ABSTRACT

TUONG, TAN DUY. Rice *rubi*3 Promoter Activity and Inheritance in Transgenic Rice Plants. (Under the direction of Dr. Rongda Qu.)

Making up of the majority of the human diet, grains from cereal crops such as rice, wheat, and maize do not only have crucial nutritional importance but also great economical importance. Over the past few decades, tremendous efforts have been devoted to improving the quality and quantity of the cereal grains through breeding practice. Recent advances in plant biotechnology, especially through genetic transformation, offer an alternative path to improve crop production and quality.

A major target in plant transformation is to produce transgenic plants that stably express the desirable gene(s) not only in the primary transformed plants, but also in their subsequent generation(s). Transgene expression and stable inheritance are affected by many factors.

This project was a continuation of Dr. Lu's studies (2006) using transgenic rice plant lines with a single transgene copy to analyze the activities of the rice *rubi*3 5' regulatory sequence in transgenic rice plants and the inheritance of the transgene expression in the T_1 progeny. The objectives of the studies consisted of: (1) quantitative evaluation of intron-mediated enhancement (IME) in T_0 rice plants transgenic of the *GFP* gene; and (2) examination of the expression of the *GUS* gene by the *rubi*3 5' regulatory sequences in T_1 offspring plants, especially within and between homozygous and hemizygous plant groups in the progeny of the transgenic rice plants.

ELISA assays showed the average level of GFP protein in both fresh root and leaf samples was about 2.3 fold higher in the intron-containing lines than in the intronless lines. Similar magnitude increase in GFP mRNA accumulation was observed in northern hybridization. In addition, we observed that the expression level of *GFP* in fresh roots was approximately 7 fold higher than in leaf samples on per unit protein basis. Although the *rubi3* intron-mediated enhancement (IME) effect was similar in leaf for both *GUS* and *GFP* genes, the results revealed a profound difference of IME in root between the two genes, suggesting that IME is affected by both tissue and the gene itself.

In the *GUS* gene expression inheritance study, a significant difference in GUS activity was observed within homozygous and hemizygous plant groups in all the three transgenic rice lines analyzed, indicating an instability of the inheritance of transgene expression. In addition, in one line, the hemizygous plants significantly outperformed the homozygous plants whereas the *GUS* expression was similar between the two groups of plants in two other lines, suggesting there was no clear correlation between expression level and the transgene dosage among the progeny.

Before this project, I attempted wheat transformation with a trehalose biosynthetic fusion gene (*TPSP*) derived from *E. coli otsA* and *otsB* genes for improved drought tolerance. A transgenic wheat plant was recovered, which was resistant to the selection herbicide bialaphos but did not express the *TPSP* gene. The results are included in the Appendix of this thesis.

Rice *rubi*3 Promoter Activity and Inheritance in Transgenic Rice Plants

by

TAN DUY TUONG

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APPROVED BY:

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Dr. Rongda Qu
Chairman of Advisory Committee

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Dr. Paul Murphy Dr. Candace Haigler

Biography

Tan was born in Bien Hoa, Vietnam. He came to the United States when he was about 12 years of age. He graduated from Athens Drive High School, where he was involved in many extracurricular activities.

North Carolina State University became his destination for his undergraduate studies. Tan came to NC State with the intent of getting a degree in Computer Engineering but quickly changed his mind after taking a Botany class. He graduated with a Bachelor of Science in Biological Science with minors in Genetics and Botany. He also held leadership positions during this time in service to on-campus organizations.

After graduation he worked as a lab technician in the Qu Lab at North Carolina State University. His work focused on the transformation of cold season crops. He began his Master of Science studies in Crop Science in August 2004, and will conclude his studies upon the approval of his thesis.

He is currently working for the United States Department of Agriculture – Agricultural Research Service where he is studying the winter hardiness in cereal crops.

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Abbreviations

ABA	abscisic acid
ANOVA	analysis of variance
bar	herbicide bialaphos resistance gene
CaMV 35S	Cauliflower Mosaic Virus 35S transcript
CAT	chloramphenical acetyl transferase
dap	day after pollination
dCTP	deoxy-cytidine triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EJC	exon-exon junction complex
ELISA	enzyme-linked immunosorbent assay
et al.	et alia (Latin; and others)
FAO	the Food and Agriculture Organization of the United Nations
Fig.	figure
GFP	green fluorescent protein
GFP	green fluorescent protein gene
GUS	β-glucuronidase (gene product)
GUS	β-glucuronidase gene
g	gram
g/l	gram per litre
IME	intron-mediated enhancement
M	molar
MARs	matrix attachment regions
mg/ml	milligram per millilitre
ml	millilitre
mM	millimolar
MOPS	4-morpholinepropanesulphonic acid
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog (1962) basal medium
MU	4-methylumbelliferone
MUG	4- methylumbelliferyl-D-glucuronide
NAA	1-naphthaleneacetic acid
nmole	nanomole
N:P:K	ratio of nitrogen (N), phosphorus (P) and potassium (K) in fertilizer
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	a measure of the acidity or alkalinity of a solution
pmole	picomole
PNPP	p-nitrophenyl phosphate disodium salt

Abbreviations (continued)

RIAS	plant contained *rubi3* promoter + *rubi3* intron + the first 27 nucleotides of the coding sequence of the *rubi3* gene fused to the *GUS* in frame
RIGS	repeat-induced gene silencing
RIP	plant transformed with *GFP* driven by rubi3 promoter plus the 5' UTR intron
RIS	plant contained the *rubi3* promoter + *rubi3* intron + *GUS*
RNA	ribonucleic acid
RP	plant transformed with *GFP* driven by rubi3 promoter without the 5' UTR intron
rRNA	ribosomal ribonucleic acid
RS	plant contained the intronless *rubi3* promoter + *GUS*
rubi	rice polyubiquitin gene
Ubi	maize polyubiquitin gene
UC703	wheat variety from University of California - Davis
V	volt
v/v	volume to volume
WT.	wild-type plant
w/v	weight to volume
T-DNA	transfer-DNA
TPP	Trehalose-6-phosphate phosphatase
TPS	Trehalose-6-phosphate synthase
TPSP	trehalose-6-phosphate synthase/phosphatase gene
T_0	regenerated transgenic plant
T_1	progeny derived from self-pollination of T_0
T_2	progeny derived from self-pollination of T_1
T_3	progeny derived from self-pollination of T_2
Ti	Tumor inducing
5' UTR	5' untranslated region
µl	microlitre
µg	microgram

Introduction

The importance of cereal crops

Besides being a multi-billion dollar industry, the production of cereal crops is the global foundation of human nutrition. The majority of the human diet consists of grains coming from cereal crops such as rice, wheat, and maize. The production of cereal crops has increased steadily since the 1970s; however, that trend has stopped in recent years. In 2006, the Food and Agriculture Organization of the United Nations (FAO) estimated 1,995.1 million tons of cereals were produced worldwide, 19 million tons lower than that of 2005. The shortage in cereal production poses serious issues in many developing countries. More than 39 countries are facing food emergency due to civil strife, economic crisis, flood, drought, harsh winters, tsunami, earthquakes, and the shortage of farmable land (*Crop prospect and food situation* #4, 2006). These natural disasters, change in the climate and economic situations have increased the demand for the production of crops. In addition, it is estimated that by 2025, the world population will be 8 billion people and that the crop production must rise 1.2% annually to meet the demand of the increased world population (Rosegrant et al., 1999).

Improvement of cereal crops via biotechnology

The efforts to improve the quantity and quality of crops to match the growing demand became more intensive in the past four or five decades, as represented by the "Green Revolution" (Conway, 1998). Among them, traditional breeding played a vital role in the improved productivity of the crops through extensive crossing, mutagenesis, and plant

selection. Plant breeding has made tremendous advances in developing improved cultivars and contributed most of the gains in crop production. However, the conventional breeding is limited by the germplasm pool and the sexual compatibility of the plants involved. Recent advances in plant biotechnology, especially through genetic transformation, offer an alternative way to improve crop production and crop quality. Plant transformation involves identification of the desirable characteristics or traits, isolation of gene(s) responsible for those characteristics or traits from a variety of plant, animal, or microorganism, and introducing them into the targeting crops. There are many reported plant transformation methods: microinjection, pollen-tube pathway, silicon carbide- or liposome-mediated transformation, biolistic, electroporation of cells and tissues, and A*gobacterium*-mediated transformation. Two of the most common methods of plant transformations are the biolistic, and the *Agrobacterium*-mediated transformation methods (Veluthambi et al., 2003).

The biolistic, or the "gene gun" method, is a versatile method of transformation that can be used for both monocots and dicots. This method employs the delivery of DNA coated tungsten or gold particles into the plant cells using compressed helium gas (Klein et al. 1987). *Agrobacterium*-mediated transformation technique utilizes the natural soil-born bacterium, *Agrobacterium tumefaciens.* The bacterium transfers a copy of their T-DNA (transfer DNA), which is a small section of DNA carried on their Ti (tumor inducing) plasmid into the plant genome. The T-DNA is flanked by the right and the left borders which consists of a 25 bp repeats. The gene(s) contained within the borders will be transferred to the host cell (Zupan et al., 2000). The *Agrobacterium*-based system has become more popular due to its relatively high transformation efficiency and the integration of a small

number of transgene copies into the plant genome, and the transformation procedures are simple and equipment cost is lower than the biolistic system (Nadolska-Orczyk et al., 2002). These transformation methods have been widely improved to transform a variety of crops and other plants species. However, there are still challenges that these gene transferring techniques must overcome. Once the desired gene(s) is delivered into the plant cells, there are many factors that can influence the expression of the transgenes as well as the stability of the transgenes inheritance in the progeny.

Transgene expression and inheritance

The economical or agronomical applications of the transgene(s) depend on the stable expression of the transgenes from generation to generation. Many studies were carried out to examine the integration and expression of transgene(s) in cereal crops. These studies looked at cereal crops transformed via direct DNA transfer (biolistic) (Spencer et al., 1992, Goto et al., 1993, Peng et al., 1995, Svitashev et al., 1999, Vain et al., 1999, Miroshnichenko et al., 2007) or via *Agrobacterium*-mediated transformation (Hiei et al., 1994, Mohanty et al., 1999, Azhakanandam et al., 2000, Vain et al., 2003). Successful incorporation of foreign genes through biolistic transformation was reported by Spencer et al. (1992) in maize, Christou et al. (1991), Goto et al. (1993). Peng et al. (1995) and Vain et al. (1999) in rice, and Weeks et al. (1993) in wheat. Mohanty et al. (1999) transformed the economically important rice cultivar Pasa Basmati 1 with *GUS* using *Agrobacterium* and observed inheritance of the gene in the T_1 generation. Azhakanandam et al. (2000) examined the effect of plant genotypes and *Agrobacterium* strain on integration and inheritance patterns of rice plants and found stable integration of *GUS* gene in rice plants transformed with *Agrobacterium* strains

LBA4404(PTOK233) and 1065. They also found a correlation between the gene dosage and the level of expression of *GUS*.

Transgene copies often integrate at a single genetic locus and are inherited in a Mendelian manner (Budar et al., 1986, Zhang et al., 1991, Spencer et al., 1992, Register et al., 1994, Srivastava et al., 1996, Miroshnichenko et al., 2007). For example, both Spencer et al. (1992) and Register et al. (1994) reported maize plants transformed by the biolistic method contained multiple copies of the transgene(s). However, the multiple copies of the transgenes tended to integrate at a single locus, and a Mendelian inheritance pattern was observed in the progeny. The transgene copy number in plants transformed by the *Agrobacterium*-mediated method is often low and inheritance in progeny is often in a Mendelian manner, too (Budar et al., 1986, Zhang et al., 1991, Srivastava et al., 1996, Baruah-Wolff et al., 1999, Azhakanandam et al., 2000, Miroshnichenko et al., 2007). However, non-Mendelian segregation had also been observed. For example, segregating patterns of 1:1 and 1:3 were observed in *GUS* transformed maize (Ishida et al., 1996). Distorted (non-Mendelian ratios where there was a greater number of 1 set of plant containing the gene of interest or vice versa) segregation ratios (among the plants containing the gene or genes of interest) was observed in transgenic cereal crop by Pawlowski et al. (1998), Park et al. (1996), Vain et al. (1998), and Altpeter et al. (1999). In many studies, both Mendelian and non-Mendelian patterns were observed over multiple generations. When looking at the inheritance pattern over three generations of transgenic *GUS* rice plants by analyzing the activity of GUS and NPT II, Peng et al. (1995) observed that the T_1 generation followed Mendelian inheritance and the T_2 and T_3 were non-Mendelian with segregation

ratio ranging from 1:1 to 1:2 and in some cases 0:all. Similar findings that Mendelian segregation was only in the transgenic T_1 rice plants and not the T_2 plants were observed by Hiei et al. (1994), and Chen et al. (1998).

There are a number of factors that account for the inheritance pattern and performance of transgenes. Variation in the inheritance pattern could be due to the poor expression of the transgene(s), transgene(s) silencing, or the unstable transmission of gene(s) (Spencer et al., 1992, Register et al., 1994, McCabe et al., 1999). Register et al. (1994) suggested that the nature of the crop genome (maize, in this case, possibly due to epigenetic control) was responsible for the lack of transgene inheritance in the T_2 generation. The transformation methods (biolistic vs. *Agrobacterium*-mediated) could also contribute to the integration of transgene(s). The biolistic method often produces plants with higher copy number of transgene(s) and higher frequency of rearrangement of transgene(s) (Veluthambi et al., 2003). The multiple transgene(s) insertion could lead to the silencing of transgene or co-suppression (Jorgensen, 1993, Register et al., 1994, Hammond et al., 2001). The site of gene insertion is another factor that influences the performance of transgenes (Walter et al., 1992). Ye and Singer (1996) found that silencing of transgene(s) might occur when integration took place in repeat-sequence regions of the heterochromatin (RIGS, repeat-induced gene silencing). Other factors such as selection system (Bhattacharyya et al., 1992), gene deletion (Spencer et al., 1992), and construct components such as type of promoter and intron (McElroy et al., 1990, Vain et al., 1996) may all play roles in the inheritance pattern and performance of transgenes.

Understanding the inheritance pattern and performance of transgenes in subsequent generations of transgenic plants allows for selection of lines of desired trait(s). In the development of agronomically important cereal crops, a homozygous population is often preferred. Studies have shown that inheritance pattern and performance of transgene(s) may change from generation to generation, and transgene expression could also vary between homozygous and hemizygous progeny plants. Observations of differences between the performance of hemizygous and homozygous plants are controversial since findings from many studies do not agree with each other. Studies examining the difference in transgene expression between homozygous and hemizygous transgenic cereal crops show that, in many cases, the homozygous plants outperformed the hemizygous plants. Duan et al. (1996) demonstrated that higher accumulation levels of the PINII protein were found in homozygous transgenic plants. Similarly, Baruah-Wolff et al. (1999) found approximately 25% higher firefly luciferase gene activity in association with the homozygous T_1 transgenic rice plants. However, there are studies showing no difference in transgene expression level between homozygous and hemizygous plants. No difference in the GUS activity was found between GUS homozygous and hemizygous rice plants (Peng et al., 1995). Similar results were reported by Fearing et al. (1997) when analyzing hemizygous and homozygous transgenic maize lines expressing the cryIA(b) gene.

In a more in-depth study looking at the relationship between homozygous and hemizygous plants in transgenic rice carrying the *GUS* gene, James et al. (2002) found no clear cut difference in *GUS* expression in homozygous and hemizygous T_2 rice plants. Instead, the performance of hemizygous and homozygous depended on the transgenic line studied. Their

study examined T_2 plants of three independent transgenic rice plant lines transformed by the biolistic method. The progeny of all three lines segregated in Mendelian fashion. Fluorometric assay (MUG assay) for the T_2 generation of one of the lines showed the GUS activity in the homozygous plants was 1.7-fold higher than the hemizygous plants. However, for the T_2 of the second line, they found that the homozygous plants performed significantly lower than the hemizygous ones. Yet for the analysis of the T_2 plants of the third line, no distinct advantage or disadvantage was detected for the GUS activity between the homozygous and hemizygous plants. James et al. (2002) reported all three lines had multiple copies of *GUS* transgene. As mentioned above, multiple copies of the transgene resulted from the biolistic method could lead to gene silencing, and they thought analysis of transgenic plants with a single transgene copy may help further provide insight on transgene inheritance in plants.

Constitutive promoters for transgene expression

A promoter is a specific DNA sequence located upstream of a transcribed sequence that is recognized by the transcription factors to allow RNA polymerase to make transcription. A promoter can be specific to only express at certain time or in specific tissue or to be constitutive, expressing in all the tissues and at all the time. One of the most commonly used constitutive promoters is the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al. 1985). The CaMV 35S promoter has been found to express well in dicot plants (Schledzewski and Mendel, 1994). In the transformation of monocots the maize polyubiquitin 1 (*Ubi1*) promoter is more widely used. Christensen et al. (1992) found that in maize, the expression level of chloramphenicol acetyl transferase (CAT) driven by the maize

*Ubi*1 promoter was about 10 times higher than the one driven by the CaMV 35S promoter. In a bombarded maize cell culture, the *Ubi*1 promoter, when paired with its 5' UTR intron, reportedly enhanced the expression level of *GUS* gene 71 fold higher than the CaMV 35S promoter (Vain et al., 1996).

