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Efficient Genetic Transformation of Rice for CRISPR/Cas9 Mediated Genome-Editing and Stable Overexpression Studies: A Case Study on Rice Lipase 1 and Galactinol Synthase Encoding Genes

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Abstract: Rice is a staple food crop for almost half of the world's population, especially in the developing countries of Asia and Africa. It is widely grown in different climatic conditions, depending on the quality of the water, soil, and genetic makeup of the rice cultivar. Many (a)biotic stresses severely curtail rice growth and development, with an eventual reduction in crop yield. However, for molecular functional analysis, the availability of an efficient genetic transformation protocol is essential. To ensure food security and safety for the continuously increasing global population, the development of climate-resilient crops is crucial. Here, in this study, the rice transformation protocol has been effectively optimized for the efficient and rapid generation of rice transgenic plants. We also highlighted the critical steps and precautionary measures to be taken while performing the rice transformation. We further assess the efficacy of this protocol by transforming rice with two different transformation constructs for generating galactinol synthase (*GolS*) overexpression lines and CRISPR/Cas9-mediated edited lines of lipase (Lip) encoding the *OsLip1* gene. The putative transformants were subjected to molecular analysis to confirm gene integration/editing, respectively. Collectively, the easy, efficient, and rapid rice transformation protocol used in this present study can be applied as a potential tool for gene(s) function studies in rice and eventually to the rice crop improvement.

Keywords: rice (*Oryza sativa*); galactinol synthase; lipase; *Agrobacterium tumefaciens*; transformation

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1. Introduction

Plant breeding is a well-known strategy for the introgression of desirable traits and has played a significant role in ensuring food security for the ever-increasing human population [1]. However, in recent years, the demand for the food supply has been increased tremendously as the world's population has rapidly increased. The situation is exacerbated by the ongoing radical changes in global climatic conditions and the increasing incidences of (a)biotic stresses that further widen the gap between actual and attainable yields. It is expected that the world population will reach nearly 10 billion by 2050 (datatopics.worldbank.org); therefore, it is of the utmost urgency to devise strategies to feed this massive population in the future. Although recent scientific developments in the field of plant breeding provide new opportunities [2], the prerequisites of sexual reproduction and compatible interspecies crossing are among the key limitations of plant

breeding. In addition, the crops developed via traditional breeding further necessitate the elimination of undesirable genes (appearing via linkage drag), which further demand multiple rounds of crossbreeding, thus making this approach laborious and time-consuming. Therefore, the biotechnological interventions possible in crop improvement strategies are now gaining interest among the scientific community and the stakeholders as a way to satisfy the ever-increasing demands for plant-sourced food products.

Recent advances in molecular tools and the advent of modern NGS platforms further open up avenues for the genetic improvement of crop plants, which has a significant impact on agriculture [3–5]. Among the other technologies, the genetic modification (GM) of crop plants has, by far, the greatest impact on global agriculture. GM technology is now widely adopted for commercial applications and is quite instrumental in a wide range of fundamental studies for the understanding of molecular mechanisms pertaining to abiotic and biotic stress tolerance and the importance of biosynthetic pathways, among others [6,7].

Rice is among the top staple foods and accounts for 16.5% of the global calorie intake (https://www.worldatlas.com/articles/most-important-staple-foods-in-the-world.html, assessed on 25 October 2021). Rice domestication is claimed as far back as nearly 8000 to 9000 years ago. Since then, selective breeding for high-yielding and stress-tolerant rice cultivars has restricted the genetic diversity of cultivated rice, rendering it more susceptible to plant pathogens and less resilient to the effects of climate change [8]. In this context, GM technology far outstrips other available breeding technologies. However, the availability of efficient and rapid gene transfer and having subsequent plant regeneration methods in place are imperative for the successful implementation of GM technology. Several methods have been successfully employed in the genetic transformation of rice, including biolistic methods, electroporation, and the PEG-mediated and *Agrobacterium*-mediated delivery of genetic material [9–12]. Among these, *Agrobacterium*-mediated rice transformation is a widely adopted method, due to its low cost, simplicity, and stable and low copy number integration of transgenes [13,14]. However, the major constraints in the *Agrobacterium*-mediated rice transformation method are low transformation efficiency, the poor regeneration potential of the transformed calli, genotype specificity, etc. [14,15]. To address these problems, several studies have been performed on *Agrobacterium*-mediated rice transformation using different explants (such as embryogenic callus, immature embryo and shoot meristem), a different regime of media components, and different strains of *Agrobacterium*, yet these protocols suffer from one or other of many limitations [16–19].

