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Original Article

Knock down *Os1bglu1* β-glucosidase in rice by *Agrobacterium*-mediated transformation

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Abstract

This research attempted to study the function of *Os1bglu1* by RNAi technique. The suppression of *Os1bglu1*gene was done using the 3'UTR region. The target gene fragment was cloned into the pHELLSGATE8 vector. The high percentages of effective callus induction of 93% were obtained when the seeds were cultured on N6D medium for 4-6 weeks at 28°C. The suitable transformation conditions were to incubate the calli with *Agrobacterium* (OD_{600} = 0.02) and blot dry to remove excess bacteria cells, then transferred to co-cultivation medium (pH 5.2) with 200 µM acetosyringone and incubate for three days at 25°C. The 20% transformation efficiency was obtained from the transformed calli with control plasmid, while transformation efficiency of only 15% was obtained from pHELLSGATE8 *Os1bglu1* constructs. The transformed calli with control construct showed higher growth rate than the transformed calli with pHELLSGATE8 *Os1bglu1*construct. The expression of *Os1bglu1* mRNA was not found in the transformed calli and siRNAs were found in the transformed calli. However no siRNAs were detected in the control transformed calli. The regeneration efficiencies of 6% were obtained from only the calli transformed with the control construct. The calli transformed with the knock down *Os1bglu1* constructs were not able to regenerate. This may indicated that *Os1bglu1* is involved in regeneration of rice from callus tissue.

Keywords: RNA interference, β-glucosidase, *Agrobacterium*-mediated transformation, pHELLSGATE8, siRNA

1. Introduction

Koshihikari is japonica rice (*Oryza sativa* L.) subspecies with sticky and short-grained seed (Crawford and Shen, 1998). Koshihikari was first created in 1956, by combining the Nourin no. 1 and Nourin no. 22 strains at the Fukui Prefectural Agricultural Research Facility. Koshihikari is one of the most popular strains of rice cultivated in Japan and other parts of the world because it has high cooking quality and taste. So it has been used as the crossing parent for breeding of high-eating quality cultivars (Daigen *et al.*, 2000, Tian *et al.*, 2006, Wakasa *et al.*, 2007). However, it is difficult to achieve high yields because of its low resistance and high susceptibility to disease and insects that cannot be controlled without chemical applications. The conventional breeding of these cultivars takes quite a long time to select, check, and release a new variety. Therefore, tissue culture method has been developed to breed new rice cultivar in a shorter period. Generally, it is not easy to culture and regenerate monocot plants, including agronomically important crops such as rice, wheat, and maize (Nishimura *et al.*, 2005). The success of rice tissue culture is influenced by many factors such as culture medium composition, explant source, genotype and environment (Khanna and Raina, 1998; Torbert *et al.*, 1998). Koshihikari has been shown to have low transformation and regeneration efficiency (Nishimura *et al.*, 2005, Wakasa *et al.*, 2007, Ozawa, 2009). Therefore, the nutrient composition and culture technique were studied in this research.

Os1bglu1 is one of the 36 isozymes of β-glucosidase gene family in rice (Opassiri *et al.*, 2006). The β-glucosidase