Rice *rubi*3 gene promoter and intron-mediated enhancement of gene expression (IME)

Introns are sections of DNA located between the expressed sequences (exons) within a gene. Introns do not function in coding for protein synthesis and are spliced out in the transcripts. Many studies showed that the intron often enhances gene expression in higher plants (Callis et al., 1987, Simpson and Filipowicz, 1996, Bourdon et al., 2001, Clancy and Hannah, 2002). Recently, intron mediated enhancement (IME) of gene expression was reported by Sivamani and Qu (2006) from studies on the rice *rubi*3 promoter and its 5' UTR intron isolated from a rice polyubiquitin gene. The isolated *rubi*3 promoter is 808 bp in length. Transient assay of bombarded rice suspension cells showed that the 1140 bp 5' UTR intron of the *rubi*3 gene enhanced the *rubi*3 promoter expression by over 20 fold as measured by the GUS enzyme activity. The expression of *GUS* gene was further elevated when the gene was fused with the coding sequence of the first 9 amino acids (aa). Transient assay revealed that the *GUS* expression was about 4-5 fold greater when the 9 aa was fused with the GUS protein, and about 2.2 fold higher than that of the *GUS* gene driven by the maize *Ubi1* promoter (Sivamani and Qu, 2006). To further investigate the activity of the *rubi*3 promoter and its 5' UTR intron region at whole plant level, Dr. Jianli Lu made several CAMBIA1300-based binary vectors containing the *GUS* or *GFP* reporter gene driven by various sequences of the

*rubi*3 5' regulatory sequences and transformed rice cultivar Taipei 309 with *Agrobacterium* strains harboring these gene constructs (Lu, 2006).

Plasmids pJLU2, pJLU6, pJLU7 (Fig. 1) were used for the transformation of rice plants with *GUS* reporter gene while pJLU12 and pJLU13 (Fig. 1) were used for the transformation with *GFP* reporter gene. To minimize the complicated gene expression effects caused by multiple transgene copies, only single-copy transgene plants were analyzed. Analysis of rice plants transformed with *GUS* and *GFP* with the *rubi*3 promoter with its 5' UTR intron showed a strong and constitutive expression of the reporter genes. Studies also confirmed a strong enhancement of the *rubi*3 promoter by its 5' UTR *rubi*3 intron at the whole plant level. With the intron, the *GUS* enzyme activity was about 3.3-fold higher in leaf, 26.5-fold in root, and 51.1-fold greater in callus tissue, suggesting that the intron mediated enhancement of the gene expression (IME) was tissue-dependent. Enhancement at the mRNA level was correlated to the GUS enzyme data. Detailed analysis revealed that the lower IME in leaf was mainly caused by higher expression level of the intronless construct in this tissue. The strong enhancement of the *rubi3* promoter activity by its 5' UTR intron presented a good system to further our understanding of the IME mechanism and activity.

Objectives of this study

When this project was started, there was no information regarding the inheritance of the *rubi*3 promoter expressing activity including the IME by its 5' UTR intron. Moreover, although the IME by the *rubi*3 5' UTR intron has been studied in detail with the *GUS* gene,

whether similar effects would be observed for other genes was not clear. Thus, in this project, our objects were:

1. To quantify and evaluate IME of the *rubi*3 5' UTR intron on *GFP* reporter gene in T0 transgenic rice plants.

2. To study the inheritance of activity of the *rubi*3 promoter and its 5' UTR intron on *GUS* reporter gene in T_1 transgenic rice plants with emphasis on the GUS enzyme activity within and between homozygous and hemizygous lines among the progeny of the transgenic rice plants.

Materials and Methods

Plant materials

To evaluate the intron enhancing effects on the *GFP* reporter gene, 6 independently transformed T_0 plants with the *GFP* gene (RP32, RP84A, RP31A, RIP20, RIP10A, and RIP17A) were obtained from Dr. J. Lu. The RP plants were transformed with the *GFP* gene driven by the *rubi3* promoter without the 5' UTR intron (pJLU12, Fig. 1). The RIP plants contained a similar construct, but had the intron between the *rubi3* promoter and the *GFP* gene (pJLU13, Fig. 1). All these plants were estimated by Southern blot analysis to have a single copy of the *GFP* transgene (Lu, 2006).

Ten independently transformed rice plant lines with the *GUS* reporter gene (RIAS2B, RIAS5B, RIAS8B, RIAS12A, RIAS24A, RIS32B, RIS33, RS33C, and RS35A) were used in a preliminary inheritance study. Each line included a T_0 plants as well as a hemizygous and one or two homozygous plants of the T_1 generation (obtained from Dr. Lu). As shown in Figure 1, the RIAS lines contained *rubi3* promoter + *rubi3* intron + the first 27 nucleotides of the coding sequence of the *rubi3* gene fused to the *GUS* gene in frame (pJLU6). The RIS lines were derived from a construct containing the *rubi3* promoter + *rubi3* intron + *GUS* (pJLU2), while the RS lines were transformed with the intronless *rubi3* promoter + *GUS* (pJLU7).

For further inheritance analysis, 20 T_1 plants from a line of each *GUS* gene construct were grown and analyzed. They were lines RIAS2B, RIS32B, and RS35A.

Growth conditions for rice plants

Transgenic T_0 *GFP* rice plants were grown in the greenhouse. The temperature was a constant at 25°C with natural light and day/night periods. Plants were grown in Metro Mix© 200 potting mix and fertilized with Osmocote 14:14:14 (N:P:K) (The Scotts Company, Marysville, OH). Pots were placed in trays filled with water. *GFP* plants were cut back to 3 inches above soil surface after seed collection to allow new tiller growth.

T_0 and T_1 GUS transgenic rice plants used for the preliminary inheritance experiment were maintained in the growth chamber at North Carolina State University (NCSU) Phytotron under the following conditions: 11.5 hours of daylight, 30°C/25°C light/dark period, light intensity of 575 $\mu mols^{-1}m^{-2}$ from both cool white fluorescent and incandescent lamps, and 50% relative humidity. Plants were watered every other day with nutrient solution. A detailed description of the composition of the nutrient solution as well as the operation protocol of the growth chamber can be found at http://www.ncsu.edu/phytotron/manual.pdf. Once seeds were collected, the T_0 plants were cut back to 3 inches above soil surface to allow for new growth.

For the more in-depth inheritance experiment, T_1 seeds from transgenic *GUS* rice plants were germinated in Petri dishes with 20 ml of water. Plates were incubated in a growth chamber at 25°C with 16 hour light period for 2 weeks. The water in the plates was refreshed every 3 days. Plantlets were transferred to sterilized surface soil and allowed to grow in growth chambers at the NCSU phytotron under the same conditions as the T_0 *GUS* plants.

ELISA assay to quantify GFP protein

Leaf samples were collected from the youngest leaves at the 5-leaf stage of the new tillers after cut back of the mature plants. About 0.7 g of leaf tissue or 1.2 g of fresh roots was collected per sample. The collected roots were washed with water and blotted to dry on paper towels, and then ground to powder in liquid nitrogen using pestles and mortars. Leaf tissues were frozen in liquid nitrogen before being ground to powder using a Silamat S5 amalgamator (Ivoclar Vivadent, Amherst, NY) for 15 seconds. Three hundred µl of PBS buffer (8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l KH_2PO_4, 1.44 g/l Na_2HPO_4, 0.2 g/l NaN_3, pH 7.4) was added to each leaf sample for protein extraction while 500 µl of PBS buffer was added to each root sample. The lysate was centrifuged for 2 minutes and the supernatant was collected for the assay. After 100 µl supernatant of each sample was added to a well of the 96-well microtiter plate and was incubated overnight at 4°C, the plate was blocked with 150 µl of 3% non-fat dry milk solution per well and incubated at 37°C for 1 hour. The plate was washed with PBST (PBS buffer + 0.5 ml/l Tween 20) and incubated at 37°C for another hour with 100 µl of *GFP* Ab-2 mouse monoclonal antibody (1:1000 dilution, Lab Vision Corp., Fremont, CA) per well, and then incubated with 100 µl of AP conjugated anti-mouse IgG (1:7500 dilution, Promega, Madison, WI) per sample at 37°C for 1 hour. Color was developed with an addition of 100 µl of p-nitrophenyl phosphate disodium salt (PNPP) (1-Step™ PNPP, Pierce, Rockford, IL). Absorbance readings were taken at 15 minutes at 405 nm wavelength using EL312e microplate bio-kenetics reader (Bio-Tek Instruments, Winooski, VT). The total protein concentration was determined using Protein Assay Kit I from Bio-Rad (Hercules, CA) according to the manufacturer's protocol.

MUG assay to quantify GUS enzyme activity

The quantitative MUG assays, as described by Jefferson et al. (1987) and Gallagher (1992), were followed. Leaf samples (T_0 and T_1) were collected from youngest leaves of the tillers at the 4-5 leaf stage. Four leaf-discs of approximately 32 mm^2 each were collected from each plant in a 1.5-ml Eppendorf® tube containing three glass beads (0.55 mm in diameter). Leaf tissue was quickly frozen in liquid nitrogen and homogenized to powder using a Silamat S5 amalgamator (Ivoclar Vivadent) for 10 seconds. Leaf powder was further ground in 2X LB solution (200 mM Tris-phosphate, pH7.8, 16 mM $MgCl_2$, 2 mM DTT, 2mM EDTA, 0.2 % v/v Triton X100, 30% v/v of 80% glycerol), and centrifuged at max speed (12,000 rpm) for 2 minutes and supernatant was collected for the assay. To keep the reading within the range of the standard curve, the original protein extracts were diluted with the same buffer 3X for extracts from plants of pJLU2, and 5X for extracts from plants of pJLU6 (Lu, 2006). The total protein concentration was determined using the Bio-Rad Protein Assay Kit I according to the manufacturer's protocol using an EL312e microplate bio-kenetics reader (Bio-Tek Instruments). Ten µl of the diluted extracts were added to 130 µl of MUG assay buffer (8.3 mg MUG in 20 ml 1X LB solution), and incubated at 37°C for 20 minutes. After incubation, 100 µl of the reaction was added to 100 µl of stop buffer (21.1 mg/ml Na_2CO_3). Fluorescence from the MUG reaction was read using the Fluostar® (BMG Labtech, Inc., Durham, NC) setting in fluorescence mode with excitation at 355 nm, emission at 460 nm, and gain at 0. MUG and protein assays were done in triplicate. The amount of MU released was calculated using a MU standard curve made with 25, 50, 250, 500, 2500, 5000 nmole of MU. The final *GUS* activity was measured as the nmole MU released per minute per mg of the total protein.

Northern blot analysis

Two hundred mg of young leaf tissue was collected, and total RNA was isolated using TRizol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommended protocol. RNA samples were dissolved in 20 μl of formamide, quantified using spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Uppsala, Sweden), and confirmed by gel electrophoresis. Thirty μg of RNA per sample was used for electrophoresis on a 1% (w/v) MOPS agarose gel. A PCR amplified product from pRESQ4 using *GUS*-IF (5'-CAA CGAACTGAACTGGCAGA-3') and *GUS*-IR (5'-TTTTTGTCACGCGCTATCAG-3') primer, or an amplified product from pJLU11 using Fs*GFP* (5'-GACCCGGGCCATGGGATCGATGCATCATC-3') and Rs*GFP* (5'-GGAGCGACTCTTACTTGTACAGCTCGTCCATGC-3') primers, was utilized as probes for detection of *GUS* and *GFP* mRNA, respectively. The PCR products were labeled with [α-^{32}P]-dCTP (Amersham Biosciences, Piscataway, NJ) and the Prime-it II® kit (Stratagene, Cedar Creek, TX) following the manufacturer's protocol. Gel was blotted onto the Hybond-N$^{+®}$ nylon membrane (Amersham). Blotting, hybridization, and washing of membrane were carried out according to the manufacturer's protocols. The washed membrane was exposed to a phosphor screen for 4 days. *GFP* mRNA level in each plant was quantified using the Storm™ 840 Image system (Amersham). Normalization of *GFP* mRNA level was done using *GFP* rRNA data. *GFP* rRNA level in each plant was also quantified using the Storm™ 840 Image system. Using the highest value of *GFP* rRNA as a reference point, we calculated the ratio between the highest *GFP* rRNA value and the rRNA value of each plant. *GFP* mRNA data was adjusted proportionally to those ratios.

Real-Time PCR

One inch segments of a fresh leaf were collected as a sample and placed in a well of a 96-well deep-well round bottom Costar 3958 plate with a Costar 3080 96-well cover. Samples were collected in duplicate. The zygosity of the T_1 plants was determined using Real-Time PCR (Ingham et al., 2001) by Syngenta Biotechnology, Inc. (Research Triangle Park, NC).

Data Analysis

Each plant extraction was read in triplicate for each MUG assay. The MUG assay was repeated twice so that six readings from each plant extraction were collected. The readings from ELISA assays for GFP plants were collected in a similar manner.

All calculations of MUG, ELISA and RealTime-PCR were done using the MS Excel software (Microsoft 2003). Analysis of variance (ANOVA) and Kruskal-Wallis test were performed using SAS V. 9.1.3 (SAS Institute, Cary, NC).

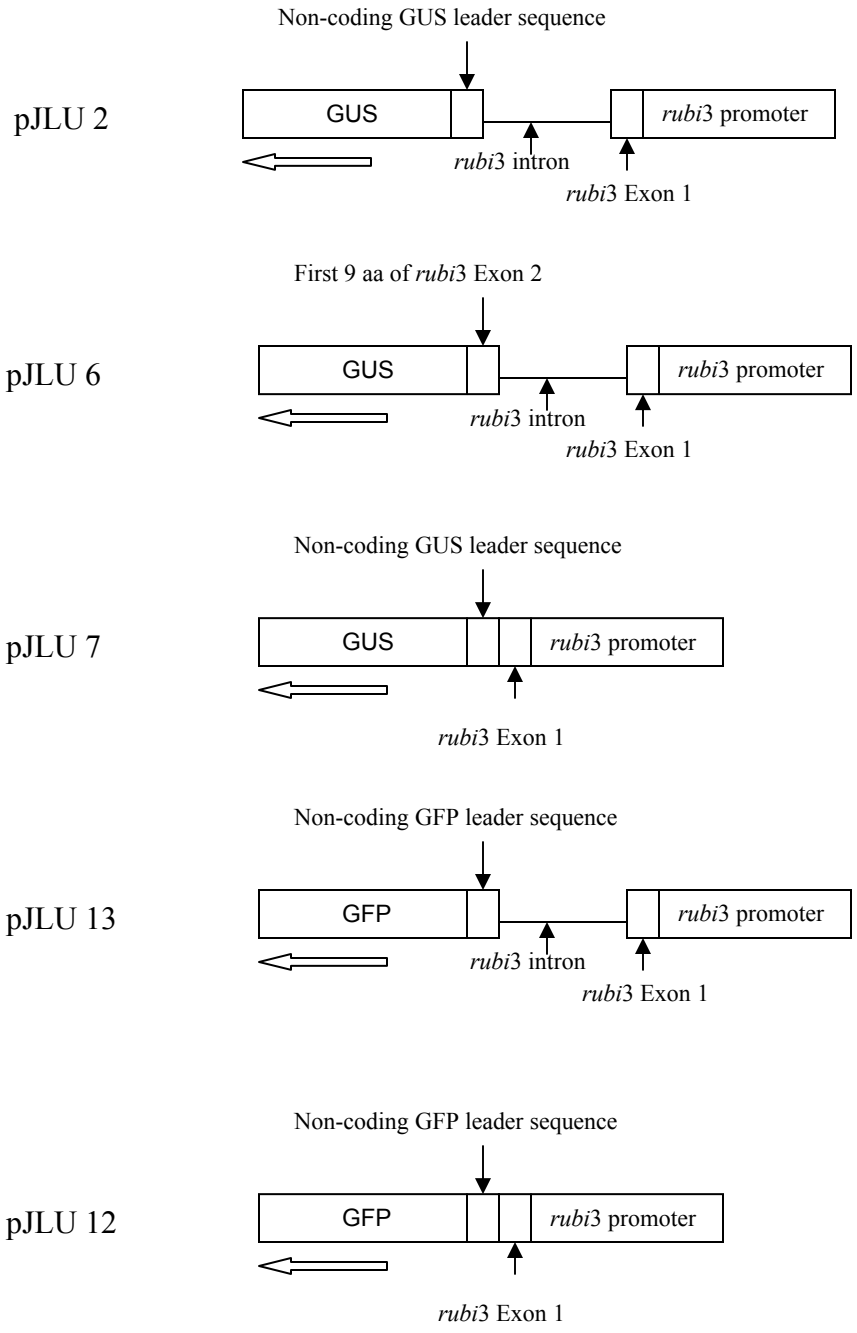


Figure 1. Constructs used in transgenic rice plants analyzed in this thesis. Plasmid details were described in Lu, 2006.

Results

Quantitative analysis of GFP in T_0 transgenic plants

In previous experiments, only visualization of GFP protein in transgenic plants was conducted. We were interested in quantitatively examining the *rubi3* promoter activity and the IME effect of its 5'UTR intron at whole plant level with a gene differing from *GUS*. Out of the 6 T_0 *GFP* transgenic plants obtained, 3 of them contained the 5' UTR intron (RIP lines from pJLU13, Fig. 1). The other 3 plants contained the construct without the *rubi3* 5' UTR intron (RP lines from pJLU12, Fig. 1). GFP protein levels in both fresh root and leaf samples were analyzed by ELISA assays (Richards et al., 2003).

