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Research Article

Efficiency of transformation mediated by *Agrobacterium tumefaciens* using vacuum infiltration in rice (*Oryza sativa* L.)

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Abstract *Agrobacterium*-mediated gene transfer has recently been developed to improve rice transformation. In this study, 3 different transformation methods were tested including soaking, co-cultivation, and vacuum infiltration. *Agrobacterium tumefaciens* GV3101 harboring the binary vector *pGreen:: LeGSNOR* was used in this experiment. This study aimed to identify the most appropriate method for transferring *LeGSNOR* into rice. Vacuum infiltration of the embryonic calli for 5 min in Ilpum resulted in high transformation efficiency based on confirmation by PCR, RT-PCR, and qRT-PCR analyses. In conclusion, we described the development of an efficient transformation protocol for the stable integration of foreign genes into rice; furthermore, the study results confirmed that PCR is suitable for efficient detection of the integrated gene. The vacuum infiltration system is a potentially useful tool for future studies focusing on transferring important genes into rice seed calli, and may help reduce time and effort.

Keywords *Agrobacterium tumefaciens*, Transformation, Soaking seeds, Co-cultivation, Vacuum infiltration, Rice

Introduction

Rice (*Oryza sativa* L.) is a cereal that is consumed by almost every population worldwide, especially in Asia. The consumption of rice as a staple food has increased with the increasing rate of global population growth, particularly in Asia. Asia is a continent with a large population, which is known as a center of rice production (Kubo and Purevdorj 2004). The current world population of 7 billion inhabitants is predicted to increase to 9.2 million by 2050, whereas demand for food will increase by up to 50% (Duan et al. 2012). However, the current supply of rice would not be able to meet this level of consumer demand. There are several reasons for the limited supply of rice, including biotic and abiotic stress on the land, which disturb plant cell metabolism. The use of genetic engineering to manipulate host chromosomes by transferring genes to alter host characteristics is one way to solve this problem. Gene manipulation may be achieved by conventional and modern methods. Transformation is one method used to manipulate and transfer genes isolated from other sources (including plants, viruses, bacteria, and animals) onto a new background in order to obtain new characteristics. *Agrobacterium tumefaciens* has been used for transformation in many plant species because it is able to stably transfer intact high molecular weight DNA into the plant genome (Hamilton et al. 1996). Utilization of this bacterium is easy in the laboratory (Toki 1997; Nishimura et al. 2007), and it is easy to attain high efficiency of rice transformation (Sahoo et al. 2011; Shri et al. 2013; Hoque et al. 2005). Furthermore, *A. tumefaciens* is able to transform plants with high efficiency, with relatively large numbers of gene copies being potentially inserted into plant chromosomes. In nature, wild type *A. tumefaciens* is a natural pathogen of many dicotyledonous species, which causes crown gall disease in the host (Tzfira et al. 2004; De La Riva et al. 1998). Crown gall arises from the transfer of genes in *A. tumefaciens* into plant cells and their expression therein, leading to uncontrolled cellular proliferation and the synthesis of compounds that are specifically metabolized by *A. tumefaciens* (Escobar and Dandekar 2003). The process of transferring T-DNA to plant chromosomes has been adopted to introduce foreign DNA from other sources into target plants to manipulate plant characteristics. Hiei et al. (2014) demonstrated that monocotyledonous plants might also be manipulated by gene insertion mediated by *A. tumefaciens.* Nitric oxide (NO) signals in defense responses in organisms, particularly in plants. The

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regulation of NO production in plants is similar to that occurring in mammals. In plants, L-arginine is used as a substrate to produce L-citrulline and NO (Hancock 2012). Furthermore, S-nitrosoglutathione reductase (GSNOR) is an enzyme present in plants and, in other organisms as formaldehyde dehydrogenase (FALDH), which is a type III alcohol dehydrogenase. GSNOR activity can be regulated under biotic and abiotic environmental stresses (Gong et al. 2015). Based on the function of GSNOR in other plants, we isolated the GSNOR gene from tomato (Kubienová et al. 2013), namely *LeGSNOR,* for the transformation of *japonica* rice. Rice was one of the first monocotyledonous plants used by researchers to transfer genes into its chromosomes (Hiei et al. 1994). Subsequently, rice transformation has been the subject of extensive study, with methods that use *A. tumefaciens* being improved to obtain the highest efficiency. Moreover, Cheng et al. (2004) identified some factors that influence transformation efficiency in monocotyledonous plants, including plant genotype, explant type, and the strain of *A. tumefaciens* used. Recently, vacuum infiltration was trialed to improve the high-throughput of transgenic *indica* rice plants via *A. tumefaciens* transformation (Lin et al. 2009). Vacuum infiltration showed 9.45% efficiency when transferring the target gene in soybean (Mariashibu et al. 2013). In this study, a method of transformation using vacuum infiltration was developed using different timeframes to determine the efficiency of rice callus transformation.

