge for the scientific community is clearly to assign a biochemical, cellular, developmental or adaptive function to the majority of the rice genes. There are several well established approaches to reach this great goal: targeted/random gene disruption, gene detection, gene activation and/or gene silencing methods to discover and validate the gene function in rice. RNA interference (Fire et al., 1998) has been proved to be effective to inactivate the expression of a target gene or a gene family. Homologous recombination (HR) was reported to be valuable in the targeted gene disruption or gene replacement in rice (Terada et al., 2002). All those functional genomics resources will benefit from a highly efficient transformation system.

Characterization of transgenic rice can be conducted through both molecular analysis and genetics approaches. PCR has proved to be very limited for the determination of transgene copy numbers. Genomic Southern blot analysis is valuable in characterization of transgenic rice, although it is time-consuming. The characterization for the number and location of genomic inserts in transgenic plants has to be performed through genetics analysis in their progeny. It needs at least one generation and considerable examination including molecular tests on each of many progeny. In this chapter, a simple approach was described to characterize the T-DNA locus number and the copy number on original transgenic rice (T_0).

MATERIALS AND METHODS

Plant material and tissue culture media

Japonica rice (*Oryza sativa* L. cv Taipei 309 and Nipponbare) plants were grown in a greenhouse as described previously (Battraw and Hall, 1990). Immature seeds or mature seed was used for callus induction. Media used for tissue culture and transformation are listed in table 2-1.

Plasmid and Agrobacterium strains

The binary plasmid pUbi1F (Figure 2.1) was constructed from pJD7 (Dong et al., 2001), in which the GUS (*uidA* gene) reporter was replaced with an mGFP5-ER reporter and the *35S* promoter was replaced with *mUbi1* promoter that was amplified from pJD4 (Dong et al., 2001). The binary vector pJD7 (a derivative of pRPA-BL-150a that confers gentamycin resistance for plasmid selection and extra copies of a DNA fragment encoding virulence genes of *Agrobacterium*: virC, virD, virG and the 3' part of the virB operon from pTVK291 were used in the transformation experiments. The T-DNA of pUbi1F contains selectable marker genes for hygromycin resistance (*35S-hpt-35S*) and a *gfp* reporter gene construct (*mUbi1-gfp-nos*) for monitoring transformation events. The tri-parental mating procedure was used to mobilize pUbi1F and pTVK291 into *Agrobacterium* strain LBA4404. *Agrobacterium* strains

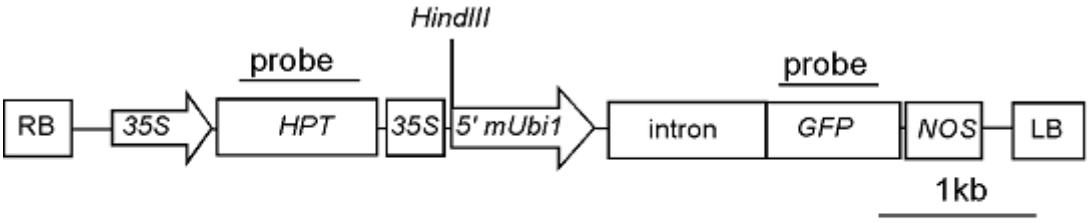

Figure 2.1. Schematic diagram of the T-DNA of the binary vector pUbi1F. Black horizontal bars indicate the *hpt* and *gfp* probes used for molecular analysis. The arrows indicate the transcriptional orientation of each gene. RB, T-DNA right border; LB, T-DNA left border; *35S*, the 35S promoter from cauliflower mosaic virus; *mUbi1*, the ubiquitin-1 promoter from maize; *hpt*, the coding sequence of the gene for hygromycin phosphotransferase conferring resistance to hygromycin B; *GFP*, the coding sequence of the gene for green fluorescent protein; *nos*, the 3' *nos* terminator. The single *Hin*dIII site facilitates molecular analysis of gene insertion number.

Table 2.1 Media for scutellum-derived callus induction and Agrobacterium-mediated transformation procedure

Medium	Composition
N6M Basic	Chu (N6) Basal salt mixture (Sigma), MS vitamins (Murashige and Skoog 1962), 30g/l sucrose
N6M	N6M Basic, 500mg/l proline, 500mg/l glutamine, 300mg/l casein acid hydrolysate, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2.5g/l Phytagel, PH 5.8
2N6M	N6M Basic+500mg/l proline, 500mg/l glutamine, 300mg/l casein acid hydrolysate, 2mg/l 2,4-D, 2.5g/l Phytagel, PH 5.8
2N6M-AS	2N6M+10g glucose and 100μM acetosyringone, pH 5.2
AAM	AAM (Hiei et al. 1994)
2N6M-CH	2N6M+350 mg/l cefotaxime, 50 mg/l hygromycin.
PRN	2N6M Basic+500mg/l proline, 500mg/l glutamine, 300mg/l casein acid hydrolysate, 3 mg/l 6-Benzylaminopurine (BAP), 0.5mg/l α-Naphthaleneacentic acid (NAA), 5 mg/l ABA, 4g/l Phytagel, PH 5.8.
RN-1	MS and vitamins (Murashige and Skoog 1962), 30 g/l sucrose, 2g/l BAP, 0.2g/l NAA, 4 g/l Phytagel, PH 5.8
RN-2	MS and vitamins (Murashige and Skoog 1962), 30 g/l sucrose, 0.5g/l BAP, 0.1g/l NAA, 3 g/l Phytagel, PH 5.8
MS0	MS and vitamins (Murashige and Skoog 1962), 30 g/l sucrose, 2 g/l Phytagel, PH 5.8

LBA4404(pUbi1F) was grown on AB minimal medium with 50 mg/L of gentamycin and 100 mg/L of kanamycin for 2 to 3 days at 28 ^{O}C. The bacteria were collected and resuspended in AAM medium for co-cultivation with immature embryo or mature embryo-derived callus. pJD7 and pJD4 in LBA4404 were also used for transformation of embryo-derived callus as control plasmid.

Induction of embryo-derived callus

Immature seed of each cultivar were collected, dehusked and surface-sterilized essentially as described previously (Dong et al., 1996).The immature embryo was excised with sterile forceps and callus was induced on a modified N6 medium (Chu et al., 1975) at 26°C in the dark. After 4 weeks (wk), callus had developed and was ready for transformation.

Mature seeds from each cultivar were dehusked and rinsed with 70% ethanol for 1 min, then soaked in 50% (v/v) bleach for 45 min on a shaker at 120rpm. The sterile seeds were rinsed five times with sterile distilled water and placed on N6M medium (Chu et al., 1975)for two weeks at 26°C in the dark. After 4 weeks (wk), callus had developed. The harvested calli was cultured for another 2 weeks at 26°C in the dark. The class I type of callus (Figure 2.2) was selected using a dissecting microscope. The morphological examination of three type of callus was conducted using an ESEM microscope purchased under National Science Foundation grant No. ECS-9214314.

Transformation procedure

Class I type of embryo-derived callus was subcultured on 2N6M medium modified from previous research (Hiei et al., 1994; Dong et al., 1996) at 28°C in dark for 3 days. Actively growing Class I callus with turgid cells on the surface (Figure 2.2A) was chosen for co-cultivation. About 50 pieces of callus, each with a diameter of approx. 5 mm were immersed in 10 ml of liquid co- cultivation medium (AAM) containing *Agrobacterium* cells at a density of 2-3x 10^8 cells/ml (OD^{595} = 0.5) in a 50 ml sterile centrifuge tube for 30 min.

Callus pieces were blotted dry on sterile paper towels, transferred to solid co-cultivation medium (2N6M-AS) and incubated for 3 days at 21°C in the dark. GFP-positive cells could be detected by fluorescence microscopy after two days of co-cultivation(Figure 2.3). After co-cultivation, the callus pieces were gently and briefly rinsed twice with 250 mg/l cefotaxime in liquid 2N6 medium. Rinsed calli (25 pieces, approx. dia. 5 mm) were individually transferred to one 100 mm-diameter Petri dish containing selection medium (250 mg/l cefotaxime; 50 mg/l hygromycin B) (2N6M-CH) for two weeks at 28°C in dark. After two weeks selection, resistant, light brown calli were evident on the surface of dead and dying (dark brown) calli(Figure 2.3). The light brown calli (with some dark brown callus attached) were transferred to fresh selection medium (2N6-CH) for 1 wk at 28°C in the dark. The surviving light brownish globular calli were gently removed from the dead calli and placed in the medium surrounding the original callus and cultured for another 5-7 days at 28°C in the dark. Four weeks after the co-cultivation, the surviving calli were ready for regeneration.

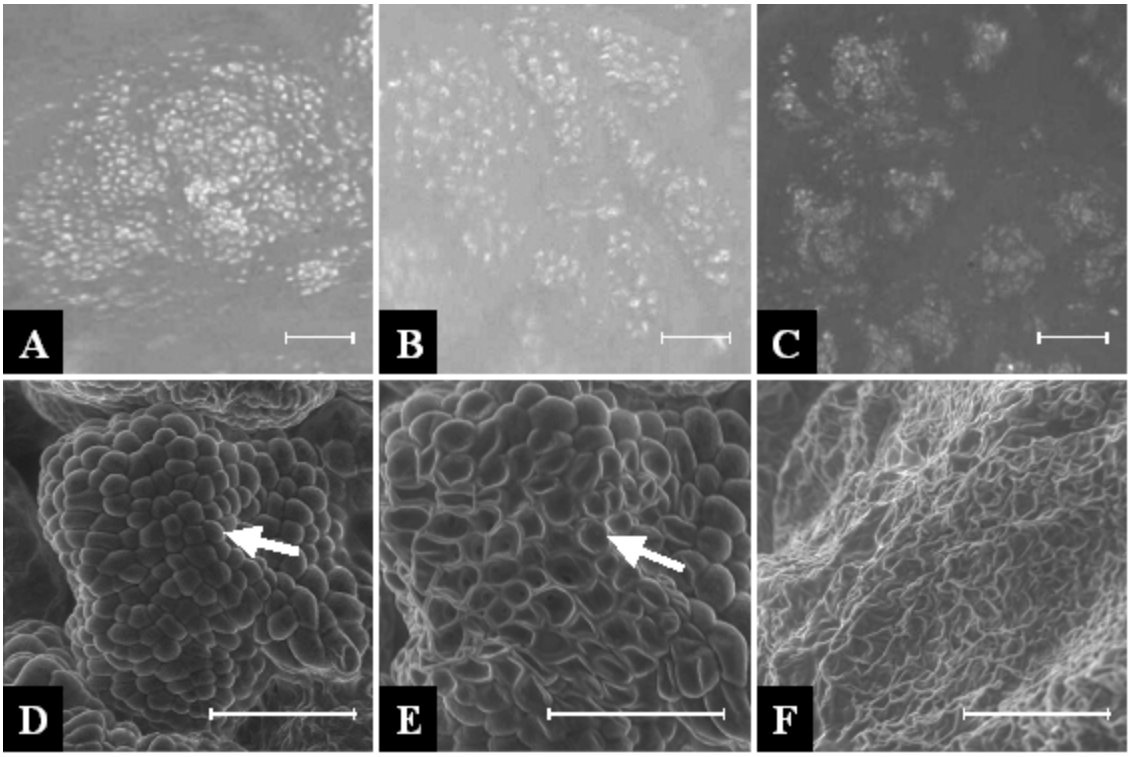

Figure 2.2. Classification of embryo-derived calli. Class I callus is shown in panels A and D, class II in B and E and class III in panels C and F. Images in panels A-C were taken under dissecting microscope and panels D-F are scanning electron micrograph(SEM) images at 270x. The arrows point to a robust, turgid cell characteristic of type I callus in panel D and to a shrunken cell in type II callus in panel E.

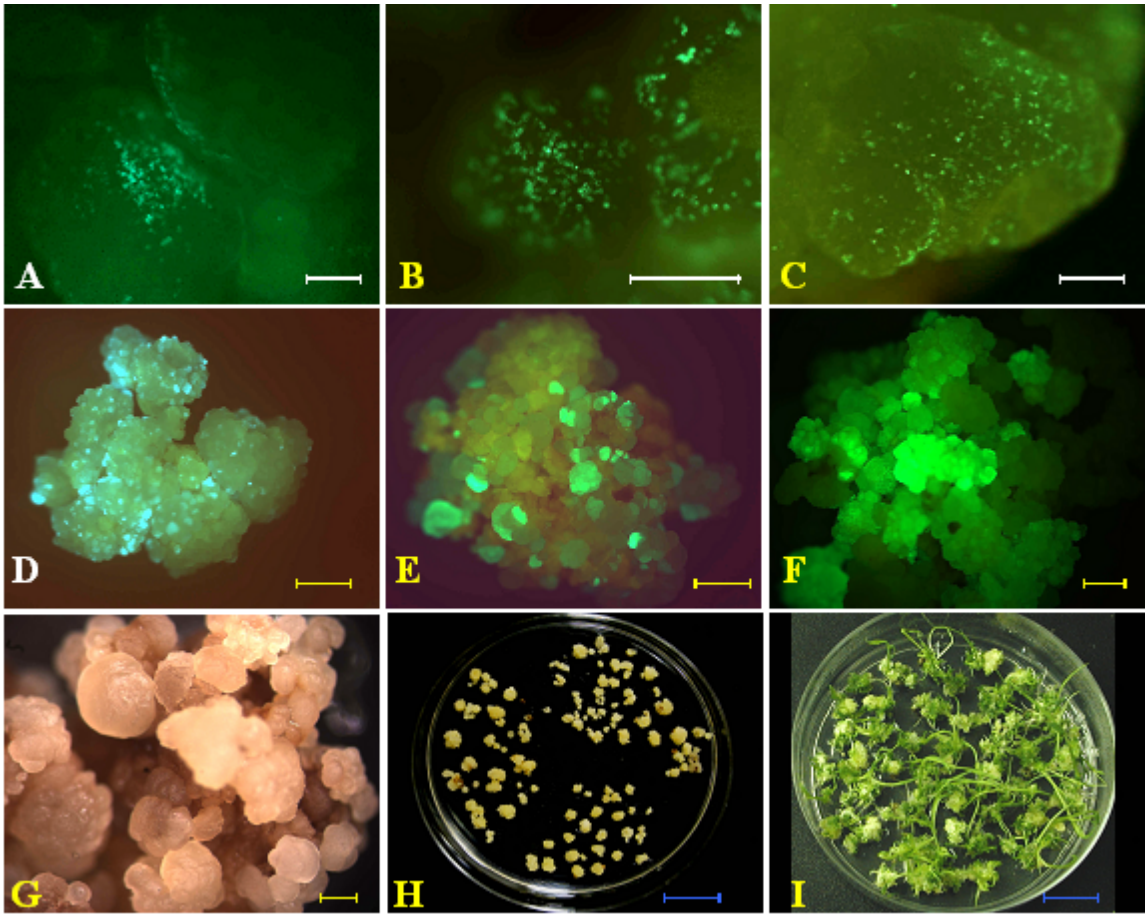

Figure 2.3. Stages in class I callus transformation. Panels A-F show images taken under illumination with 500 nM UV light. Panels A and B show calli 2d and 3d, respectively, after co-cultivation. Panel C shows calli 10d after co-cultivation (7d on selection). Panels D, E and F show calli after 14d, 21d and 28d of selection, respectively. Panel G shows calli morphology under white light after 28d of selection and immediately prior to transfer to pre-regeneration medium. Panel H shows calli 7d (white light) after placement on pre-regeneration medium and immediately prior to placement on regeneration medium. Panel I shows calli 7d after transfer to RN-2 regeneration medium (white light). White bar: 0.2mm; yellow bar: 1mm; blue bar: 2cm.

The transformation efficiency and development of hpt-resistant callus was followed throughout the selection step by fluorescence microscopy(Figure 2.3). Round, yellowish Class I calli with turgid cells on the surface were chosen for culture on pre-regeneration medium (PRN: (Sallaud et al., 2004))for 1 wk at 26°C in the dark. All of the surviving calli from a single co-cultivated piece of callus were grouped into individual portion on the PRN Petri dish so that they would not co-mingle with calli surviving from other co-cultivated calli. After 1 wk of culture on PRN medium, all remaining calli from the same original co-cultivated callus piece were individually transferred to a single 100 mm dia. Petri dish containing RN-1. The dish was kept in the dark at 26°C for one day, then at 26°C for 2 wk with a 16/8-h light/dark cycle. Multiple green shoots, with or without small roots, regenerated from the light brown surviving calli. All calli with green shoots derived from the original co-cultivated callus were individually transferred to a 100 mm-diameter/25 mm-height Petri dish containing medium (RN-2) for the second round of regeneration for 2 wk. The plantlets were then individually transferred to rooting medium (MS_0) for one wk. The resulting plantlets were cataloged and transferred to soil in the greenhouse.