Considering the importance of rice genetic transformation in crop improvement and fundamental genomic studies, in this paper, we have developed a rapid, efficient and reproducible protocol for *Agrobacterium*-mediated genetic transformation for rice. We ameliorated the amount and type of plant growth regulators and the carbon source used for rapid and efficient plant regeneration. We demonstrated the applicability of the present rice genetic transformation protocol for the overexpression of the rice galactinol synthase encoding gene (*GolS*) and the knockout of the rice lipase (Lip) gene via the CRISPR/Cas9 genome-editing approach. The protocol we developed here may be helpful in the search to overcome the key hurdles of rice genetic transformation.

2. Materials and Methods

2.1. Plant Material and Bacterial Strains

Rice (*Oryza sativa*) seeds (var: *Taipei-309*) grown at the National Agri-Food Biotechnology Institute, Mohali (Punjab) were used as an explant for *Agrobacterium*-mediated transformation. For cloning experiments, the *Escherichia coli* DH5α strain was used, while *Agrobacterium tumefaciens* LBA-4404 was used for agro-infection to rice callus. The vectors used in this study are pENTR™/D-TOPO™ (Thermo Fisher Scientific, San Francisco, CA, USA), pANIC6B (CD3-1708) and pRGEB31 (Addgene, Watertown, MA, USA).

2.2. Construct Generation

To generate the overexpression lines of *GolS* in rice, full-length *GolS* without a stop codon was cloned in the gateway destination vector pANIC6B (ABRC, Ohio State University, Columbus, OH, USA), using an LR clonase reaction mixture (Invitrogen, California, USA), according to the manufacturer's instructions. The pANIC6B:*GolS*:*hpt* was transferred into the *Agrobacterium tumefaciens* strain LBA-4404, which was subsequently used for the genetic transformation of the Japonica rice (*Oryza sativa*) cultivar, Taipei-309 variety.

In order to generate CRISPR/Cas9-edited rice plants, an expression vector containing the gRNA targeted to *OsLip1* was constructed. An oligo duplex was formed using the primers specific to gRNA, which was further ligated with the *Bsa*I digested binary vector, pRGEB31, following the procedure used in [20]. The ligated product was transformed in *E. coli* (DH5α) and the isolated colonies were used to extract plasmids. To confirm the gRNA sequence, the constructs were subjected to Sanger sequencing using the M13 reverse primer.

2.3. Embryogenic Callus Induction

The mature and healthy rice seeds (de-husked) were thoroughly rinsed with distilled water 4–5 times, then sterilized with 70% ethanol for 2 min, then with 4% sodium hypochlorite (v/v) and 1 drop of Tween-20 for about 20 min. The sterilized rice seeds were then blotted dry on sterile blotting sheets, which were then placed on callus-induction media and incubated for the induction of embryogenic callus at 28 °C in a dark growth chamber for 10 days. After 10 days, the calli were ready to subculture; thus, radical and seed remainders were carefully expelled with the help of a flame-sterilized blade and scalpel. The calli were sub-cultured on fresh plates of callus induction media, wrapped with Micropore tape, and incubated at 28 °C for 5 days in a growth chamber in the dark.

2.4. Agrobacterium Infection and Co-Cultivation

After 5 days in the chamber, calli were infected with an overnight-grown *Agrobacterium* culture. The culture was prepared from a single colony of *Agrobacterium*, transformed with the binary vectors harboring our gene of interest. The calli were transferred in the autoclaved flask containing the callus induction media (CIM) broth and were then kept in the incubator shaker for 30 min at 28 °C. After 20–30 min, the supernatant was disposed of and the calli were blotted dry on sterile blotting-paper sheets. After drying, the treated calli were placed on round Whatman filter paper kept on solidified agar co-cultivation media plates. The calli were co-cultivated for 36 h at 28 °C in the dark in the plant growth chamber.