Materials and methods

Plant materials, *Agrobacterium tumefaciens* strain, and binary vector

The mature seeds of japonica rice (*Oryza sativa* L.) cultivars Ilpum and Ilmi used in this study were grown during summer 2013, in the fields of the Affiliated Experimental Practice, Kyungpook National University, Korea. The seeds were manually de-hulled and sterilized in 70% ethanol (EtOH) for 1 min with vigorous shaking. They were then washed three times with autoclaved sterile distilled water, followed by sterile distilled water containing 1% sodium chloride (NaOCl) for 30 min at 120 rpm. The samples were then washed three times with sterile water, after which they were dried on sterile filter paper for 1 h at room temperature before they were ready to use. The *A. tumefaciens* strain GV3101 harboring binary plasmid pGreen I 0029 was used as the transfer vector in this study. The binary vector contains *LeGSNOR* as a reporter gene and neomycin phosphotransferase II (NPTII) as a selective

Supplementary 1 List of the media used in this study

Medium	Composition
Soaking	Luria-Bertani (LB) broth[a], 50 μg/mL kanamycin[b], 10 μg/mL rifampycin[b], 50 μg/mL gentamycin[b]
Seedling MS	4.41 g/L Murashige and Skoog (MS) vitamin powder[c], 30 g/L sucrose[c], 4 g/L gerlite[c], 100 μM acetosyringone[b] (pH 5.2)
Regeneration MS	4.41 g/L MS vitamin powder, 30 g/L sucrose, 5 mg/L kinetin and 1 mg/L 1-naphthaleneacetic (NAA)[d], 250 mg/L carbenecillin[c], 50 mg/L geneticin[e], 4 g/L gerlite
Growing MS	4.41 g/L MS vitamin powder, 30 g/L sucrose, 250 mg/L carbenecillin, 50 mg/L geneticin, 4 g/L gerlite
Callusing	4.14 g/L MS vitamin powder, 2 mg/L 2.4-dichlorophenoxyacetic acid (2,4-D)[b], 30 g/L sucrose, 2 g/L casein hydrolysate[c], 4 g/L gerlite
Co-cultivation	4.41 g/L MS vitamin powder, 5 mg/L kinetin, 1 mg/L NAA, 2 g/L casein hydrosalate, 30 g/L sucrose; 5 g/L silver nitrate[b], 10.5 mg/L L-cysteine[b], 1 mM sodium thiosulfate[b], 4 g/L gerlite, 100 μM acetosyringone (pH 5.2)
Infection liquid	MS + 100 μM acetosyringone
Callusing vacuum	4 g/L N_6 powder[c], 30 g/L sucrose, 2.878 g/L L-proline[b], 0.3 g/L casein hydrolysate, 0.1 g/L myo-inositol[b], 2 mg/L 2,4-D, 4 g/L gelrite
Co-cultivation vacuum	4 g/L N_6 powder, 30 g/L sucrose, 10 g/L D-glucose[b], 0.3 g/L casein hydrolysate, 2 mg/L 2,4-D, 4 g/L gelrite, 100 μM acetosyringone (pH 5.2)
Selection and elimination	4 g/L N_6 powder, 30 g/L sucrose, 2.878 g/L L-proline, 0.3 g/L casein, 2 mg/L 2,4-D, 4 g/L gelrite, 250 mg/L carbenecillin, 150 mg/L kanamycin
Regeneration	4.41 g/L MS vitamin powder, 30 g/L sucrose, 30 g/L sorbitol$_b$, 2 g/L casein hydrolysate, 2 mg/L kinetin, 1 mg/L NAA, 0.1 g/L myo-inositol, 4 g/L gelrite, 500 mg/L carbenecillin, 50 mg/L geneticin

[a]Becton, Dickinson and Company, New Jersey, United States, [b]Sigma-Aldrich, St. Louis, Missouri, United States, [c]Duchefa Biochemie, BH Haarlem, Netherlands, [d]Junsei Chemical co., Ltd, Tokyo, Japan, [e]Phytotechnology Laboratories, Overland Park, United States [f]Yakuri Pure Chemical, Kyoto, Japan

marker gene. The genes are driven by the CaMV 35S (P35s) promoter (Fig. 2). *A. tumefaciens* was maintained on solid Luria-Bertani (LB) agar medium supplemented with 50 mg/L kanamycin, 10 mg/L rifampicin, and 50 mg/L gentamycin.