Since there was no commercially available GFP to make a standard curve, we used the GFP values of six various amounts of total protein to construct the relative standard curves (Fig. 2A-D). These curves displayed a linear relationship between the GFP readings and the total protein amounts tested. Subsequent determination of GFP protein levels were all within the linear relationship range. Although moderate variations were present among lines from the same constructs, most likely caused by "positional effect", the average level of GFP protein in both fresh root and leaf samples was about 2.3 fold higher in RIP lines than in RP lines (Fig. 2E, F) in the ELISA assays. The assays also revealed that the expression level of GFP in fresh roots was approximately 7 fold higher than in leaf samples on per unit protein basis in each plant.

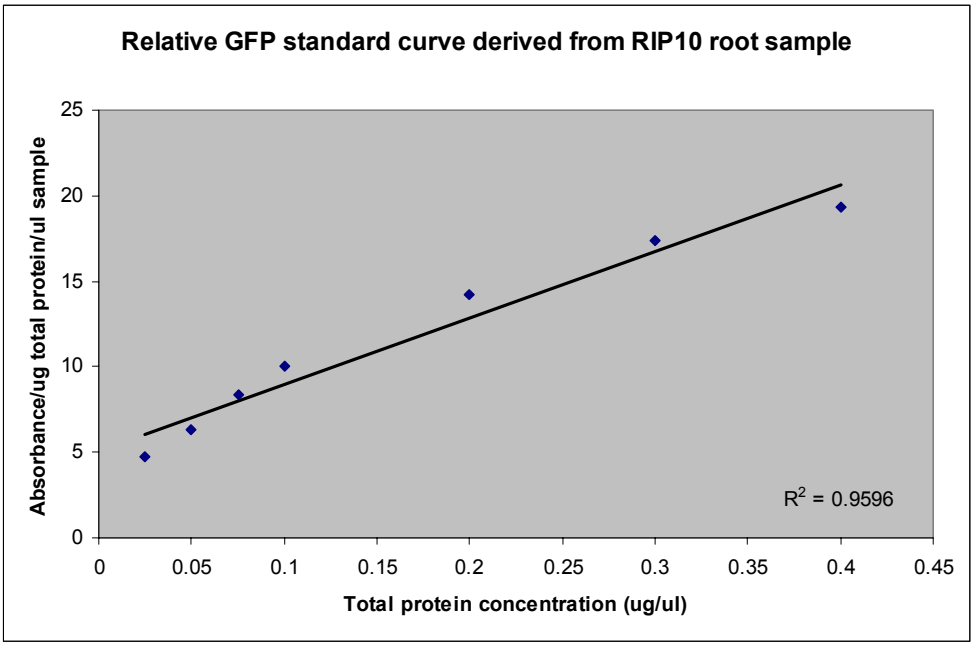
Northern analysis of the GFP *mRNA level in T_0 transgenic rice plants*

A Northern blot hybridization of total RNA from leaves of these plants was performed to determine whether the higher *GFP* expression in RIP plants was caused by higher levels of *GFP* mRNA. Phosphorimaging analysis revealed that on average, the amount of *GFP* mRNA of plants from the intron containing construct was approximately 2 fold higher than the *GFP* mRNA level in plants of the intronless construct (Fig. 3), suggesting the observed higher GFP protein level can be mostly attributed to the enhanced mRNA accumulation level of *GFP* in plants.

It is interesting to note that the IME of the *GFP* gene is very different from the IME of the *GUS* gene in these transgenic plants. Lu (2006) observed that in rice plants transgenic of the *rubi*3 promoter and the *GUS* gene, IME was 3.3 fold in leaf, but 26.5 fold in roots, and 51.1 fold in callus. He concluded that IME is tissue-dependent and the lower IME in leaves was caused by a relatively higher *GUS* mRNA level from the intronless construct. In our GFP plants, IME in roots was very similar to IME in leaves, and very different from the *GUS* gene case, this suggested that the extent of IME is affected by both tissue and, most likely, the gene sequence itself.

Figure 2. ELISA assays of GFP protein level in transgenic rice plants. All absorbance readings were taken after 30 minutes of incubation. To make relative standard curves, various protein concentrations were used from extraction supernatant. Each well was filled with 100 µl of the supernatant, and the absorbance unit was arbitrarily determined. A. Standard curve for GFP RIP root samples constructed using absorbance reading from plant RIP10A. B. Standard curve for GFP RP root samples constructed using absorbance reading from plant RP32. C. Standard curve for GFP RIP leaf samples constructed using absorbance reading from plant RIP10A. D. Standard curve for GFP RP leaf samples constructed using absorbance reading from plant RP32. E. ELISA of T_0 *GFP* leaf samples. F. ELISA of T_0 *GFP* fresh root samples. RIP = lines contain *rubi3* promoter + *rubi3* intron + *GFP*. RP = lines contain *rubi3* promoter alone + *GFP*.

A


Relative GFP standard curve derived from RIP10 root sample
Absorbance/ug total protein/ul sample
Total protein concentration (ug/ul)
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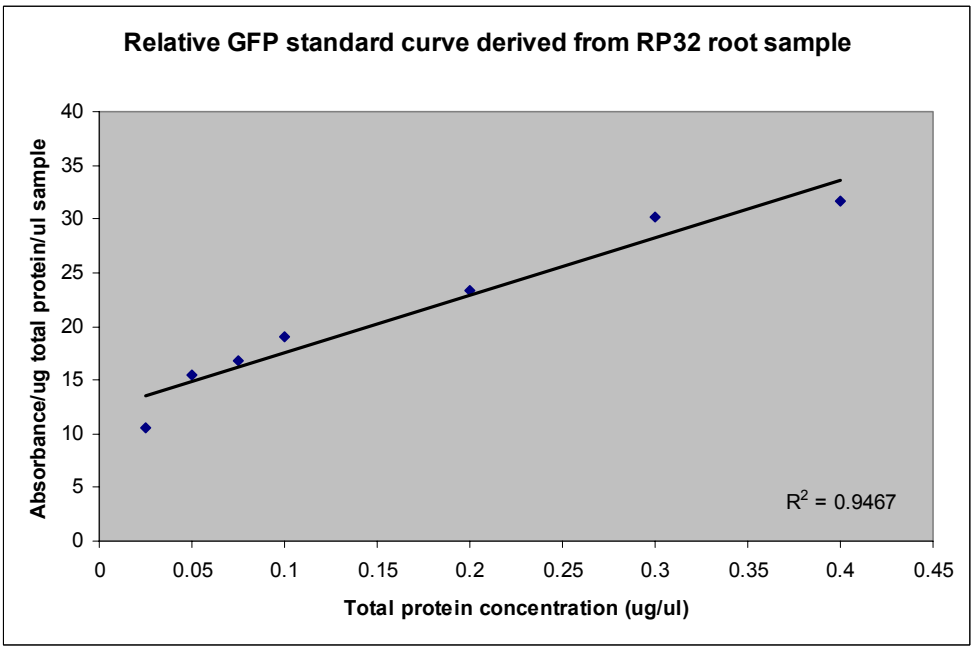
5
10
15
20
25
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45

B


Relative GFP standard curve derived from RP32 root sample
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Total protein concentration (ug/ul)
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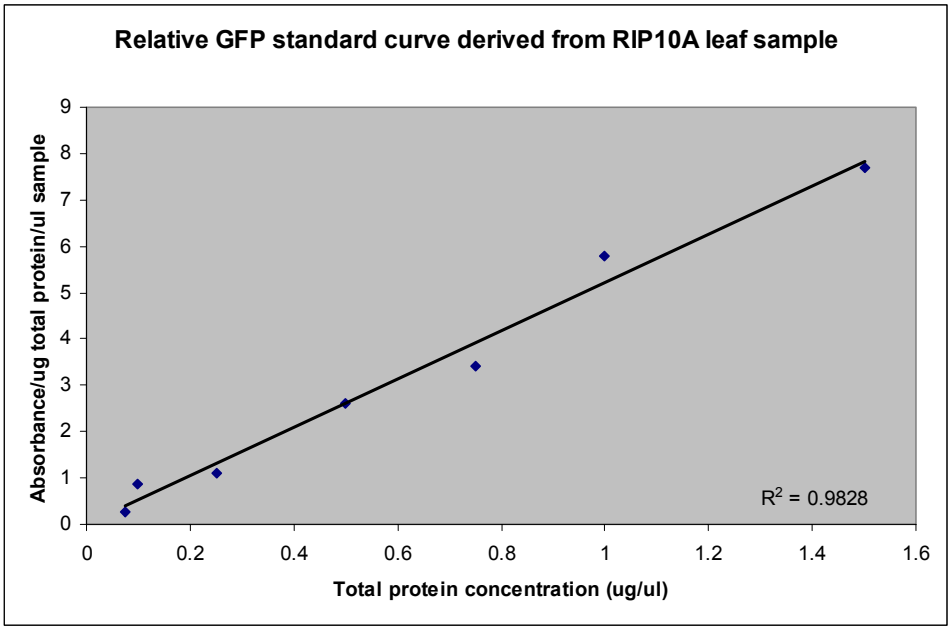
0.4
0.45

C


Relative GFP standard curve derived from RIP10A leaf sample
Absorbance/ug total protein/ul sample
Total protein concentration (ug/ul)
R2 = 0.9828
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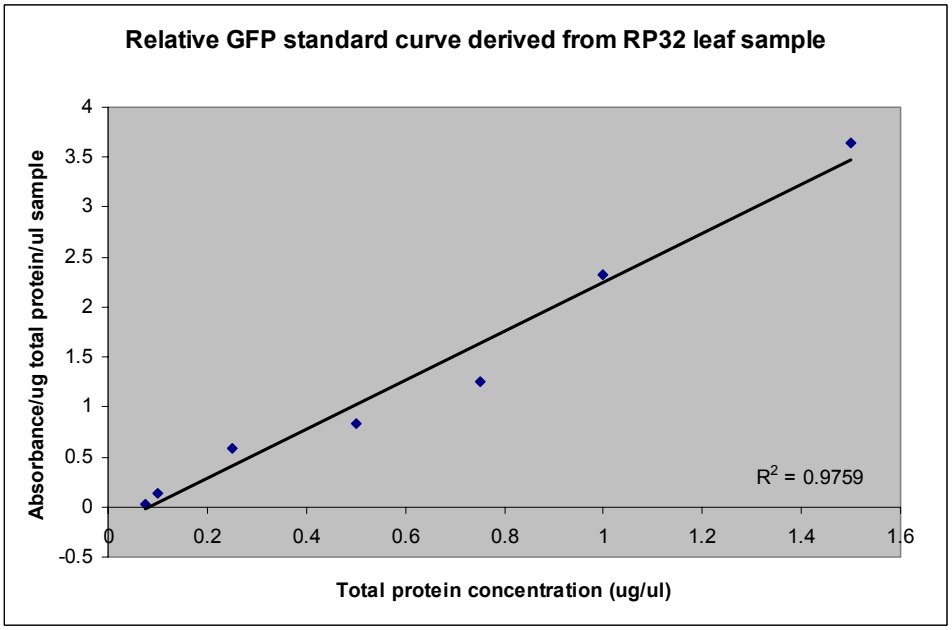
2
3
4
5
6
7
8
9
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6

D


Relative GFP standard curve derived from RP32 leaf sample
Absorbance/ug total protein/ul sample
Total protein concentration (ug/ul)
R2 = 0.9759
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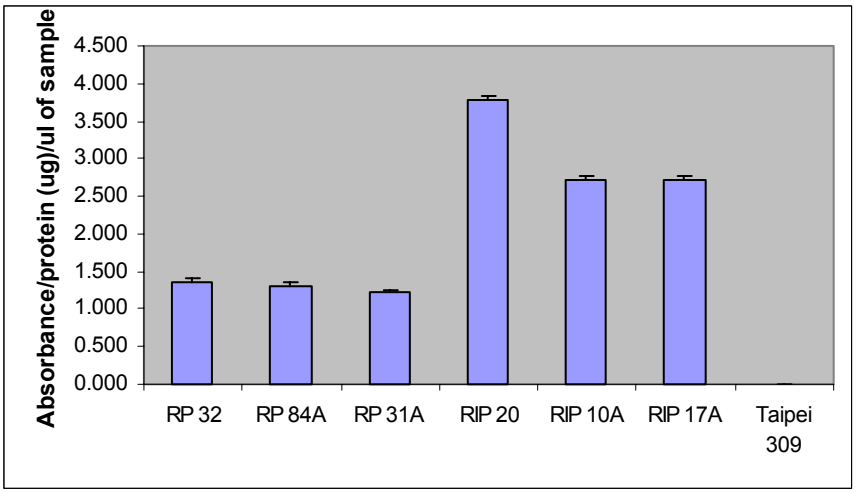
0
0.5
1
1.5
2
2.5
3
3.5
4
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6

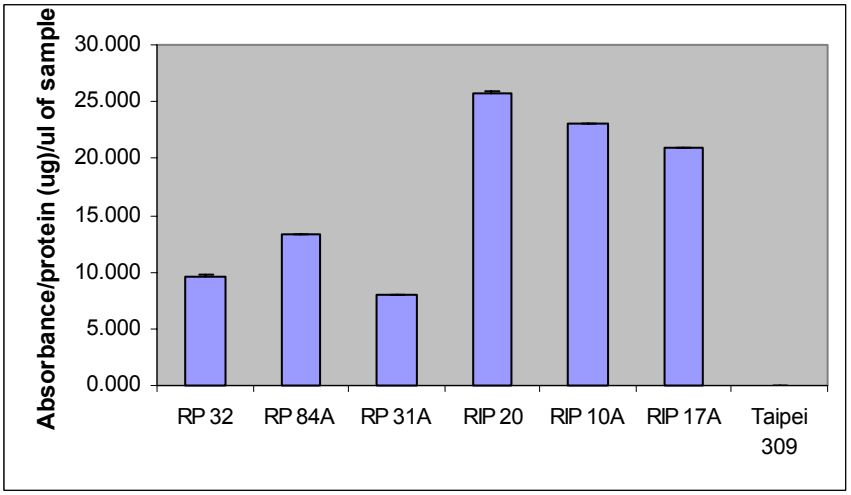
E

ELISA of T_0 GFP leaf samples

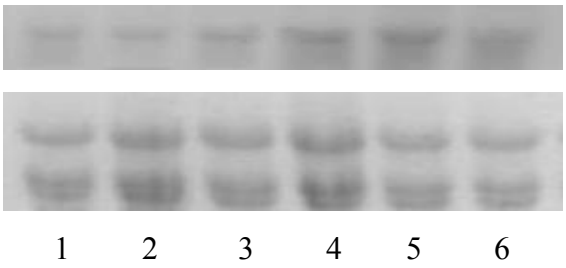


F

ELISA of T_0 GFP fresh root samples



A



B

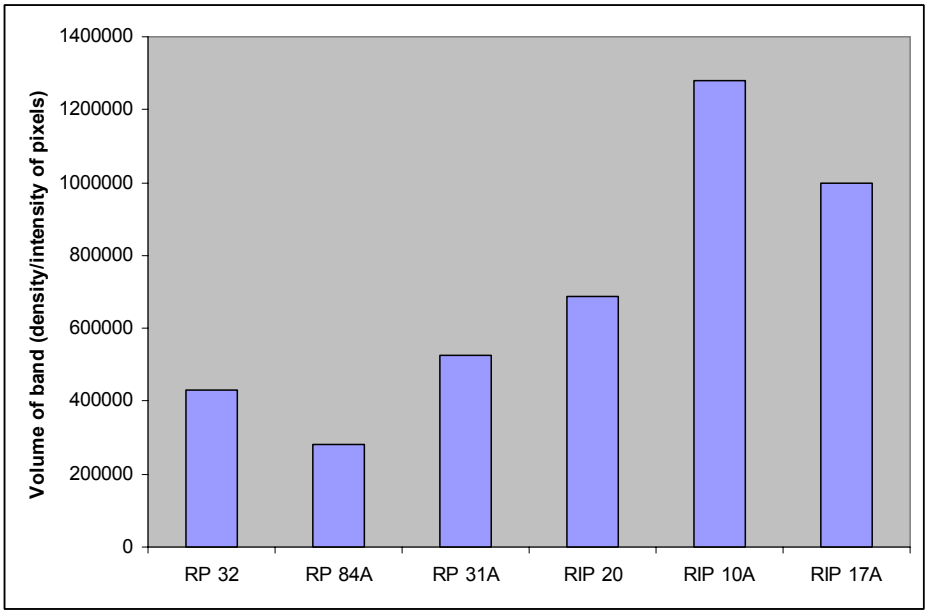


Figure 3. Northern analysis and normalized quantification of *GFP* mRNA levels in leaves of T_0. Volume was measured as density/intensity of pixels. Normalization was performed based on the quantification of the rRNA loading controls. A. Northern analysis *GFP* mRNA levels in leaves of T_0. Image was taken using phosphor-screen. 1=RP32, 2=RP84A, 3=RP31A, 4=RIP20, 5=RIP10A, 6=RIP17A. B. Normalized quantification of *GFP* mRNA levels.