Detection of gfp reporter genes

The expression of the gfp gene in transformed callus and plant tissues was observed using a Stemi SV11 APO Microscope (Zeiss) fitted with an AxioCam HRc camera. Calli that expressed GFP were imaged using a 500 nm filter and an exposure time of 3 sec. Pollen that expressed GFP were imaged at an exposure time of 2 sec. The mean value of green

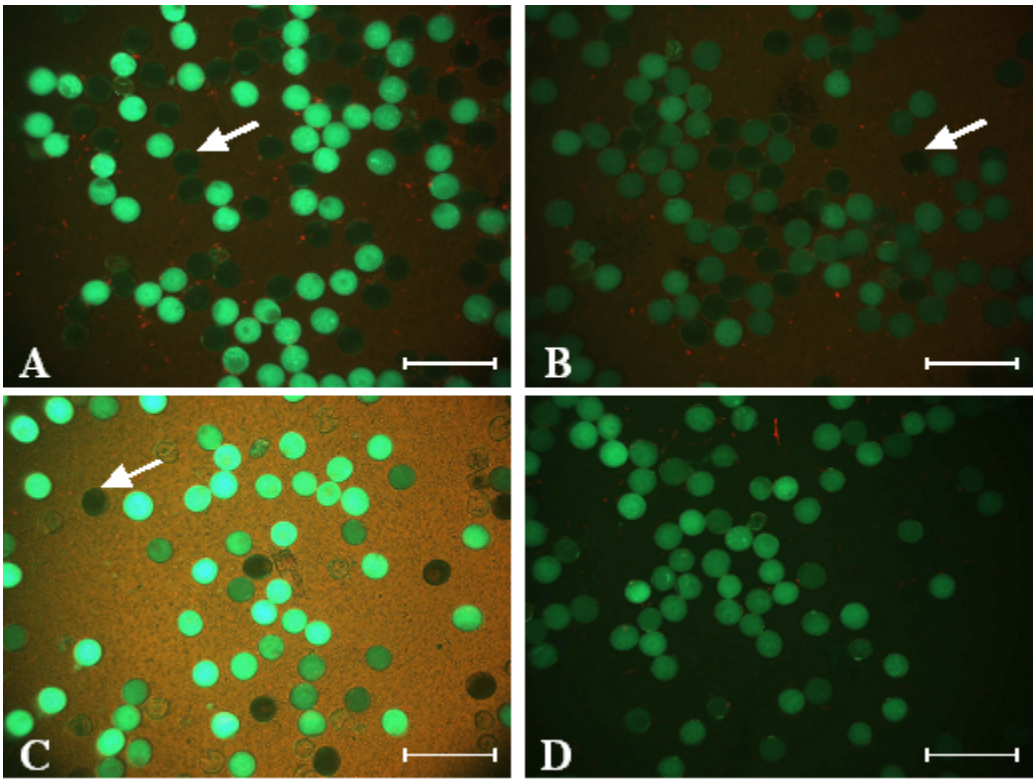

Figure 2.4. Locus number of T-DNA insertion on rice chromosome revealed by pollen *GFP* expression. Panel A: Single genetic locus of T-DNA insertion on rice chromosome revealed by pollen *GFP* expression. In accord with the segregation of the *GFP* reporter in pollen, 1:1 ratios for GFP positive : negative pollen (arrow) showed under fluorescent microscope ($\chi 2$ test for 1:1 of this transgenic plant, P=0.488). Panel B: Two genetic loci of T-DNA insertion on two non-homologous chromosomes revealed by pollen *GFP* expression. In accord with the segregation of the *GFP* reporter in pollen, $\chi 2$ test for 3:1 ratios for GFP positive : negative pollen (arrow), P=0.796. Panel C: Three genetic loci of T-DNA insertion on three non-homologous chromosomes revealed by pollen *GFP* expression.$\chi 2$ test for 7:1 ratios for GFP positive : negative pollen (arrow), P=0.546. Panel D: Three genetic loci of T-DNA insertion on three chromosomes(both members of one pair of homologous chromosomes and one non-homologous chromosome) revealed by pollen *GFP* expression.$\chi 2$ test for 1:2:1 ratios for 1GFP+++:2GFP++:1GFP+ , P=0.789.

fluorescence for each pollen was measured using the interactive measurement module of AxioVision 3.0 (Carl Zeiss). Each reading was normalized by subtracting the nearby background fluorescence reading. Seeds that expressed GFP were imaged at an exposure time of 1 sec.

T1 segregation analysis for GFP activity of pollen from T_0 plants

Five random chosen field of view of pollen from T_0 plants were observed using a Stemi SV11 APO Microscope (Zeiss) fitted with an AxioCam HRc camera. The image were taken using an exposure time that does not saturate the exposure. Segregation for pollen GFP activity was calculated by a statistical method using χ2-test (Figure 2.4).

T1 segregation analysis for hygromycin resistance and GFP activity of seeds

Fifty to one hundred were dehusked and observed for GFP activity under a Stemi SV11 APO Microscope (Zeiss) fitted with an AxioCam HRc camera. The seeds were then surface-sterilized, placed on a petri dish containing 30 mg/ml hygromycin and allowed to germinate under light at 26°C. After 7-10 days, seeds were scored for germination. Segregation for seed GFP activity and hygromycin resistance was calculated by a statistical method using χ2-test.

Nucleic acid extraction and Southern blot analysis

Rice genomic DNA was isolated from fresh leaves as described previously (Buchholz et al., 1998). Genomic DNA (2 μg) was digested with 20 units of *Hind*III for 12 h. After electrophoretic separation in a 0.8% agarose gel, the DNA fragments were transferred to Hybond-N+ membrane (Amersham). DNA probes were labeled using a DECAprime II™ kit (Ambion). Hybridizations were performed using ULTRAhyb™ (Ambion) according to the manufacturer's recommendations. Autoradiography of the membrane was done using a PhosphorImager (Fuji, Stamford, CT).

RESULTS

Classification of embryo-derived callus

Embryo-derived callus was examined under a dissecting microscope after 2 weeks of subculture . The calli were classified into three types of callus according to morphological characteristics of the surface cell structure (Figure2-2). Approximately 38% of the embryo-derived calli were class I type of callus with many turgid cells on the surface (Figure 2-2A). The callus surface was dry and no liquid could be detected. Anthoer 34% of embryo-derived calli were class II type of callus with a few of turgid cells on the surface. Liquid-like surface covered a small portion of the callus (Figure 2-2B). The final 28% of embryo-derived calli

were class III type of callus with no detectable turgid cells on the surface. Most of the surface was covered with liquid.

Time course of production of transgenic plants from transformation of class I callus

Embryo-derived calli of cv.Taipei 309 and Nipponbare, were co-cultured with Agrobacterium strain LBA4404 harbouring the pUbi1F, pJD4 and pJD7 binary plasmid (Figure 2-1) according to the procedure described in Materials and Methods. GFP expression in transgenic cells or callus was detected from 2 days after co-culture of the embryo-derived callus pieces, until plant regeneration (Figure 2.3). Two days following the co-cultivation of callus with pUbi1F on 2N6-AS at 21°C in dark, a few portion of GFP positive cells were detected using fluorescent microscopy on every co-cultivated class I type of callus (Figure 2.3). The number of GFP positive cells or callus increased when the time of co-cultivation and selection increased (Figure 2.3). One week after transfer to selective medium, growth of the co-cultured callus was completely inhibited by hygromycin. However, the GFP positive cells could be detected all over the top surface of the co-cultivated callus. Two weeks following the selection, hundreds of translucent globules with different GFP fluorescence intensity under UV light excitation could be detected all over the callus surface (Figure 2.3). Whitish resistant cell lines from structurally independent regions of the callus surface were potentially independent transgenic calli. Most of transgenic calli exhibited different GFP activity at this stage (Figure 2.3).

After one more week of selection on the selection medium, the surviving transgenic calli were examined under a light dissecting microscope (Figure 2.3). Class I type of transgenic callus could be detected(Figure 2.3). The transgenic calli were classified into three types as previously described (Figure 2.2). The selected calli were pre-regenerated for 1 week at 26°C in dark Then all calli were transferred to regeneration medium for 2 weeks at 26°C in 16h light/8 h dark. About 8 weeks was needed to produce transgenic plantlets from co-cultivation using class I type of embryo-derived callus.

Exceptionally efficient transformation of class I type of embryo-derived callus

The results of the co-culture experiments of three type of callus from each cultivar are summarized in Table 2-2. Every class I type of co-cultivated calli (100%) yielded multiple transgenic calli while ~15% class II type of calli yielded at least one transgenic callus. Only ~1% of class III type calli developed at least one transgenic callus. Transformation of the three type of callus together (mixture) resulted in very low efficiency (~23%) not only in transgenic callus production but also in transgenic plantlets per co-cultivated callus (Table 2-2).

Exceptionally efficient transformation of embryo-derived callus was observed only on class I type of callus. The mean number of hygromycin-resistant cell lines for both cultivars were 23.6 on each co-cultivated callus. The mean number of GFP positive cell lines reached 45.3 on each co-cultivated callus. ~40 plantlets could be regenerated on those transgenic cell line from a single piece of co-cultivated class I type of callus. More than one thousand

independent transgenic rice plants could be obtained using this exceptionally efficient transformation protocol.

Table 2.2 Summary of transformation using different types of callus

Callus Type	Cultivar	Number of co-cultured calli	Percentage of co-cultured calli yielding resistant cells	Mean (±SD) number of GFP^+ calli per co-cultured callus	Mean (±SD) number of resistant calli (GFP^-)per co-cultured callus	Mean (±SD) number of plantlet per co-cultured callus
I	T309	62	100.0	47.2 ± 13.4	25.4 ± 9.2	43.1 ± 15.2
	Nip	52	100.0	43.4 ± 11.3	23.6 ± 8.3	40.3 ± 12.7
II	T309	54	15.6	1.5 ± 0.83	1.1 ± 0.06	1.8 ± 1.12
	Nip	58	13.4	1.2 ± 0.46	1.3 ± 0.04	1.6 ± 0.86
III	T309	150	0.6	0.2 ± 0.08	0.3 ± 0.12	0.04 ± 0.02
	Nip	165	1.2	0.3 ± 0.12	0.2 ± 0.07	0.06 ± 0.04
Mix	T309	220	22.3	2.4 ± 1.1	2.8 ± 1.6	0.42 ± 0.23
	Nip	216	18.7	2.8 ± 1.5	3.2 ± 1.1	0.51 ± 0.34

Exceptionally efficient regeneration of class I type of transgenic cell line

The results of regeneration experiments of three types of transgenic callus from each cultivar are summarized in Table 2.3. An average of 67.6% of class I type of transgenic calli from both cultivars yielded at least one green plantlet (Figure 2.5). Among those class I type

Table 2.3 Summary of regeneration using different types of callus

Callus Type	Genotype	Number of survived calli for regeneration	Number of calli with regenerated plant	Percentage of regeneration
I	T309	562	387	68.9
	Nipponbare	558	370	66.3
II	T309	758	36	4.75
	Nipponbare	588	32	5.44
III	T309	850	2	0.24
	Nipponbare	678	0	0
Mix	T309	1228	199	16.2
	Nipponbare	1167	249	21.3

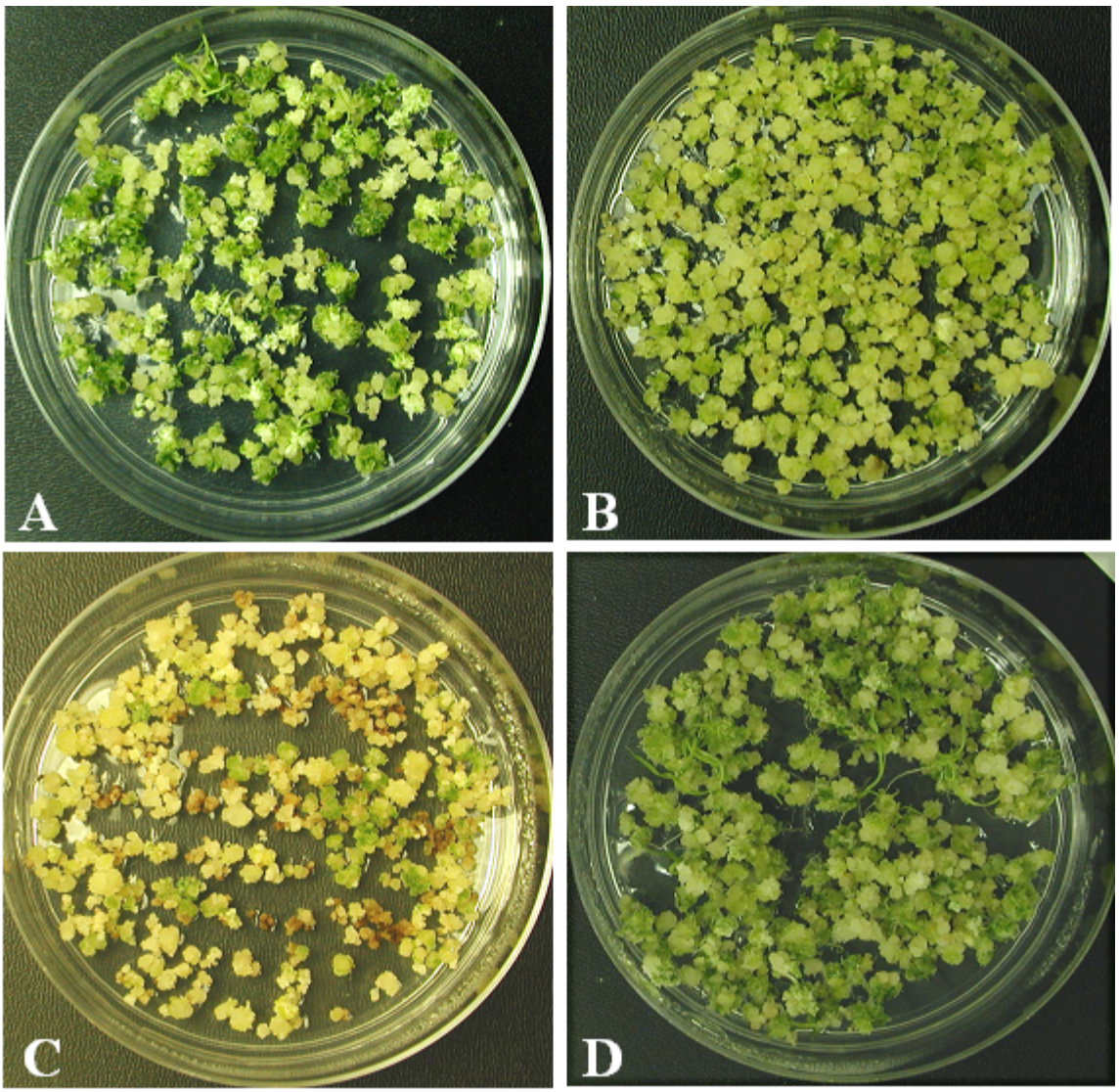

Figure 2.5 Regeneration of three type of transgenic callus. Most of class type I of transgenic calli were able to develop at least one green plantlet(Panel A). Only a few green plantlets could be recovered from class II type of transgenic calli(Panel B). Almost no green plantlet could be regenerated from class III type of calli(Panel C). Regeneration of the mixture of three type of callus was presented in panel D.

of calli with regenerated plantlets, ~20% bore multiple green plantlets (Figure 2.5A). Only ~5% of class II type transgenic calli yielded at least one transgenic plantlet. Most of those class II type of calli with regenerated plant only bore one recovered plantlet (Figure 2.5B). None of class III type of transgenic calli from Nipponbare yielded any transgenic plantlets (Table 2.3). Only 2 out of 850 class III type transgenic calli from Taipei 309 yielded regenerated plants. ~20% of the mixture of three types of transgenic calli gave rise to regenerated plants (Figure 2.5D and Table 2.3).

Analysis of the transgenic plants regenerated from the same co-cultured callus

The green plants recovered from each co-cultivated callus were numbered and grown in a greenhouse essentially as described previously (Battraw and Hall, 1990). 200 transgenic plants from both cultivars were analyzed by Southern blotting as described in Materials and Methods. Genomic DNA was isolated from a single leaf of each plant, digested by a restriction enzyme (*HindIII*) that cut once in the middle of the T-DNA (Figure 1) and hybridized with the

Table 2.4 Summary of the number of independent transgenic plants obtained from individual callus transformed with pUbi1F

Calli No.	Number of resistant calli (Survived)	Number of regenerated plants	Plants analyzed by Southern	Plants analyzed by pollen	Independent plants by Southern: Number	(%)	Independent* plants by pollen: Number	(%)
1	73	52	47	38	44	(93.6)	36	(94.7)
2	56	39	36	34	36	(100)	34	(100)
3	50	32	32	26	32	(100)	26	(100)
4	65	41	37	30	36	(97.3)	29	(96.7)
5	82	54	48	32	48	(100)	32	(100)
Total	326	218	200	160	196	(98.0)	157	(98.1)

*Percentage of independent events from the number of transgenic plants analyzed.

hpt and *gfp* probes localized at both T-DNA ends. As illustrated in Figure 2.6 for callus no. 1 (Table 2.4), all but one of the 25 plants was a transgenic plant. All but one of those transgenic plants exhibited a distinct hybridization pattern, indicating that 23 plants were all derived from independent events, whereas plants 17 and 18 were of clonal origin (sibling). Overall~98% were independent transgenic events (Table 2.4). Only two plants were from callus that escaped from the selection and turned out to be wild type. For those multiple plants recovered from a single co-cultivated callus, Southern blot anlysis indicated almost all of them were independent transformants.

T-DNA integration pattern

The Southern blot analysis described above also allowed us to distinguish integration patterns among those independent plants of both rice cultivars. Most of the transgenic plants bore more than one T-DNA copy. Many of the plants with multiple T-DNA copies showed a putative tandem structure. As an example in Figure 2.6, plants numbered 3, 5, 8, 9, 11, 15, 19, the number of bands hybridizing to the *hpt* (RB) and the *gfp* (LB) probes were different (Figure 2.6). Head-to-head tandem repeat or truncated T-DNA copies could result in this kind of band pattern. The highest number of bands observed either with the *hpt* or the *gfp* probe was used for the purpose of estimating the T-DNA copy number. For many practical purposes, single T-DNA insertion is desired. As illustrated in Figure 2.6, plants numbered 2, 4, 6, 7, 10, 12, 14, 24, both probes indicated one copy of T-DNA insertion. Overall, an average of ~34%

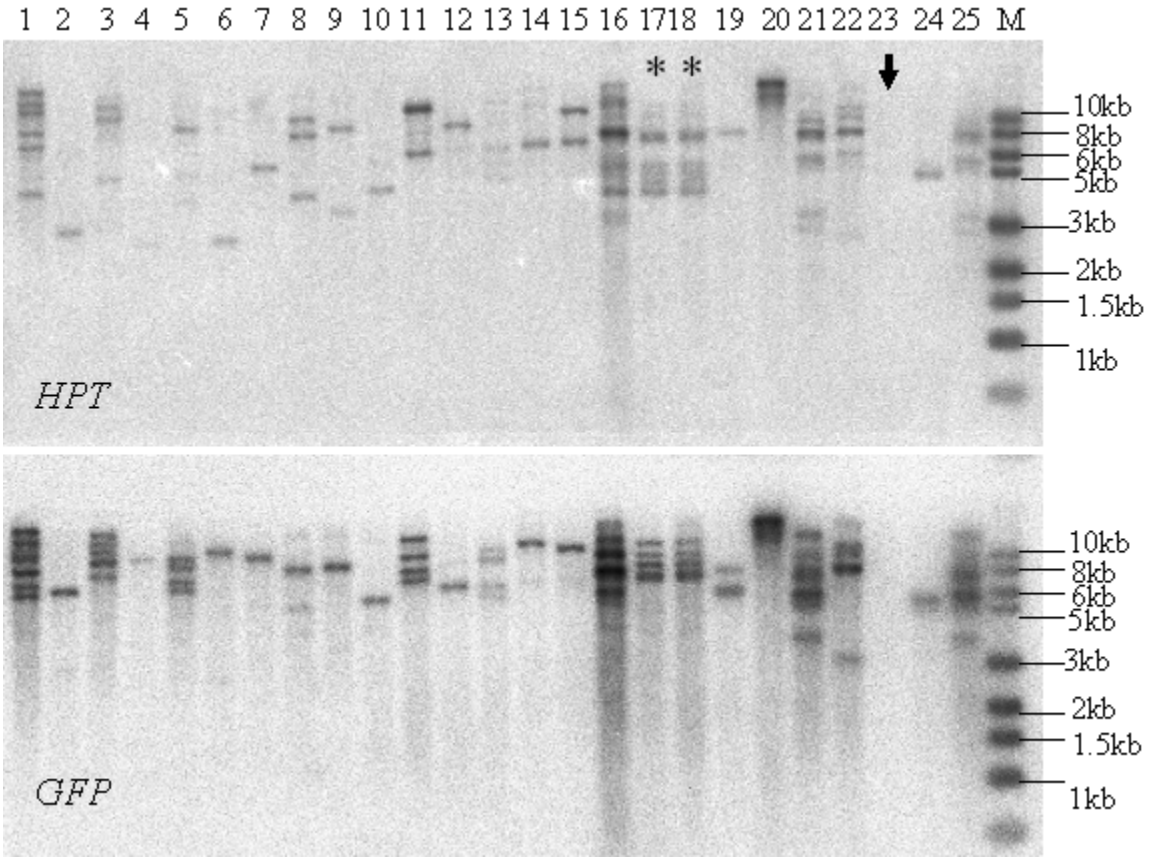

Figure 2.6 Genomic blot analysis of T_0 lines of transgenic T309. Genomic DNA from lines 1 to 25 (all from one piece of co-cultivated callus) was digested with *Hind*III, and hybridization was performed with the *hpt* or *gfp* probe. The sizes of the bands visualized for each plant with either the hpt or gfp probes were different, indicating that T-DNA integrated at different locations in the rice genome and that 23 out of 25 transgenic plants were independent transformation events. (*) indicates the two primary transformants showing the same pattern of hybridization with *hpt* and *gfp* probes. Black arrow indicates the plant without transgenic insertion.

of the transgenic plants for both cultivars bore one copy of T-DNA. The number of T-DNA inserts on each plant averaged 2.6 in a population of 200 transgenic plants as determined by Southern analysis.