Agrobacterium-mediated preparation

A primary culture of the *A. tumefaciens* strain GV3101 *pGreen::LeGSNOR* was prepared by inoculating a single colony from a freshly streaked plate in 10 mL of autoclaved liquid LB broth containing 50 mg/L kanamycin, 10 mg/L rifampicin, and 50 mg/L gentamycin. The culture was incubated for 1 day on a rotatory incubator shaker at 200 rpm in the dark at 28°C. Once the OD_{600} reached ~1.0, *A. tumefaciens* cells were collected by centrifugation at 13,000 rpm for 10 min.

Media used for rice transformation

Different media were used at different stages of the experiment. Supplementary 1 presents a list of the media that were used. The pH of the medium was 5.8, unless otherwise stated in the table.

Method of soaking seeds

In this experiment, both cultivars were used in the transformations. The transformation procedure began after the sterilized seeds had been soaked in soaking medium for 1, 2, and 3 days at 28°C under dark conditions. The seeds were then dried and placed on seedling MS medium under continuous dark conditions for 3 days and under 16/8 h light/dark conditions for a further 2 days. Subsequently, the seeds were placed on regeneration medium under 16/8 h light/dark conditions for 2 weeks. Regenerated seedlings were transferred to growing MS medium for 1 week, and the leaves were cut for use as putative transformant samples.

Different co-cultivation methods

In this experiment, Ilpum calli were used as transformation explants. The sterilized seeds were cultured on callusing medium at 28°C under continuous dark conditions for 30 days. The embryogenic calli were separated from the endosperm and shoot (Fig. 1), at which point they were considered ready for infection. The explants were shaken in infection liquid at 28°C for 30 min in a rotary incubator and dried on autoclaved filter paper for 45 min at room temperature. The dried calli were placed in co-cultivation medium under continuous dark conditions for different durations and at different temperatures as follows; A:

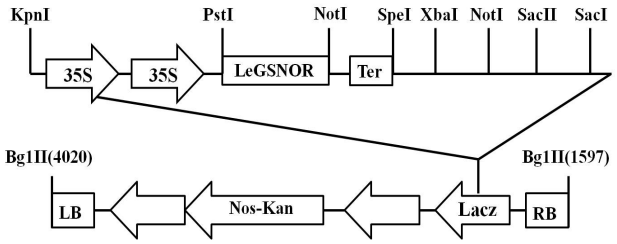

Fig. 1 Embryogenic callus ready for use as an explant transformant; the lines (a and b) show the cut side

28°C for 3 days, B: 28°C for 1 day followed by 25°C for 2 days, and C: 25°C for 3 days. Each treatment was repeated three times. The calli were inoculated on selection and regeneration medium for 45 days, and were subcultured 3-4 times in the same selection and regeneration media at 2-week intervals. Subsequently, the calli were transferred to a test tube containing the selection and regeneration media and left for 45 days. After this time-period, the leaves were cut and used as samples for the putative transformant.

Vacuum infiltration method

Both cultivars were used for transformation. The sterilized seeds were cultured on callusing vacuum medium at 32 ± 1°C under continuous dark conditions for 10 days, and were then separated from the endosperm and shoot to prepare the explants for infection. Subsequently, the calli were transferred to a falcon tube containing the *A. tumefaciens* suspension, and were vacuum infiltrated for 0, 5, 10, and 10 min at 80 kPa using a vacuum infiltrator (Hanil R&D, Korea) (Lin et al. 2009). After treatment, the calli were shaken at 28°C for 30 min in a shaker incubator and dried on sterile filter paper for 45 min at room temperature. Thereafter, the infected calli were incubated on co-cultivation media at 28°C for 1 day and 23°C for 3 days under dark conditions. In this study, 0 min was used as the control treatment, with no vacuum being used. Each treatment was repeated three times. After 4 days of co-cultivation, the explants were eliminated from *A. tumefaciens* by washing five times with sterilized distilled water. Subsequently, the explants were washed with 250 mg/L carbenicillin, and dried on sterile filter paper for 1 h at room temperature. The explants were inoculated on elimination medium for 7 days, after which they were transferred to regeneration medium. Infected calli were sub-cultured on fresh selection and regeneration media, three times over a 2-week interval. After 1 month of final sub-culturing, the putative transformants were transferred to a test tube containing regeneration media for complete rooting.

DNA isolation and polymerase chain reaction

Genomic DNA was isolated from the rice leaves of transgenic and non-transgenic plants for molecular analysis. DNA was isolated from rice leaves using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For molecular confirmation, and to confirm the presence of the transgene in *LeGSNOR* positive rice, the transformed and non-transformed rice samples were analyzed by PCR analysis. PCR was performed using *Ex Taq* DNA polymerase (TaKaRa, Otsu-shi, Japan) in a total volume of 30 μL. Total genomic DNA from various independent transgenic lines was used for PCR using *LeGSNOR*-specific primers (5′-AGCACTGCAGATGGCTACACAAGGTCAAGT-3′ and 5′-AGCAGG CGGCCGCTCATACAAACATATCCA-3′), generating a *LeGSNOR* fragment of 1163 bp. The PCR conditions were 94°C for 10 min; 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were visualized by electrophoresis on a 0.8% agarose gel (Nihon Eido Co. Ltd, Tokyo, Japan).