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enzyme acts in cell wall remodeling, responses to abiotic and biotic stress, defenses against herbivores and activation of hormones, for example abscisic acid and gibberelin. The β-glucosidases are involved in plant germination, growth and development in every stage (Cicek and Esen, 1998; Cicek *et al.*, 2000; Rodo *et al.*, 2008). However, up to now the exact functions of *Os1bglu1* gene and even other isozymes are yet well studied. In this research, the function of *Os1bglu1* by RNA interference (RNAi) was determined. Recently, the function of many genes in rice has been studied by RNAi (Prasad and Vijayaraghavan, 2003; Xiao *et al.*, 2003; Wang *et al.*, 2004; Islam *et al.*, 2005; Miki *et al.*, 2005; Lin *et al.*, 2005; Luo *et al.*, 2005; Hu *et al.*, 2005; Zhong *et al.*, 2007; Xu *et al.*, 2008; Yang *et al.*, 2008; Wang *et al.*, 2009; Li *et al.*, 2009). RNAi is one of the most preferable gene knock down methods of high power to study the function of genes in many organisms (Cottrell and Doering, 2003). Double strand RNA (dsRNA) is the major activator of the RNAi mechanism (Fire *et al.*, 1998; Waterhouse *et al.*, 1998). Dicer, the enzyme that cuts dsRNA, will generate small interfering RNAs (siRNA) of 21-25 bp (Bernstein *et al.*, 2001). The RNA induced silencing complex (RISC) will combine with siRNA to unwind the siRNA from double strand RNA to single strand RNA (Hammond *et al.*, 2000). The siRNA-RISC complex will search and destroy the mRNA target with the sequence complementary with the siRNA. The RNAi mechanism will be activated automatically after the dsRNA is produced in the cell, which leads to the knock down of the target gene by destroying the target mRNA before it can be translated to protein (Matzke *et al.*, 2001; Waterhouse *et al.*, 2001; Hannon, 2002; Plasterk, 2002; Meister and Tuschl, 2004). Therefore, the function of the target gene can be observed and predicted from the phenotype or biological and chemical pathway.

2. Material and Method

2.1 Plant material

Koshihikari seeds were obtained from Rice Research Center, Chiang Mai, Thailand. Newly harvest seeds were used within six months.

2.2 Plasmid preparation

The 3'UTR was used as the target sequence region to knock down the *Os1bglu1* gene. The primers used to amplify DNA fragment encoding RNAi target were *Os1bglu1*-forward 5' **CACC**CTCGAGGGCTACTTCGCCTGGTCC and *Os1bglu1*-reverse 5'- GAATTCCAATCTTGAATGATG. The CACCCT CGAG underling sequence was added to the 5'end of the forward primer for directional cloning into pENTR™/D-TOPO and *XhoI* sites for cloning the first piece into pHELLSGATE8. GAATTC underling sequence was *EcoRI* site added to the 5'end of the reverse primer for cloning the first piece into pHELLSGATE8.

The genomic DNA extracted from 2-weeks old rice leaf was used as template to amplify target gene fragment and then cloned into pENTR™/D-TOPO vector. The target gene fragments in pENTR™/D-TOPO were sequenced and then cloned into pHELLSGATE8 (CSIRO, Australia) by standard restriction enzyme digest with *XhoI* and *EcoRI* for the first piece and by LR clonase enzyme (Invitrogen) for the second piece. pHELLSGATE8 is a plasmid that contained an intron (pyruvate orthophosphate dikinase; pdk) flanked by two specific multiple cloning sites (MCSs). The complete *Os1bglu1*-pHELLSGATE8 that contains two pieces of target genes (Figure 1) was transformed into *Agrobacterium* strain EHA105. The pHELLSGATE8 without of target gene insertion was used as plasmid control.

Figure 1. Diagram of T-DNA region of pHELLSGATE8 construct. The target gene fragments are in opposite orientation under the control of 35S promoter.

2.3 Seed sterilization

Mature rice seeds were dehusked and surface sterilized by soaked 70% ethanol for 1 min and washed three times with sterile water. Three percent of sodium hypochlorite was used to sterilize the seeds under shaking condition for 30 min and the seeds were washed six times with sterile water. The seeds were blotted and dried in a petri dish for 30 min and then the seeds were moved to the N6D medium.

2.4 Callus induction

The sterile seeds of Koshihikari were cultured on N6D medium (pH 5.8) containing 30 g/L of sucrose or 30g/L of maltose, 3.98 g/L of CHU basal salt mixture (phytotechnology, USA), 300 mg/L of casamino acids, 2.878 g/L of L-proline, 5 mL/L of 100X N6 vitamin, 2 mg/L of 2, 4-D and 4 g/L of gellengum (phytotechnology, USA) for callus induction. The cultures were incubated at 25 to 33°C in dark condition. The secondary calli were obtained from 4-6 weeks.