Because the GFP reporter gene driven by pUbi1F was expressed in pollen (Figure 2.3 and 2.6), it also could be used to characterize the transgenic plants. To estimate the number of functional inserts, segregation analysis for *gfp* expression of pollen in T_0 plants was conducted as described in Material and Methods. According to pollen segregation analysis of *gfp,* 217 out of 339, ~64% transgenic plants with *gfp* expression in pollen bore one T-DNA insertion at a single locus (Table 2.5). ~22% plants bore two independent loci. About 14% of plants bore 3 or more independent loci(Table 2.5).

GFP expression was observed in mature seed on those plants with GFP expression in pollen. The estimate of locus number was analyzed by seed GFP segregation in F_1 progeny along with the pollen segregation on T_0 original transgenic plants (Table 2.6). For the single locus transgenic plants, the segregation for hygromycin resistance was analyzed by germinating seeds on media as described previously. All tested T_1 lines were found to exhibit a 3:1 segregation ratio for hygromycin resistance (data not shown), thereby confirming the presence of one locus insertion.

The copy number of T-DNA insertion at a single locus varies from one to four copies (Table 2.7). Southern blot analysis of those transgenic plants with single T-DNA insertion that was confirmed by pollen segregation revealed that ~55% of single locus T-DNA plants bore

Table 2.5. T-DNA locus number on chromosome revealed by pollen segregation

	One locus	2 loci (2 non-homologous chromosomes)	3 loci (3 non-homologous chromosomes)	4 loci (4 non-homologous chromosomes)	≥2 loci (both of a pair of homologous chromosomes)	Total
Plant No.	217	75	34	10	3	339
%	64.0	22.1	10.0	3.0	0.9	100

Table 2.6 Segregation analysis for GFP activity in pollen of T0 and seed of T1 progenies

Plant No.	GFP + pollen number	GFP- pollen number	Expected segregation ratio	χ2 value (P)	GFP+ seed number	GFP- seed number	Expected segregation ratio	χ2 value (P)	Number of independent integration loci
1	443	28	15:1	0.784	323	1	255:1	0.261	4
2	346	328	1:1	0.488	212	71	3:1	0.972	1
3	455	148	3:1	0.796	326	19	15:1	0.569	2
4	254	239	1:1	0.499	158	56	3:1	0.693	1
5	186	175	1:1	0.563	86	26	3:1	0.663	1
6	283	292	1:1	0.707	122	38	3:1	0.715	1
7	415	398	1:1	0.551	97	30	3:1	0.720	1
8	276	92	3:1	0.928	286	15	15:1	0.363	2
9	365	347	1:1	0.501	184	58	3:1	0.711	1
10	183	196	1:1	0.504	85	32	3:1	0.557	1
11	479	0	0		337	0	0		3
12	244	236	1:1	0.715	156	47	3:1	0.543	1
13	511	486	1:1	0.429	176	62	3:1	0.708	1
14	437	412	1:1	0.391	214	68	3:1	0.731	1
15	276	295	1:1	0.427	181	53	3:1	0.407	1
16	169	21	7:1	0.546	285	3	63:1	0.476	3
17	354	105	3:1	0.293	214	12	15:1	0.559	2
19	346	312	1:1	0.185	165	60	3:1	0.564	1
20	234	199	1:1	0.321	196	58	3:1	0.426	1
21	431	127	3:1	0.222	257	15	15:1	0.617	2
22	338	107	3:1	0.642	169	9	15:1	0.511	2

Table 2.7 T-DNA copy number at one locus of chromosome

	one copy	two copies	three copies	4 copies	total
Plant No.	78	36	20	8	142
%	55.0	25.3	14.1	5.6	100

one T-DNA Copy, and ~25% of single locus T-DNA plants bore two T-DNA copies. However, about 20% of the transgenic plants have multiple T-DNA copies (three or more) inserted at one locus.

Additive gene expression of gfp driven by pUbi1F

Additive transgene expression was observed in pollen segregation on plants that bore two or more independent loci (Figure 2.4). In those transgenic plants with multiple independent loci of T-DNA insertion, the higher intensity of relative green fluorescence was displayed in pollen that bore more T-DNA copies (Figure 2.4). The pollen with different GFP intensity segregated in a Mendelian fashion on T_0 plants that could be used to estimate the T-DNA insertion loci number (Table 2.6). Additive gene expression of gfp in pollen was confirmed by analysis of the relative green fluorescence in pollen along with the Southern blots of each T_0 transgenic plant and its progeny (Figure 2.7). The relative green fluorescence (RGF) of pollen bearing both copies of T-DNA was equal to the addition of RGF of pollen in plants number 1 and 3 bearing a single copy of T-DNA (Figure 2.7 Panel C).

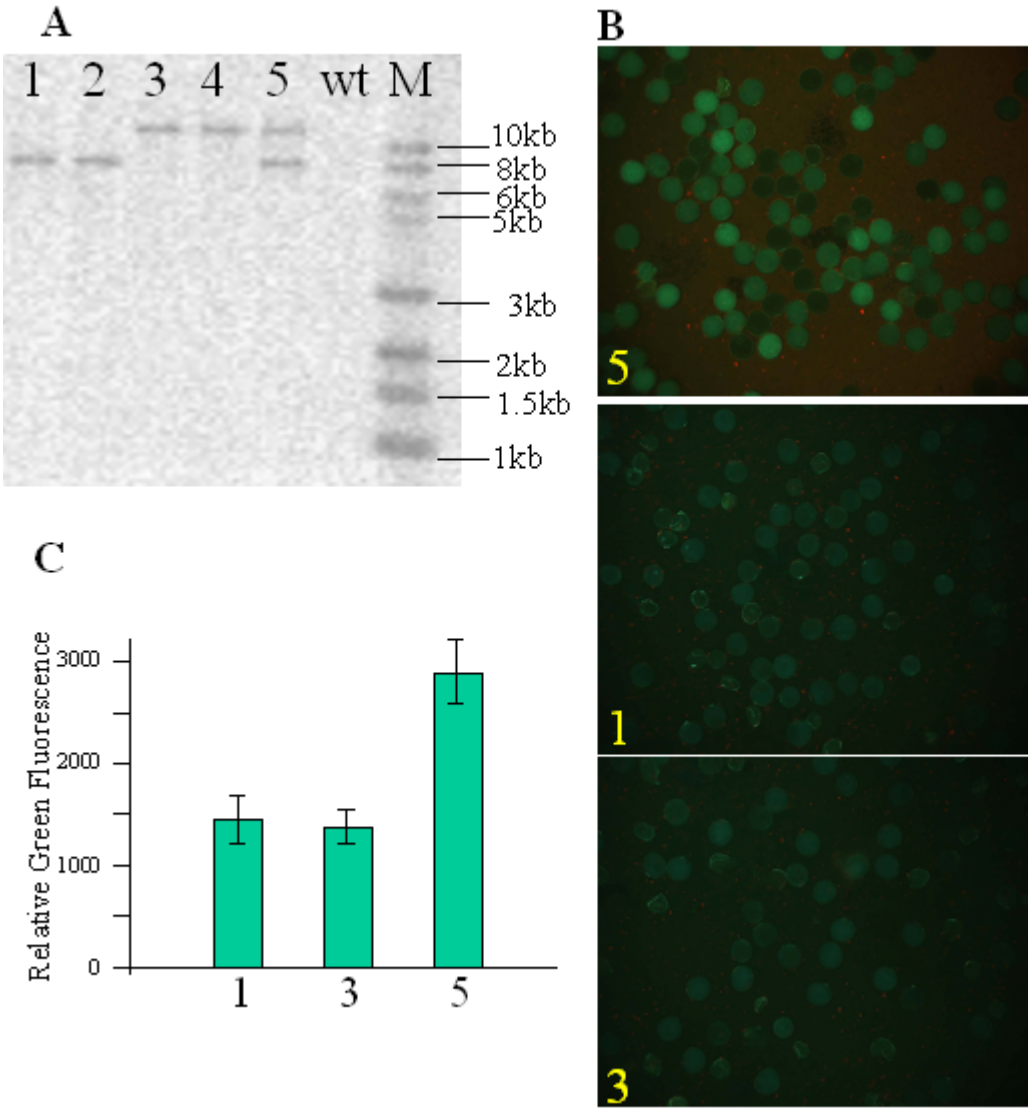

Figure 2.7 Additive gene expression of *gfp* in pollen. Panel A: Southern blot of a T_0 plant (number 5) with two independent single copy insertions, and its progeny(number 1, 2 and number 3, 4) that bore a single copy insertion. Panel B: *gfp* expression pollen in the plants corresponding to Southern blot. The relative green fluorescence of pollen bore both copies of T-DNA (Panel C, number 5, corresponding to pollen with higher GFP intensity), one copy of T-DNA(Panel C, number 1 and 3).

DISCUSSION

An exceptionally efficient transformation procedure on class I type of embryo-derived callus for japonica rice

A highly efficient transformation procedure has been established on the basis of class I type of callus that was chosen using a dissecting microscope. With the help of the *gfp* reporter gene driven by pUbi1F, transformation could be evaluated at a very early stage. Usually gfp expression in transformed class I type of callus could be readily detected only two days after co-cultivation. In some cases, gfp expression in transformed cell was detected 36 hours after co-cultivation. Numerous transgenic cells with strong gfp expression were found all over the surface of co-cultured class I type of callus. The technique is suited either for stable transformation or for transient gene expression studies. *Agrobacterium*-mediated transformation could be conducted in a simple laboratory without requiring much equipment. With such high efficiency at the early stage of transformation, the procedure could replace particle bombardment at a much lower cost for measuring transient gene expression.

The most attracting aspect of this improved procedure is the high efficiency for stable transformation. 100% of co-cultivated class I type of callus resulted in multiple transgenic hygromycin resistant calli. Furthermore, the class I type of transgenic callus was found to be regenerated into green plant at very high percentage compared to class II or class III type of callus. It was discovered that transformation of cells in class I type of callus can be detected

early during the selection process. As the selection culture time extended, class I type of callus was gradually transformed to Class II type or Class III type of callus. Frequent subculture proved to be helpful to maintain the class I type of callus.

Additive gene expression of gfp driven by pUbi1F

Additive transgene expression was previously reported mainly based on the studies between homozygous transgenic plant and hemizygous plants that was derived from same transgenic plant (Halfhill et al. 2003)(Niwa et al., 1999; Stewart, 2001; James et al., 2002). In our transgenic plants, gfp expression was presented in the pollen at different level of green fluorescence(Figure 2.4). Additive gene expression of gfp in pollen was confirmed by analysis of the relative green fluorescence in pollen along with its Southern blot of T_0 transgenic plant and its progeny(Figure 2.7). The relative green fluorescence of pollen in single locus T-DNA plants also shows additive gene expression according to the T-DNA copy number(Figure 2.8). It is interesting that the pollen gfp expression for one copy at single locus varied from each other among a few of independent one copy T-DNA plants(Figure 2.8 Panel A,B,C). The possible interpretation for this is the different insertion position on chromosome that affect the gene expression.

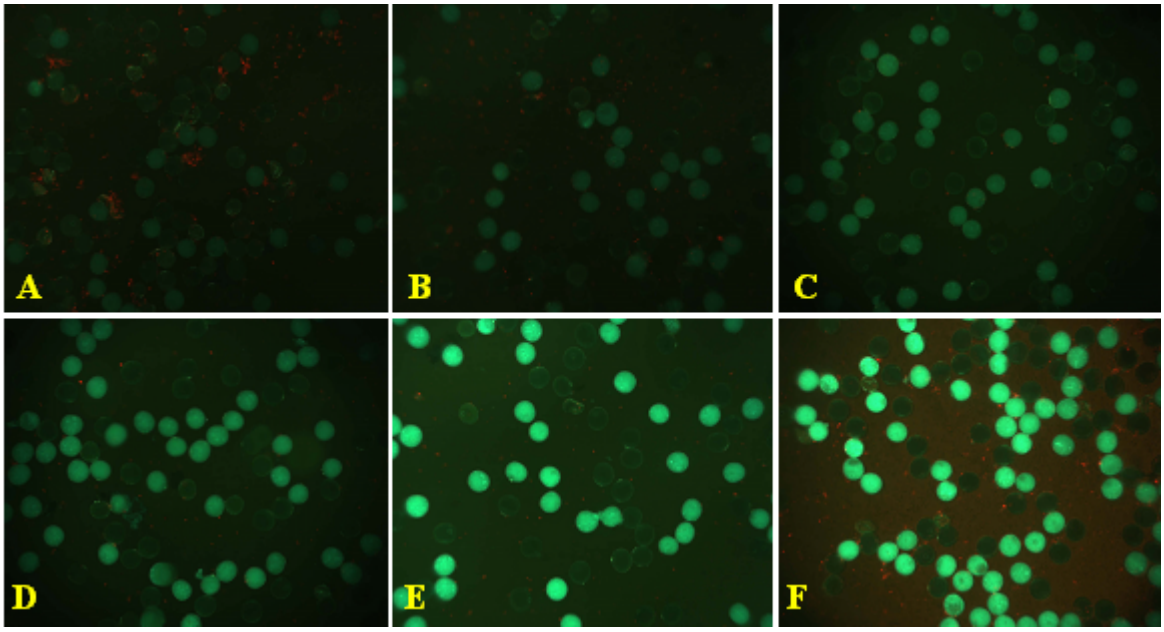

Figure 2.8 GFP expression in pollen of transgenic plants with one chromosome insertion (1 locus). Panel A, B, C, are pollen images from transgenic plants with one copy of T-DNA; Panel D shows pollen from plant with two copies; Panel E shows pollen from plant with three copies; Panel F shows pollen from plant with four copies.

Rapid characterization of transgenic plant via pollen gfp expression

The additive gene expression in pollen could be used to rapidly to characterize the transgenic plants. There are many pollens in a single T_0 transgenic plant creating a large segregating population for genetical analysis on the T-DNA integration pattern without extra cost. Only one or two florets from plants at the flowering stage could provide enough pollen for the characterization of original transgenic plants.

In previous studies, the average number of integration loci was difficult to estimate in multiple-copy plants due to gene silencing (Hiei et al., 1994; Sallaud et al., 2004). T_1 segregation analysis for hpt was limited when both members of a pair of homologous chromosomes bear at least one copy of T-DNA insertion(Figure 2.4D). In this case, there would be no segregation for hpt. With the help of statistical analysis on the pollen data, we could estimate the T-DNA insertion locus number and the copy number on a single locus without much extra cost of the study.

CHAPTER III

HIGHLY EFFICIENT PRODUCTION OF HAPLOID AND DOUBLED HAPLOID TRANSGENIC RICE VIA *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION

INTRODUCTION

Initial success with rice transformation was attained using naked DNA introduced into rice cells by physical means, such as electroporation of protoplasts (Fromm et al., 1986) and particle bombardment of callus tissues (Cao et al., 1992). However, it became evident from studies in our lab and others that the transgenes inserted by these approaches were typically rearranged and frequently silenced (Kumpatla and Hall, 1998b; Svitashev et al., 2002). In contrast, Agrobacterium-mediated transformation generally resulted in insertion of intact transgenes and relatively infrequent silencing (Dong et al., 1996; Hiei et al., 1997). However, whereas homozygous lines are highly desirable for both practical and basic studies, all of these procedures yield hemizygous plants.

The production of haploid rice through anther/microspore culture is widely used in rice breeding since doubling of the chromosome compliment results in a homozygous plant (Afza et al., 2000). Therefore, it would seem the transformation of haploid cells, such as microspores, should provide an attractive route for production of homozygous plants and it is surprising that very few articles exist describing the use of this approach. Indeed, we have located only one paper (Brisibe et al., 2000) describing transformation of a monocot (wheat) via anther culture

bombardment. There are several articles describing the production of homozygous rice plants by anther culture, but these are quite different as they deal with plants that are already transformed (Baisakh et al., 2001; Zeng et al., 2002). While an article describing the adaptation of rice anther culture on gene transformation was found during the preparation of this manuscript, the highest transformation efficiency was only 11.27% for hygromycin resistant calli (Chen, 2006).

Successful Agrobacterium-mediated transformation of microspore-derived embryos has been described for the dicot genera Datura and Nicotiana (Sangwan et al., 1993); a patent application exists for transformation of flax by this approach (Chen and Dribnenki, 2003). However, for all three examples, transformation efficiency and production of homozygous transgenic plants were very low.

In this study, a highly effective transformation procedure of microspore-derived callus has been established. Hundreds even thousands of transgenic haploid and doubled haploid plants could be generated in a single transformation experiment using high quality microspore-derived callus. In this protocol, the high quality microspore-derived callus is readily selected under a dissecting microscope. The modified protocol for selection and regeneration is easy to handle for a high efficient transformation. The homozygous doubled haploid plants could be simply to identified by the examination of pollen from the T_0 plants without growing and examining the next generation. Many phenotypic mutations even lethal or semi-lethal transgenic haploid and doubled haploid have been detected.