RNA isolation and reverse transcriptase PCR (RT-PCR)

Total RNA from the rice leaves was extracted and isolated from transgenic and non-transgenic plants (Qiagen, Hilden, Germany). The extracted total RNA samples were used for RT-PCR analysis. A SuperScript® III Reverse Transcriptase kit (Invitrogen, Carlsbad, United States) was used for the analysis. RT-PCR primer pairs used to amplify *LeGSNOR* were 5′-AGCA CTGCAGATGGCTACACAAGGTCAAGT-3′ and 5′-AGCAGGCGGCCGCTC ATACAAACATATCCA-3′, generating a *LeGSNOR* fragment of 1,163 bp in length. The RT-PCR conditions were 1 cycle at 55°C for 30 min to synthesize cDNA, followed by 94°C for 2 min for denaturation, and then 40 cycles at 94°C for 15 s, 48°C for 30 s, and 68°C for 1 min, and final extension at 68°C for 5 min. The PCR products were visualized by electrophoresis on 0.8 % agarose gels.

cDNA and quantitative real time PCR (qRT-PCR)

Samples of total RNA were used to synthesize cDNA. Then, the samples were used for qRT-PCR. For the synthesis of cDNA from RNA, a qPCR SyGreen 1-Step kit from the PCR biosystem was used to analyze the expression of *LeGSNOR*. A 10-μL volume of 1000 ng total RNA was mixed with 4 μL of synthesized 5X cDNA, 1 μL RTase, made up to 20 μL with nuclease free water. Quantitative Eco Real Time PCR (Illumina, California, United State) was used in this study. PCR primer pairs used to amplify *LeGSNOR* were 5′-AGCACTGCAGA TGGCTACACAAGGTCAAGT-3′ and 5′-AGC AGGCGGC CGCTCATACAAACATATCCA-3′. The primer pairs used to amplify the reference gene (OsActin) were 5′-TCCATC TTGGCATCTCTCAG-3′ and 5′-GTACCCGCATCAGGCATC-3′. qRT-PCR conditions were 95°C for 2 min in order to activate the polymerase, followed by 40 cycles of 95°C for 10 s, 48°C for 30 s, 72°C for 15 s, and final melting curve generation at 95°C for 15 s, 55°C for 15 s, and 95°C for 15 s.

Flanking analysis

About 100 ng of genomic DNA was digested with 4-5 units of *Bfa*I (New England Biolabs, Frankfurt, Germany) in a total volume of 20 μL for approximately 3 h at 37°C, following which, *BfaI* was inactivated at 65°C for 20 min. An aliquot of 5−10 μL from the restriction reaction was used for ligation [20 μL total volume, approximately 3U of T4 DNA ligase (New England Biolabs, Frankfurt, Germany), for 11 h at 2°C], with 12.5 pmol of the *Bfa*I adaptor. Subsequently, the ligation was inactivated by incubation at 65°C for 10 min. The first linear amplification was performed in a 50 μL reaction mix, with the whole *BfaI* digestion process using the AP1 primer (5′-GGATTCTAATACGACTCACTATAGGGC-3′) and primer TDNA3 (for LB) (5′-CCACATTGCAATAGTCGAACGTA-3′) or primer AP1-R (for RB) (5′-CCTAGGATTATGCTGAGT GATATCCCG-3′) at a concentration of 200 nM, 250 μM of each dNTP, and 1.5 Unit *Taq* DNA polymerase (TaKaRa, Otsu-shi, Japan). Amplification was performed under the following conditions: 94°C for 10 min, 40 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min, and then elongation at 72°C for 5 min. One-microliter from the first amplification reaction was used as a template for the second PCR amplification in a total volume of 50 μL with AP2 primers (5′-TATAGGGCTCGAGCGGC-3′), nested T-DNA primers (TDNA4-p Green (5′-AACCGTCCTATATAACACCACA TT-3′) for LB and AP2-R (5′-AT ATCCCGAGCTCGCCG-3′) for RB), and adaptor primers. The PCR reagents and cycling parameters were identical to those used in the first amplification step. Results from the second set of PCR products were checked, along with the sequences of the region flanking the RB and LB of the T-DNA insert. The sequences of the flanking regions were analyzed using a BLASTN search routine against sequences of the binary vector, the genomic sequence assemblies, and all other existing sequences in the online database.