2.5. Selection and Regeneration of Transformed Calli

After 36 to 48 h of co-cultivation with the *Agrobacterium* culture, the infected calli were transferred to a selection medium, containing hygromycin (50 µg/L), for 10 days in the dark growth chamber at 28 °C. After three rounds of the selection step, the successful healthy calli were transferred to a regeneration medium for the development of shoots and kept in the dark at 28 °C for 8 days. In the *in vitro* regeneration process, the transformed calli were then shifted to light conditions, with a 16/8-h day/night photoperiod, in the next round. It took about 20 days for the shoot to develop. The regenerated shoots of 1–2 cm were transferred to fresh regeneration media, stored in phyta jars (Himedia, India), and incubated for a 16/8-h day/night photoperiod at 28 °C for 10 days, to obtain 4–5 cm-long shoots. After shoot development, the plantlet was transferred in sterile phyta jars containing the rooting medium for root development for 10–12 days. Phytagel (Merck, Darmstadt, Germany) or CleriGel (HiMedia, India) was used as a gelling agent throughout the preparation of the media.

After the shoot and root development, the plants were maintained hydroponically. The hydroponic system comprises a nutrient solution, i.e., Yoshida solution, for pre-hardening and plants are then incubated in the growth chamber. After incubating in hydroponic

conditions for 10 days at 28 °C in the plant growth chamber, the transgenic plant was transferred to soil to grow in glasshouse conditions.

2.6. Molecular Analysis of Putative Transgenic Plants

For genomic DNA extraction, leaf samples were collected from the putatively transformed plants obtained after the hygromycin selection. The genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method [21], and the isolated DNA was subjected to RNase and proteinase K treatment. The genomic insertion of T-DNA in the putatively transformed plants was confirmed by the PCR amplification of *Cas9* (for CRISPR) and *hptII* (for overexpression) genes. The editing of the target *OsLip1* gene locus was determined via Sanger sequencing using the primers specific to the gRNA flanking region. For overexpression in transgenic lines, the transcript abundance of the gene of interest was determined by using semi-quantitative PCR analysis. The primers used for the molecular analysis of putative transformants are listed in Supplementary Table S1.

3. Result and Discussion

3.1. Brief Overview of the Transformation Protocol

Among the Asian cultivated rice species (*Oryza sativa* L.), there are plenteous genomic resources available for japonica (*O. sativa* L. ssp. *japonica*) subspecies compared to indica (*O. sativa* L. ssp. *indica*) subspecies. Therefore, cultivars of japonica subspecies are mostly used for "omics" and functional studies. Considering the significance of having an efficient rice transformation protocol in genomics studies, we optimized the rice transformation protocol. Although the japonica rice cultivar (*TP-309*) has a high regeneration potential [22], *Agrobacterium* infection and genetic transformation reduce the regeneration potential of totipotent calli [14]. Therefore, in the present study, we optimized the regeneration media composition to enhance the plant regeneration efficiency from the transformed calli. We used dehusked mature rice seed as the explant to induce the generation of embryogenic calli via CIM (Table 1). The advantage of using rice seeds as an explant is their availability throughout the year; moreover, the embryogenic calli are supposed to have higher regeneration potential [23]. Although 98–99% of the seeds developed scutellar calli within the 8 days of inoculation when on the callus induction medium (Figure 1), only the embryogenic scutellar calli (93–96%) were sub-cultured on fresh CIM for 4 days (Figure 1) (see "Detailed protocol with critical points highlighted for troubleshooting"). The sub-cultured calli were then subjected to Agro-infection using *Agrobacterium* (LBA4404), harboring T-DNA plasmids (CRISPR-Cas9 and overexpression construct). In comparison to previous reports, instead of using 3–4-week-old calli for *Agro*-infection, we used 10–12-day-old calli [24–26]. Moreover, the use of early-stage calli has been previously reported to have improved transformation efficiency [27]. The infected calli were then co-cultivated for 36 h on Whatman filter paper (Grade 1) placed on co-cultivation medium (CCM) (Figure 1). This arrangement helps the calli in terms of nutrient acquisition, without direct contact with the CCM, and reduces the contamination events further during selection. After co-cultivation, the calli were thoroughly washed with sterile water containing cefotaxime and carbenicillin antibiotics, then air-dried on filter paper before placing them on selection medium (SM). For the first round of selection, the selection plates were supplemented with cefotaxime and carbenicillin, which further reduced the occurrence of *Agrobacterium* contamination. During the course of selection when on hygromycin, some of the calli developed a brownish color, while the calli that remained creamy in color were transferred until the third round of selection (Figure 1). After this, the third selection when on hygromycin, 48–52% of the co-cultivated calli were available for regeneration. Likewise, Singh et al. [24] recently reported a comparable percentage of viable calli in TP309 after antibiotic selection (hygromycin). However, in contrast to Singh et al. [24], we used N6 medium instead of MS-medium, along with a higher ratio of 2,4-D to BAP in SM. We also observe that instead of transferring the whole callus to the regeneration medium (RM), the transfer of microcalli (developed on the surface of mother calli) greatly enhanced the proliferation of microcalli on RM.