2.5 Co-cultivation

The secondary calli were separated and subcultured on fresh N6D medium for 3-7 days. *Agrobacterium* strain EHA105 harboring control plasmid and pHELLSGATE8 Os1bglu1 were streaked on solid AB medium composed of 100 mg/L spectinomycin, 5 g/L of Glucose, 50 mL/L of 20X AB buffer, 50 mL/L of 20X AB salt (Chilton *et al.*, 1974) and 15 g/L of bactoagar. The bacterial cultures were incubated in the dark at 28°C for three days.

The bacterial culture on the AB plates were resuspended in IF medium (pH 5.2) (1 mL/L of A1, 1 mL/L of A2, 1 mL/L of A3, 1 mL/L of A4, 1 mL/L of A5, 5 mL/L of A6, 1 mL/

L of Asol, 0.5 g/L of casamino acid, 68.5 g/L of sucrose, 39 g/L of glucose, 0.90 g/L of L-glutamine, 0.30 g/L of L-aspatic acid, 3 g/L of KCl) with 100 or 200 µM of acetosyringone. The IF medium was shaken to disperse the bacterial clump and the density of the bacterial suspension was adjusted to OD_{600} of 0.02.

The pre-culture secondary calli were moved dried on a petri dish for 30 min and then immersed in the bacterial suspension for 5 min. The excess bacterial suspension was removed by blotting the calli on sterile tissue paper. The calli were transferred to co-cultivation medium (CN6, pH 5.2) that contains 30 g/L of sucrose, 10 g/L of glucose, 3.98 g/L of CHU basal salt mixture, 300 mg/L casamino acid, 5 mL/L of 100X N6 vitamin, 2 mg/L of 2, 4-D and 4 g/L of gellengum with 100 and 200 µM of acetosyringone. The infected calli were incubated in the dark at 25°C or 28°C for three days.

2.6 Callus selection

After three days, the infected calli were washed four times with sterile water followed by three times with 300 mg/L of timentin. The calli were blotted on sterile tissue paper and transferred to N6D medium (8 g/L of agar A, Biobasic science Inc.) containing 100 mg/L of paromomycin and 300 mg/L of timentin. The calli were then incubated in the dark at 28°C for two weeks. The healthy calli were moved onto fresh selection medium every two weeks.

2.7 Plant regeneration

After four rounds of selection, actively growing calli were transferred to regeneration medium (pH 5.8) containing 30 g/L of sucrose, 30 g/L of sorbitol, 4.33 g/L MS basal salts mixture vitamin (phytotechnology, USA), 2 g/L casamino acid, 0.5 mg/L of NAA, 2 mg/L of kinetin, 30 mg/L of paromomycin and 8 g/L of agar A (Biobasic science Inc.). The calli were incubated under a photoperiod of 16/8 hrs (light/dark). Shoot and root regeneration were observed after six weeks and the calli were transferred to a rooting medium (MS medium, pH 5.8, containing 4.33 g/L of MS basal salt mixture vitamin, 30 g/L of sucrose and 4 g/L of gellangum). The 2–week old plantlets were then transferred to soil. The plantlets were covered with plastic bags to maintain moisture for seven days and then the plastic bags were removed and the plantlets were transferred to greenhouse.

2.8 PCR amplification in transformed calli and plantlet

The resistant calli on selection medium and the transgenic plants were collected for DNA extraction. The *nptII* gene primers (forward primer: GCTATTCGGCTATGA CTG and reverse primer: CGGCCATTTTCCACCATG, specific product size 730 bp) were used to confirm the integration of the T-DNA into the rice genome. The PCR reaction mix included DNA template, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 2.5 µL of 10X buffer (Promega), 40 pM forward and reverse primer, 0.5 µL of Taq DNA polymerase (homemade) and 16.9 µL of sterile distilled water to make the final volume 25 µL. The PCR amplification was done at 94°C for 5 min for the initial denaturation step, followed by 35 cycles of 94°C for 30 seconds, annealed at 55 °C for 30 seconds, and at 72°C for 35 seconds and then the final extension of 5 min at 72°C was done after the last cycle.