Results

In this experiment, the soaking seeds method affected germination of infected seeds (Supplementary 2). The highest

Supplementary 2 Effect of the soaking method on seed germination

Cultivar	Duration of soaking (days)								
	1			2			3		
	No. of inoculated seeds	No. of germinated seeds	No. of transformant seeds	No. of inoculated seeds	No. of germinated seeds	No. of transformant seeds	No. of inoculated seeds	No. of germinated seeds	No. of transformant seeds
Ilmi	328	54 ± 28.3[a]	0 ± 0	312	33 ± 15.6	0 ± 0	312	28.5 ± 12.0	0 ± 0
Ilpum	334	114 ± 63.6	0 ± 0	360	99 ± 43.8	0 ± 0	247	68.5 ± 20.5	0 ± 0

[a]Mean ± standard deviation

Supplementary 3 Effect of different types of co-cultivation methods on calli development

Type of co-cultivation	No. of inoculated calli	No. of green spots	No. of regenerated plants	No. of transformants
A	204[a]	9.3 ± 3.1[b]	1.6 ± 0.9	0 ± 0
B	251	30.3 ± 31.4	6.0 ± 4.2	0 ± 0
C	229	11.7 ± 7.6	4.2 ± 1.3	0 ± 0

[a]No. of inoculated calli, [b]Mean ± standard deviation, A: 28°C for 3 days, B: 28°C for 1 day and 25°C for 2 days, C: 25°C for 3 days

Table 1 Number of calli regenerated under the vacuum treatment method

Cultivars	Vacuum treatment (min.)											
	0			5			10			15		
	N[a]	PR[b]	T[c]	N	PR	T	N	PR	T	N	PR	T
Ilmi	249[d]	8.5 ± 12.0[e]	0	253	31.7 ± 16.0	0	266	55.3 ± 21.6	0	264	19.0 ± 17.3	0
Ilpum	269	26.3 ± 45.6	0	276	41.7 ± 33.2	9	275	0 ± 0	0	287	0 ± 0	0

[a]Number of inoculated calli, [b]number of regenerated plants, [c]number of transformants confirmed with PCR, [d]sum of calli with each replication, [e]mean ± standard deviation

germination rate (114 ± 63%) was obtained by soaking in Ilpum for 1 day. Our data showed that Ilpum led to higher germination rates than Ilmi in all of the soaking treatments. For both cultivars, soaking treatment for 1 day was the optimal duration for germinating seeds. When using this method, multiple shoots were produced following 2 and 3 days of soaking.

Supplementary 3 shows that different types of co-cultivation had different effects on the initiation of the green spot and the number of plants that regenerated. The B-type co-cultivation method conditions (28°C for 1 day followed by 25°C for 2 days) were found to be optimal, but transformants were not produced by this method. Rice development under the different co-cultivation methods showed that 30-day-old calli were inducted in the callusing medium. The embryogenic callus was first cut (Fig. 1) for use as the explant. The initiation of a green spot was detected after 7 days in the regeneration medium, which initiated leaf growth 2 weeks later.

The duration of vacuum infiltration had a notable effect on the number of calli that regenerated throughout the whole experiment (Table 1) and in every subculture (Supplementary 4) of both cultivars. Vacuum infiltration for 5 min led to the production of 41.7 ± 33.2 plant regeneration (Table 1) for Ilpum, whereas 10 min of vacuum treatment produced 55.3 ± 21.6 plant regeneration for Ilmi. Calli were observed to regenerate at different subculture durations. Kim et al. (2012) suggested that the somatic embryos could be continuously proliferated for years through the repetitive subculture of embryogenic calli. In this study, plant regeneration greatly

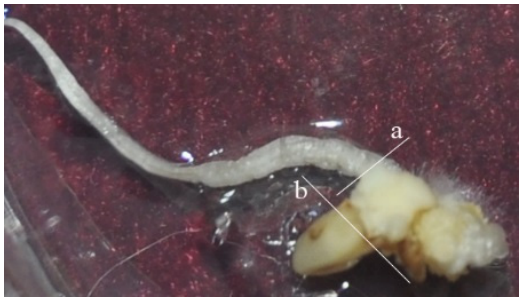

Fig. 2 Schematic of the plasmid vector *pGreen::LeGSNOR*. The binary vector harbors *LeGSNOR*, CAMV 35S as as promoter, and neomycin phosphotransferase II (NPTII) as a selective marker gene

Supplementary 4 Number of calli regenerated after subculturing times

Sub-culture time (day)	cultivar	Vacuum treatment (min.)											
		0			5			10			15		
		N[a]	PR[b]	T[c]	N	PR	T	N	PR	T	N	PR	T
14	Ilmi	249[d]	-	-	253	0.3 ± 0.6[e]	-	266	-	-	264	-	-
	Ilpum	269	-	-	276	0.3 ± 0.6	-	275	-	-	287	-	-
28	Ilmi	249	-	-	253	1.3 ± 2.3	-	266	1.0 ± 1.7	-	264	1.3 ± 2.3	-
	Ilpum	269	1.3 ± 2.3	-	276	1.7 ± 2.9	-	275	-	-	287	-	-
42	Ilmi	249	1.0 ± 1.7	-	253	1.7 ± 2.9		266	2.7 ± 4.7	-	264	1.3 ± 2.3	-
	Ilpum	269	2.0 ± 3.5	-	276	5.1	9	275	-	-	287	-	-

