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 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
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\(^d\), 100 µM acetosyringone (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)\(^e\), 250 mg/L carbenecillin\(^f\), 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 hydrolysat\(^e\), 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\(^e\), 4 g/L gerlite, 100 µM acetosyringone (pH 5.2) |
| Infection liquid        | MS + 100 µM acetosyringone |
| Co-cultivation vacuum  | 4 g/L N\(_6\) powder\(^b\), 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 gerlite |
| 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 gerlite, 250 mg/L carbenecillin, 150 mg/L kanamycin |
| Regeneration            | 4.41 g/L MS vitamin powder, 30 g/L sucrose, 30 g/L sorbitol\(^c\), 2 g/L casein hydrolysate, 2 mg/L kinetin, 1 mg/L NAA, 0.1 g/L myo-inositol, 4 g/L gerlite, 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:

**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′-AGCAGGCGGCCGCTCATACAAACATATCCA-3′ and 5′-AGCAGGCGGCCGCTCTACATACAAACATATCCA-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′-AGCAGG CGGCCGCTCATACAAAACATATCCA-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 activate the polymerase, followed by 40 cycles of 95°C for 10 s, 48°C for 30 s, 72°C for 15 s, and then elongation 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).

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′-AGCAGGCGGCCGCTCTACATACAAACATATCCA-3′ and 5′-AGCAGG CGGCCGCTCTACATACAAACATATCCA-3′. The primer pairs used to amplify the reference gene (OsActin) were 5′-TCCATCTTGGCATCTCTCAG-3′ and 5′-GTACCGGACATCAGGCATC-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 BfaI (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 BfaI 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 API primer (5′-GGATTCGAGACGACTATAGGAAAGG-3′) and primer TDNA3 (for LB) (5′-CCACATTGCAATAGTCGAACGTA-3′) or primer AP1-R (for RB) (5′-CTAGGATTATGCTGAGTGTATCAGGTCGTA-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, 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′-TATTAGGGTCAGACGTCGAG-3′), nested T-DNA primers (TDNA4-p Green (5′-AACCAGCTCTATATAACACACAT GT-3′) for LB and AP2-R (5′-ATCCCGAGTCGCCGCGG-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) | 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 |
|----------|---------------------------|-------------------------|------------------------|--------------------------|-------------------------|------------------------|--------------------------|-------------------------|------------------------|--------------------------|
|          | 1                         |                         |                        |                          |                         |                        |                          |                         |                        |                          |
| 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                  |                          |

^aMean ± 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                |

^aNo. of inoculated calli, ^bMean ± 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         |
| 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         |

^aNumber of inoculated calli, ^bnumber of regenerated plants, ^cnumber of transformants confirmed with PCR, ^dsum of calli with each replication, ^emean ± 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
Table 1: Number of calli regenerated after subculturing times

| Subculture time (day) | Cultivar | Vacuum treatment (min.) |
|-----------------------|----------|-------------------------|
|                       |          | 0                      | 5       | 10      | 15      |
|                       |          | N^a        | PR^b   | T^c    | N        | PR   | T    | N      | PR   | T    |
| 14                    | Ilmi     | 249^d      | -      | -      | 253      | 0.3 ± 0.6 | -      | 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

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) | Chromosome | Matching (%) | Matching Length | Chr Start | Chr End | Type | Gene_ID | Description |
|------------|------------------|--------------------------|---------------------|-------------|--------------|----------------|-----------|---------|------|---------|-------------|
| 74_A       | 778              | 778                      | -1                  | 778         | 99.7         | 774            | 5777561   | 5776789 | Intergenic | Os10t0187366-01 downstream | Hypothetical protein. |
| 74_B       | 507              | 495                      | 496                 | 495         | 99.2         | 495            | 24411663  | 24412156 | 5'Upstream-1000 | Os04t0477900-01 upstream | Similar to Cysteine proteinase EP-B 1 precursor (EC 3.4.22.-) |
| 90_A       | 766              | 766                      | -1                  | 766         | 99.6         | 759            | 5777560   | 5776802 | Intergenic | Os10t0187366-01 downstream | Hypothetical protein. |
| 90_B       | 436              | 424                      | 425                 | 424         | 98.6         | 424            | 24411733  | 24412156 | 5'Upstream-1000 | Os04t0477900-01 upstream | 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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