2.9 RT-PCR analysis

RT-PCR was performed to check the mRNA expression of *Os3bglu8* genes. Total RNA was extracted from resistant calli using TRIzol reagent (Invitrogen). After the treatment with RNase-free DNaseI, total RNA was reverse transcribed with oligo (dT) primers and SuperscriptIII reverse transcriptase (invitrogen) according to the manufacturer's instructions. The cDNA was subjected to PCR reactions using the *Os1bglu1* primer pairs as mention above. β-actin gene wAS used as control. The PCR reactions were done under the following conditions: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, annealed for *Os1bglu1* at 55°C and 53°C for β-actin for 30 second, and extension at 72°C for 30 second. The final extension of 72°C for 5 minutes was done after the last cycle.

2.10 Northern blot analysis for detect siRNA

Total RNA from resistant calli were extracted by Trizol reagent according to the standard protocol (Invitrogen). The RNA was precipitated at -20°C overnight with isopropanol. The RNA samples were mixed with 6X loading buffer (Fermentas) and denatured at 65°C for 10 minutes and load in 15% denaturing polyacrylamide gel (7 M urea). The gel was run at 50V till the dyes enter the gel (30 minutes). The well was then washed to remove high molecular weight RNA and then continue the electrophoresis at 100 V until the bromophenol blue reached the bottom of the gel. The gel was capillary blotted on Nylon membrane (Roche) overnight (Molnar, 2007). For probe preparation the pENTR™/D-TOPO containing of *Os1bglu1* 3'UTR was digested with *Not*I. The digested plasmid was then used as template for T7 RNA polymerase in vitro transcription to make RNA probes labeled with DIG-11-UTP (Roche) according to the manufacturer's instructions. The probe/hybridization mixture were added to the membrane and incubated at 42°C overnight with gentle agitation. The solution was removed and washing buffer was added briefly followed by blocking solution, washing buffer, and detection buffer. Visualization of the signals on blots was done using chemi DOC (BIORAD).

3. Result and Discussion

3.1 Callus induction in Koshihikari

The callus induction is the first step of rice transformation. After one month, the rice seeds on callus induction medium generated secondary calli to use for *Agrobacterium*

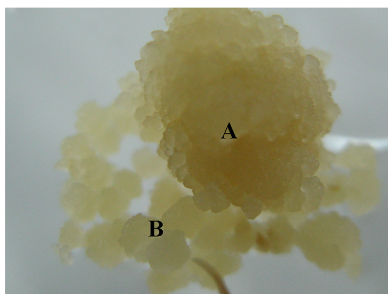

Figure 2. Primary calli (A) and secondary calli (B).

transformation (Figure 2). Mechanically breaking off of primary calli to induce the secondary calli leads to browning and poor callus formation. Therefore, the secondary calli should be grown from the primary calli until the secondary callus separated itself from the primary calli onto the medium.

The percent of callus induction was 93%. Light is a very important physical factor for callus induction, cell growth, and production of plant secondary metabolites (Vom Endt *et al.*, 2002). However the level of responsiveness to light depends on cell type, plant species, and cultivar. The secondary calli were induced in light/dark (16/8 hrs) and dark condition under constant temperature of 28°C in a growth chamber. From this research we found that light was not an important factor for Koshihikari secondary callus induction (data not shown). However, in dark condition, the calli show a less browning rate when compared to light condition. Qian *et al.* (2004) reported that light influences the rate of callus induction and callus grow faster but also increase the browning rate in rice variety Pei'ai64s. Pipatpanukul *et al.* (2004) reported that the effect of light and dark conditions mainly depended on the concentration of 2, 4-D in the medium.

Incubation temperature on calli growth showed no difference from 25-30°C. However, temperatures of higher or lower than 25-30°C can affect the secondary calli induction in this research. The calli, grown at temperatures higher than 30°C, were soft, light brown in color, friable, and died under high humidity from the evaporation of the water in media, and they gave low transformation efficiency. The calli morphology at temperatures lower than 25°C was dry and yellowish in color with compact appearance. The highest amounts of secondary calli were obtained at 28°C in a constant temperature dark chamber. These conditions gave good quality secondary calli for transformation.