No. of inoculated calli, [b]No. of regenerated plants, [c]No. of transformants confirmed with PCR, [d]Sum of calli with each replication, [e]Mean ± standard deviation, -: no data

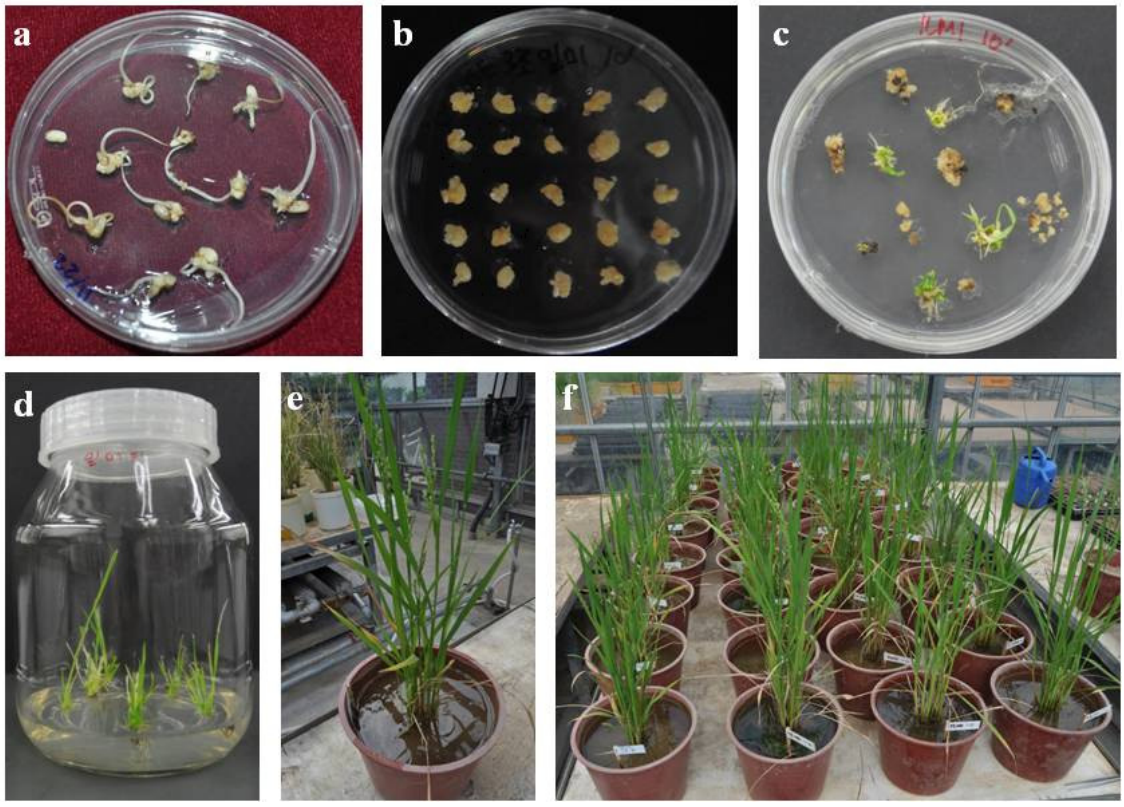

Fig. 3 Noticeable plant regeneration. (a) Calli induction, (b) co-cultivation of calli, (c) regeneration of calli, (d) plant regeneration in a bottle, (e) individual plant in pot, and (f) transgenic plant after acclimatization in a green house

increased in the two cultivars at different subculture durations. Supplementary 4 shows that Ilpum was faster than Ilmi at initiating plant regeneration in the 0-min vacuum treatment after 28-days subculture.

Plant regeneration was initiated after 14-days subculture for both cultures in the 5 min vacuum treatment group, but not the 10 and 15 min vacuum treatment groups. It was easy to regenerate Ilmi plants using this method. The notable plant regeneration observed in this study is shown in Figure 3. Vector backbone DNA sequences in transgene loci were detected at both left and right borders by flanking analysis. Nine transformed plants were tested and transformant numbers 74 and 90 were inserted into chromosome number 10 and 4 (Supplementary 5).

The number of plants integrating the transgene based on PCR is shown in Supplementary 4 and Table 1. Approximately 125 plants, nine plants were positive for *LeGSNOR* amplification. PCR results from some of the samples are presented in Figure 4a. The quantity of the mRNA transcript of a single gene is a direct reflection of the level of *LeGSNOR* transcription.