Preliminary assay of the rubi*3 promoter activity on* GUS *expression in T_0 and T_1 plants*

To study the inheritance of the *rubi*3 promoter activity, a preliminary experiment was performed using *GUS* transgenic rice plants. Ten single-copy, independent transgenic lines from 3 gene constructs were examined. Five lines were derived from pJLU6 containing the *GUS* gene driven by the *rubi* promoter + the *rubi*3 5' UTR intron + 9 aa (RIAS lines). Two lines were from pJLU2 with the *GUS* gene driven by the *rubi*3 promoter + the *rubi*3 5' UTR intron (RIS lines), while 3 lines had the *GUS* gene driven by the *rubi*3 promoter alone (RS lines).

Due to space limitations and based on the assumption that all homozygous (or hemizygous) T_1 plants in the same transgenic line had an identical genetic background and would perform the same, only 1 hemizygous and 1 or 2 homozygous T_1 plants from each line were maintained and examined. The *GUS* enzyme activity in leaf differed among the lines transformed with different constructs. Expression levels in T_0 and T_1 plants of RIAS lines were higher than that of RIS and RS lines. MUG assays indicated that, on average, *GUS* enzyme activity in RIAS lines was 2 fold higher than RIS lines and 5 fold higher than RS lines (Fig. 4).

Six readings were collected from each plant for the GUS enzyme activity. The mean and the standard error of each plant analyzed are presented in Figure 4. ANOVA (p=0.05) was performed to examine whether there was significant difference among the plants (T0, and T1 homozygous and hemizygous) in each line. The results suggested a complicated picture of inheritance of *GUS* transgene expression. The analysis indicated that there was no significant

difference for the plants within line RIAS12A (p=0.12), line RIS33 (p=0.081), RS32A (p=0.088) and RS35A (p=0.235), implicating no difference in *GUS* gene expression between homo- and hemizygous plants in T1 generation and between T0 and T1 generations. The analysis revealed a significant difference in GUS enzyme activity between the plants within each line for lines RIAS2B (p=0.025), RIAS5B (p=0.0144), RIAS8B (p=0.021), RIAS24A (p=0.028), RIS32B (p=0.044), and RS33C (p=0.013).

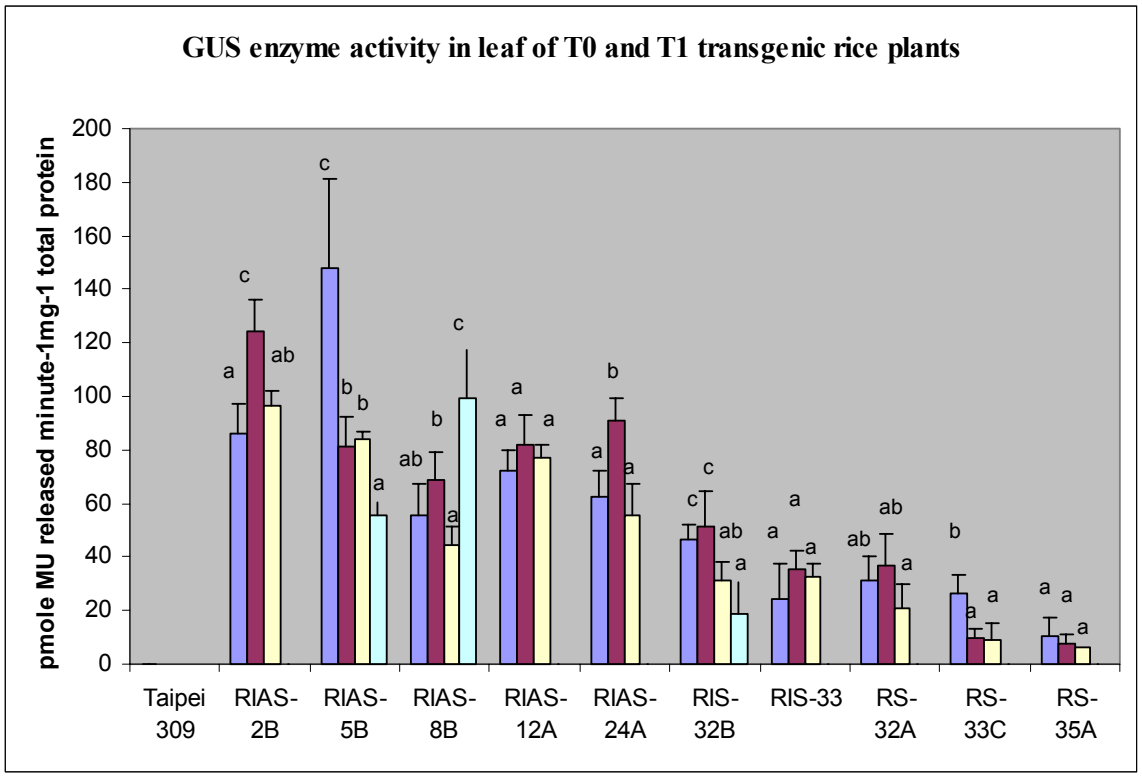


Figure 4. Preliminary assay of the *rubi*3 promoter activity on GUS expression in T_0 and T_1 plants. RIAS lines contain *rubi3* promoter + *rubi3* intron + 27 nucleotides of the first exon2 of the *rubi3* gene fused to the *GUS*. RIS lines contain *rubi3* promoter + *rubi3* intron + *GUS*. RS lines contain *rubi3* promoter alone + *GUS*. Blue columns are T_0 plants. Red columns are T_1 hemizygous plants. Yellow and green columns are T_1 homozygous plants. Bars are standard errors. Columns with the same letters within each line are not significantly different.

From this experiment, some unexpected phenomena were observed.

1. There were two homozygous T_1 plants in lines RIAS5B and RIAS8B. The *GUS* expression levels of the homozygous T_1 plants within each line differed significantly from each other, indicating that identical genetic background did not warrant similar transgene expression.

2. Except in RIAS8B, where a homozygous plant had significantly higher GUS activity than the hemizygous T_0 and T_1 plants, the hemizygous T_1 plants generally had similar or higher GUS enzyme activity when compared to the T_1 homozygous plants within the same line (Fig. 4), suggesting transgene expression in homozygous plants was generally not correlated to the transgene dosage.

3. In two lines (RIAS5B and RS33C), the GUS levels in T_1 homozygous and hemizygous plants were only about half of the T_0 parent plants, which may suggest progressive transgene silencing even in single-copy plants.

4. In two other lines (RIAS2B and RIAS24A), T_1 hemizygous plants had significantly higher GUS activity than their T_0 hemizygous parents.

Further inheritance study in T_1 transgenic rice plants

The above observation prompted us to further investigate the inheritance of the *rubi*3 promoter activity among T_1 transgenic plants. Sixty or so T_1 seeds from each of the 10 lines were germinated. However, the germination rates were quite low. Only 20-40 percent of the seeds from each line geminated. Only lines RIAS2B, RIS32B, and RS35A had more than 30 percent germination rate and over 20 plants per line, and thus were used for further analysis of the inheritance of the *rubi*3 promoter activity. Twenty plants from each line were randomly chosen for the analysis.

Histochemical assay of the leaves of T_1 plants showed a darker blue color for wells containing leaf samples from line RIAS2B (Fig. 5). *GUS* expression in wells containing samples from lines RIAS2B and RIS32B leaked into the buffer resulting in blue wells whereas the expression of *GUS* was contained onto the samples themselves for line RS35A. The assay visually illustrated a considerable difference in expression level between offspring plants transformed with different constructs. The observation was confirmed and further quantified by the MUG assay results (Fig. 6A, 7A, 8A).

PCR results from the plants analyzed matched with the histochemical assay and MUG data and showed the expected *GUS* fragment of 799 bp (Fig. 6B, 7B, 8B). Samples with positive results for one assay were also positive for the other two. Six out of 20 T_1 RS35A plants (30%), 3 out of 20 T_1 RIAS2b plants (15%), and eight out of 20 T_1 RIS32B plants (40%) were negative for the *GUS* enzyme activity assays and PCR (Fig. 5, 6B, 7B, and 8B). These plants were segregated with no transgene.

We used the chi-square test to determine whether the segregation ratio between the offspring containing *GUS* to those without *GUS* follows the 3:1 ratio under Mendel's law. The test looks at the difference between the predicted results and the observed results to determine the probability (P) of whether the difference is due to random chance alone. The higher the probability, the more likely it is that any differences from the results are just due to random chance and that the results support the prediction that the plants segregated according to Mendelian fashion. A lower probability means that it is less likely that the differences from the results are due to random chance, so the differences are significant and the results do not support the prediction. Using the standard 0.05 probability level as our critical value, Chi-square tests suggested that the offspring plants containing *GUS* to those without *GUS* were consistent with the expected 3:1 ratio under Mendel's law with p-value of 0.3 for RIAS2B line, 0.1-0.2 for RIS32B line, and 0.5-0.7 for RS35A.

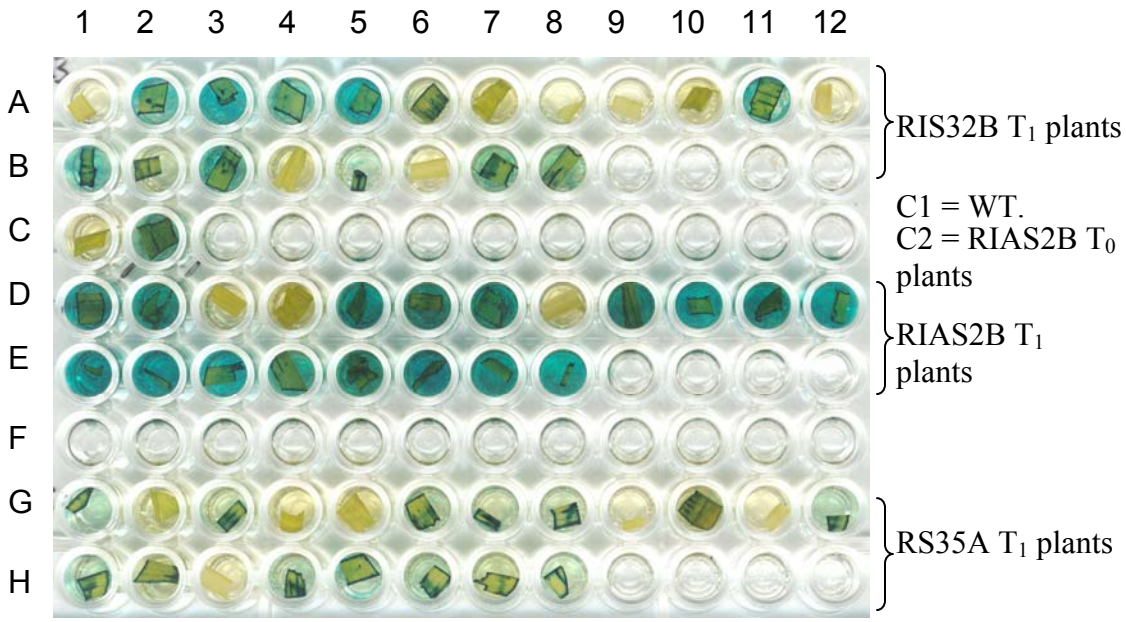


Figure 5. Histochemical assay of T_1 transgenic rice leaf. Leaf samples were incubated in GUS assay buffer at 37°C overnight. As indicated, rows A1-A12 and B1-B8 were leaf samples from RIS32B T_1 plants. C1 was the non-transformed negative control Taipei 309 (WT). C2 was the RIAS2B T_0 plant. Rows D1-D12 and E1-E8 were leaf samples from RIAS2B T_1 plants. Rows G1-G12 and H1-H8 were leaf samples from RS35A T_1 plants.

The zygosity of T_1 *GUS* plants was determined using Taqman RealTime PCR. Plants having a relative copy number from 0.4 – 1.2 are considered hemizygous while plants containing relative copy number between 1.5 and 2.4 are considered homozygous.

We included plants numbered RIAS2B, RIAS2B7E, RIAS2B17O, RS35A, RS35A9E, RS35A10O as control plants to test the accuracy and repeatability of the relative *GUS* copy number generated by RealTime PCR. The zygosity of these six plants was previously determined using the same method (Lu, 2006). The zygosity of these control plants in this test was consistent with previous findings, indicating that the method was reliable. However, the relative *GUS* gene copy number of a few plants were not consistent with PCR and MUG assays (RIS32B7, RS35A11) or too high for even homozygous plants (RIS32B19 and RIS32B20), and they were not included in further analysis.

Only GUS enzyme activity in leaf tissue of the T_1 homozygous and hemizygous plants were measured and analyzed. The trend of expression continued to emphasize the difference among the constructs. Overall, on average, the *GUS* expression among the RIAS2B T_1 plants was 2.5 fold higher than RIS32B T_1 plants and 6.7 fold higher than RS33A T_1 plants (Figs. 6-8).

In line RIAS2B, there were 9 hemizygous and 8 homozygous plants among 20 T_1 plants analyzed. Significant variations on GUS enzyme activity were observed within each category (Fig. 6D, Table 1, 2). In hemizygous plants, 4 plants clustered at higher levels (82-106 nmole MU/min/mg protein, the unit is the same for values described below), about 2 fold

of the levels (42-64) of another cluster of five plants (Fig. 6D). The clustering in homozygous plants was not very obvious, but the expression levels among these plants also differed significantly (Table 2). The mean value of hemizygous plants was 67.2, slightly higher than 61.8 of the mean of homozygous plants in this line. However, a Kruskal-Wallis test ($P=0.6009$) indicated that there was no significant difference at $P>0.05$ between the mean GUS enzyme activity of the hemizygous and the homozygous plants. Thus, the gene dosage did not appear to have an effect on the expression level for this line.

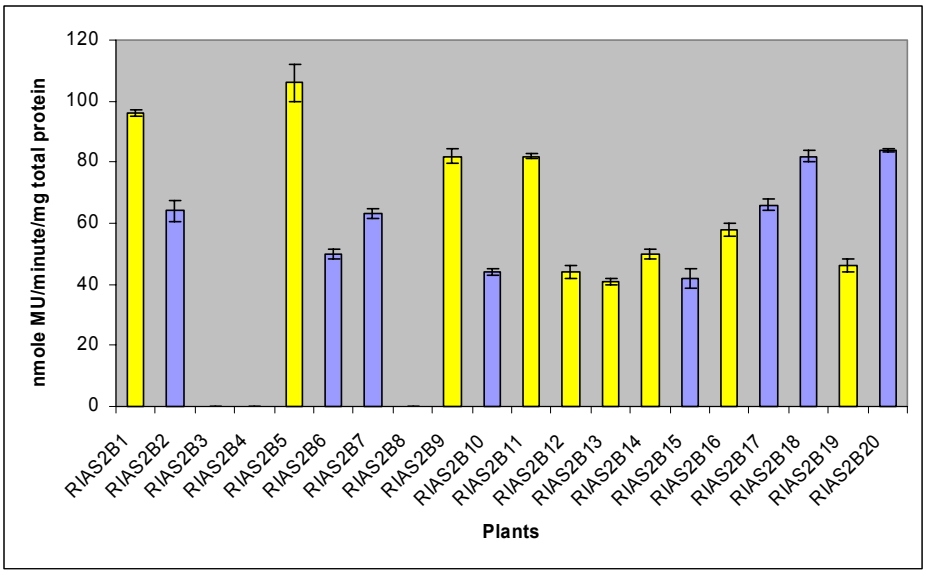
In line RIS32B, all 5 hemizygous plants had relatively high expression and the levels were close to each other (30-38) (Fig. 7C) but statistical analysis still indicated significant difference ($p<0.05$, Table 3). The mean GUS activity of hemizygous plants was 35.2 (Table 7). However, in the five homozygous plants analyzed, four clustered between 16 to 21 with one being lower than 8. Significant variation within the homozygous category was also observed ($p < 0.0001$, Table 4) and the mean value was 18.6 (Table 7). Overall, the GUS expression level in homozygous plants was only nearly half of the level of the hemizygous plants in this line. A Kruskal-Wallis test indicated that the mean GUS enzyme activity of the hemizygous plants differed highly significant ($p<0.001$) from the mean of the homozygous plants.

Among T_1 plants of line RS35A, significant variations of GUS enzyme activity were observed within each category ($p < 0.0001$, Table 5,6). In 11 hemizygous plants, the values ranged from 4 to 14 with a mean of 8.7 (Table 7). There were only 3 homozygous plants in 20 T_1 plants of this line, with 2 having GUS activity at 15 to 16 and the other at 7. The mean

was 12.7 (Table 7), which was about 50% higher than the mean of hemizygous plants (8.7). Despite the difference in the means, statistical analysis (Kruskal-Wallis test P=0.1064) of the means of GUS activity between hemizygous plants and homozygous plants suggested that there was no significant difference at P=0.05.

Figure 6. PCR and MUG assays of RIAS2B T_1 plants. A. GUS enzyme activity for RIAS2B T_1 plants. B. PCR results for RIAS2B T_1 plants. C. Controls for PCR data. C1, C2 = Taipei 309 non-transformed plants. P = positive plasmid control. Expected band size = 799 bp. D. The MUG assay data in A were reorganized for clearer visual evaluation of the *GUS* gene expression in the two zygosity categories. Yellow bars represent hemizygous plants. Blue bars represent homozygous plants in A and D.