After 7 days of regeneration in the dark, the majority of microcalli were developed into a somatic embryo with a small protruding structure that eventually develops into plantlets (Figure 1). After the first round of regeneration in the dark, the proliferating microcalli were shifted into the light in the second round of regeneration and kept in the light thereafter. The shifting of microcalli in the light conditions turned the majority of the creamy-white protruding structures on microcalli into green spots that eventually led to the emergence of plantlets (Figure 1). After 14 days of regeneration in the RM under light conditions, the emerging plantlets were shifted sequentially, first to shooting and then to rooting media, for the faster growth of transgenic plants (Figure 1). The plantlets with developed roots and shoots were kept on a hydroponic medium for 4–5 days before shifting them to potting soil (Figure 1). The protocol developed here is highly efficient as well as fast, generating the transgenic plants in >60 days to pot level. The intricacy of the protocol is given stepwise in Section 3.5, "Detailed protocol with critical points highlighted for troubleshooting".

Table 1. Composition of the different media used in rice transformation, and the calculated regeneration percentage.

Media Components	CIM	CCM	SM	RGM				RM
				RGM_1	RGM_2	RGM_3	RGM_4	
MS salts	-	4.4 g/L	-	4.4 g/L	4.4 g/L	4.4 g/L	4.4 g/L	4.4 g/L
N6 salts	4 g/L	-	4 g/L	-	-	-	-	-
Maltose	30 g/L	30 g/L	30 g/L	30 g/L	30 g/L	30 g/L	30 g/L	-
Sucrose	-	-	-	-	-	-	-	30 g/L
Glucose	-	10 g/L	-	-	-	-	-	-
L-proline	0.6 g/L	0.6 g/L	0.6 g/L	-	-	-	-	-
Casein Hydrolysate	0.3 g/L	0.3 g/L		-	-	-	-	-
2,4-D	4 mg/L	4 mg/L	4 mg/L					
NAA	-	-	-	4 mg/L	0.2 mg/L	0.2 mg/L	0.5 mg/L	0.2 mg/L
BAP	0.25 mg/L	0.25 mg/L	0.25 mg/L	-	-	2 mg/L	3 mg/L	-
Kinetin	-	-	-	0.25 mg/L	2 mg/L	2 mg/L	1 mg/L	-
Hygromycin	-	-	50 mg/L	30 mg/L	30 mg/L	30 mg/L	30 mg/L	30 mg/L
Cefotaxime	-	-	250 mg/L	-	-	-	-	-
Carbenicillin	-	-	250 mg/L	-	-	-	-	-
Phytagel	3 g/L	3 g/L	-	3 g/L	3 g/L	3 g/L	3 g/L	3 g/L
pH	5.8	5.2	5.8	5.8	5.8	5.8	5.8	5.8
Plant regeneration efficiency				52%	49%	56%	58%	