Supplementary 5 Results summary of flanking analysis

Query name	Read Length (bp)	Insert to	Adaptor Start	Insert Length (bp)	Chro-mosome	Mat-ching (%)	Mat-ching Length	Chr Start	Chr End	Type	Gene_ID	Description
74_A	778	778	-1	778	10	99.7	774	5777561	5776789	Intergenic	Os10t0187366-01 downstream 8.416kb	Hypothetical protein.
74_B	507	495	496	495	4	99.2	495	24411663	24412156	5'Upstream-1000	Os04t0477900-01 upstream 0.394kb	Similar to Cysteine proteinase EP-B 1 precursor (EC 3.4.22.-).
90_A	766	766	-1	766	10	99.6	759	5777560	5776802	Intergenic	Os10t0187366-01 downstream 8.417kb	Hypothetical protein.
90_B	436	424	425	424	4	98.6	424	24411733	24412156	5'Upstream-1000	Os04t0477900-01 upstream 0.324kb	Similar to Cysteine proteinase EP-B 1 precursor (EC 3.4.22.-).

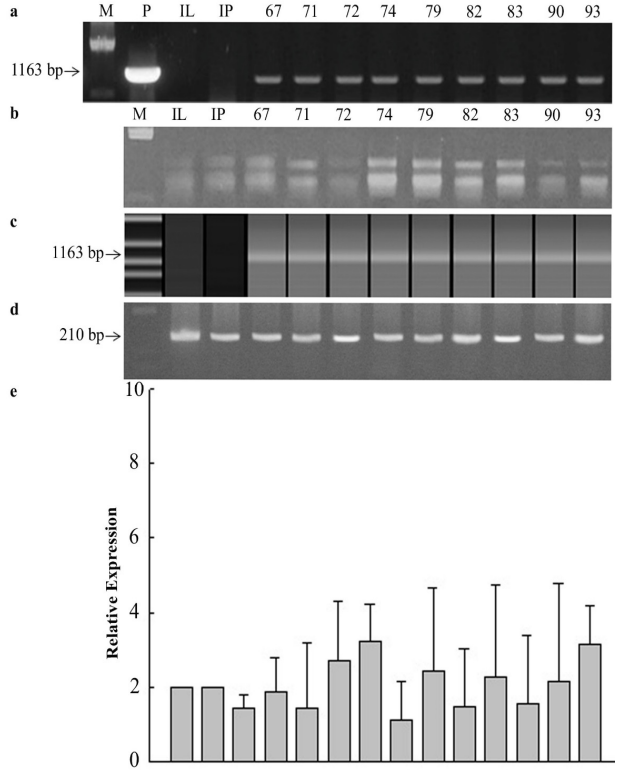

Fig. 4 PCR analysis of leaf tissue from a regenerated plant. *Lane M* Lambda DNA/*Hind* III marker, *Lane P* Plasmid DNA, *Lane IL* Ilmi, *Lane IP* Ilpum, *Lane 67*, *71*, *72*, *74*, *79*, *82*, *83*, *90*, *and 93* transformants in Ilpum. (a) Amplification of *LeGSNOR* by PCR, (b) total RNA, (c) amplification of *LeGSNOR* by RT-PCR, (d) amplification of *OsActin* and (e), qRT-PCR analysis

Reverse transcriptase-PCR (RT-PCR) of total RNA was used to confirm the presence of mRNAs in each of the transformants (Fig. 4b). To compare the levels or changes in *LeGSNOR* expression, quantitative real time PCR was used for further analysis. Figure 3c shows that gene expression in all nine transformants was higher than that in non-transformed rice (Ilmi and Ilpum). Out of the transformants, sample number 90 had the highest expression, followed by 74, 72, 93, 83, 82, 67, 79, and 71.

Discussion

Successful rice transformation has been achieved via *Agrobacterium*-mediated gene transfer (Aldemita and Hodges 1996) using various methods. However, the development of high-throughput transformants following the genetic transformation of monocotyledonous plants remains limited, because these plants do not serve as a natural host for *A. tumefaciens* (Aldemita and Hodges 1996). Consequently, in recent years alternative procedures have been developed for *Agrobacterium*-mediated transformation in monocotyledons. Such procedures include vacuum infiltration and the piercing method (Lin et al. 2009), co-cultivation (Hei et al. 1994), utilizing of different *A. tumefaciens* strains (Balaji et al. 2003), and the use of different cultivars (Huang et al. 2001; Saika and Toki 2010; Chen et al. 2004) and explants (Manimaran et al. 2013; Karthikeyan et al. 2011; Dey et al. 2012). In our study, three types of methods were developed for transferring *LeGSNOR* into *japonica* rice.