MATERIALS AND METHODS

Plasmid and agrobacterium strains

The binary plasmids pUbi1F (Figure 1), pJD4 and pJD7 in LBA4404 were used in this study. It was constructed as described in Chapter II.. *Agrobacterium* strains LBA4404(pUbi1F) was grown on AB minimal medium with 50 mg/L of gentamycin and 100 mg/L of kanamycin for 2 to 3 days at 28 ^{O}C. The bacteria were collected and resuspended in AAM medium (Hiei et al., 1994)for co-cultivation with microspore-derived callus.

Plant materials and tissue culture media

Rice (*Oryza sativa* ssp Japonica, cv Nipponbare and Taipei309) plants were grown in Redi–earth® in 5" pots in a greenhouse at 25°C-32°C under natural light from March to October; supplemental light was provided from November to February to give 16 h day and 8 h night. Water was supplied by an automatic system and the plants perpetually produced tillers for anther culture at any season in a year. The media used for tissue culture and transformation in this study was listed in Table 2.1. The medium used for anther culture was supplemented with 20g/L Sorbital in the N6M.

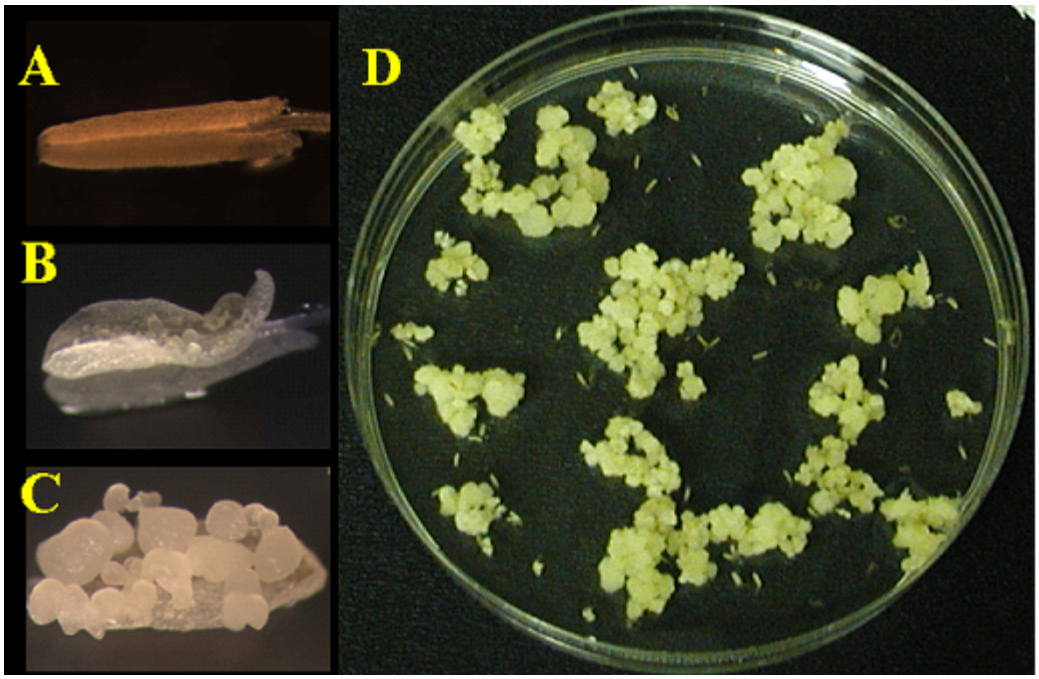

Figure 3.1 Microspore-derived callus. A. Anther on modified N6 medium. B. After two weeks on N6M-A medium, globular calli, derived from individual microspore, are visible using a dissecting microscope. C. Individual microspores from a single anther give rise to many active calli after another two weeks. D. After 6 weeks of callus induction, about 50% of cultured anthers produced multiple calli ready for transformation.

Induction of microspore-derived callus

In order to maximize collection of microspores at the mid-uninucleate to early binucleate stages of development, disease-free panicles (boots) were collected from tillers of ratooned plants when the distance of the flag leaf auricle to that of the next leaf was 2-7 cm. Collections were made between 8 and 10 a.m. or between 4 and 5 p.m. as microspore mitosis is maximal at those times. The panicles were wrapped with foil, accurately labeled with the date and time of collection and placed in an incubator at 4-8°C. After 7 days the panicles were surface-sterilized by surface-spraying with 70% ethanol. Boots were removed carefully with sterile forceps and florets with anthers that did not exceed half of the floret length were selected. The anthers were excised with sterile forceps and callus was induced on a modified N6 medium (Chu et al., 1975) at 26°C in the dark. After ~6 weeks (wk), haploid callus had developed and was ready for transformation (Figure 3.1).

Transformation procedure

Microspore-derived callus was subcultured on 2N6 medium (Hiei et al., 1994; Dong et al., 1996) at 28°C in dark for 3 days. Actively growing Class I callus with turgid cells on the surface (Figure 3.2) was chosen for co-cultivation. About 50 pieces of callus, each with a diameter of approx. 5 mm were immersed in 10 ml of liquid co- cultivation medium (AAM) containing *Agrobacterium* cells at a density of 2-3x 10^8 cells/ml (OD^{595} = 0.5) in a 50 ml sterile

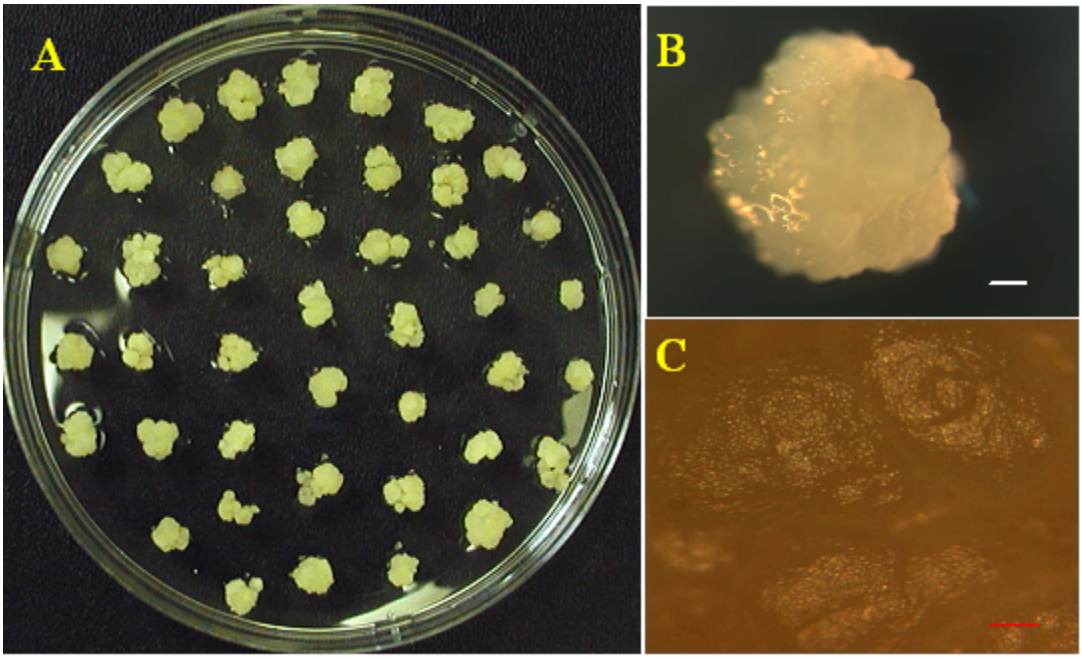

Figure 3.2 Class I type of microspore-derived callus for transformation. A. Class I type of callus at diameter ~5mm was chosen and subcultured on 2N6M medium. B and C was the image under dissecting microscope, many active turgid cells on the callus surface that could be transformed during the co-cultivation. White bar: 1mm; Red bar: 0.1mm.

centrifuge tube for 30 min. Callus pieces were blotted dry on sterile paper towels, transferred to solid co-cultivation medium (2N6-AS) and incubated for 3 days at 21°C in the dark. After co-cultivation, the callus pieces were gently and briefly rinsed twice with 250 mg/l cefotaxime in liquid 2N6 medium. Rinsed calli (~20 pieces, approx. dia. 5 mm) were individually transferred to one 100 mm-diameter Petri dish containing selection medium (250 mg/l cefotaxime; 50 mg/l hygromycin B) (2N6M-CH) for two weeks at 28°C in the dark. After two weeks selection, resistant, light brown calli were evident on the surface of dead and dying (dark brown) calli. The light brown calli (with some dark brown callus attached) were transferred to fresh selection medium (2N6M-CH) for 1 wk at 28°C in the dark. The surviving light brownish globular calli were gently removed from the dead calli and placed in the medium surrounding the original callus and cultured for another 5-7 days at 28°C in the dark. Four weeks after the co-cultivation, the surviving calli were ready for regeneration.

The transformation efficiency and development of hpt-resistant callus was followed throughout the selection step by fluorescence microscopy. Round, yellowish Class I calli with turgid cells on the surface (see Figure 3.2C) were chosen for culture on pre-regeneration medium (PRN) (Sallaud et al., 2004) for 1 wk at 26°C in the dark. All of the surviving calli from a single co-cultivated piece of callus were grouped into individual sectors of the PRN Petri dish so that they would not co-mingle with calli surviving from other co-cultivated calli. After 1 wk of culture on PRN medium, all remaining calli from the same original co-cultivated callus piece were individually transferred to a single 100 mm dia. Petri dish containing RN. The dish was kept in the dark at 26°C for one day, then at 26°C for 2 wk with a 16/8-h light/dark cycle. Many green shoots, with or without small roots , regenerated from the light brown surviving calli. All

calli with green shoots derived from the original co-cultivated callus were individually transferred to a 100 mm-diameter/25 mm-height Petri dish containing medium (RN-2) for the second round of regeneration for 2 wk. The plantlets were then individually transferred to rooting medium (MS_0) for one wk. The resulting plantlets were cataloged and transferred to soil in the greenhouse.

Detection of gfp reporter genes

The expression of the gfp gene in transformed callus and plant tissues was observed using a Stemi SV11 APO Microscope (Zeiss) fitted with an AxioCam HRc camera. Calli that expressed GFP were imaged using a 500 nm filter and an exposure time of 3 sec. Pollen that expressed GFP were imaged at an adjusted exposure time so that none of pollen images was saturated in pixels. Seeds that expressed GFP were imaged at an exposure time of ~1 sec.

T1 segregation analysis for hygromycin resistance and GFP activity of seeds

Fifty to one hundred were dehusked and observed for GFP activity under a Stemi SV11 APO Microscope (Zeiss) fitted with an AxioCam HRc camera. The seeds were then surface-sterilized, placed on a petri dish containing 30 mg/ml hygromycin and allowed togerminate under light at 26C. After 7-10days, seeds were scored for germination. Segregation for GFP activity and hygromycin resistance was calculated by a statistical method using $\chi 2$-tests.

T1 segregation analysis for GFP activity in pollen from T_0 plants

Five random chosen field of view of pollen fromT_0plants were observed using a Stemi SV11 APO Microscope (Zeiss) fitted with an AxioCam HRc camera. The image were taken using an exposure time that does not saturate the exposure. Segregation for pollen GFP activitywas calculated by a statistical method using χ2-tests.

Nucleic acid extraction and Southern blot analysis

Rice genomic DNA was isolated from fresh leaves as described previously (Buchholz et al., 1998). Genomic DNA (2 µg) was digested with 20 units of *Hind*III for 12 h. After electrophoretic separation in a 0.8% agarose gel, the DNA fragments were transferred to Hybond-N+ membrane (Amersham). DNA probes were labeled using a DECAprime II™ kit (Ambion). Hybridizations were performed using ULTRAhyb™ (Ambion) according to the manufacturer's recommendations.

Ploidy determination by chromosome counting

The protocol used for chromosome counting was modified from the protocol described by Dong et al. (2001). The root tips from haploid tiller and doubled haploid seedling were treated with 0.035% hydroxyquinoline for 2 hours and fixed in 4:1 100% ethanol: acetic acid solution. Fixed root tips were then digested with 5% w/v cellulase Onozuka R-10 (Yakult

Honsha, Japan) and 1% w/v pectolyase Y-23 (Seishin Pharmaceutical, Japan) in 0.05M citrate buffer pH 4.5 for 52 min at 37 °C. Chromosomes were stained with *SlowFade*® Gold antifade reagent containing DAPI (Invitrogen) and their karyogram were revealed using Olympus FV1000 confocal microscope .

Amplification and sequencing of T-DNA left border flanking regions

The amplification of T-DNA left border flanking region was carried out using the Adaptor ligated PCR described in Siebert et al. (Siebert et al., 1995). The protocol consists of three steps: ligation, polymerase chain reaction (PCR)1 and PCR2. Each DNA sample (1.5µg) was digested with *EcoR*V and precipitated using a phenol/chloroform/isoamyl alcohol protocol. The purified digested genomic DNA was ligated in 10µl volume with the ADP1/ADP2 adaptor using T4 DNA ligase at 16 °C overnight. The adaptor was prepared by annealing the complementary oligonucleotides, ADP1 (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3') and ADP2 (5'-P-ACCTGCCC-NH2-3') by incubation at 97 °C for 5 min in NEB restriction enzyme buffer no 2 followed by gradual cooling to room temperature (~24 °C). PCR1 was conducted with a specific adaptor primer, AP1 (5'-GGATCCTAATACGACTCACTATAGGGC-3'), AP2 (5'-AATAGGGCTCGAGCGGC-3'), and a specific T-DNA left border primer,

LB1: 5'-GAATTAGTCGAGACACGTCGAAATAAAGATTTCCG-3').

LB2: 5'-CGACGGATCGTAATTTGTCGTTTTATCAAAATGTAC-3'

LB3: 5'-CCATATTGACCATCATACTCATTGCTGATCCATG-3'

If the previous PCR failed, the a specific T-DNA right border primer was used,

RB1: 5'-GCTGAAAGCGACGTTGGATGTTAACATCTACAAATTG-3'

RB2: 5'-CGTAAGCGCTTACGTTTTTGGTGGACCCTTGAGG-3'

RB3: 5'-GATGGGGGGCATCGCACCGGTGAGTAATATTGTAC-3'

The thermocycling conditions were 5 min at 94 °C, followed by 29 cycles of 94 °C 30 s, 62 °C for 45s, and 72 °C for 2 min, with a final polymerization step at 72 °C for 10 min. PCR2 was performed with a nested specific adaptor primer, AP2 (5'-CTATAGGGCTCGAGCGGC-3'), and a nested specific T-DNA left border primer, LB2 or RB2. PCR2 was performed with a 1/50 dilution of the PCR1 product using the same conditions as for PCR1, except for the final volume of the reaction which was adjusted to 100 ul. A 4-ul aliquot of the PCR2 reaction was loaded on a 1.2% agarose gel for electrophoresis. After gel staining with ethidium bromide, PCR products showing a unique band were then directly sequenced with a third nested specific primer, LB3 or RB3.

RESULTS

Induction of microspore-derived callus of rice

Microspore-derived callus was induced using the method described as in Materials and Methods. Globular callus derived from individual microspores in the anther could be observed under a dissecting microscope after two weeks culture on N6M-A medium (Figure 3.1). Many independent microspore-derived calli could be observed without use of a dissecting microscope

after 4 weeks culture. Approximately 50% of anthers cultured from Taipei309 and Nipponbare could produce microspore-derived callus (Figure 3.1). After 6 weeks of anther culture, microspore-derived callus was ready for transformation. The modified medium used was based on N6 medium (Chu et al. 1975) supplemented with 500mg/l proline, 500mg/l glutamine, 300mg/l casein acid hydrolysate, and 30g/l sucrose and 20g/l D-sorbital.

Transformation of class I type of microspore-derived callus at exceptional efficiency

The transformation protocol was essentially described in previous reports (Hiei et al., 1994; Dong et al., 1996) . Class I type of microspore-derived callus (Figure 3.2) was pre-cultured on 2N6M medium at 28 °C in the dark for 3 days, followed by co-cultivation on

2N6M-AS medium at 21 °C in the dark for 3 days, first selection on 2N6M-CH medium at 28 °C in the dark for 14 days, and second selection on 2N6M-CH medium at 28 °C in the dark for 14 days. Four weeks after the co-cultivation, the surviving calli were generally ready for regeneration. A highly efficient transformation procedure was established using class I type of callus. All three gene constructs, pJD4, pJD7 and pUbi1F, resulted in almost 100% transformation rate for co-cultivated class I type callus. Typically each co-cultivated callus at approx. dia. 5mm could produce dozens of transgenic calli and multiple transgenic plants (Figure 3.3).

Time course for the transformation of class I type of microspore-derived callus is similar to that of embryo-derived callus. ~8 weeks was needed from the time callus was inoculated with Agrobacterium to the recovery of regenerated green plants from class I type of microspore-derived callus.

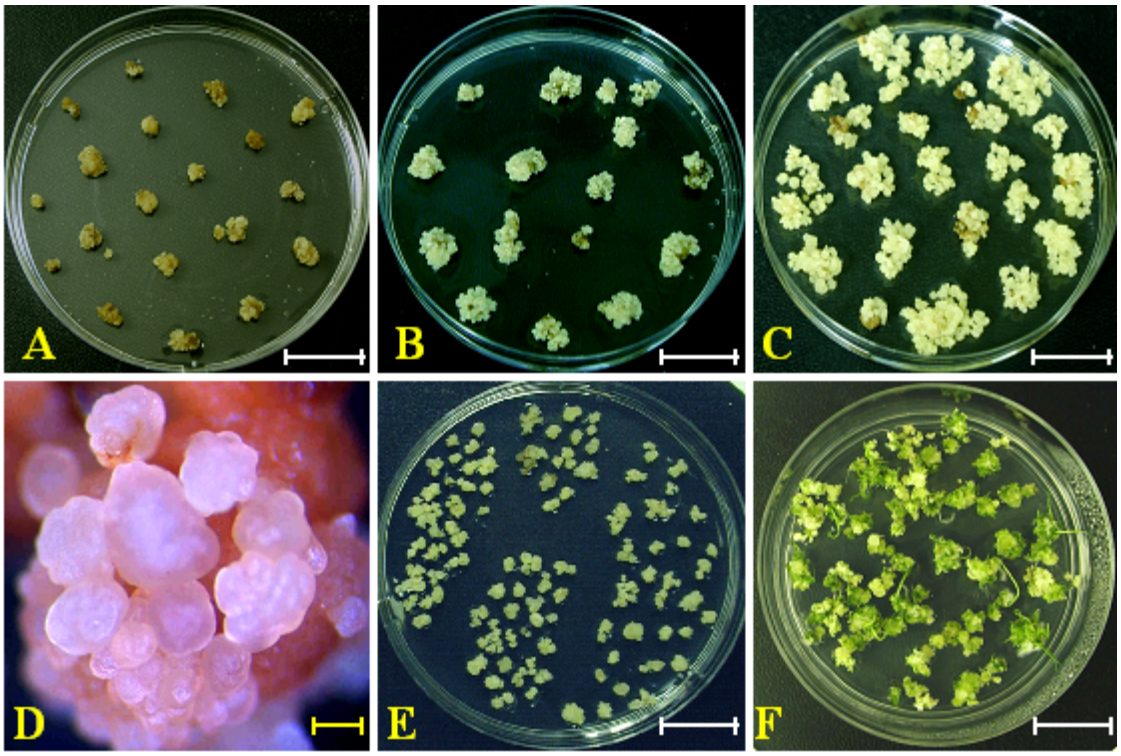

Figure 3.3 Exceptionally efficient Transformation of class I type of microspore-derived callus. A. After 3 days of co-cultivation, the co-cultivated callus was placed on first selection medium. B. After two weeks of selection, the surviving transgenic cell lines could be observed on every co-cultivated callus surface without help of a microscope. C. After two weeks of second selection, dozens of surviving transgenic cell lines could be obtained on each co-cultivated callus. D. Dozens of class I type of transgenic calli could be observed under a dissecting microscope, Panel D indicates the active class I callus in high quality for regeneration. E. Surviving calli from each co-cultivated callus were harvested and subcultured on the pre-regeneration medium. All of the surviving calli from a single co-cultivated piece of callus were grouped into individual sectors. F. Calli derived from the same co-cultivated callus were placed on one petri-dish containing regeneration medium; most of those class I type of transgenic calli could be regenerated into green plants.