A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100
80
60
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RIAS2B6
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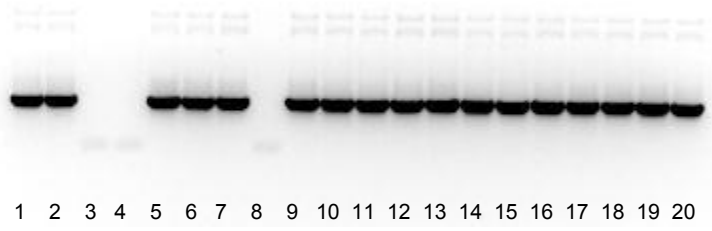
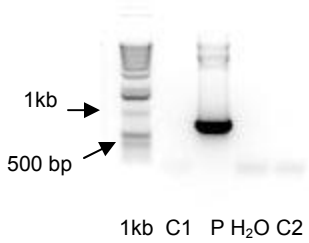
RIAS2B8
RIAS2B9
RIAS2B10
RIAS2B11
RIAS2B12
RIAS2B13
RIAS2B14
RIAS2B15
RIAS2B16
RIAS2B17
RIAS2B18
RIAS2B19
RIAS2B20
Plants

.

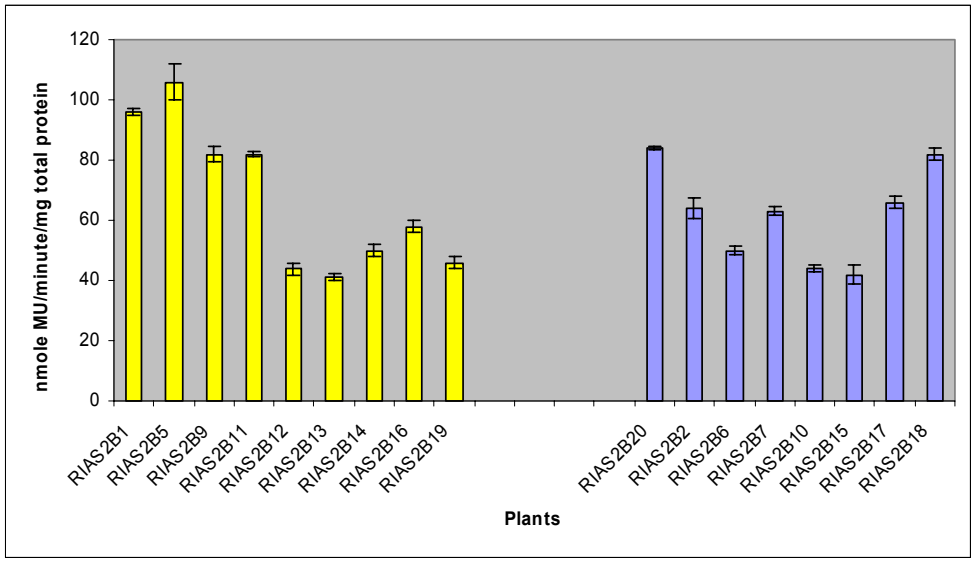
B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

C


1kb
500 bp
1kb C1 P H2O C2

D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100
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60
40
20
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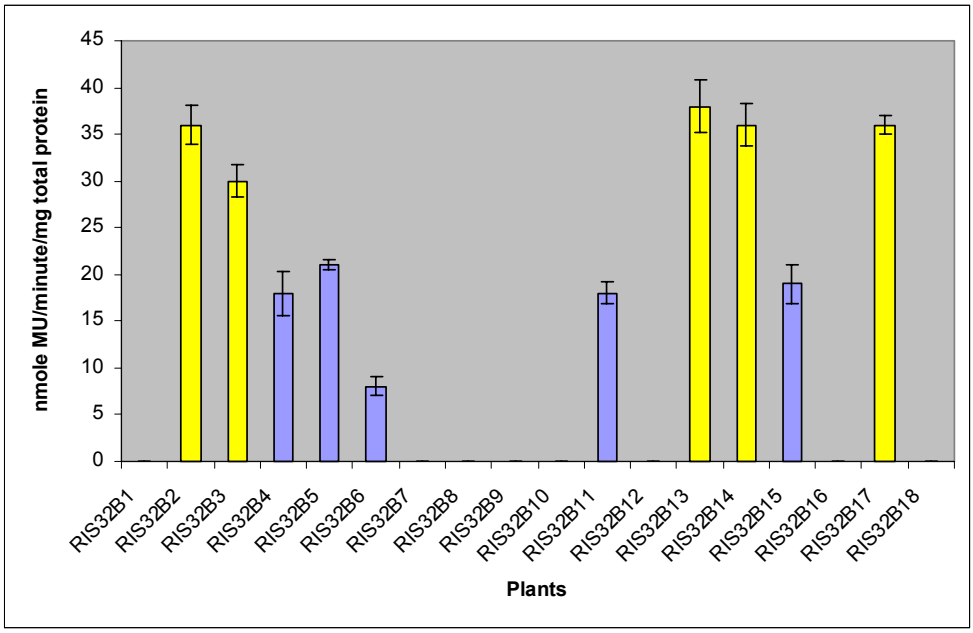
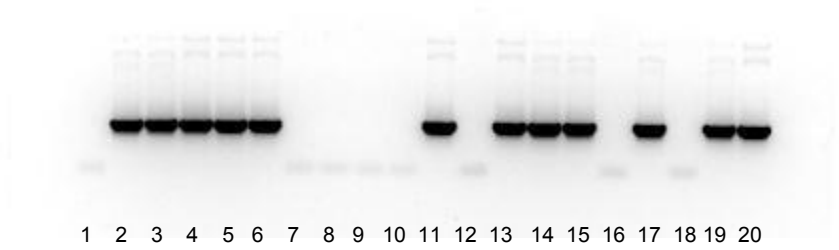
RIAS2B13
RIAS2B14
RIAS2B16
RIAS2B19
RIAS2B20
RIAS2B2
RIAS2B6
RIAS2B7
RIAS2B10
RIAS2B15
RIAS2B17
RIAS2B18
Plants

Figure 7. **PCR and MUG assays for RIS32B T_1 plants**. A. GUS enzyme activity for RIS32B T_1 plants. B. PCR results for RIS32B T_1 plants. C. The MUG assay data in A were reorganized for clearer visual evaluation of the *GUS* gene expression in the two zygosity categories. Yellow bars represent hemizygous plants. Blue bars represent homozygous plants in A and D.

A



B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

C

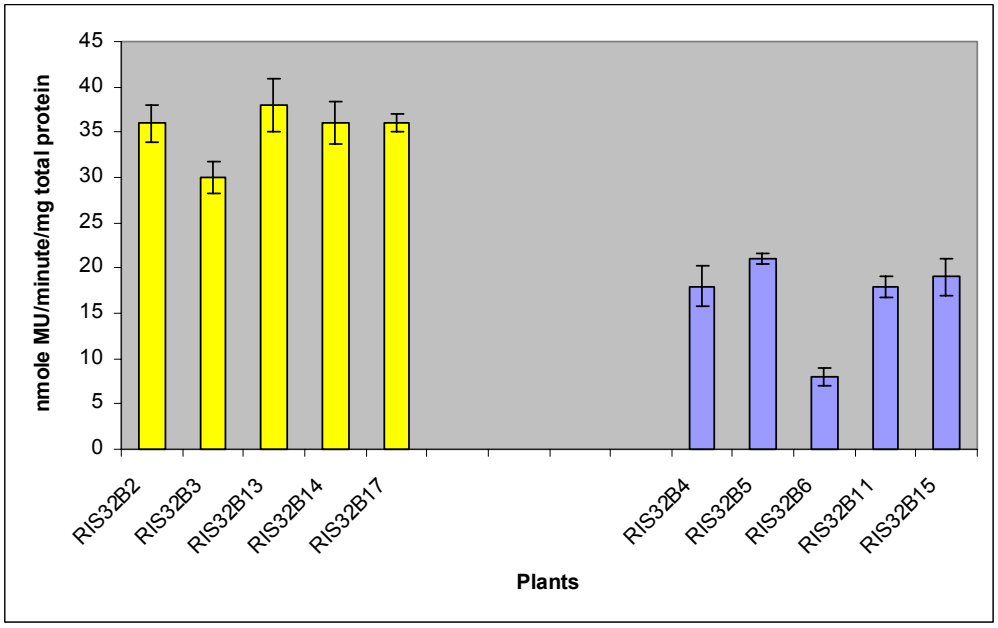
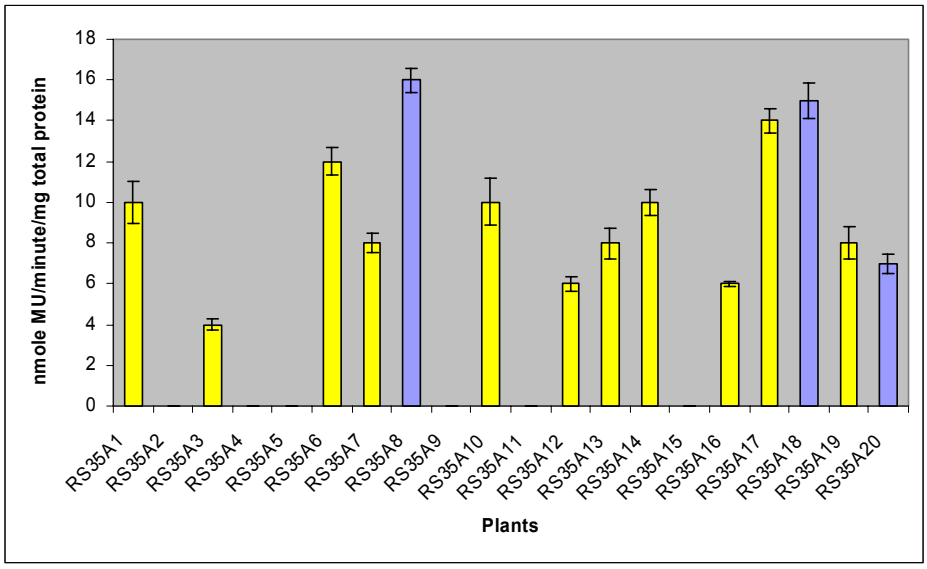
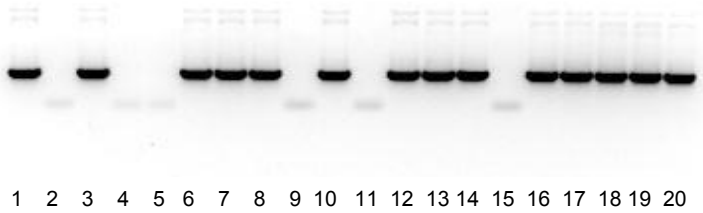

nmole MU/minute/mg total protein
45
40
35
30
25
20
15
10
5
0
RIS32B2
RIS32B3
RIS32B13
RIS32B14
RIS32B17
RIS32B4
RIS32B5
RIS32B6
RIS32B11
RIS32B15
Plants

Figure 8. **PCR and MUG assays of RS35A T1 plants**. A. GUS enzyme activity for RS35A T_1 plants. B. PCR results for RS35A T_1 plants. C. The MUG assay data in A were reorganized for clearer visual evaluation of the *GUS* gene expression in the two zygosity categories. Yellow bars represent hemizygous plants. Blue bars represent homozygous plants in A and C.

A



B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

C

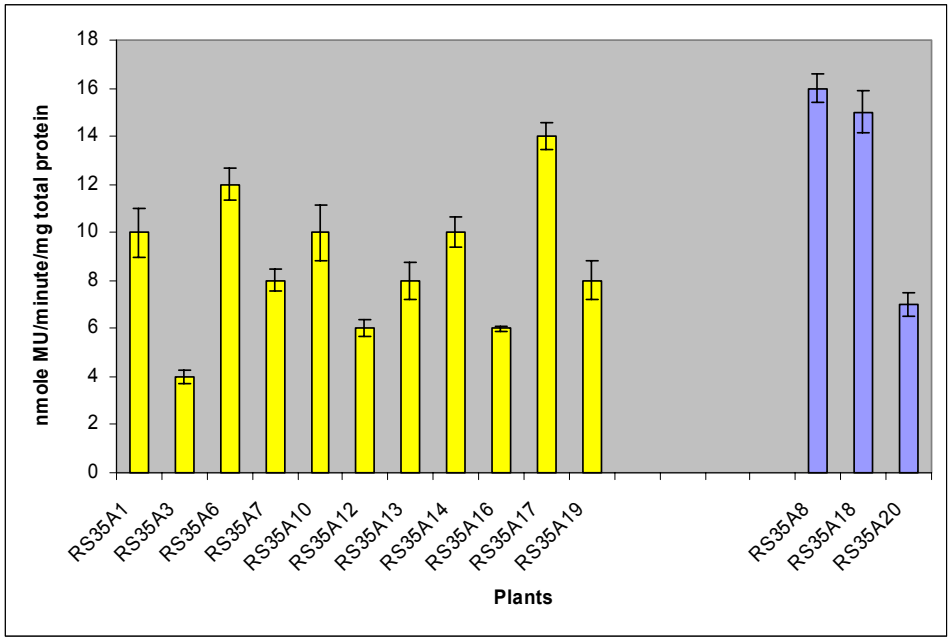

nmole MU/minute/mg total protein
0
2
4
6
8
10
12
14
16
18
RS35A1
RS35A3
RS35A6
RS35A7
RS35A10
RS35A12
RS35A13
RS35A14
RS35A16
RS35A17
RS35A19
RS35A8
RS35A18
RS35A20
Plants

Table 1. ANOVA analysis of T_1 hemizygous RIAS2B plants

Source	DF	Mean Square	F Value	Pr > F
Plant	8	1820.33	255.98	<.0001
Error	18	7.11		

Table 2. ANOVA analysis of T_1 homozygous RIAS2B plants

Source	DF	Mean Square	F Value	Pr > F
Plant	7	773.97	185.75	<.0001
Error	16	4.16		

Table 3. ANOVA analysis of T_1 hemizygous RIS32B plants

Source	DF	Mean Square	F Value	Pr > F
Plant	4	26.433	6.01	0.0099
Error	10	4.40		

Table 4. ANOVA analysis of T_1 homozygous RIS32B plants

Source	DF	Mean Square	F Value	Pr > F
Plant	4	77.17	31.28	<.0001
Error	10	2.47		

Table 5. ANOVA analysis of T_1 hemizygous RS35A plants

Source	DF	Mean Square	F Value	Pr > F
Plant	10	26.86	57.19	<.0001
Error	22	0.47		

Table 6. ANOVA analysis of T_1 homozygous RS35A plants

Source	DF	Mean Square	F Value	Pr > F
Plant	2	62.12	143.72	<.0001
Error	6	0.43		

Table 7. Analysis of GUS enzyme activity in T_1 hemizygous and homozygous rice plants of each line. Column entries for mean GUS activity with different letters in a line are significantly different (P<0.05) by the Kruskal-Wallis test.

Plant line	Zygotic state	Number of T_1 plant	Mean GUS activity	LSD	Coefficient of Variation
RIAS2B	Hemizygous	9	67.2**a**	7.6	3.9
	Homozygous	8	61.8**a**	5.8	3.3
RIS32B	Hemizygous	5	35.1**a**	5.6	5.9
	Homozygous	5	16.7**b**	4.2	9.4
RI35A	Hemizygous	11	8.7**a**	2	7.8
	Homozygous	3	12.5**a**	1.6	5.3

Discussion

The effect of intron on gene expression

Intron-mediated enhancement (IME) of gene expression has been reported and studied for over 20 years. The enhancement is often quite large, and the mechanism was not well understood for quite a while. In the past several years, studies mostly in mammals revealed that transcription and mRNA processing, including intron splicing, is a coupled process (Bentley, 2002, Proudfoot et al., 2002), and the intron does enhance transcription to some extent (Furger et al., 2002) when the intron is proximal to the promoter. A quantitative analysis on the IME in mammals revealed that introns primarily enhance cytoplasmic mRNA accumulation, but not the nucleocytoplasmic mRNA distribution. IME, however, does not enhance the mRNA stability while the spliced mRNA also had higher translational yield (Nott et al., 2003). A protein complex, called exon-exon junction complex or EJC, was found to deposit 20-24 nucleotides upstream of mRNA splicing sites (Le Hir et al., 2000), and it is the EJC and not the mRNA splicing itself that is responsible for the observed intron-mediated enhancement of gene expression (Wiegand et al., 2003). Moreover, intron also enhances translation, which can be attributed to some EJC proteins or other related proteins accompanying the spliced mRNA to the cytoplasm and shuttling back to the nucleus (Nott et al., 2004, Sanford et al., 2004).

In dissecting the intron effects on enhanced gene expression of the rice *rubi3* promoter, Samadder et al. (2007) used stably transformed rice cell lines and found that the *rubi3* 5' UTR intron enhanced GUS enzyme activity by 29 fold. Nuclear run-on experiments revealed

a 1.9 fold increase at the transcriptional level while the *GUS* mRNA accumulation increased by nearly 20 fold. The data also suggested about 50% enhancement at translational level. Lu (2006) analyzed the *rubi3* promoter in transgenic rice plants, and observed a tissue-dependent pattern of the IME on the *GUS* transgene expression. Intron enhanced GUS enzyme activity by 3.3 fold in leaves, 26.5 fold in roots, and 51 fold in calli. While the intron-containing construct had high *GUS* expression in all the tissues, the low enhancement in leaves was mostly due to the higher GUS activity in leaves (6.2 fold higher than in roots) of the intronless transgenic plants, which was mainly caused by the higher accumulation of the *GUS* mRNA in the leaves.