3.2 Transformation efficiency

The secondary calli of Koshihikari co-cultivated with *Agrobacterium* for three days gave the best transformation efficiency. Co-cultivation for more than three days tends to decrease the transformation frequency and more than 80% calli died. This result was similar to the results of Samiphak and Siwarungson (2006) that three days co-cultivation of KDML105 rice gave approximately two fold higher green fluorescent protein (GFP) positive calli than two days co-cultivation. Hoque *et al.* (2005) reported that GUS activity was only observed from the co-cultivated calli from 2 to 5 days, although calli co-cultivated for 4 and 5 days showed GUS activity but the tissue were harmfully affected by prolonged cultivation with *Agrobacterium*.

Co-cultivation of Khosihikari with *Agrobacterium* in the dark for three days at 25 or 28°C indicated that incubation at 25°C led to higher transformation efficiency (21%) when compare to the callus co-cultivated at 28°C (10%) (see Table 1). This result was similar to Ozawa (2009), indicated that the best conditions for co-cultivation were 25°C.

The concentration of *Agrobacterium* is one of the important factors. The high concentrations of *Agrobacterium* caused overgrown and calli damage. In this experiment, the *Agrobacterium* concentration at OD_{600} = 0.02 in AAM medium was used to co-cultivation for 5 min. Other concentration of *Agrobacterium* were also performed but the results were not as good as the *Agrobacterium* concentration at OD_{600} = 0.02. This is confirms the earlier observations of Kumria *et al.* (2001) that high bacterial density (OD_{600} = 0.7-1.0 with 10 min infection) or prolonged infection time (15-30 min with the optimal OD_{600} = 0.3-0.6) are harmful and affect the growth and regeneration of indica rice MDU 5 callus. Similarly, Chakrabarty *et al.* (2002) reported that high amounts of *Agrobacterium* (OD_{600}=0.5) resulted in severe necrosis, whereas diluted culture (1:10 and 1:20 dilution) reduced necrosis. Therefore, the amount of *Agrobacterium* should be controlled by immersing the callus in a low concentration of bacterial suspension for a short time and blotting the calli dry on sterile paper before putting the calli on co-cultivation medium.

In monocots, acetosyringone are not synthesized to support the gene transfer. In this research, the 200 µM concentration of acetosyringone resulted in higher numbers (19%) of transformanted calli when compared to 100 µM (15%) (Table 2). Hiei *et al.* (1994) reported that transformation efficiency was extremely low when acetosyringone was omitted. The optimum concentration of acetosyringone in co-cultivation medium may vary between different cultivars of rice (Vijayachandra *et al.*, 1995; Hiei *et al.*, 1997).

3.3 The callus screening on selection medium and regeneration medium

Three days after the washed-calli were transferred onto the selection medium, yellow color was observed (Figure 3A). After two weeks, 50% of the calli turned brown color and more than 70% of non–transformed calli could not grow on the selection medium and died (Figure 3B). The surviving calli were separated on a new selection medium with about 30 calli/plate (Figure 3C). After one month of selection, only the transformed calli were grew and the non transformed calli were died (Figure 3D). Then the transformed calli were transferred into the new selection medium to increase the amount of transformed calli to be enough for subculture on

Table 1. Transformation efficiency of Koshihikari callus with 200 μM acetosyringone at 25 °C and 28 °C .

Replication	25°C			28°C		
	No. calli*	Resistant Calli**	Transformation efficiency (%)***	No. calli*	Resistant Calli**	Transformation efficiency (%)***
1	150	24	16.00	180	14	7.78
2	150	31	20.67	180	11	6.11
3	150	28	18.67	180	8	4.44
4	240	51	21.25	180	15	8.33
5	240	46	19.17	300	39	13.00
6	240	53	22.08	300	41	13.67
7	300	69	23.00	300	27	9.00
8	300	71	23.67	300	35	11.67
9	300	56	18.67	300	46	15.33
10	300	68	22.67	300	29	9.67
Average	237	50	21 ± 2^a	252	27	10 ± 3^b

* The calli after 2 weeks on selection medium after co-cultivation.
** The survival calli on selection medium at 1 month.
*** Transformation efficiency = No. resistant callus** x 100 / No. callus*
Within a column, values with the different superscripts (a, b) are significantly different ($P \leq 0.05$).