The first method involved soaking seeds, which was inferred by phenomenal seed imbibition in nature. Imbibition is the phenomenon of water absorption, or any other liquid, by the solid particles of substances, without forming a solution. In plants, imbibition is one of the steps that triggers the activation of enzymes during the early germination stage. In the current study, we attempted to transfer *LeGSNOR* into the rice chromosome by infecting seeds through germination in *A. tumefaciens* suspension. The longest shoots were formed after 3-days soaking, followed by 2 days and 1 day. The longest shoots may have been infected by *A. tumefaciens*, thus facilitating *LeGSNOR* transfer; however, no transformants could be amplified by PCR. This method could be improved

to obtain transformed rice by germinating the seeds in medium containing 2,4-D in order to multiply shoots. Multiple shoots were assumed to occur because of the effect of *LeGSNOR* insertion in the rice chromosome. Thus, one of the multiple shoots could potentially be used as the transformant; however, no transformant was produced from this method. Germination is necessary to obtain a shoot and facilitate *A. tumefaciens* infection of the explants. In this method, the development of germinating seeds was initiated by the uptake of water from the environment and the rehydration of seed tissues by imbibition. Water passes through the embryo, and picks up the germination signal, which is provided by the hormone, gibberellic acid. The water moves the hormone from the embryo to the aleurone layer of the endosperm. The water activates the hydrolysis enzymes that degrade the storage protein into amino acids. Gibberellic acid activates the gene encoding the enzyme amylase in the aleurone cells. The radicle protrudes from the seed, and germination is accomplished (Hopkins and Huner 2009). In the current study, after soaking, the seeds were transferred to seedling MS medium and maintained under dark conditions for 3 days. The shoot was not green in color because of the lack of photosynthesis under dark conditions. Thus, it was then transferred to 16/8 h light/dark conditions for 2 days. Under the light condition, the leaf turned green and photosynthesis was initiated leading to shoot and plant growth.

The second method involved different types of co-cultivation, whereby different temperatures were tested based on the optimal conditions for both *A. tumefaciens* and explants. In this method, calli were used as transformation explants. Temperature has been documented to affect *A. tumefaciens* Phytochrome Agp1 (Njimona and Lamparter 2011), and it is critical for ensuring the transfer of *A. tumefaciens* machinery to the plant (Fullner and Nester 1996). In this study, three different co-cultivation methods were tested: 28°C for 3 days, 28°C for 1 day followed by 25°C for 2 days, and 25°C for 3 days. We selected 28°C because it is the optimal temperature for *A. tumefaciens* growth, whereas 25°C was selected because it is the optimal temperature for the induction of *vir* genes in *A. tumefaciens.* The number of transformants was obtained from this method. Temperature was observed to affect the development of rice calli. Based on our data, the combined temperature treatment (28°C for 1 day followed by 25°C for 2 days) resulted in the highest green spot initiation and plant regeneration.

Vacuum infiltration is an *Agrobacterium*-based transformation system, in which plant tissue is submerged in *A. tumefaciens* suspension and subjected to vacuum at the appropriate pressure (Bechtold et al. 1993; Bechtold and Pelletier 1998; Tague and Mantis 2006). A pressure of 80 kPa is used for this method (Lin et al. 2009) for different lengths of time (0, 5, 10, and 15 min). The data collected in the current study (Table 1) show that the two cultivars, Ilmi and Ilpum, responded differently at several stages of transformation. In this study, calli were shown to differ in responsiveness at different times in both cultivars (Ilmi and Ilpum) of *japonica* rice by improving the efficiency of transformation. The transformant was obtained following 5 min vacuum treatment of Ilpum. During 5 min vacuum treatment, *A. tumefaciens* can infiltrate into the Ilpum to enable transformation. To detect the inserted gene of interest, PCR was used by designing the *LeGSNOR* primer. At the DNA level, conventional PCR was used to detect transformed plants. Based on the results of PCR analysis, nine transformants *LeGSNOR* primer pair transformants were amplified in 9 plants. Traditionally, integrated genes were analyzed in transformed plants using northern blot analysis, and copy number was estimated using southern blot analysis. In this study, RT-PCR analysis was used to confirm the transcript levels of the original RNA samples and qRT-PCR was used to estimate the copy number. Figure 3 shows that the level of *LeGSNOR* transcription in each rice transformant differed. This result was confirmed by qRT-PCR analysis, in which *LeGSNOR* expression differed to that of the reference genes. In non-transformant Ilmi and Ilpum rice, there was no *LeGSNOR* expression compared with that detected for the reference OsActin gene. However, the expression of all transformants differed to that of the reference gene. Scientists have used RT-PCR and qRT-PCR to determine whether a gene has successfully inserted into a plant genome (Tellier et al. 1996; Santos et al. 2004; Shiao 2003; Dean 2002). We believe that it will be easy to analyze integrated genes using PCR-based systems in transformants.

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