The enhancement of rubi3 5' UTR intron on GFP *gene*

The goals of this project were to investigate whether IME on *GFP* transgene by the *rubi3* 5' UTR intron is similar to that on *GUS* reporter gene, and to study the inheritance of the *rubi3* promoter activity, including the IME caused by its 5' UTR intron. For the first goal, we observed similarity of IME in leaves but a clear difference in roots between the *GUS* and the *GFP* reporter genes. The GFP protein levels in the leaves and roots were both enhanced by the intron by only 2.3 fold. Similar to GUS results, data from leaves suggested that the low GFP enhancement can mostly be attributed to the elevated mRNA accumulation in plants from the intronless construct. However, the IME of *GFP* expression in roots was quite different from the IME of *GUS* expression in the same tissue. In the *GUS* gene case, the IME effect in roots was high (26.5 fold) whereas the IME of *GFP* expression was only 2.3 fold. The results indicate that IME is not only affected by tissues, as observed by Lu (2006), but also by the genes. That IME is gene dependent was first proposed by Rethmeier et al. (1997)

when the authors observed the rice *salT* intron enhanced mRNA and protein expression of the *cat* gene but not the *bar* gene expression in comparable constructs using the CaMV 35S promoter in transient assays with maize suspension cells. Our observation suggests that both tissues and the genes are effectors of IME. Further analysis of mRNA accumulation in root tissues of plants transformed with intron-containing and intronless GFP constructs is important to understand whether the low IME in roots at protein level is based on mRNA accumulation level.

The activity and inheritance of rubi*3 5' UTR intron in transgenic* GUS *rice plants*

Another goal of the project was to study the inheritance of the *rubi3* promoter, especially the intron-containing one and the one with further enhancement by fusing the first 9 amino acid coding sequence to the *GUS* gene. Commercialization of transgenic cultivars demands the transgene expression be stably transmitted to progeny over generations. For self-pollinated species, such as rice, it is a common practice that homozygous plants are used for transgenic breeding programs and cultivar release. To consider the application of the *rubi3* promoter for commercial use, it is important to collect information on its expression in offspring generations. In this study, use of plants with single transgene copy minimizes the potential silencing effect of multiple transgene copies (Nadolska-Orczyk et al., 2002). In inheritance study of the *rubi3* promoter activity, a preliminary experiment indicated that enhancements of the *GUS* gene expression by the 5' UTR intron and the first 9 amino acid coding sequence of the *rubi3* gene were generally transmitted to the next generation. However, it was also observed that homozygous plants from the same transgenic line may not express the *GUS* gene at the same level even though they had identical genetic background. Moreover, quite

often homozygous plants did not express the *GUS* transgene higher than the hemizygous plants, as usually assumed. The observations prompted us to further investigate the expression pattern in the T_1 generation. Poor seed germination rates in most of the transgenic lines hindered our efforts but we eventually were able to study three lines, one from each of the three constructs, with each line having more than 20 seedlings.

Analysis of GUS activity in T_1 rice plants

Chi-square tests (critical p-value = 0.05) suggested that the segregation of the seedlings was in accordance with the expected 3:1 ratio under Mendel's law with a p-value of 0.3 for the RIAS2B line, 0.1-0.2 for the RIS32B line, and 0.5-0.7 for the RS35A. Among the plants containing the transgene, the ratios of the homozygous versus the hemizygous plants were skewed (Table 7). This could be an attribute of the small sample number. From the data collected from the experiment, significant difference in GUS activity was observed within homozygous and hemizygous plants in each line analyzed (Table 1-6), indicating transgene expression instability in offspring plants even though they had identical genetic background. Among the three lines analyzed, there was no correlation between expression level and transgene dosage (Table 7). The hemizygous plants significantly out-performed the homozygous plants in line RIS32B. However, statistical analysis suggests no significant difference on the performance between the T_1 hemizygous plants and the homozygous plants in two other lines (Kruskal-Wallis test, P=0.6009 for RIAS2B, and P=0.1064 for RS35A).

The relationship between homozygous and hemizygous of the T1 transgenic rice plants

It appears that the stability of transgene expression in offspring generations is generally low, and MARs can improve it to some extent (Ulger et al., 1999; Vain et al., 1999). Vain et al. (2002) studied transgene expression over two generations in 95 transgenic rice plants obtained by particle bombardment (usually yielding multiple transgene copies), and observed that only 7 % of the plants without MARs and 17% of the transgenic plants with the MARs had stable transgene expression over two generations. In a more detailed study on the relationship between homozygous and hemizygous transgene expression levels over generations in transgenic rice plants with multiple transgene copies, James et al. (2002) also observed substantial variations of transgene expression within each category of plants. Moreover, although some lines showed stable transmission of transgene expression, segregation, and gene dosage effect (i.e., homozygous plants expressing higher than hemizygous plants), lower expression of transgene in homozygous offspring plants were observed in many other lines, and homozygosity appears to be often disadvantageous in transgene expression. Together with the results from our experiments, it seems this is not an uncommon phenomenon. This observation suggested that when applying genetic engineering to a breeding program, one may need to generate a large number of transgenic plants, and also look at a relatively large population in the offspring generations to identify individuals which have not only ideal transgene expression level at the T_0 generation but also stable inheritance in transgene expression.

The various transgene expression levels within the homozygous and hemizygous categories seem to be very common no matter whether the plants have multiple transgene copies or

have just one (James et al., 2002; this report), and the causes are unknown. Considering the plants within each category share identical genetic background, the variation in expression is most likely caused by epigenetic effect, such as DNA methylation, which sometimes takes place on transgenes. It is known that DNA methylation is a progressive and uneven process and is reset at every generation (Bender, 2004). Thus the variation on the extent and pattern of the transgene methylation among individual offspring plants could generate various transgene expression levels.

Although it appears superficially from our data that different constructs may vary in their transgene expression inheritance pattern, such as variations between and within homozygous and hemizygous categories, it may be too early to draw such a conclusion since we analyzed only one line from each construct mostly due to the poor T_1 seed germination rates of the transgenic lines. To draw a reliable conclusion on transgene expression inheritance regarding various gene constructs used in this experiment, dozens more transgenic lines from each construct probably would need to be analyzed.

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Appendix:

Introduction of the Trehalose-6-phosphate Synthase/Phosphatase (TPSP) Gene into Spring Wheat (*Triticum aestivum* L.)

Introduction

Wheat has always played a vital part in human diet. The first domesticated wheat can be dated back to 10,000 years ago in the area known as the Fertile Crescent in southwest Asia (Kingfisher, 2004). Einkorn and Emmer wheat were two of the earliest wheats cultivated. Over the several hundred years of cultivation, these wheats crossed with wild relative grasses growing in the same fields and eventually gave rise to the free-threshing tetraploid (*T. durum*), and hexaploid (*T. aestivum*, or common wheat) as known of today (Hancock, 2004). According to the UN Food & Agriculture Organization (FAO), 626 million metric tons of wheat was grown worldwide in 2005. In the US alone, more than 57 million metric tons of wheat was produced for the year 2005/2006 (Vocke and Allen, 2006). With such a huge impact both agriculturally and economically, wheat improvement has been a priority for countless breeders and other scientists, and a focus for those in the field of plant genetic engineering in recent years.

Plant genetic engineering has opened doors for rapid improvement of many of wheat traits with great agronomical and economic importance. Quality of wheat flour was improved by the introduction of high molecular weight glutenin subunit (1Dx5) into *T. aestivum* L. (Rooke et al., 1999). Hu et al. (2003) obtained wheat resistance to herbicide (Roundup®) by transforming the plant with the glyphosate tolerant aroA: CP4 gene encoding a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), isolated from *Agrobacterium* sp. strain CP4. A more extensive list of important genes that were transferred to wheat through genetic transformation was reviewed by Janakiraman et al. (2002). Improved wheat drought

resistance level was achieved by the introduction of *HVA*1 gene (Sivamani et al., 2000; Patnaik and Khurana, 2003) and *DREB*1A gene (Pellegrineschi et al., 2004). Although a milestone has been reached in wheat transformation, there is still a major need to improve the technology due to the low transformation efficiency and the genotype-dependent response of wheat to tissue culture.

The main purpose of this project was to introduce the trehalose-6-phosphate synthase/phosphatase (*TPSP*) gene (Garg et al., 2002) into spring wheat (*Triticum aestivum* L.). *TPSP* gene is a fusion gene containing the *E. coli ots*A gene which encodes TPS (trehalose-6-phosphate synthase) and *ots*B gene which encodes TPP (trehalose-6-phosphate phosphatase). When the *TPSP* fusion gene was expressed in rice, Garg et al. (2002) found an increase (3-10 times higher than wild type control) in the accumulation of trehalose, a non-reducing disaccharide of glucose that has found to help protecting cells during abiotic stresses. When subjected to salt, drought, or low temperature stress tolerance tests, transgenic *TPSP* rice plants survived longer, displayed more vigorous root and shoot growth, and had fewer symptoms of stress (Garg et al., 2002).

In addition to introducing the *TPSP* gene into wheat, we also evaluated existing tissue culture protocols, different wheat varieties, and factors that might influence efficiency of wheat transformation.

Materials and Methods

Plant Materials

Three spring wheat genotypes, Bobwhite, Hi-line, and UC703, were utilized for transformation experiments. Bobwhite is a breeding line often used for genetic transformation experiments (Weeks et al., 1993). The hard red spring wheat (*Triticum aestivum* L.) cultivar Hi-line was developed by the Montana Agricultural Experiment Station and released in 1991 (Lanning et al. 1992). The UC703 variety seed was provided by Dr. Calvin Qualset, University of California-Davis. Seeds were sown in Metro Mix© 200 (SunGro, Bellevue, WA). About 120 seeds were germinated every two weeks with four seeds per 6-inch pot. Plants were maintained at the greenhouse at North Carolina State University Phytotron. The temperature was maintained at 25ºC with natural day/night light conditions and light intensity. Plants were watered every other day with nutrient solution prepared according to http://www.ncsu.edu/phytotron/manual.pdf.

Plant materials for transformation were also collected from plants grown in greenhouse unit located on Method Road. The temperature setting was 25°C with natural light and day/night periods. Plants were grown in Metro Mix© 200 and fertilized with a slow released fertilizer (Osmocote®, The Scotts Company, Marysville, OH).

Immature seeds were collected from spikes at 7 days, 12 days, 14 days, 18 days, and 21 days after pollination (dap). Seeds were removed from rachis within hours after harvest, washed with 70% ethanol for 2 minutes followed by sterilization in 25% Clorox (6.15% Sodium

hypochlorite, Clorox, Oakland, CA) solution for 15 minutes. Only whole embryos were used in the experiments. Once isolated from immature seeds, embryos were place with the scutellum side up on culture medium.

Tissue Culture

Seed age

Immature embryos from 7-day, 12-day, 14-day, and 21-day after pollination (dap) seeds were placed on pre-culture medium (W1, Table A) for 5 days in a dark incubator at 25ºC. Roots and shoots that developed were removed. Calli were subcultured onto fresh W1 medium and incubated in the dark for another 3 weeks. Developed calli were transferred to regeneration medium (W4, Table A) and allowed for generation of shoots and roots under light condition at 25ºC.

Protocol evaluation

Four different protocols were evaluated (Table A). Protocol 1 was described by Sivamani et al. (2000). Protocol 2 was described by Altpeter et al. (1996). Protocol 3 was a modification of protocol 1. The pre-culture time was increased to 7 days instead of 5 days. Protocol 4 was described by Wright et al. (2001). Immature embryos from 14-dap seeds were used. After isolation, embryos were placed on respective media and under culturing conditions of the protocols (Table B).

Table A: Protocols 1&2 for Wheat Transformation

Protocol 1 (Sivamani et al. 1999)

Pre-culture embryo in dark @ 25°C for 5 days

W1

1.	MS basal salt	
2.	L-Asparagine	150mg/L
3.	Thiamin HCl	40mg/L
4.	Maltose	20g/L
5.	Ammonium nitrate	1.6g/L
6.	2-4,D	2mg/L
7.	ADS	50mg/L
8.	Phytagel	3g/L
9.	pH 5.7	

Prebombarded treatment: place calli on high osmoticum (W2) in dark for 4hrs.

W2

W1 + 0.4M mannitol

Bombardment

Postbombardment treatment: calli remain on W2 in dark for 20 hrs

Calli on selection medium (W3) in dark @ 25°C for 3 weeks

W3

W1 + 5mg/L bialaphos

Regeneration on W4, incubated **under light** @ 25°C for 3 weeks

W4

1.	MS basal salt	
2.	L-Asparagine	150mg/L
3.	Thiamin HCl	40mg/L
4.	Maltose	20g/L
5.	Ammonium nitrate	1.6g/L
6.	BAP	2mg/L
7.	Kinetin	1mg/L
8.	Phytagel	3g/L
9.	Thidiazuron	0.15mg/L
10.	Bialaphos	1mg/L
11.	pH 5.7	

Rooting: W5 **under light** @ 25°C for 3 weeks

W5

1.	½ MS basal salt	
2.	NAA	05mg/L
3.	Bialaphos	2mg/L
4.	Maltose	10g/L
5.	Phytagel	3g/L
6.	pH 5.7	

Transfer plantlets to soil.

Protocol 2 (Altpeter et al. 1996)

Pre-culture embryo in dark @ 25°C for 7 days

MS+

1.	MS basal salt	
2.	2-4,D	2mg/L
3.	Sucrose	20g/L
4.	Glutamine	500mg/L
5.	Casein hydrolysate	100mg/L
6.	Gelrite	0.25%
7.	MS vitamins (after autoclaved)	
8.	pH 5.7	

Prebombarded treatment: place calli on high osmoticum (W2) in dark for 4hrs.

MS+Osmoticum

MS+ with 0.2M mannitol + 0.2M sorbitol

Bombardment

Postbombardment treatment: calli remain on MS+Osmoticum in dark for 16 hours

Transfer calli onto selection medium in dark @ 25°C for 2 weeks

DB3

1.	MS basal salt	
2.	2-4,D	2mg/L
3.	Sucrose	20g/L
4.	Gelrite	0.25%
5.	Bialaphos	3mg/L
6.	MS vitamins (after autoclaved)	
7.	pH 5.7	

Regeneration **under light** @ 25°C for 10 days

LB3

1.	MS salt	
2.	Sucrose	20g/L
3.	Zeatin	10mg/L
4.	Bialaphos	3mg/L
5.	Gelrite	0.25%
6.	pH 5.7	

Elongation for 2x 14days in light

LB5

1.	½ MS + vitamins	
2.	Sucrose	15g/L
3.	Bialaphos	5mg/L
4.	Gelrite	0.25%
5.	pH 5.7	

Transfer plantlets to soil.

Table A (continue): Protocols 3&4 for Wheat Transformation

Protocol 3 (modified from Protocol 1)

Pre-culture embryo in dark @ 25°C for 7 days

W1

1.	MS w/ maco&micro salt	
2.	L-Asparagine	150mg/L
3.	Thiamin HCl	40mg/L
4.	Maltose	20g/L
5.	Ammonium nitrate	1.6g/L
6.	2-4,D	2mg/L
7.	ADS	50mg/L
8.	Phytagel	3g/L
9.	pH 5.7	

Prebombarded treatment: place calli on high osmoticum (W2) in dark for 4hrs.

W2

W1 + 0.4M mannitol

Bombardment

Postbombardment treatment: calli remain on W2 in dark for 20 hrs @ 25°C

Calli on selection medium (W3) in dark @ 25°C. Subculture every 2 weeks (3X)

W3

W1 + 5mg/L bialaphos

Regeneration on W4. Light @ 25°C for 3 weeks (until plantlets regenerated)

W4

1.	MS basal salt	
2.	L-Asparagine	150mg/L
3.	Thiamin HCl	40mg/L
4.	Maltose	20g/L
5.	Ammonium nitrate	1.6g/L
6.	BAP	2mg/L
7.	Kinetin	1mg/L
8.	Phytagel	3g/L
9.	Thidiazuron	0.15mg/L
10.	Bialaphos	1mg/L
11.	pH 5.7	

Rooting: W5 **under light** @ 25°C for 3 weeks

W5

1.	½ MS w/ macro&micro salt	
2.	NAA	05mg/L
3.	Bialaphos	3mg/L
4.	Maltose	10g/L
5.	Phytagel	3g/L
6.	pH 5.7	

Transfer plantlets to soil.

Protocol 4 (Wright et al. 2001)

Pre-culture embryo in dark @ 28°C for 7 days

3MS3S

1.	MS medium + vitamins	
2.	L-Asparagine	75mg/L
3.	Glutamine	150mg/L
4.	Sucrose	30g/L
5.	2-4,D	3mg/L
6.	Phytagel	3g/L
7.	pH 5.7	

Prebombarded treatment: place calli on high osmoticum in dark for 4hrs.