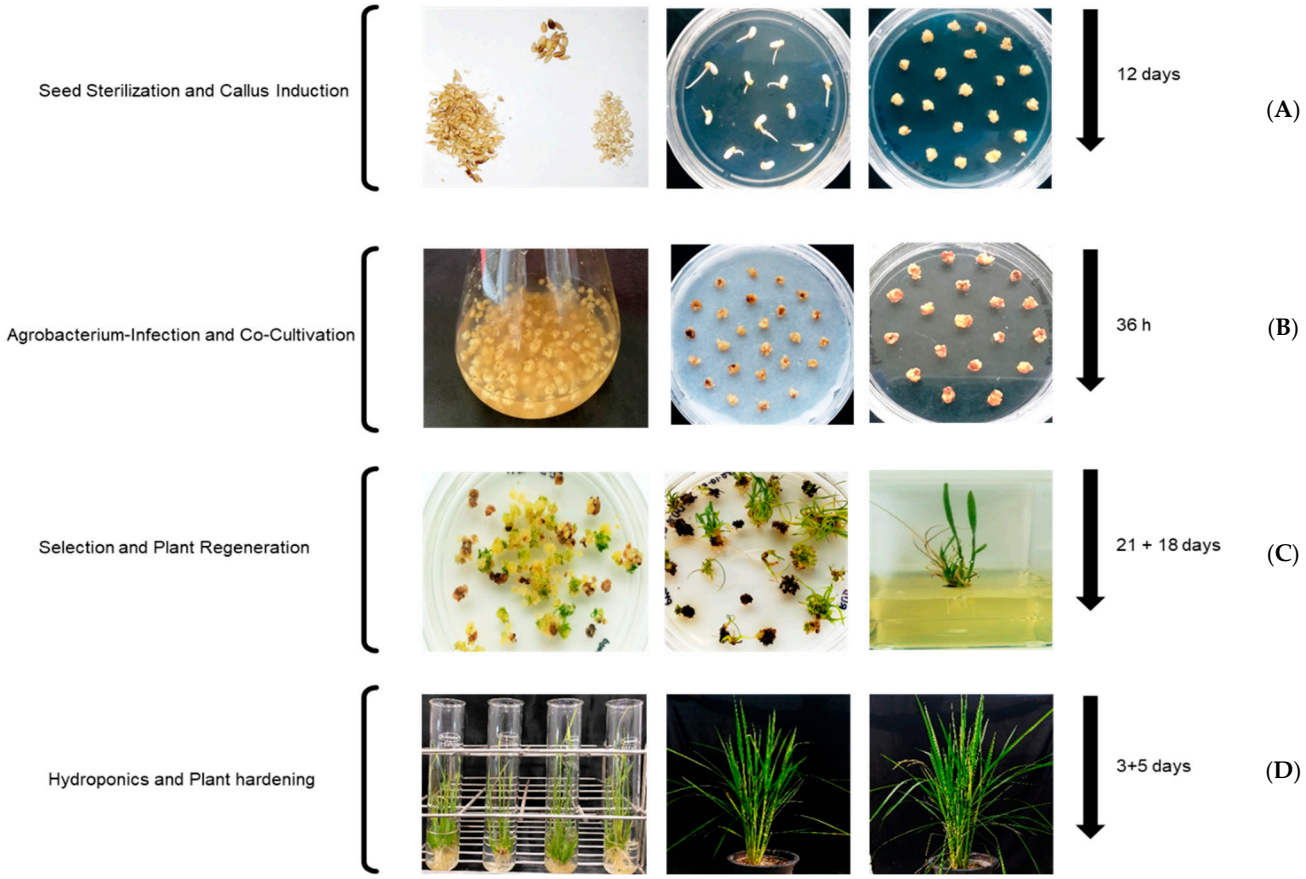

Figure 1. The stepwise *Agrobacterium*-mediated rice transformation and regeneration. (**A**) Dehusked seeds were surface-sterilized before callus induction on callus induction media. (**B**) Calli were *Agro*-infected and co-cultivated with *Agrobacterium tumefaciens*. (**C**) The selection of transformed calli on hygromycin selection and their regeneration for shoot and root development. (**D**) Plantlets were grown hydroponically before hardening in soil, and subsequently grown till maturity.