Evaluation of transformation via GFP

The transformation efficiency and development of *hpt*-resistant callus was followed throughout the selection by fluorescence microscopy (Figure 3.4). Figure 3.4 illustrates the efficiency of the protocol that I have developed for *Agrobacterium*-mediated transformation of microspore-derived callus. Moreover, the use of pUbi1F (Figure 2.1) permits an early evaluation of how successful the transformation event was. Indeed, several hundreds of GFP-positive cells can normally be detected in any small region of virtually all calli by 3 days after co-cultivation (Figure 3.4A). In most cases of the experiments, GFP cells could be detected under fluorescence microscope only two days after co-cultivation with Agrobacterium on 2N6M-AS medium. More GFP positive cells could be observed 5 days after co-cultivation (Figure 3.4B). Even though the number of cells with GFP decreased at 7 days after the first selection (2N6M-CH), overall, hundreds of cell lines with GFP at different intensity of fluorescence level were observed on small portions of a piece of co-cultivated callus (Figure 3.4C). Hundreds of globular calli expressing GFP were visible at different position all over the surface of the co-cultivated callus after surviving on the selection medium for 2 weeks (Figure 3.4D). Most of those GFP cell lines survived another weeks of selection (second selection). The GFP cell lines could be observed throughout the selection course (Figure 3.4E and F).

As illustrated in Figure 3.3, dozens of green transgenic plants could be regenerated

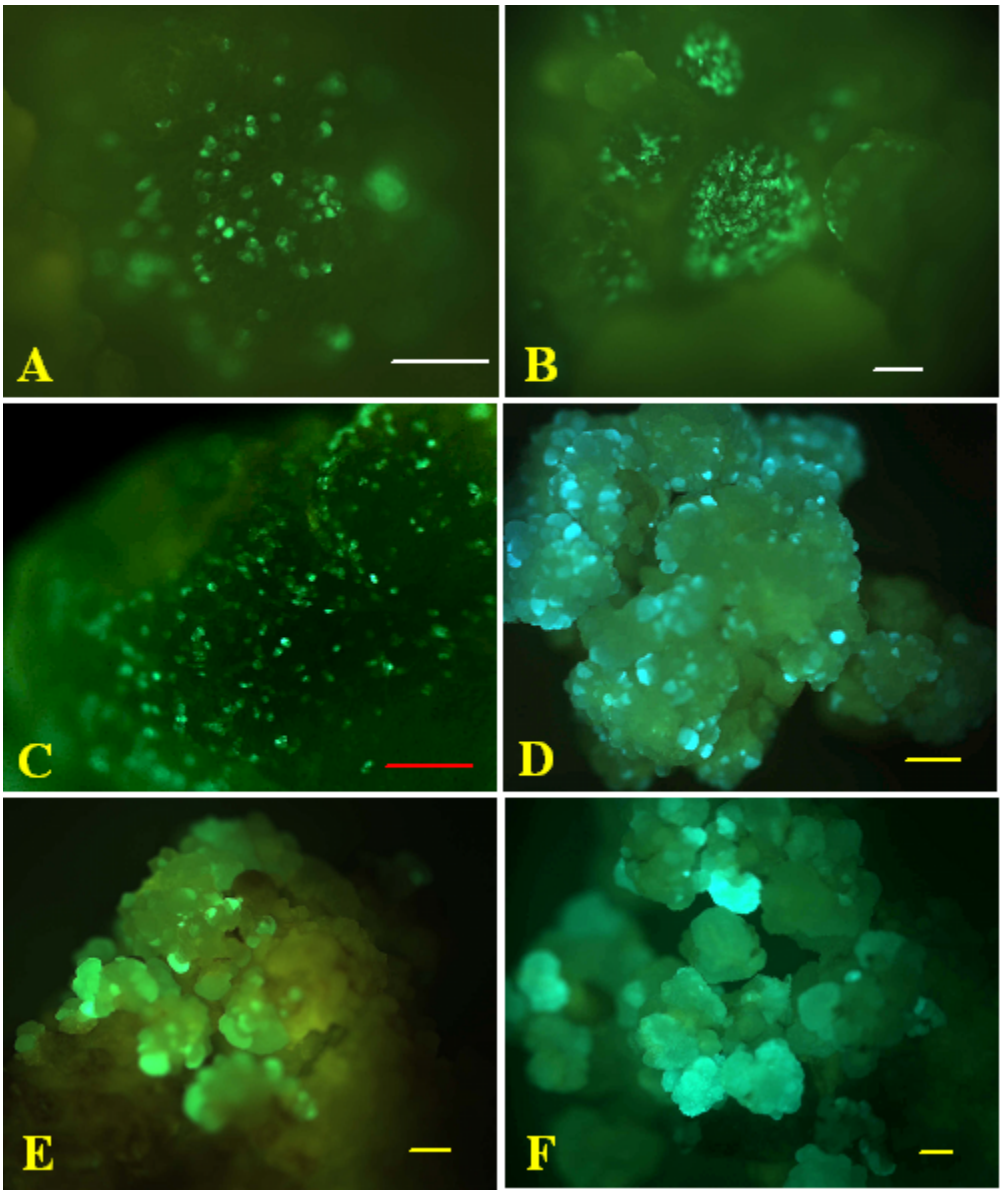

Figure 3.4 Highly efficient transformation of class I type of microspore-derived callus of rice revealed by GFP. A. 3 days after co-cultured; B. 5 days after co-cultured; C. 7 days after selection; D. 2 weeks after selection; E. 3 weeks after selection; F. 4 weeks after selection. White bar: 0.1mm; Red bar: 0.5mm; Yellow bar: 1mm.

Table 3.1. Transformation efficiency for microspore-derived callus (cv. Nipponbare)

Experiment	Number of class I calli used for transformation	Number of independent transgenic plants	Transgenic plants/ co-cultivated callus	Transformation rate (%)
1	20	913	45.65	4,565
2	46	1,862	40.48	4,048
3	50	1,173	23.46	2,346

from a single piece of co-cultivated type I callus. The production of high numbers of transgenic plants from a single co-cultivation is exemplified by the data shown in Table 3.1 for three typical experiments. Thousand of transgenic plants could be obtained from a single transformation experiment on dozens of class I type of microspore-derived callus. The calculation on the transformation rate (%) based on the number of transgenic plant for each co-cultivated callus reached well above 100% (Table 3.1). We prefer to use the number of transgenic plants per co-cultivated callus to indicate the transformation efficiency for each transformation experiment.

The regenerated plants from each co-cultivated callus were cataloged and transferred to soil in the greenhouse. Genomic DNA was isolated from a single leaf of each transgenic plant. Southern blot analysis was conducted as described previously. That the plants are overwhelmingly independent transformants is clear from the Southern blot analyses shown in Figure 3.5. To date, we have analyzed over 200 individual plants and have only encountered 4 sibling plants.

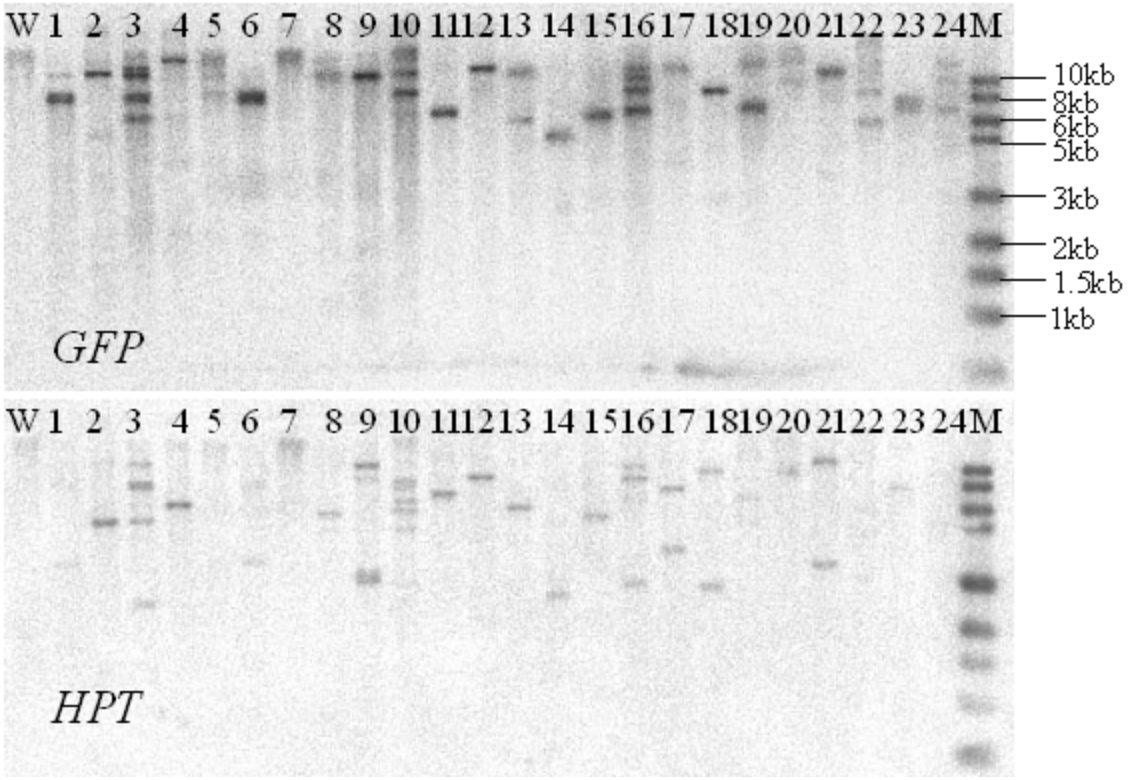

Figure 3.5 Genomic blot analysis of T_0 lines of transgenic plants from microspore-derived callus. Genomic DNA from lines 1 to 24 (all from one piece of co-cultivated callus) was digested with *Hind*III, and hybridization was performed with the *hpt* or *gfp* probe. The sizes of the bands visualized for each plant with hpt or gfp probe were different, indicating that T-DNA integrated at different locations in the rice genome and that all 24 transgenic plants from a single co-cultivated callus were independent transformation events.

Identification of homozygous plants in T_0 transgenic lines

Anthers were isolated from florets of T_0 plants and crushed gently on a slide in one drop of ddH2O. The pollen was immediately examined using a Zeiss SV-11 fluorescence microscope and imaged using an AxioCam HR camera (Carl Zeiss, Jena, Germany). The exposure time was adjusted so that the pixels in the spot images were not saturated. All pollen grains from homozygous doubled haploid (DH) plants had a uniform green fluorescence, revealing the absence of wt pollen grains that lack GFP (Figure 3.6A). The accuracy of the visual test was confirmed by genomic blot analysis of progeny (Figure 3.6B). Hemizygous DH transgenic plants show (wt) pollen and GFP pollen segregation (Figure 3.6B; wt pollen is present as dense black grains) that can be used to determine the number of chromsomes bearing a T-DNA insertion as described in Chapter II. From our preliminary experiment results, about 60% of the hemizygous transgenic DH plants have one T-DNA insertion locus (Table 3.2).

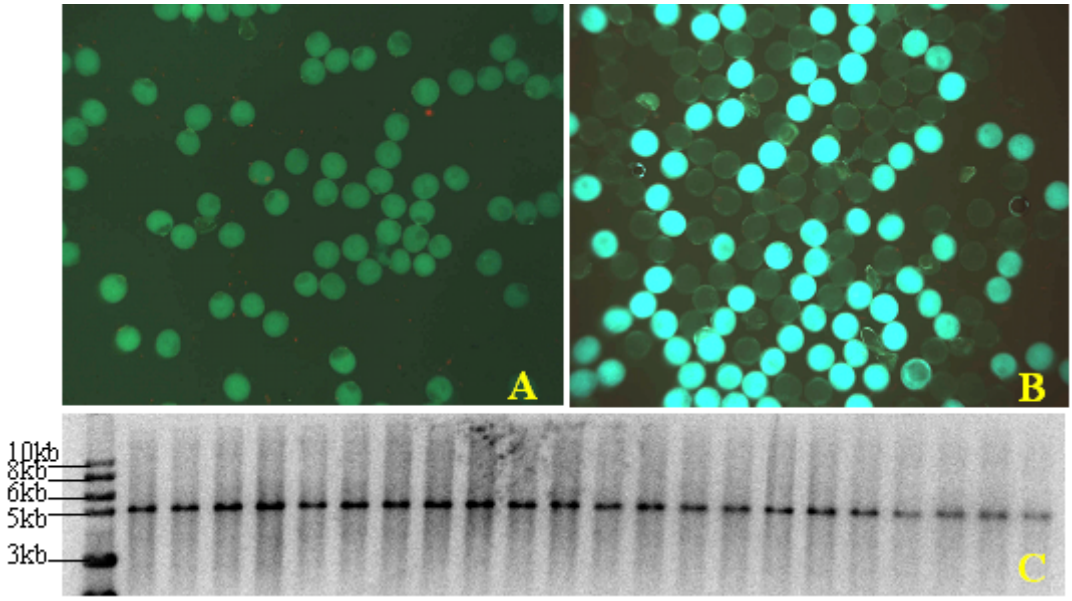

Figure 3.6 Homozygous T-DNA doubled haploid plant revealed by GFP pollen. A. All pollen grains from homozygous doubled Haploid (DH) plants had a uniform green fluorescence; B. Half of the pollen from a hemizygous DH transgenic plant had green fluorescence, indicating a single locus T-DNA insertion after chromosome doubling; C. A homozygous T-DNA DH plant as confirmed by Southern blot. All progeny from one regenerated plant had the same pattern of T-DNA insertion.

Table 3.2. Independent locus of chromosomes bearing T-DNA insertion revealed by pollen segregation

	1 locus insertion	2 loci insertion	3 loci insertion	4 loci insertion	BHC* insertion	Total
No. of plants	242	96	44	14	3	399
%	60.7	24.1	11.0	3.5	0.7	100

*BHC: Both members of a pair of homologous chromosome have one T-DNA insertion

Distribution of haploid and doubled haploid transgenic plant in transformation of microspore-derived callus

Although haploid plants are not readily distinguishable at the time they are transplanted to soil from the Magenta box, visual differences are usually evident after culture in soil for 1 month. Haploid plants are sterile, have reduced floret size and are typically substantially smaller in stature than are wt diploid plants (Figure 3.7A and B). The accuracy of the visual test of haploid and DH plants were confirmed by chromosome counting (Figure 3.8), as described in Materials and Methods. Without colchicine treatment, about 45% of the transgenic plants were haploid (Table 3.3, Control). About 46% of the transgenic plants were hemizygous T-DNA plants, indicating that nearly half of the haploid cells had already spontaneously doubled the chromosomes before the time of transformation.

In order to increase the number of homozygous transgenic DH plants in the T_0 transformants, several experiments were conducted on the colchicine treatment protocol during selection (Table 3.3). 0.03% colchicine (Sigma) (w/v) was added in selection medium (2N6-CH). The experiments were conducted by keeping the co-cultivated calli on 2N6-CH with colchicine supplement for the first selection only, for both selections (first and second) and a control (2N6-CH with no colchicine supplement). Colchicine treatment following the co-cultivation step significantly increased the frequency of recovery of transgenic homozygous plants and reduced the haploid plants for both experiments. Up to 26% of the plants recovered were homozygous transgenic doubled haploids (see Table 3.3).