3MS3S high osmoticum

3MS3S + 150g/L maltose, no sucrose

Bombardment

Postbombardment treatment: calli remain on high osmoticum in dark for 24hrs @ 28°C

Culture calli on 3MS3S for three weeks in dark

Regeneration/selection (NG) in light @ 20°C for 2 weeks

NG

1.	MS medium + vitamins	
2.	L-Asparagine	75mg/L
3.	Glutamine	150mg/L
4.	Sucrose	30g/L
5.	Gibberellic acids	1mg/L
6.	NAA	1mg/L
7.	Phytagel	3g/L
8.	Bialaphos	5mg/L
9.	pH 5.7	

Regeneration (MS2S0.5). Light @ 20°C for 3 weeks (until plantlets regenerated)

MS2S0.5

1.	MS medium + vitamins	
2.	Sucrose	20g/L
3.	Mannose	5g/L
4.	Glutamine	300mg/L
5.	Asparagine	150mg/L
6.	Phytagel	3g/L
7.	Bialaphos	5mg/L
8.	pH 5.7	

Rooting (1/2 MS): Under light @ 20°C

½ MS

1.	½ MS medium	
2.	NAA	0.05mg/L
3.	Mannose	15g/L
4.	Phytagel	3g/L
5.	pH 5.7	

Transfer plantlets to soil.

For each tissue culture experiment, immature seeds from all three genotypes, Bobwhite, UC703, and Hi-line were harvested at the same time and embryos were cultured on the same day. The experiment was carried out in January, February, June, July, and October, 2006.

Table B: Tissue culture conditions

Protocol 1	**Protocol 2**	**Protocol 3**	**Protocol 4**
Incubate calli in dark @ 25ºC for 5 days on W1	Incubate calli in dark @ 25ºC for 6 days on MS+	Incubate calli in dark @ 25ºC for 7 days on W1	Incubate calli in dark @ 28ºC for 7 days on 3MS3S
Roots and shoots are removed. Calli are sub-cultured on W1 for 3 weeks in the dark.	Roots and shoots are removed. Calli are sub-cultured on MS+ for 2 weeks in the dark.	Roots and shoots are removed. Calli are sub-cultured on W1 every 2 weeks for 6 weeks in the dark.	Roots and shoots are removed. Calli are sub-cultured on 3MS3S for 3 weeks in the dark at 28ºC.
Calli are transferred onto regeneration medium (W4) and incubated under light @ 25ºC for 3 weeks.	Calli are transferred onto regeneration medium (LB3) and incubated under light @ 25ºC for 10 days	Calli are transferred onto regeneration medium (W4) and incubated under light @ 25ºC for 3 weeks.	Calli are transferred onto regeneration-selection medium (NG), without selection reagent, and incubated under light @ 20ºC for 2 weeks.
Plantlets are transferred to rooting medium (W5) and incubated under light @ 25ºC for 3 weeks or until root develops.	Plantlets are transferred to elongation medium (LB5) and incubated under light @ 25ºC for 4 weeks with subculturing every 2 weeks.	Plantlets are transferred to rooting medium (W5) and incubated under light @ 25ºC for 3 weeks or until root develops.	Calli are transferred onto regeneration medium (MS2S0.5), and incubated under light @ 20ºC for 3weeks.
Transfer to soil	Transfer to soil	Transfer to soil	Plantlets are transferred to rooting medium (1/2 MS) and incubated under light @ 25ºC for until root develops.
			Transfer to soil

Evaluation of bialaphos concentration for selection

Bialaphos was used as a selecting agent for the transformation experiments. We performed an experiment to test the effectiveness of different concentration of bialaphos. Eight concentrations were evaluated, i.e., 0, 1, 2, 3, 4, 5, 6, and 7 mg/l. Fourteen dap immature Hi-line embryos were used. After 7 days of pre-culture in the dark, calli were transferred to fresh culture media. Fifty calli (10 /plate with 5 plates) were used for each concentration of bialaphos. The calli from each plate were weighed and incubated in the dark. After 3 weeks of incubation in dark, they were subcultured onto fresh culture medium for another 3 weeks and the weight was measured at the end of the incubation period. Effectiveness of bialaphos concentration on inhibiting callus growth was judged based on the growth of calli during the 6 week incubation.

Transformation

Particle-bombardment transformation of cells was done using the Bio-Rad PDS-1000He Biolistic™ gene gun. The four tissue culture protocols were also tested for transformation experiments. For protocols 1, 2, and 3, each shot contained 37.5 μg of gold particle and about 1.25 μg of DNA. Preparation of 1-micron gold particle was performed according to manufacturer's instruction. Five μg of DNA was added to 10 μl of gold particle solution (15 mg/ml stock concentration). DNA/gold mixture was vortexed briefly followed by an addition of 10 μl of 0.1 M spermidine and 10 μl of 25% PEG 1000, and was vortexed briefly again. Ten μl of 2.5 M $CaCl_2$ was added preceding a 15 minute incubation period at room temperature on a medium-speed shaker. After incubation, tube was centrifuged at 12,000

rpm in a microfuge for 5 seconds. The supernatant was removed and DNA-coated particles were vortexed with 500 μl of ice-cold 100% ethanol, followed by another 5 seconds of centrifugation. The supernatant was removed and particles were resuspended in 100 μl of 100% ethanol. Twenty five μl of the resuspended DNA-coated gold particles were loaded onto macrocarrier for each shot. Calli were incubated on high osmotic medium (culture medium added with 0.4 M mannitol) for 4 hours before the bombardment. Each plate contained about 50-70 pieces of calli arranged closely together within a circle with the diameter of 2.5 cm in the center of the plate. The circle of calli was centered 7 cm underneath the stopping screen. Each plate was shot once using 1,500-psi rupture disks. Each plate in protocol 4 was shot twice with each shot containing 0.83 μg of DNA and 37.5 μg of gold particles using 1,100-psi rupture disks.

Gene Construct

Plasmid pSB109-TPSP (Fig. A) was provided by Dr. R. Wu from the Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY. The plasmid contained a fusion gene (*TPSP*) consisting of the coding sequences of the *E. coli otsA* and *otsB* genes. The gene was driven by an abscisic acid (ABA)-inducible promoter. The plant selectable marker was the *bar* gene driven by the CaMV 35S promoter (Garg et al., 2002).

Plasmid pRESQ48 was used for the GUS transient assay. pRESQ48 was provided by Dr. Sivamani from the Department of Crop Science at North Carolina State University. It contained a *GUS* gene fused with the first 3 aa coding sequence of the *rubi3* gene. The gene was driven by a *rubi3* promoter and its 5' UTR exon and intron (Sivamani and Qu 2006).

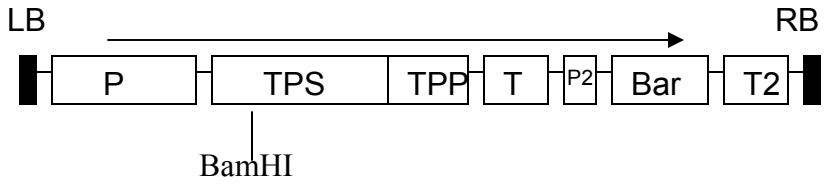


Figure A. Diagram of pSB109-TPSP (modified from Garg, et al., 2002). P = Promoter consisting of 4ABRC1 of barley HVA22, rice actin *Act*1 basal promoter and intron 1-exon 2-intron 2 of HVA22. TPS-TPP = *E.coli* trehalose-6-phosphate synthase/phosphatase. T = 3' PinII terminator. P2 = 35S promoter. Bar = *bar* gene coding sequence. T2 = 3' NOS terminator. LB = left border. RB = right border.

Transient Assay

Fifty 14-dap immature embryos from each wheat genotype were used for all the transient assay experiments. After bombardment with gold particles coated with pRESQ48, calli went through the post-bombardment treatment (~ 16 h osmotic treatment on the 0.4 M mannitol-containing medium) and were cultured on respective pre-culture media (Table A and B) for 4 days in a dark incubator at 25°C. Calli were then incubated in GUS buffer solution (Jefferson et al., 1987) overnight at 37°C and scored visually.

PCR and Southern Blot Analysis

Genomic DNA from plants transformed with pSB109-TPSP was extracted as described by Dong and Qu (2005). DNA samples were quantified using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Uppsala, Sweden). Bioneer AccuPower PCR Premix (Bioneer, Inc., Alameda, CA) kit was used for PCR for *bar* and *TPSP* gene fragments. About 100 ng of genomic DNA was used per reaction. PCR of *bar* gene was performed using forward primer (BAR1): 5'-TGCACCATCGTCAACACTA-3', and the reverse primer (BAR2): 5'-ACAGCGACCACGCTGTT-3'. The PCR conditions were as follows: initial denaturation at 94°C for 3 minutes, denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds, and the final extension at 72°C for 7 minutes. The PCR was set for 35 cycles. PCR product of the *TPSP* gene coding sequence was amplified by two primers, TPSP1 and TPSP2 (the sequences of primers are described below) using the same conditions.

For Southern analysis, leaves from a T_0 plant that was PCR positive (P2), and 4 progeny (T_1) plants of the P2 plant, together with a non-transformant Hi-line control plant (CN), and a PCR-negative T_0 plant (P5-1), were ground in liquid nitrogen using pestles and mortars, and DNA extracted as described above. Forty μg of genomic DNA was digested with *Bam*HI restriction enzyme overnight. Digested DNA was fractionated on 0.8% agarose gel at 25 V overnight. Blotting was done using Hybond-N^+ nylon membrane (Amersham) following manufacturer's instructions. A 1.4 kb PCR product of the *TPSP* gene coding sequence amplified by two primers, TPSP1 (5' CGG GAA TTC ATG ACT ATG AGT CGT 3') and TPSP2 (5' GCA AGC TTT GGA AAG GTA GCA AC 3'), was used as probe and was labeled with α-^{32}P dCTP. Labeling of probe, hybridization, and washing of membrane was done according to instruction manuals of the Prime-It random primer labeling kit (Stratagene) and the Hybond-N^+ nylon membrane. Labeled membrane was exposed to Kodak BioMax MS film.

Leaf-painting Assay

Finale (AgrEvo® USA Company) is a non-selective herbicide with the main ingredient of glufosinate-ammonium (11%). Plants transformed with pSB109-TPSP would contain the *bar* gene, and thus be resistant to Finale. When wheat plants reached about 12 inches tall and had four to five leaves, the leaves were subjected to a leaf-painting assay. Two leaves were chosen randomly for each plant. A section of 1.5 inches long was marked in the middle of the leaf. A 0.5% (v/v) Finale solution was applied to the back and front of the leaf in the marked area using a Q-tip. Plants were placed in water trays and watering was done by

refilling of the trays to avoid water washing away Finale solution on leaves. Leaves were scored 7 days after application.

Results

The embryos varied significantly in size and color at 7, 14, and 21 days dap (Fig. B-2). After 5 days of incubation in the dark at 25°C with the embryo axis facing down on the respective pre-culture media and protocols, no difference was noticeable in the callus development among the three wheat cultivars and among the different pre-culture media. Embryos isolated from 21 dap seeds developed small white shoots and spongy and clear, in appearance, calli. Most of the 7 dap embryos did not develop any callus. The calli developed from 14 dap embryos were yellowish and compact in texture. Some also developed spongy and watery calli in addition to the yellow calli.

The regeneration rate of the induced calli from 14 dap embryos were high, ranging from 76-100%. There was no apparent difference in regeneration among different cultivars within a protocol. However, there was a trend among different experiments that were done in different time periods. The regeneration rates of all three cultivars, Bobwhite, Hi-line, and UC703, for all 4 protocols from January, February, and October were overall ~10-15% higher than the ones that were done in June and July. Immature seeds collected in January, February and October were more uniform in size and appearance. It was possible that variations of temperature and sunlight during June and July had an effect on the seed quality for the tissue culture response.

Previous studies on wheat transformation used 5 mg/l of bialaphos in selection media (Sivamani et al., 2000, Altpeter et al., 1996). We tested the effectiveness of different concentrations of bialaphos using Hi-line wheat embryos and measured the growth of calli in a 6 week period. We also found that, at this concentration, bialaphos inhibited the growth of calli effectively (Fig. C-1 and 2) although the calli cultured at this concentration still retained their color and texture (Fig. C-1).

Transient assays for the three wheat genotypes tested using each protocol exhibited no obvious difference among the wheat varieties and the protocols. Calli from all 3 different varieties of wheat developed blue spots in GUS transient assays (Fig. D). There was no blue spot on non-transformed control calli (picture not shown). The intensity of the blue spots varies among individual callus from different varieties and protocols.

A total of 72 transformation experiments with more than 21,000 immature embryos were performed. A typical process of the wheat transformation procedure from immature embryo culture, and selection of the bombarded tissues, to plantlet regeneration was shown in Figure E. Putative *TPSP* transformants (bialaphos resistant) were screened by PCR for presence of the *TPSP* gene and the *bar* gene. No TPSP-transformed Bobwhite or UC703 plant was recovered. However, we were able to recover a transgenic plant, P2, from Hi-line. PCR of the P2 T_0 and T_1 plant leaf samples resulted in the expected 1.4 kb fragment amplified with primers TPSP1 and TPSP2 (Fig. F-A). Southern blot confirmed integration of TPSP gene in P2 T_0 plant and transmission of the gene to the T_1 plants (Fig. F-B).

The *TPSP* and *bar* gene constructs are linked in pSP109-TPSP (Fig. A). PCR for amplification of the *bar* gene was done for plant P2 showing that P2 was also positive for the *bar* gene (data not shown). Since plants transformed with the *bar* gene should confer resistance to bialaphos or glufosinate, a leaf painting assay would provide information regarding the *bar* gene expression in the P2 plant. Leaf painting results (Fig. G) showed that leaves from a non-transformed control plant (CN) and a PCR-negative plant (P5-1) dried up and turned yellow from the marked area up to the tip of the leaves. Leaves from T_0 and T_1 plants of the P2 line that were PCR positive for *bar* genes displayed no injury after 7 days treated with Finale solution (Fig. G). Sixty T_1 seeds from the P2 T_0 plant were germinated and subjected to leaf painting assay. Seventeen out of 60 T_1 plants tested (approximately ¼ of the plants) showed herbicide injury (such as plant 6-10 in Fig. G) while the rest 43 were resistant as expected with Mendelian segregation.

Since *TPSP* gene was driven by an ABA-inducible promoter. T_0 and T_1 plants of the P2 line positive from PCR and Southern analyses were sprayed with 100 μM of ABA solution. Leaf samples were collected after 0 hour, 1 hour, 3 hours, 5 hours, 12 hours, 24 hours, and 48 hours and were kept immediately in liquid nitrogen for RNA extraction. Reverse transcription-PCR of plants sprayed with ABA solution and northern blot analysis (results not shown) indicated that no *TPSP* mRNA was expressed.

For transformation experiments, regeneration rate of calli were about 30-50%. Many calli turned brown during selection and did not regenerate. The ones that regenerated during selection developed good root and shoot systems in rooting media. However, almost all of

them were negative in PCR assays for *TPSP* or *bar* gene, indicating the selection system was not tight and was ineffective.

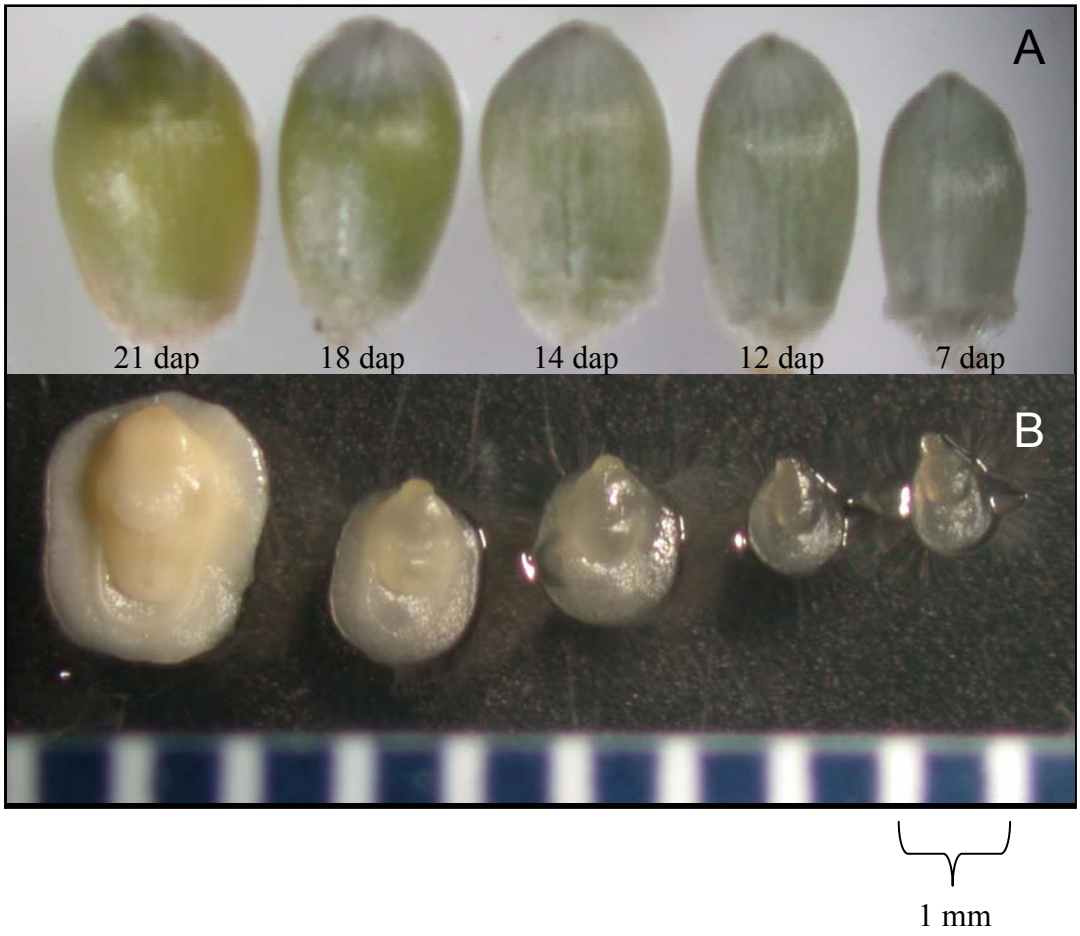


Figure B. Collection of immature seeds and embryos at different stages after pollination. A. Immature seeds from cv. Hi-line collected at 21, 18, 14, 12, and 7 dap (day after pollination). B. immature embryos isolated from their respective immature seeds.