Table 2. Transformation efficiency of Koshihikari callus with 100 μM and 200 μM acetosyringone at 25°C.

Replication	100 μM			200 μM		
	No. calli*	Resistant calli**	Transformation efficiency (%)***	No. calli*	Resistant calli**	Transformation efficiency (%)***
1	240	21	8.75	210	34	16.19
2	240	32	13.33	210	43	20.48
3	240	27	11.25	240	39	16.25
4	270	35	12.96	240	45	18.75
5	270	41	15.19	240	42	17.50
6	300	47	15.67	300	57	19.00
7	300	53	17.67	300	61	20.33
8	300	64	21.33	300	72	24.00
Average	270	40.00	15 ± 4^b	255	49	19 ± 3^a

* The calli after 2 weeks on selection medium after co-cultivation.
** The survival calli on selection medium at 1 month.
*** Transformation efficiency = No. resistant callus** x 100 / No. callus*
Within a column, values with the different superscripts (a, b) are significantly different ($P \leq 0.05$).

regeneration medium, DNA, RNA, and siRNA extraction (Figure 3E). The number of calli does not increase after several rounds of subculture and after about three months (4-6 subcultures) the calli turned brown with soft texture and died. Therefore, high quality calli were moved onto regeneration medium within two months.

To confirm the integration of T-DNA fragments, the analysis of PCR amplification of genomic DNA of transformed calli and plantlets were amplified with *nptII* primers (Figure 4 and 5). The expected 730 bp fragment band was found in all calli and plantlets after transformation with constructs for knock down *Os1bglu1* and control. No *nptII* band was found in the non-transformed calli and plantlet (Figure 4 and 5, lane 2).

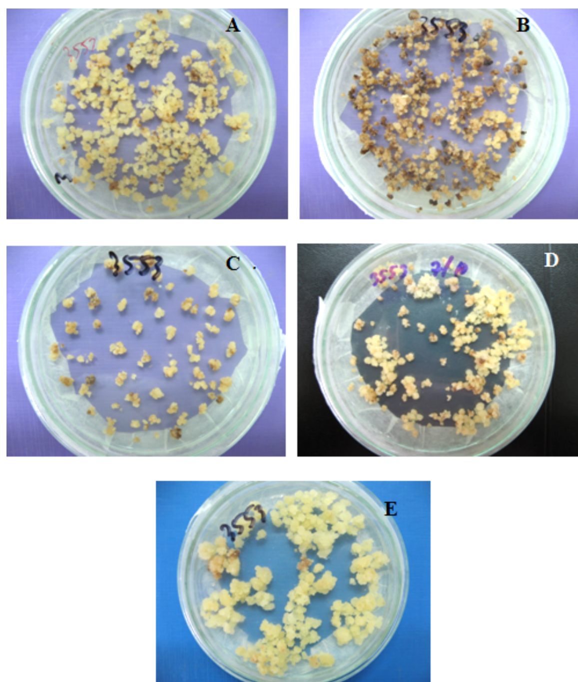

Figure 3. Koshihikari calli on selection medium. Three days after transfer on to selection medium (A), at week 2 on new selection medium (B), the surviving calli after 2 weeks separated on new selection medium (C), the transformed calli at week 4 (D), and the transformed calli at week 8 (E).

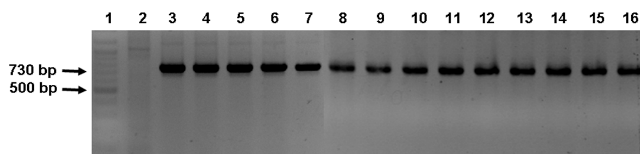

Figure 4. The *nptII* gene PCR products from genomic DNA of transformed calli. Lane 1: 100 bp marker; Lane 2: control genomic DNA of non–transformed calli; Lane 3-7: PCR products of *nptII* gene from different transgenic calli transformed with construct to knock down *Os1bglu1*; Lane 8-16: PCR products of *nptII* gene from different transgenic calli transformed with control construct.