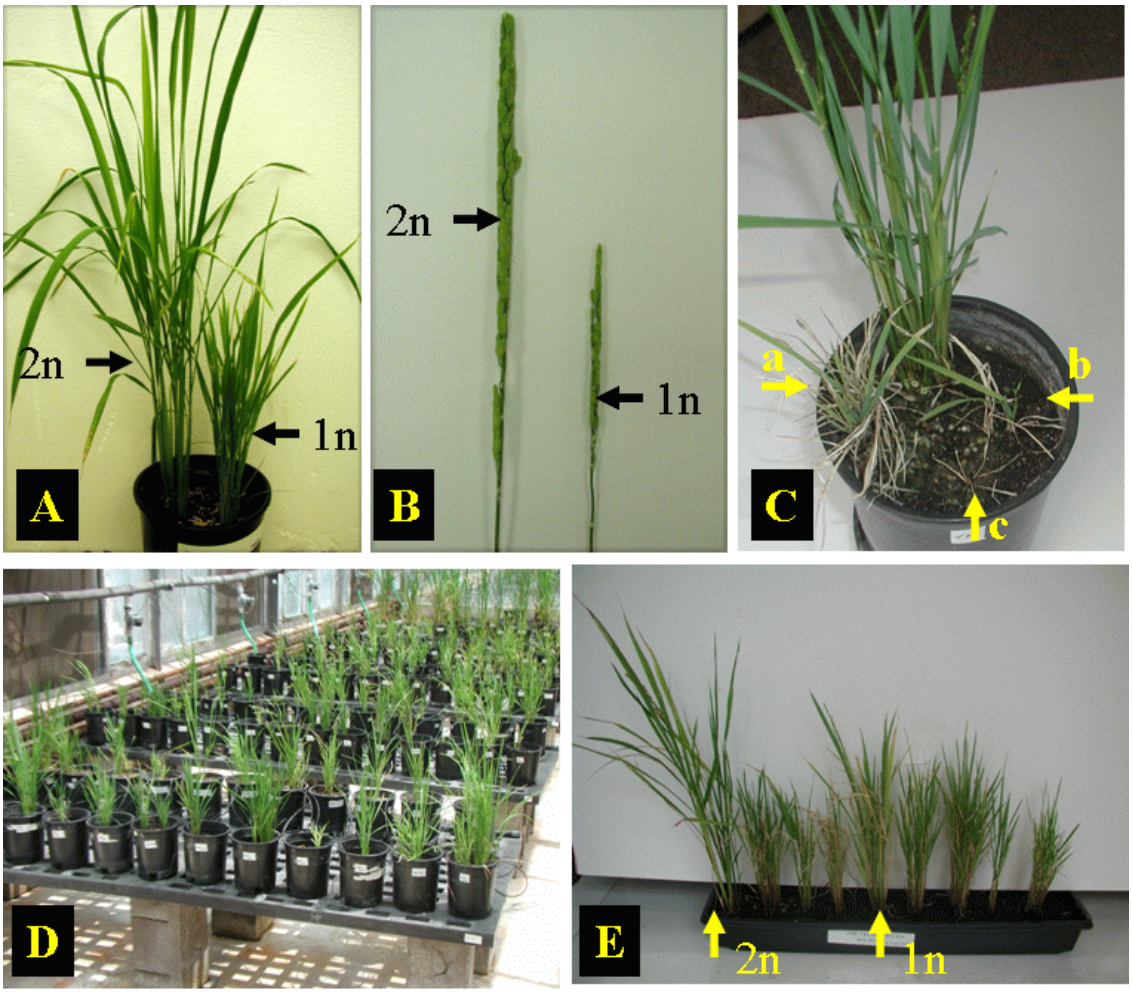

Figure 3.7. Transgenic haploid plants in greenhouse. A. The stature of transgenic haploid (1n) plants is usually shorter than that of a diploid (2n) wt plant. B. Compared with the wt diploid (2n) panicle, the haploid (1n) panicle is shorter and floret size is greatly reduced. C. Haploid plants with abnormal (a), semi-lethal (b) and lethal (c) phenotypes. The tall plant is a "wt" haploid control. D. Transgenic haploid plants from a single co-cultivation were labeled and planted in greenhouse. E. Transgenic haploid plants growing in the same tray depict various phenotypic aberrancies (arrows indicate wild type).

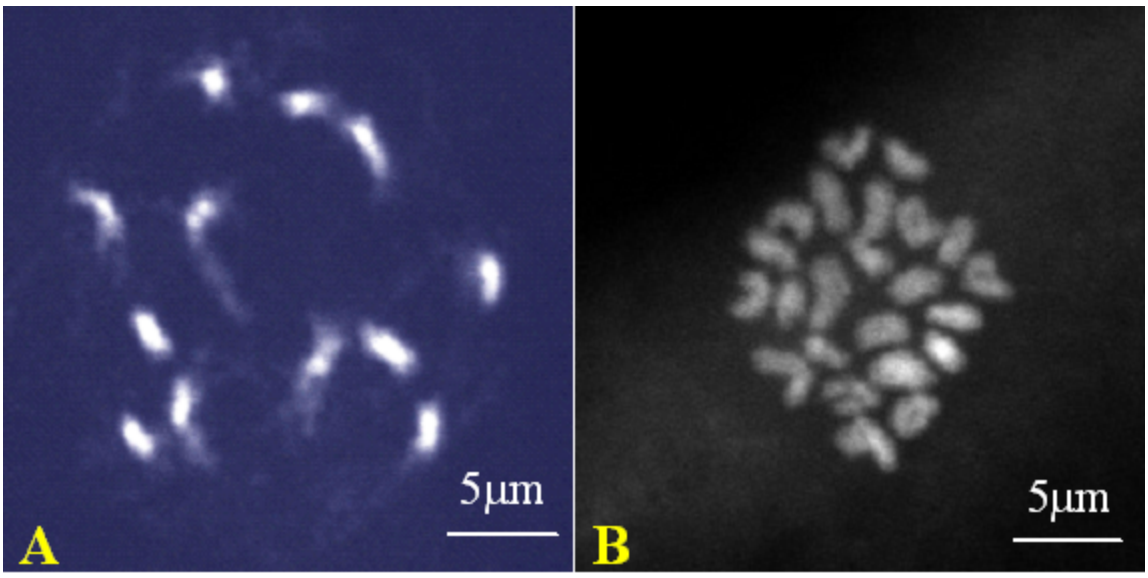

Figure 3.8. Chromosome examination of haploid and doubled haploid transgenic plants. A. Representative karyogram from a transgenic haploid plant containing 12 chromosomes (1N = 12); B. Representative karyogram from a transgenic doubled haploid plant containing 24 chromosomes (2N=24).

Table 3.3. Transgenic plants from transformation of microspore-derived callus

Treatment	Haploid plants (survived)		Doubled haploid (hemizygous)		Doubled haploid (homozygous)		Semi-lethal or lethal plants		Total	
	Number	%	Number	%	Number	%	Number	%	No.	%
Control	416	45.56	425	46.55	33	3.62	39	4.27	913	100
I	185	35.1	258	48.8	67	12.7	18	3.4	528	100
II	74	22.8	158	48.6	85	26.2	8	2.4	325	100

I. Colchicine treatment for 1st selection period (14 d). II. Colchicine treatment over 2 selection regimes (28 d).

Phenotypical variation of haploid and DH transgenic plants

At flowering , visible phenotypic differences between transgenic haploid plants and wt haploid plants often become apparent. Transgenic haploid plants with visible phenotypes are readily identified from the original transgenic haploid plants by comparing them with non-transgenic haploid plants that were regenerated and planted in greenhouse as controls.

Three months after transfer to the greenhouse, about 25% of the T_0 haploid plants were visibly different from haploid plants recovered from calli that had not been subjected to co-cultivation ("wt" haploid plants). About 6% of the T_0 haploid plants exhibited seriously abnormal phenotypes that were lethal or semi-lethal (see Table 3.4 and Figure 3.7C).

Table 3.4. Distribution of transgenic haploid plants with visible phenotypes

Experiment	Haploid plant like wild type		Haploid plant with visible phenotype		Haploid plant semi-lethal or lethal		Haploid plant (total)	
	Number	%	Number	%	Number	%	Number	%
1	332	72.9	84	18.5	39	8.6	455	100
2	507	69.6	187	25.7	34	4.7	728	100

Identification of rice phenotypic mutants

The homozygous DH transgenic plants were planted in the greenhouse (4 plants per line using 2 wild type Nipponbare or Taipei 309 plants as a control). All plants were planted under the same cultivation and environmental conditions (two mutant plants and one wild type plant

in one pot). The growth-base phenotypic profile was collected and rice phenotypic mutants were identified by comparing to the phenotypic profile of wild type plants. From the preliminary experimental results, 57 rice phenotypic mutants were identified. Homozygous transgenic insertion was confirmed in these plants by examination of pollen GFP and genomic Southern blot analysis.

Diploidization of haploid transgenic plants by tissue culture

A large number of transgenic haploid plants have been obtained from a single co-cultivation experiment using microspore-derived callus. However, for use in comprehensive studies of novel gene function, it is highly desirable to have a seed-bearing fertile plant. For this, the chromosomes in a haploid have to be doubled. There are several published approaches for diploidization of a haploid plant (Maluszynski et al., 2003). One of these is the use of colchicine treatment on haploid tillers; another is the manipulation of haploid tissue culture.

In an exploratory experiment, haploid plants were removed from soil and washed with water. The roots of the plants were cut 3-5cm from the plant base and the upper portion trimmed to 20-30 cm before immersion in a 0.1% colchicine solution dissolved in 2% dimethysulfoxide (DMSO) containing 0.01% Tween 20 for 5 h at room temperature. The efficiency of conversion was only 30%. However, since no DH plants were recovered from the non-treated controls, the experiment was very encouraging as it established the feasability of this approach.

The inflorescence (about 1-2cm in length), leaf and stem tissues were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2mg/l 2,4-D and 1mg/l kinetin (plus

30mg/l hygromycin B for selection) for 3 weeks at 26 C in the dark. The active callus that was induced from the tissue was transferred to regeneration medium (MSD4) (Dong et al. 2001)at 26 °C in the dark for 3 weeks. 75% of young inflorescences from transgenic haploid could produce active callus. Fertile doubled haploid plants have been regenerated for all tested haploid sterile plants (see Table 3.5). This approach is especially useful to obtain doubled haploid plants from the transgenic plants with serious abnormal phenotype that are not strong enough to go through the colchicine treatment.

Table 3.5. Doubled haploid plants induced by tissue culture

Explant	Callus induction on tissue			Plant regeneration		
	No. of tissues on culture	No. of tissues with calli (%)		No.of sterile haploid plants tested	No.sterile haploid giving fertile plants	(%)
Inflorescence	64	48	(75)	12	12	(100)
Stem or shoot	30	11	(36.7)	10	3	(30.0)
Leaf	28	0	(0.0)	12	0	(0.0)

Determination of T-DNA chromosome insertion location

The amplification of T-DNA left border flanking region was performed using the Adaptor-ligated PCR described in Siebert et al. (1995). The three step PCR was proved to be effective to recover the left border flanking sequence from the tested mutant plants. A typical agarose gel showing the PCR2 product corresponding to the flanking sequence amplification

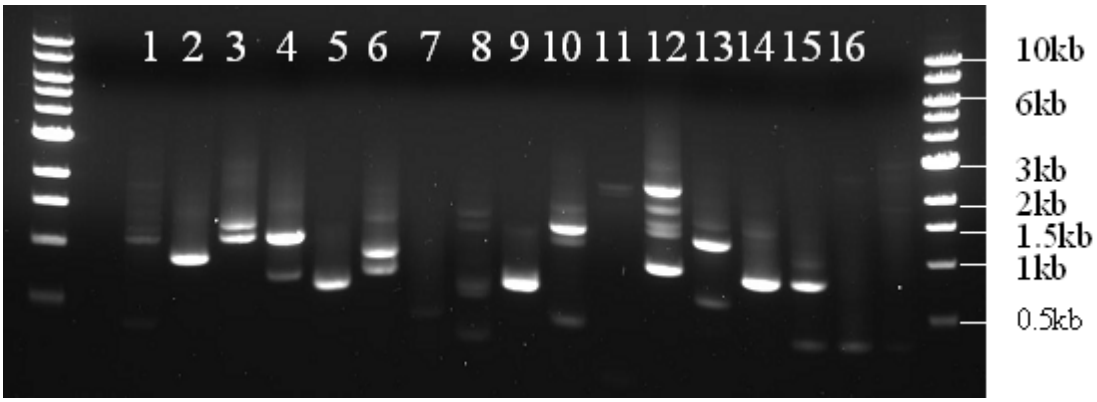

Figure 3.9 Isolation of T-DNA flanking sequences from genomic DNA of 16 Transgenic haploid plants by adaptor-ligation PCR method. Visualization of the PCR reaction product was performed by electrophoresis on 1% agarose Gel. For each plant, genomic DNA was digested with EcoRV and ligated with Adaptor. Two step PCR was performed using T-DNA left border specific primer with adaptor AP1 and AP2 primers.

from DNA isolated from 32 T-DNA lines and digested with *EcoR*V restriction enzymes is shown in Figure 3.9.

Through collaboration with Dr. Siva Kumpatla (Dow AgroSci.) and Dr. Sujata Pammi (Ocimum Biosolutions), Inverse PCR protocol and Tail PCR (2003) were used to identify the T-DNA insertion site in haploid transformants possessing aberrant phenotypes. In a preliminary screen from 32 haploid transformants, regions flanking the left border of T-DNA in 6 transformants were successfully sequenced and data obtained were used as queries to search against TIGR database. The T-DNA insertion sites in these 6 transformants were mapped to their rice chromosome loci (Table 3.6).

Table 3.6 BLAST results using TIGR databases

Line	Phenotypes	Locus number (From TIGR)	Gene Product
Mut-2	Yellow leaves, small size, semi-lethal	LOC_Os08g28930	unclassified putative retrotransposon protein
Mut-11	No flowers, yellow leaves	LOC_Os01g71040	expressed protein (unknown function)
Mut-14	No flowers, yellow leaves, semi-lethal	LOC_Os03g08680	Mitochondrial import inner membrane translocase subunit Tim17 family protein
Mut-22	short stem	LOC_Os06g46940	putative cyanogenic beta-glucosidase precursor
Mut-24	few flowers	LOC_Os05g51620.1	Unknown gene, Hypothetical protein
Mut-26	few flowers	LOC_Os05g51620.1	Unknown gene, Hypothetical protein

DISCUSSION

Rice, arguably the most important food crop worldwide, has become a model plant for molecular studies of monocot crops. A major attraction is its relatively small genome; indeed, sequencing of the entire genome of the Japonica rice, Oryza sativa L. var. Nipponbare genome is complete (International et al., 2005). A second important attraction is the ease with which it can be transformed, and our laboratory has made much use of this in studying various aspects of gene silencing (Iyer et al., 2000; Yang et al., 2005).

The simplicity of rice transformation renders it particularly suitable for T-DNA insertional mutagenesis, and, with the published sequence available for guidance, attention has rapidly turned to the use of this approach for the acquisition of genome-wide functional information. It is well established that harmful mutations are often recessive and that harmful genes in the homozygous recessive state may well be lethal. Unfortunately, in undertaking genomic screens for novel important genes, i.e. genes having a dramatic effect on the organism, mutations in recessive genes appear to be under-represented. This is not surprising. For example, if a recessive gene is mutated, the functional dominant counterpart will completely mask the effect. Thus, the presently available hemizygous insertional libraries show no phenotype in the primary transformants (T0) and at least one additional generation is required to obtain any homozygous recessive transgenic plants. Forward screening for the desired homozygous recessive mutants is in any case laborious and, compared with Arabidopsis, substantially more containment greenhouse space is needed for rice. Based on the calculation developed for the

Arabidopsis genome, over 660,000 independent rice transformants are needed to ensure a 99% probability that an insertion occurs every 3 kb or so (Krysan et al., 1999). Screening for this number of insertional mutant lines and for identifying the homozygous mutants would be a huge amount of work. If there are two or more T-DNA insertion loci, screening of homozygous mutants from hemizygous trangenic lines becomes considerably more onerous.

The problems associated with hemizygous insertional mutational libraries, mentioned above, can be overcome by the use of haploid cells as the target for transformation. Several successful experiments using a microspore culture system as the target for transformation have been reported (Palmer et al., 2005). These include Brassica napus (Pechan, 1989) , Datura innoxia and Nicotiana tabacum (Sangwan et al., 1993) Zea mays (Fennell and Hauptman, 1992) and Triticum aestivum (Leob and Reynolds, 1994).

The potential benefits of using haploid and doubled haploid (DH) plants in crop breeding has been known for over 80 years (Kasha and Maluszynski, 2003) but, until recently (Palmer et al., 2005), remarkably little effort has been put into their utilization for either applied or basic purposes. In part, difficulties in the routine production of haploid plants have decreased enthusiasm for their use. Here, we report for the first time details of procedures not only for highly effective and reproducible production of haploid cultures of a commercial rice variety but also for, to our knowledge, unprecedented high rates of Agrobacterium-mediated transformation together with very low occurrence of siblings. Further, we have pioneered the use of GFP expression in pollen for effective, rapid and inexpensive identification of homozygosity and estimation of the number of chromosomes bearing T-DNA insertions. We believe these procedures will greatly enhance progress in the functional genomics of rice, a vital crop for much

of the world's population and one of the few plants for which the genome is completely sequenced.

The routine regeneration of thousands, rather than the typical dozen or so, of independently transformed plantlets from a single co-cultivation is a major technical advance of substantial value in studying functional genomics of rice. The ability for early detection of a failed transformation vastly reduces costs incurred in maintaining failed cultures. The reliable GFP-fluorescence tests for transformation efficacy and homozygosity of individual transformants are rapid, inexpensive, very simple and exceptionally beneficial in reducing the need for radioactive materials.

While the merits of the rapidity, reliability and low cost of the transformation system we have developed are extremely beneficial, its greatest attribute is likely to be its high potential for revealing novel gene functions, especially those having lethal or near-lethal effects if they are disrupted, e.g. by insertional mutagenesis. This results from the relatively high frequency of occurrence of homozygous recessive transformants. Although we do not anticipate identifying completely lethal mutations (no regeneration), we do expect to be able to culture transformants with severely debilitating mutations long enough to obtain sufficient DNA to identify the location of the mutation.

Since haploid cells can survive and be induced to, or even spontaneously diploidize to yield doubled haploid (DH) cells that can be regenerated to yield fertile plants, a relatively high frequency of homozygous recessive plants could be recovered. The highly efficient production of transgenic haploid and DH plants offers a substantially improved method to discover novel rice phenotypic mutants of special interest in functional genomics. Indeed, success in this study

with rice would endorse its use for virtually any flowering plant species since a haploid has only one set of chromosomes (1n) instead of the 2 sets (2n) of the diploid. Consequently, recessive mutations will show up directly..

CHAPTER IV

A SMALL MODIFIED *MUBI*1 PROMOTER CONFERS HIGH LEVEL EXPRESSION IN RICE

INTRODUCTION

The development over the past decade of highly effective and reliable systems for Agrobacterium-mediated transformation of rice portends successful use of many biotechnological modifications that will alter and enhance this crop that is central to the nutrition of millions. While the transformation technologies are available, a wider range of promoters and regulatory elements to drive transgene expression in a reliable manner is vital as molecular approaches of plant improvement become more complex.

The Zea mays (maize) polyubiquitin-1 (mUbi1) promoter has been shown drive high levels of reporter activity in several monocot species (Christensen et al., 1992; Takimoto et al., 1994; Christensen and Quail, 1996). It is also widely used to drive marker genes for transgenic plant selection (Cornejo et al., 1993) and for expressing foreign genes to produce recombinant proteins (Zhong et al 1999)(Hood et al., 1997; Witcher et al., 1998). Indeed, it remains one of the most active constitutive genes characterized in plants.

The mUbi1 promoter is 1,992 bp long and includes 899 bp of promoter region, 83 bp of untranslated exon (leader) sequence and a 1,010 bp-long first intron (Christensen et al., 1992; Christensen and Quail, 1996). The 5' flanking sequence of mUbi1 includes regions with similarity to defined cis-acting elements. A TATA box is located in the consensus position, and

two overlapping sequences with similarity to the Drosophila hsp70 heat shock element are located approximately 0.2 kb upstream of the transcription start site. Modification of these elements resulted in an altered reporter gene expression pattern but high levels of heterologous gene expression in maize were retained (Streatfield et al., 2004).