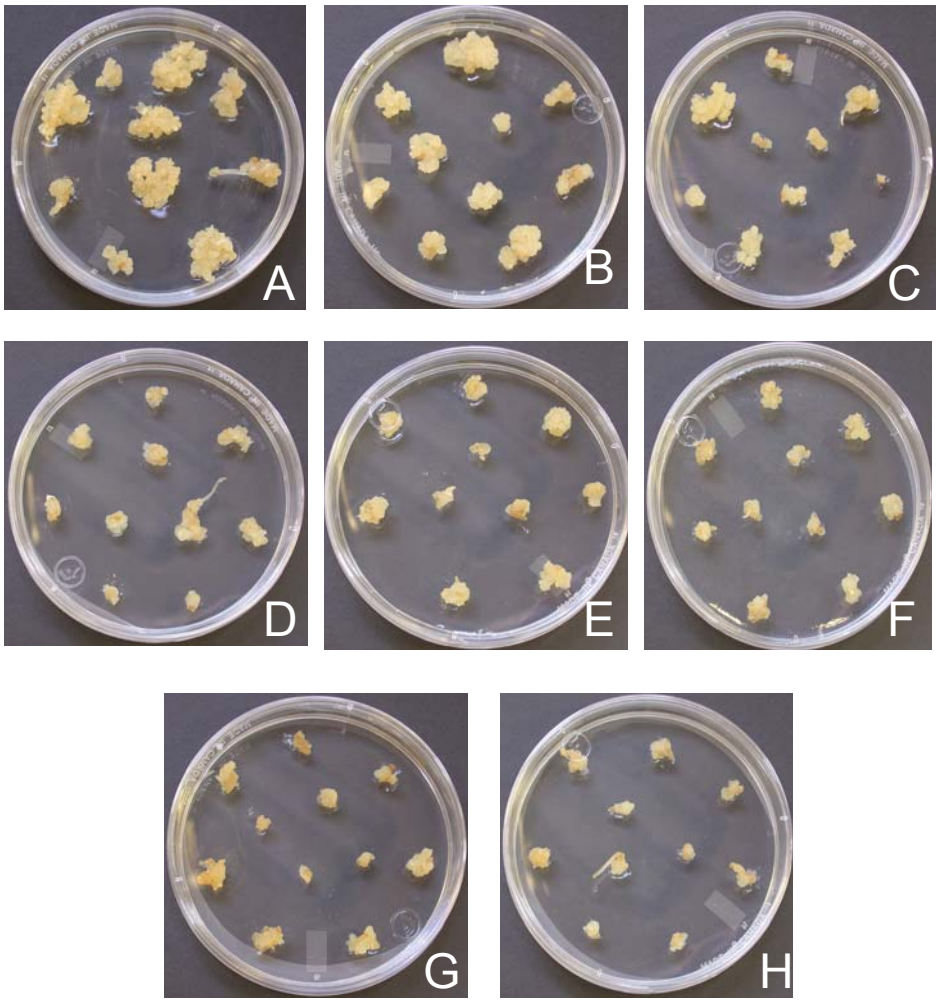


Figure C-1. Callus growth inhibition on culture medium containing various concentration of bialaphos 6 weeks after incubation in dark. A. 0 mg/l bialaphos. B. 1 mg/l bialaphos. C. 2 mg/l bialaphos. D. 3 mg/l bialaphos. E. 4 mg/l bialaphos. F. 5 mg/l bialaphos. G. 6 mg/l bialaphos. H. 7 mg/l bialaphos

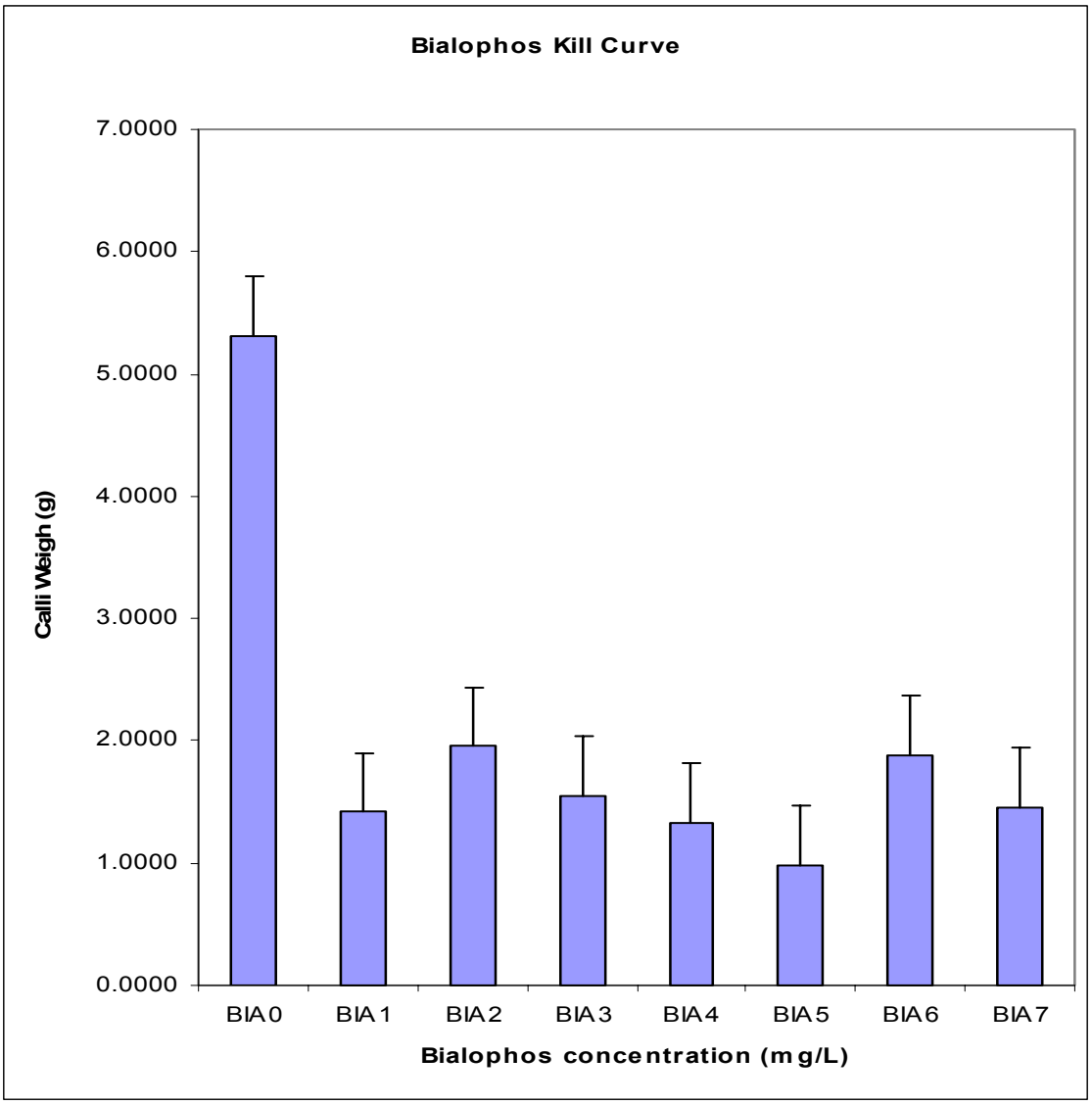


Figure C-2. Callus growth inhibition by various concentration of bialaphos. Calli weight was scored after six weeks of incubation in the dark.

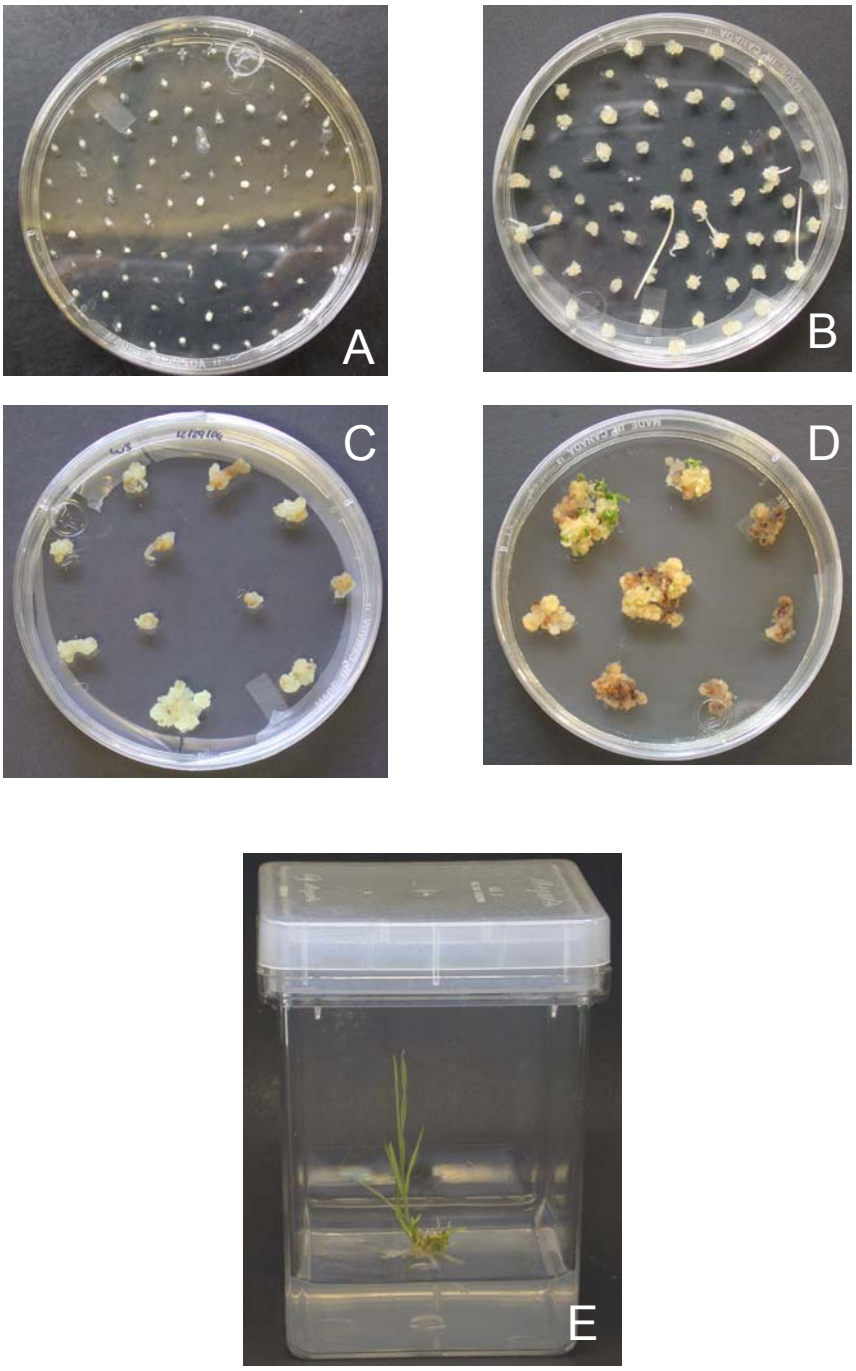


Figure D. Wheat transformation process using protocol 1. A. Embryos from 7 dap Hi-line seeds. B. Calli formation after 7 days of incubation on pre-culture medium. C. Calli after 3 weeks of dark incubation on selection medium containing 5 mg/l bialaphos. D. Calli after 3 weeks of light incubation on regeneration medium containing 1 mg/l bialaphos. E. Regenerated plantlet on rooting medium containing 3 mg/l bialaphos.

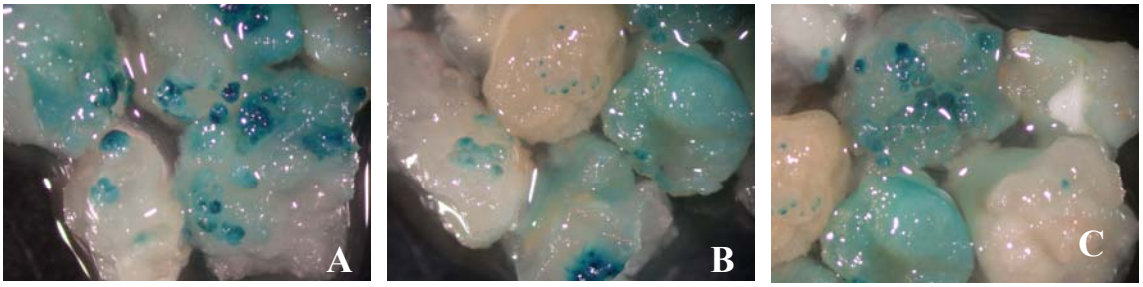
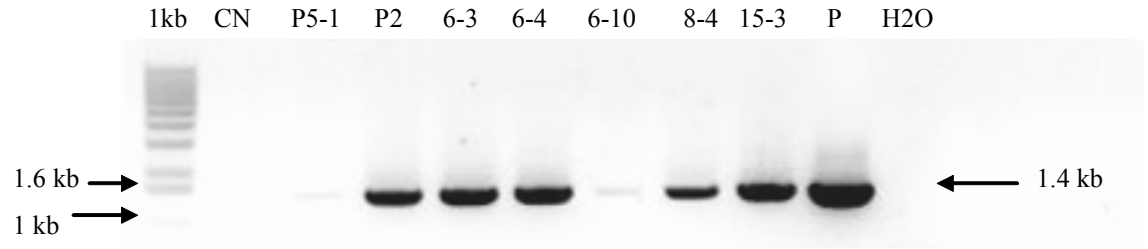


Figure E. GUS transient assays for 3 different wheat varieties. A. Hi-line. B. UC 703. C. Bob white.

A.



B.

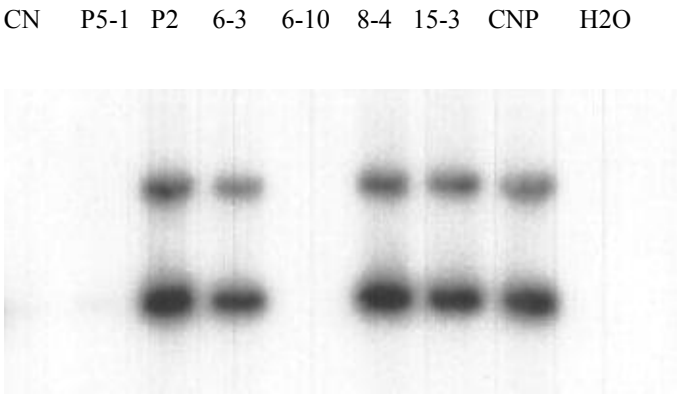


Figure F. PCR and Southern results of T_0 and T_1 plants of P2 line transformed with pSB109-TPSP. A. PCR results for *TPSP* gene; B. Southern analysis. CN = a nontransformed Hi-line plant, P5-1= a tissue culture plant, P2 = T_0 plant of transgenic P2 line, 6-3, 6-4, 6-10, 8-4, and 15-3 = T_1 segregating offspring plants of P2 line, P or CNP = pSB109-TBPS plasmid control.

Figure G. Hi-line leaf-painting assay results. Leaves were painted with 0.5% Finale and scored after 7 days. CN, nontransformant Hi-line plant, P5-1, tissue culture plant, P2, T_0 transformed plant, 6-3, 6-4, 6-10, 8-4, 15-3, T_1 offspring of P2 plant.

Discussions

The tissue culture results demonstrated that age of embryos and time of harvesting could affect the quality of callus and regeneration rate. Our selection curve study showed that 5 mg/l concentration of bialaphos inhibited the growth of callus and is suitable for selection. However, the fact that most of the recovered plants were PCR negative and susceptible in the leaf-painting assays indicated that the escape rate was quite high and the selection of bialaphos was not efficient.

The recovered transgenic plant (P-2) was positive for the presence of *TPSP* gene but no transcript of the gene was detected. Since we used biolistic method for transformation, there is a possibility that only part of the *TPSP* gene construct was integrated into the wheat genome, resulting no expression of the gene in P2 plant. In addition, multiple copies of the transgene could have integrated into the genome leading to gene silencing. It was noticed that, for unknown reasons, transgene silencing appeared to be more commonly observed among polyploid plants such as wheat (Jarakiraman et al. 2002).

The main intention of the transient assay was to test the mechanics and technique of DNA coating and bombardment. Thus, the number of calli used for the experiment was small and no callus shot with pRESQ48 was allowed to regenerate into plants. A possible future study that would shed more light on the responses of different wheat cultivars on different biolistic protocols is to follow the same protocol as described for the transient assay experiment, and allow the bombarded calli to regenerate and look at the transformation efficiency of plants transformed with pRESQ48.

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