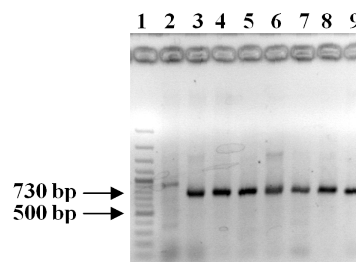

Figure 5. The *nptII* gene PCR products from genomic DNA of plantlets transformed with control construct (empty pHELLSGATE8). Lane 1: 100 bp marker; Lane 2: non-transgenic calli; Lane 3-9: PCR products of *nptII* gene from different transformed plantlets.

The transformation efficiency was observed after transformed with control construct and *Os1bglu1* construct. The results indicated that the calli that transformed with control construct showed higher transformation efficiency at about 20% when compared with the construct to knock down *Os1bglu1* at 15% (Table 3).

3.4 The detection of *Os1bglu1* siRNA and mRNA expression

To confirm the RNAi mechanism in the cell, the siRNAs were detected. siRNA is a product of RNAi after dsRNA cleaved by dicer or Dicer-like (DCL) proteins. Therefore, the presence of siRNAs indicates the occurrence of RNA silencing in cells. The Figure 6 shows the accumulation of *Os1bglu1*-siRNA in the transformed calli, which can an initiate degradation of the *Os1bglu1* transcripts with sequence complementarities. The high amounts of siRNA give more chance to combine with the RISC complex to search and destroy the mRNA target. Figure 6B shows that siRNAs were generated and should be able to completely knock down and the mRNA of *Os1bglu1*. Therefore, *Os1bglu1* mRNA was not detected in Figure 7 lane 2 to 6. This result indicated that

Table 3. Transformation efficiency of transformed calli.

Construct	Replication	No. callus ($\bar{x}$)*	No. resistant calli ($\bar{x}$)**	Transformation efficiency (%)***
Control	17	242	48.12	20 ± 2^a
Os1bglu1	10	246	39.00	15 ± 3^b

* The calli after 2 weeks on selection medium after co-cultivation.
** The survival calli on selection medium at 1 month.
*** Transformation efficiency = $\frac{\text{No. resistant callus**}}{\text{No. callus*}} \times 100$

Within a column, values with the different superscripts (a, b) are significantly different ($P \leq 0.05$).

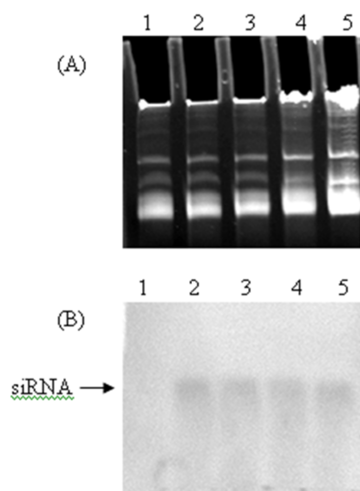

Figure 6. Northern blot analysis of siRNA using 3' UTR probes of *Os1bglu1*. (A) Total RNA sample load in 15% polyacrylamide gel (upper part of gel). (B) siRNA detection on membrane (lower part of gel was cut and moved to blot on the membrane). Lane 1 (A) and (B): paromomycin resistant calli transformed with empty pHELLSGATE8; Lane 2-5 (A) and (B): paromomycin resistant calli transformed with the construct to knock down *Os1bglu1*.

the transformed calli with *Os1bglu1*-pHELLSGATE8 was able to completed knock down of the *Os1bglu1* in calli.

3.5 Effect of knock down *Os1bglu1* in calli on selection medium and regeneration medium

The calli with the *Os1bglu1* knock down show slower growth rate when compared to the control calli (transformed with control plasmid) (Figure 8). The regeneration efficiency in Table 5 shows that only the calli transformed with control constructs could regenerate to plantlets, while the transformed calli with pHELLSGATE8-*Os1bglu1* could not regenerate to plantlet. The regeneration medium was not the factor that prevented the regeneration because the transformed control calli could regenerate to plantlet (6%) (Table 4). This research may conclude that the effect of knock down *Os1bglu1* genes inhibit regeneration of calli to plantlet.