The long promoter sequence can be inconvenient for cloning and is susceptible to gene silencing (Kumpatla, 1997; Kumpatla et al., 1997; Kumpatla and Hall, 1998b). Reducing the size of the promoter appeared to be an attractive approach to address both of these deficiencies. However, concern existed that deletion of promoter regions could affect the topology or chromatin structure (Hall et al., 1998; Li et al., 2001), thereby decreasing its high expression levels and ubiquitous spatial pattern of expression that are valuable features of this promoter. A strong enhancing effect on gene expression was shown when intron 1 was included along with the upstream region of the mUbi1 promoter by Salguerio et al. (Salgueiro et al., 2000), and similar results have been obtained for introns associated with other promoters (Sinibaldi and Mettler, 1992; Takumi et al., 1994; Vain et al., 1996). Therefore, in designing truncations of mUbi1 promoter we were careful to assess the possible effect on intron-mediated enhancement. Although inspection of the sequence motifs present in a promoter is helpful in designing mutated forms, it is still necessary to test the effects experimentally.

MATERIALS AND METHODS

Plasmid construction

The constructs used for transient expression are shown in Figureure 4.1. The mUbi-900-In (Figure 4.1A) construct (1992 bp) includes 899 bp of the 5' promoter region, 83 bp of untranslated exon (leader) sequence and 1,010 bp of the first intron (Christensen et al., 1992; Christensen and Quail, 1996). A 5' primer which contained a HindIII site (atgaagcttttcttgtttcgagtagataatg) and a 3' primer containing a BamHI site: cgggatccaggcctgcagaagtaacaccaaacaac) were used to to amplify the 5' region of the mUbi1 promoter up to base -343, thereby eliminating 556 bp of the original 5' promoter. The PCR amplification reaction was performed using PCR supermix (Invitrogen). PCR products were purified, digested with HindIII and BamHI, then cloned into BJ81 (a vector that contains the rice ubiquitin 2 promoter driving the gfp gene (Yang et al., 2001), yielding mUbi1-343-in (Figureure 4.1B), a truncated ubiquitin promoter driving the gfp coding region. To further shorten the promoter, an XbaI fragment (from +123 to +706) was excised from intron-1 of mUbi-343-In, yielding mUbi-343-tIn (Figureure 4.1C). Construct mUbi-0-In (Figureure 4.1D) has full-length intron1 but no upstream promoter sequence and was derived from mUbi-343-In by digestion with HindIII and BglII, followed by Mung Bean Nuclease treatment and subcloned into BJ81; construct mUbi-0-tIn (Figureure 4.1E) was made similarly from mUbi-343-tIn.

For stable transformation of rice calli, binary vectors pUbi-900-In, pUbi343-In , pUbi-343-tIn, were constructed by cloning the promoter and gfp fragments from the intermediate plasmid pBJ81 between the HindIII and MscI sites of pJD7 (Dong et al., 2001).

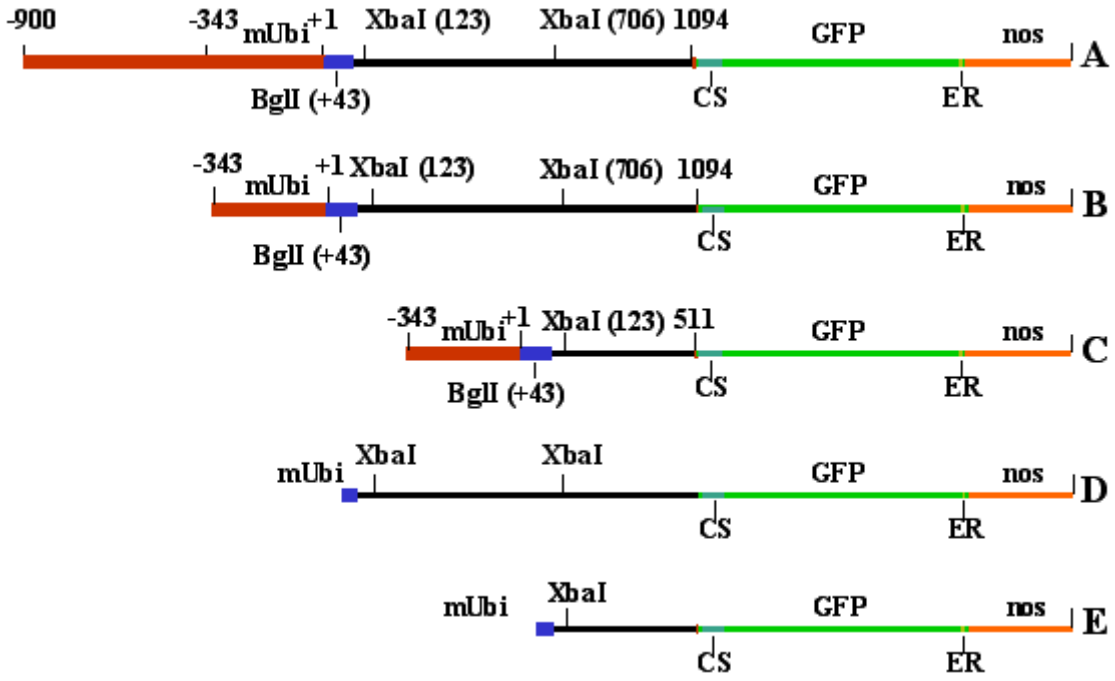

Figure 4.1. Schematic diagram of constructs. (A) Ubi-900-In is the full-length intron (In)-containing promoter originally described by Christensen et al. (Christensen et al, 1996); the promoter extends 900 bp upstream of the transcription initiation site (+1). (B) Ubi-343-In is similar to A, but the promoter is shortened to 343 bp. (C) Ubi-343-tIn is similar to B, but the first intron was truncated (tIn) by the excision of an XbaI fragment between positions 123 and 706. (D) Ubi-0-In is similar to B but lacks the 5' proximal promoter (-343 to +1) and 5' UTR (+1 to 93) regions. (E) Ubi-0-tIn is similar to D but lacks part (+123 to +706) of intron 1. All five constructs contain the entire mGFP coding sequence (Siemering et al, 1996) and nos terminator.

The resulting binary vectors were transferred into Agrobacterium strain LBA4404 by triparental mating (Ditta et al., 1980).

Initiation of scutellar calli

Mature T309 seeds were dehusked and rinsed with 70% ethanol for 1 min, then soaked in 50% (v/v) bleach for 45 min on a shaker at 120 rpm. The sterilized seeds were washed five times with sterile distilled water. The seeds were then placed embryo side face up on N6 medium (Chu et al., 1975) for two weeks in the dark at 28 C. Induced calli were separated from seed and plant tissue and cultured on N 6 medium for 10 to 14 days. During this time, many embryogenic calli developed that were subcultured, yielding many actively growing calli.

Biolistic transformation

Robust, actively growing calli were chosen and placed on high osmolarity N6 medium supplemented with mannitol and sorbitol (0.3M each) for 4 h before bombardment. Gold particles (1.0 μm, 600 μg) were coated with 1 g plasmid DNA and used for bombardment with a Biolistic Particle Delivery System model PDS-1000 using 1300 psi rupture discs (E. I. du Pont de Nemours & Co., Wilmington, DE). Bombarded calli were incubated in the dark (28 °C) and examined after 72 h.

Agrobacterium-mediated transformation and regeneration

Actively growing embryogenic calli were co-cultivated (Hiei et al., 1994) with Agrobacterium LBA4404 containing binary plasmids mUbi-900-In, mUbi-343-In and mUbi-343-tIn in the dark at 21°C for 3 days. After co-cultivation, infected calli were rinsed with sterile distilled water containing cefotaxime (250 mg/l) before being transferred to N6 selection medium containing 2, 4-D (2 mg/l), hygromycin (50 mg/l), and cefotaxime (250 mg/l). Calli were transferred onto fresh selection medium every 2 weeks. After 4 weeks on selection medium, somatic embryogenic calli were transferred to MSD4 regeneration medium (MS supplemented with 0.5mg/l 6-BAP and 0.05mg/l NAA) (Dong et al., 1996). Once shoot initiation was observed, the plates were transferred to an incubator with 16 h light and 8 h dark photoperiods at 26 °C to allow chloroplast development. Two weeks later, actively growing plantlets were transferred to Magenta boxes for rooting in MS0 (MS without plant regulators) for 2 weeks, then transferred to soil and grown to maturity in the greenhouse (Buchholz et al., 1998).

Fluorescence imaging, quantification and data processing

In transient assays, for each plasmid used, 100 green fluorescent spots were imaged using an AxioCam HR (Carl Zeiss) camera attached to a Zeiss SV11 stereomicroscope (Carl Zeiss, Oberkochen, Germany), using 470-nm excitation and 500- nm emission filters. The exposure time was adjusted so that none of the spot images was saturated in pixels and the mean value of

green fluorescence for each spot was measured using the interactive measurement module of AxioVision 3.0. Each reading was normalized by subtracting the nearby background fluorescence reading. The normalized readings from 100 spots for each plasmid were used to calculate average values. Green fluorescence in seed, roots, leaves, stamen and pollen was recorded using the same method. Measurement of fluorescence for 100 pollen grains from each of at least 50 independently transformed rice plants was performed similarly except that readings for nearby non-transgenic pollen grains were used for normalization. For the wild type (wt, non-transgenic) control, the fluorescence values for 100 pollen grains from 5 plants were normalized by subtracting the value for the nearby background fluorescence. Average values and standard errors were calculated using Microsoft Excel.

Protein extraction

Approximately 500 mg of 7 day-old etiolated seedling tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle, and transferred into an Eppendorf tube. Total leaf protein was then extracted with TE buffer (pH 8.0) for 10 min and centrifuged at 1.2 x 104 g for 20 min. The supernatant was collected and total protein concentration determined (Bradford, 1976).

Determination of GFP fluorescence in total protein

The fluorescence of plant samples (50 µg protein) was determined (Bio-Rad VersaFluor

fluorometer; Bio-Rad, Hercules, CA, USA) relative to a standard curve for a GFP (Clontech, Mountain View, CA, USA) concentration range of 100 ng/ml to 1 g/ml using 480/20 nm excitation and 510/10 nm emission filters.

RESULTS

Generation of a small functional mUbi1 promoter

Whereas a typical Pol II promoter lies entirely upstream of the transcription start site, the region commonly thought of as the mUbi1 promoter includes a sequence element that encodes a lengthy (1,010 bp) intron (black bar in Figureure 4.1A) that encompasses most of the 5' UTR and a more traditional element (-900 to+1, red bar in Figureure 4.1A). Our strategy for shortening the promoter was, therefore, two-fold with one objective being to shorten the traditional element and the second to shorten the intronic element. The 3' region of the mUbi1 promoter was amplified up to position -343 by PCR, thereby eliminating 556 bp of the 899 bp promoter 5' region. The full length (mUbi-900-In) and truncated (mUbi-343-tIn) promoter constructs were inserted into the pBJ81vector to drive GFP expression (Figureure 4.1). Bombardment of wt rice calli showed that the truncated mUbi1 promoter was functional, with a similar level of activity as the wt full length mUbi1 promoter.

In designing truncation of the intronic element, we took advantage of convenient XbaI restriction sites at positions +123 and +706 bp to excise 583 bp from the intronic region without changing its 5' or 3' ends. This yielded the mUbi-343-tIn derivative (Figureure 4.1C), in which

both the promoter and intronic elements are truncated. The size of the modified promoter was reduced to 853bp. The removal of 1,139bp from the full length mUbi1 has deleted 154 restriction endonucleases sites. This will be very helpful to further cloning of the modified promoter into different constructs. Following insertion into the pBJ81vector, it was used for bombardment of wt rice calli; green fluorescence confirmed functionality.

Comparison of transient expression driven by the truncated promoters

The activities of four modified promoters (Figureure 4.1B–E) relative to the full-length (mUbi-900-In) construct (Figureure 4.1A) were initially assessed in transient expression assays using particle bombardment of wt T309 rice calli. Four days after bombardment, discrete green fluorescent spots representing promoter activity were imaged as described previously (Yang et al., 2005). As shown in Figureure 4.2, panel A, the calli bombarded with mUbi-343-In and mUbi-343-tIn showed strong GFP expression, similar to that of mUbi-900-In; compare images (B) and (C) with image (A). Quantitative measurement, (Panel B and legend) supported the visual assessment, with mUbi-343-In having 93.2% and mubi-343-tIn having 89.3% of the activity of the full-length mUbi-900-In. A combination of the mUbi-343 truncation and the intron truncation (mUbi-343-tIn; Figureure 4.1C) resulted in a 10.7% reduction in expression compared with mUbi-900-In (Figure 4.2). However, a dramatic decrease in activity (to only

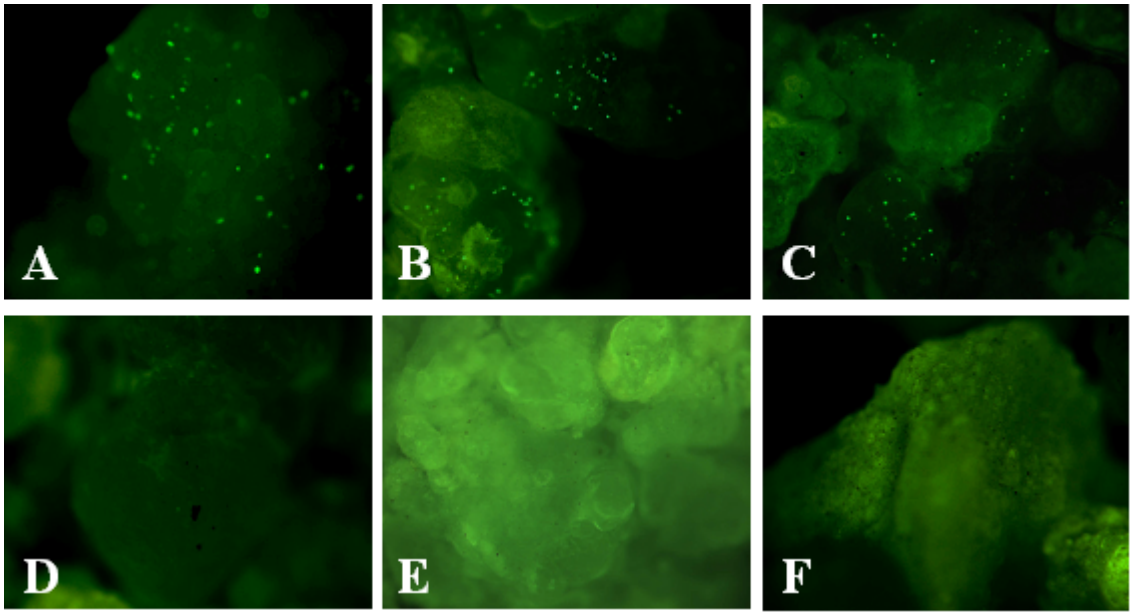

Figure 4.2. Transient expression of GFP in rice callus. Representative images of rice calli bombarded with constructs: A) mUbi-900-In; B) mUbi-343-In; C) mUbi-343-tIn; D) mUbi-0-In; E) mUbi-0-tIn or F) No construct (control).

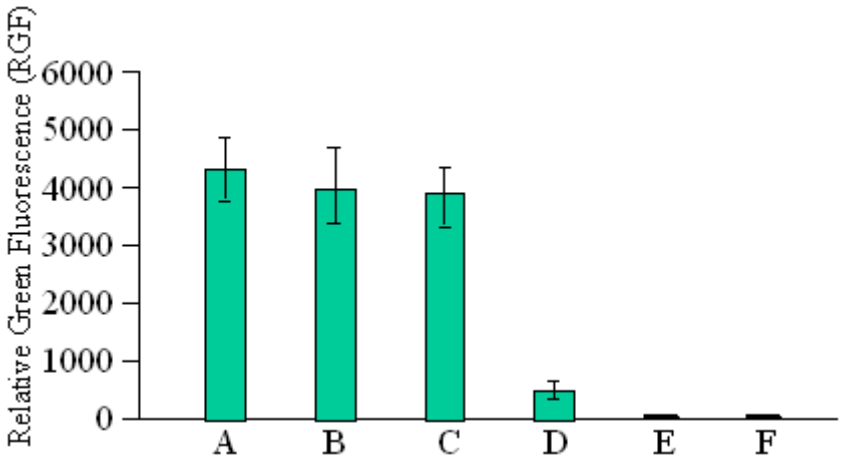

Figure 4.3 Relative green fluorescence. A) mUbi-900-In; B) mUbi-343-In; C) mUbi-343-tIn; D) mUbi-0-In; E) mUbi-0-tIn or F) No construct (control).

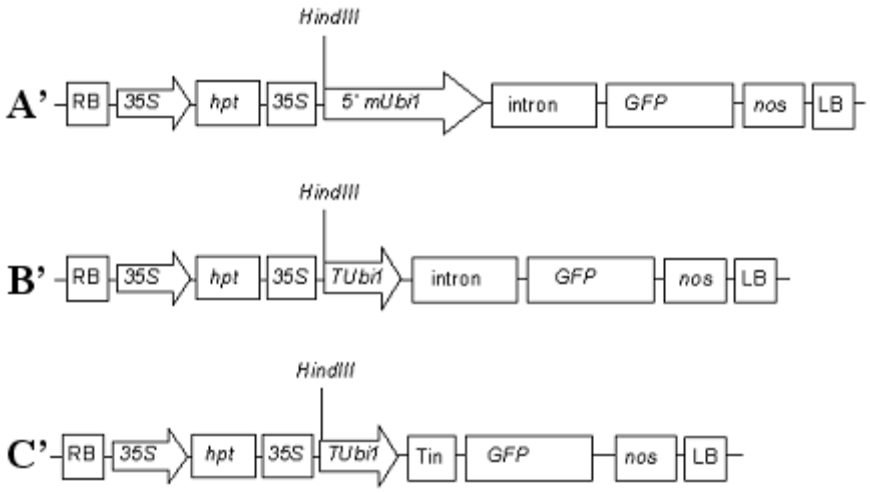

Figure 4.4 T-DNA regions of constructs for *Agrobacterium*-mediated transformation of rice. (**A'**) mUbi-900-In; (**B'**) mUbi-343-In; (**C'**) mUbi-343-tIn; The arrows indicate direction of transcription 35S, the 35S promoter from cauliflower mosaic virus; mUbi1, full-length ubiquitin1 promoter from maize; TmUbi1, truncated ubiquitin promoter from maize (Figureure 1 B); Intron, full-length first intron; tIn, truncated first intron (Figureure 1 C); hpt, the coding sequence for hygromycin phosphotransferase conferring resistance to hygromycin B; GFP, the coding sequence for green fluorescent protein; nos, 3' nos terminator. RB, T-DNA right border; LB, T-DNA left border.