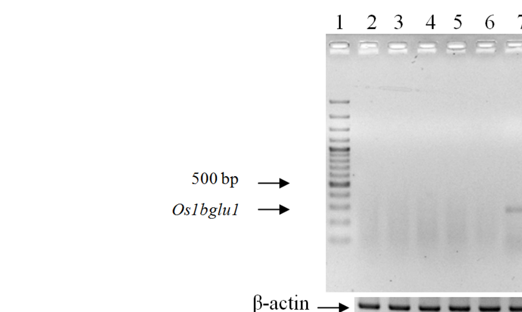

Figure 7. RT-PCR products from mRNA of β-glucosidase in transformed rice calli with construct *Os1bglu1*. Gene specific 3'UTR primers were used to generate *Os1bglu1* and β-actin was used as a control. Lane 1: 100 bp marker; Lane 2-6: paromomycin resistant calli transformed with *Os1bglu1*; Lane 7: paromomycin resistant calli transformed with empty pHELLSGATE8.

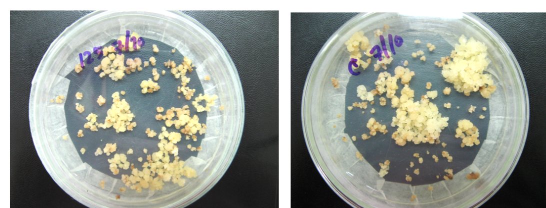

Figure 8. Calli after transformed with pHELLSGATE8- *Os1bglu1* (A) and control constructs (B) on the selection medium at 1 month.

4. Conclusion

One set of proteins that is involved in rice growth is β-glucosidases. Currently, the β-glucosidases functions in rice are yet to be known. In this study, *Os1bglu1* one of the 36 β-glucosidase gene in rice, was chosen for functional analysis by using the 3'UTR region for knock down by *Agrobacter-*

Table 4. Regeneration efficiency of transformed calli with control and *Os1bglu1*-pHELLSGATE8.

Construct	Replication	No. callus ($\bar{x}$)	No. plantlet ($\bar{x}$)*	Regeneration efficiency (%) **
Control	7	150	8.29	6 ± 2^{a}
Os1bglu1	9	178	0	0

* The plantlet on regeneration medium at 2 months.
** Regeneration efficiency = $\frac{\text{No. resistant callus*}}{\text{No. Plantlet}}$ x 100

Within a column, values with the different superscripts (a, b) are significantly different (P≤0.05).

ium transformation RNAi study. The secondary calli of Koshihikari were obtained after 4-6 weeks on N6D medium at 28°C and a light was not an important factor for callus induction. In this research, 200 µM of acetosyringone was a suitable concentration for rice transformation. The lower concentration led to decreased transformation efficiency. The co-cultivation of three days shown to be suitable time to gave high transformation efficiency. Longer time led to high percentage necrosis calli and shorter time led to low transformation efficiency. Temperature is an important factor for controlling the *Agrobacterium* growth, the plant cell infection and the T-DNA insertion into the rice callus. 25°C was suit for these co-cultivation conditions. *Agrobacterium* concentration is also a critical factor for the transformation efficiency. The high concentration of *Agrobacterium* led to calli necrosis. *Agrobacterium* concentration at $OD_{600} = 0.02$ in infection medium reduced the necrosis of calli and resulted in high transformation efficiency. The T-DNA insertion was confirmed by PCR with *nptII* primers all calli resistant paromomycin were transgenic calli. The transformed calli with control construct showed highest transformation efficiency than pHELLSGATE8-*Os1bglu1* constructs. The RT-PCR indicated a complete knock down of mRNA expression of *Os1bglu1* and siRNAs in the transgenic calli were found. Only the transformed calli with control construct were able to regenerate to the plantlet. This research indicated that the *Os1bglu1* may involve in the growing of calli and regeneration of rice cv. Koshihikari in tissue culture process.

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