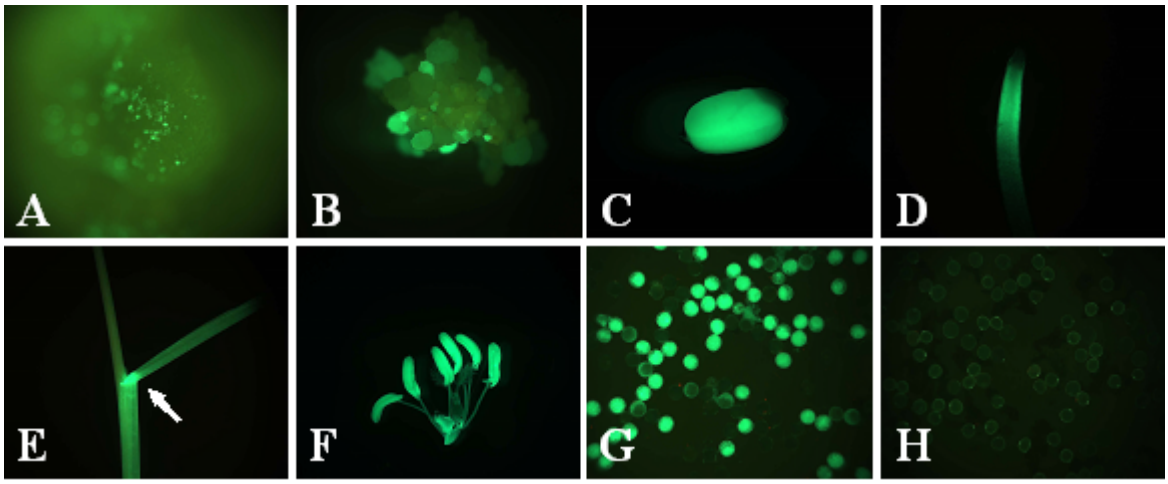

Figure 4.5. GFP expression in various rice tissues stably transformed with mUbi-343-tIn. (A) callus at 4 days after co-cultivation with Agrobacterium; (B) callus at 3 weeks after co-cultivation; (C) seed; (D) root; (E) leaf blade, ligule (arrow) and sheath; (F) stamen; (G) pollen; (H) pollen from a wt plant.

12% of the full-length promoter) was evident upon further deletion of the upstream promoter, even though the first intron remained intact (mUbi-0-In, Figureure 4.1D). Virtually all activity was lost when the 5' promoter was deleted and the intron was truncated (mUbi-0-tIn: Figureure 4.1E).

GFP expression driven by truncated mUbi1 promoters in transgenic rice plants

A binary vector containing the full length mUbi1 promoter (mUbi-900-In) and two containing promoter truncations (mUbi-343-In and mUbi-343-tIn) were constructed (Figure 4.4) and used for *Agrobacterium*-mediated transformation of wt T309 calli (Hiei et al., 1994). GFP expression was detected as early as 3 days after co-cultivation (Figure 4.5A); as the callus cells grew and divided, more highly fluorescent cells were evident (Figure 4.5B). Regeneration of plants from these stably transformed calli was as described in Materials and Methods. Bright green fluorescence was evident in all tissues (Figure 4.5 C-G).

Although visual inspection suggested that each of the promoters yielded similar levels of GFP expression, quantitative measurement of relative fluorescence intensity for seedlings revealed small but reproducible differences, with seedlings of plants transformed with the

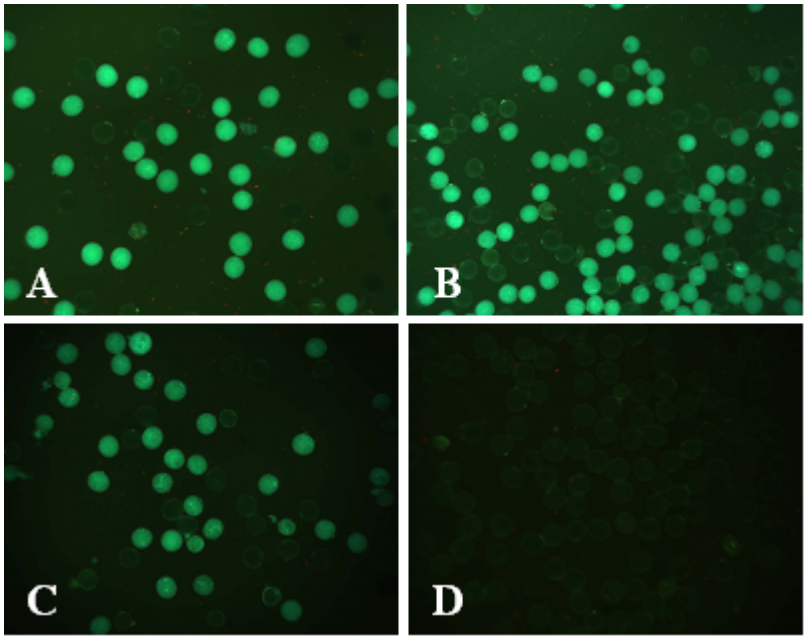

Figure 4.6 Pollen from stably transformed rice plants. Pollen is shown from representative plant transformed with (**A**) mUbi-900-In; (**B**) mUbi-343-In; (**C**) mUbi-343-tIn; (**D**) wt.

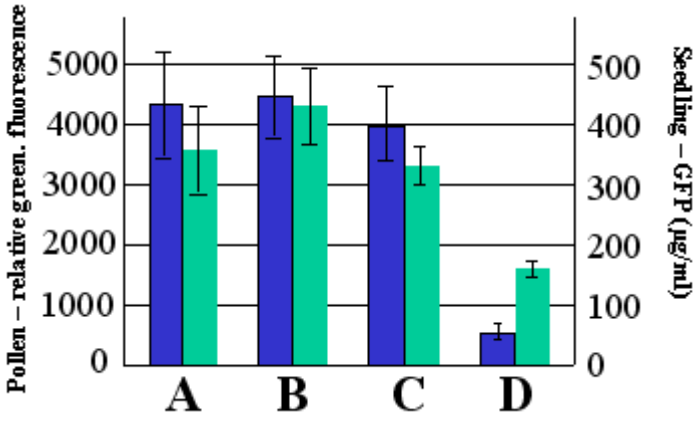

Figure 4.7. Relative green fluorescence intensity of pollen (blue bars) and etiolated seedlings (green bars) of transgenic rice plants. (**A**) mUbi-900-In; (**B**) mUbi-343-In; (**C**) mUbi-343-tIn; and (**D**) WT control: No construct. Standard errors of the mean are shown for 50 plants.

mUbi-343-In construct having a relative fluorescence of 120% (Figure 4.7). As was found for the bombardment experiments, the relative intensity of GFP expression in cells of stably transformed calli can be used to determine relative promoter activity. However, we noted that expression of GFP in pollen grains was very uniform (see for example, Figure 4.5G and Figure 4.6), making them highly suitable for quantitative experiments. These results reveal that the shortened mUbi1 promoter has similar spatial and quantitative characteristics as does the full length mUbi1 promoter. Similar results were obtained from the GFP quantitation of total proteins extracted from seedlings that had been etiolated for 7 days (Figure 4.7).

DISCUSSION

The mUbi1 promoter has been shown to be highly active in many cell types of a wide range of monocotyledonous plants (Christensen et al, 1992; Toki et al., 1992; Cornejo et al., 1993; Christensen and Quail, 1996). In carefully-conducted experiments, Salgueiro et al.(Salgueiro et al., 2000)compared GUS activity driven by an mUbi1 promoter bearing intron1 with that driven by an intronless promoter. GUS expression from the intronless promoter was essentially the same as background, revealing a strong enhancing effect on gene expression for the intron. These authors also showed that a DNA fragment that included 40 bp of the 5'UTR (part of exon 1) and the mUbi1 intron could drive transient GUS expression in tritordeum inflorescences and wheat scutellae, suggesting that cis-elements important for binding transcription factors are present in mUbi1 intron1.

The present study extends the analysis of intron function as part of a promoter (Salgueiro et al., 2000) through dissections of both upstream promoter sequences and intron1 of mUbi1. Deletion of 556 bp of upstream sequence (Figure 4.1B) reduced transient expression by 6.8% [Figure 4.2 A, B and Figure 4.3 A, B] and increased stable expression by 4% in pollen [Figure 4.6 B and Figure 4.7B] and by 20.3% in seedling tissues [Figure 4.6B and Figure 4.7B]. Interestingly, deletion of 583 bp (of a total 1010 bp) of intron1 (Figure 4. 1C) only reduced transient expression from the -343 mUbi1 promoter by 3.9% [Figure 4.2C and Figure 4.3C]; stable GFP expression in pollen decreased by 10.8% [Figure 4.6C and Figure 4.7C] and that in seedling tissues by 28.3% [Figure 4.7C].

However, the promoter activities are expected to differ in different cereal crops and different plant tissues or cell types. The results presented in this chapter suggested that the mUbi1 promoter activities were highly active in transformed callus cells both in transient expression and stable transformations. This promoter has been shown to be highly active in all cell types in the rice plant.

Transgene silencing remains an important consideration in the application of biotechnology to crop improvement. A variety of silencing effects has been described in the literature, involving single transgene loci, interactions between unlinked loci, or even interactions with or through an endogenous homologous gene (Meyer et al., 1992; Iglesias et al., 1997; Iyer et al., 2000). Epigenetic silencing of transgenes and endogenous genes can occur at the transcriptional level (TGS) or at the posttranscriptional level (PTGS) (Fagard and Vaucheret, 2000). Silencing is now known to be involved in plant defense systems against invasive DNA or RNA sequences (viruses, transposons) and surveillance processes that check the genome

integrity to suppress the expression of abnormal or alien transcription units (Jorgensen et al., 1998; Kumpatla et al., 1998; Iyer et al., 2000). Aspects such as insert location, rearrangements, multiple-copy loci, homology to an endogenous sequence, excessive level of transcription and others have been shown to trigger silencing. Whatever mechanism of silencing is involved, the presence of inserted foreign DNA frequently initiates events leading to the failure of a gene to express. For several years, the concept of homology dependent gene silencing (HDGS) was in favor (Matzke and Matzke, 1995), supported by studies showing that 300 bp(Matzke et al., 1989)and even 90 bp (Vaucheret, 1993) of sequence homology was sufficient for trans-inactivation. In consequence, the use of short transgene sequences appeared to be beneficial in avoiding induction of gene silencing. With the recognition that dsRNA, often generated from rearranged or fragmented DNA, was powerful in inciting RNAi (Mette et al., 2000), the value of reducing transgene size appeared to be diminished. Indeed, our own studies with the 35S promoter revealed that a second round of rice transformation using a transgene sequence identical to a resident, silenced, transgene, did not result in silencing of the incoming gene (Yang et al., 2005). Nevertheless, it remains possible that certain sequences or topologies favor HDGS, making the use of relatively small transgenes attractive. Another beneficial aspect of reducing the size of transgenes, especially promoter elements, is the elimination of restriction enzyme sites that complicate transgene construction.

In the experiments described here, 556 bp of -899bp in the promoter upstream region were removed. The remaining 343bp of upstream promoter contains the TATA box and a heat-shock element (Christensen, 1992; Streatfield, 2004). The activities of the resulting modified promoters (pUbi-343-In and pUbi-343-tIn) remained high compared to the full-length

mUbi1 promoter both in transient expression and stable transformed plants. The removal of 556bp of upstream region with an intact, unmodified first intron (pUbi-343-In) resulted in higher expression levels compared to the full-length mUbi1 promoter only in stably transformed plants. These results suggest that the genomic sequence at the insertion site in the transgenic plants may serve as an enhancer for mUbi1 after the removal of 556 bp promoter sequence. The lower activities of pUbi-343-In in transient expression experiments compared to the full-length mUbi1 promoter may mean that the 556 bp promoter upstream region has an enhancer function for the full-length mUbi1 promoter. With no genomic sequences flanking the pUbi-343-In , it results in a lower promoter activities in the transient expression cells. Although stable transformations are usually considered to be more reliable than transient expression for assessing promoter activity, in this case it appears preferable to use transient expression to assess the innate promoter activity of each construct since in stable transformations it is possible that genomic sequences flanking the insertion site could influence the promoter activity.

The regulatory role of introns in transcription as measured by gene expression has been reported in many cases (McElroy et al, 1990; Last et al, 1991; Luehrsen and Walbot, 1991; Sinibaldi and Mettler, 1992; Vain et al, 1996). The first intron of mUbi1 promoter may also directly drive gene expression because it is similar to typical promoter sequences (Salgueiro et al, 2000). Therefore, in this case, the first intron is recognized as a part of the mUbi1 promoter. It includes a TATA-box-like sequence (TATAA) at position +924, and a CAT-box at position+390 (CAAT), and an E-box at position +404 (CANNTG) (Salgueiro et al., 2000). After removal of all of the promoter upstream region and part of the 5'UTR, the modified mUbi1 still showed a small amount of promoter activities in the transient expression experiments. The

removal of +123 to +706 of mUbi1 first intron in pUbi-0-tIn resulted in removal of the CAT-box and E-box, and resulted in the complete loss of promoter activity. Interestingly, the truncated promoter upstream and the 5'UTR presented with the truncated first intron showed a strong promoter activity. The results seen here suggest that 343bp of the upstream promoter region plays a major role for mUbi1 function. Expression appeared to be lower in green plant tissues or mature plant leaves, most likely a consequence of severe interference of chlorophyll with GFP fluorescence (Zhou et al., 2005). In present studies, GFP fluorescence intensity in pollen with the same genetical background was shown to be uniform. As they contains no chlorophyll, pollen represents an ideal cell type for GFP quantitation.

For practical purposes, the use of a reliable promoter to drive transgene expression is highly desirable. However, many reports exist in which silencing of a transgene was attributed to the presence of more than one copy. As previous work in other labs suggested that identical sequences of ~100 base pairs rendered the transgenes susceptible to silencing, we decided to examine the effect of promoter diversification so that no two sequences were identical for >90 bp. The promoter chosen for the diversification study, mUbi1, is generally regarded as a strong constitutive promoter for monocots. As is the case for many monocot promoters, an intron is considered as part of the mUbi1 promoter. However, deletion of much of mUbi1 intron-1 did not affect promoter strength in transgenic rice, permitting the use of a substantially shorter promoter (343 bp in place of over 1 kb). Sequence substitutions were introduced at positions -100, -200 and -310 (and permutations thereof) and the diversified promoters used to drive GFP expression in transgenic rice. Strong GFP expression was detected in all tissues of rice transformed with the various diversified mUbi1 promoter - GFP reporter fusions tested, except

for the triple mutant construct -100/-200/-310-GFP (data not shown). Expression of GFP from this triple mutant construct was remarkable in that it was very strong in all tissues except pollen. This finding is very exciting since it implies that the use of this promoter to drive expression of insecticidal protein will yield protection in all tissues other than pollen. The death of beneficial insects, such as Monarch butterflies, as a result of consuming pollen that express insecticidal protein has been of great public concern. It would appear that the use of the triple mutant mUbi1 promoter would eliminate this problem.

CHAPTER V

SUMMARY

Plant transformation is an indispensable tool for plant molecular biology. Improved approaches and highly efficient transformation will facilitate functional genomics and studies of gene expression and silencing. In this study, an exceptionally effective transformation procedure has been established by the use of carefully chosen class I type of callu from both rice immature embryos and mature embryos. The procedure was effective for both japonica varieties tested (Nipponbare and T-309). Following an improved tissue culture procedure, nearly 100% of treated class I type of calli bear transformed cells within each callus clump (approximately 5 mm diameter) yielding multiple independently transformed plants (average for 62 calli was approximately 40 plantlets). The regenerated plants from each co-cultivated callus were cataloged and numbered according to the original co-cultivated callus. Genomic DNA analysis of the T-DNA integration patterns and pollen segregation with green fluorescent protein (GFP) were examined. The results in a population of more than 400 T_0 transgenic plants revealed that about 64% bore a single locus T-DNA insertion. Among those single locus insertions, approximately 55% were found to bear a single T-DNA copy. Additive transgene expression was observed for T_0 T-DNA plants with GFP driven by the mUbi1 promoter. This was found to be valuable in that a reliable estimate of the number of T-DNA insertion loci on different chromosomes could be readily obtained simply by examining the segregation of GFP in the pollen of T_0 plants. This allows identification of single locus insertion plants from a large population of T_0 transformants without the need for further generations or Southern analysis.

This highly efficient production of transgenic plants could be used to create thousands of independent transformants in one carefully prepared co-cultivation experiment. The rapid and simplified method for the characterization of transgenic plants using pollen GFP segregation and additive gene expression is a valuable breakthrough.

The use of *Agrobacterium tumefaciens*-mediated transformation of microspore-derived callus for generating large amount of T-DNA haploid and fertile doubled haploid plants has also been investigated. Using T-DNA constructs bearing the hygromycin resistance (hpt) and green fluorescent protein (gfp) genes, an exceptional transformation procedure has been established that results in approximately 100% frequency for class I type of microspore-derived calli co-cultivated with Agrobacterium. Each callus typically yields multiple independent transgenic plants. The T-DNA integration pattern in a population of more than 200 transgenic plants has been characterized by Southern blots. Approximately 98% of those transgenic plants are independent events. About 45% of the transgenic plants were identified as haploid plants, whereas about half are DH hemizygous (doubling occurred prior to integration) or homozygous transgenic plants. The pollen GFP expression segregation pattern in fertile T_0 transgenic doubled haploid plants has been analyzed along with corresponding Southern blot data. Approximately 9% of the fertile doubled haploids were homozygous transgenic plants that were confirmed by Southern blot as well. In the two cultivars studied, about 60% of T_0 transgenic plants were found to have a single locus T-DNA insertion. Among those single locus T-DNA plants, about 45% of T_0 plants were found to have a single T-DNA copy. Furthermore, in a population of over 2,000 haploid and doubled haploid T-DNA plants , approximately 25% had clear-phenotypical differences from wt haploid plants, including 5% that were seriously phenotypically abnormal,

lethal or semi-lethal mutants. This highly efficient transformation procedure using microspore-derived callus could be valuable not only in speeding up plant breeding but in new gene discovery as well.

Gene expression has been investigated using GFP driven by altering mUbi1 promoters. Diversification of the mUbi1 promoter led to discovery of a minimal mUbi1 promoter that has a similar function as the original mUbi1. The minimal promoter of *mUbi*1 was created by PCR amplifying the region of mUbi1 promoter up to base -343, thereby eliminating the 5' 556 bp of the 899 promoter region, and truncating the first intron at XbaI restriction sites at 123 and 706 bp. The resulting minimal promoter was coupled with a GFP reporter gene. Gene expression driven by this minimal promoter in transient and stable transformants showed the minimal mUbi1 promoter has a similar function as the original wild type promoter.

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