3.2. Optimization of the Regeneration Media Composition

From the available literature on transformation in japonica rice, we observed that some of the components, such as the carbon and nitrogen sources, and the gelling agents were the best at giving desirable results [23,24]. Therefore, we kept these components as they were in our protocol. For instance, we used maltose as a carbon source until the plant regenerated, whereas in the later stages of plant growth we used a ratio of maltose and sucrose (Table 1). Similarly, the proline was used as a nitrogen source and phytagel/clerigel was used as a gelling agent throughout the media preparation. Since the literature suggests the critical role of plant growth regulators in efficient plant regeneration, we optimized the concentration of cytokinins and auxins in the regeneration media. We formulated four regeneration media with different regimes of 6-benzylaminopurine (BAP), kinetin and naphthalene acetic acid (NAA), while the rest of the components were kept constant (Table 1). We observed that a higher concentration of BAP, either alone or in combination with kinetin, positively affected plant regeneration, compared to the higher concentration of NAA (Table 1). Moreover, the combination of BAP and kinetin in RM_3 and RM_4 resulted in comparable plant regeneration. Indeed, the presence of auxins (NAA) promotes somatic embryogenesis; because NAA is light-sensitive, the first regeneration in the dark became critical for somatic embryogenesis.. The highest plant regeneration efficiency we observed was in the range of 56–58%, which is in consistent with previous reports on various japonica rice cultivars [24,26,27].

3.3. Molecular Analysis of Transgenic Lines

3.3.1. Genome Editing of *OsLip1*

Around 500 metric tons (MT) of milled rice are produced annually. The rice bran layer represents ~8% of the kernel weight, equating to ~40 MT post-milling. Rice bran is rich in oil (18–22%) that is excellent for cooking; the defatted bran also contains fiber, protein, and ~60% of the micronutrients in the entire kernel. Despite this, rice bran is underexploited as a source of human nutrition and of revenue. A major problem associated with bran utilization is its instability. During milling, the bran layer ruptures and releases endogenous Lips that commence the hydrolysis of stored lipids and the release of unsaturated free fatty acids (FFAs). Lipoxygenase catalyzes the further oxidation of these FFAs, particularly linoleic acid (18:2). This reaction generates conjugated hydroperoxides of 18:2, which break down and yield secondary oxidation products that are responsible for the strong, stale off-flavors [28,29]. The outcome is a deterioration of the bran lipids and a significant loss of oil during the rice bran oil (RBO) refining process. This process can render the rice bran rancid within hours of post-milling and, if the FFA content exceeds 5%, oil extraction becomes commercially unviable. Physical processes have been developed to stabilize rice bran, but they require large capital investment and energy and are only partially effective [29].

Thus, it is necessary to adopt modern biotechnological solutions to overcome rancidity in the grain without any negative impact on other nutritional parameters. A recent addition in the biotechnologist's toolkit, the CRISPR/Cas system, holds tremendous potential to precisely and efficiently engineer the plant's genetic makeup. It has remarkably improved the potential to manipulate specific genes that are responsible for various applications in crop improvement, by creating "InDels" into genomes. With the availability of well-annotated rice genetic information, gene discovery for biotechnological applications is now possible.

Expression analyses of rice bran Lip genes revealed that *OsLip1* (LOC_Os11g43510) is the predominantly expressed gene during the onset of lipid mobilization [25]. In this study, we targeted the *OsLip1* gene in order to validate the transformation protocol. We randomly picked 10 putative transformants for DNA extraction and PCR amplification of the *Cas9* gene. Out of the 10 plants, 7 were observed to be positively transformed, as determined through the amplification of a ~1000 bp DNA fragment corresponding to the *Cas9* gene (Figure 2A). The genomic integration of *Cas9* is an indication of the possible gene editing of the target gene, *OsLip1*. In order to determine the genome editing events, we amplified and sequenced the target region using *OsLip1*-specific primers, flanking the PAM sequence using genomic DNA (Supplementary Table S1). The sequence obtained from the transgenic plants was then aligned to the wild-type sequence to identify any indels or substitutions. In one of the representative transgenic plants shown here (Figure 2B), we found a deletion of an adenosine (A) 4bp upstream of the PAM sequence. The sequencing chromatogram confirms the deletion of adenosine, as it exhibits two sharp peaks of Thymine (T) and no indication of adenosine at the DNA double-strand break site (Figure 2B). In order to check whether the deletion of a single base disrupts the protein structure, the mutated *OsLip1* sequence was translated *in silico*. The single adenosine base deletion in the coding sequence of *OsLip1* leads to a frameshift mutation of the protein structure. Furthermore, the protein structures were predicted, and models were generated for the wild-type and mutated OsLip1 proteins using Swiss-Model (https://swissmodel.expasy.org/, assessed on 25 October 2021). The predicted structures showed complete disruption of Lip structure in the mutated OsLip1 protein (Figure 2C).

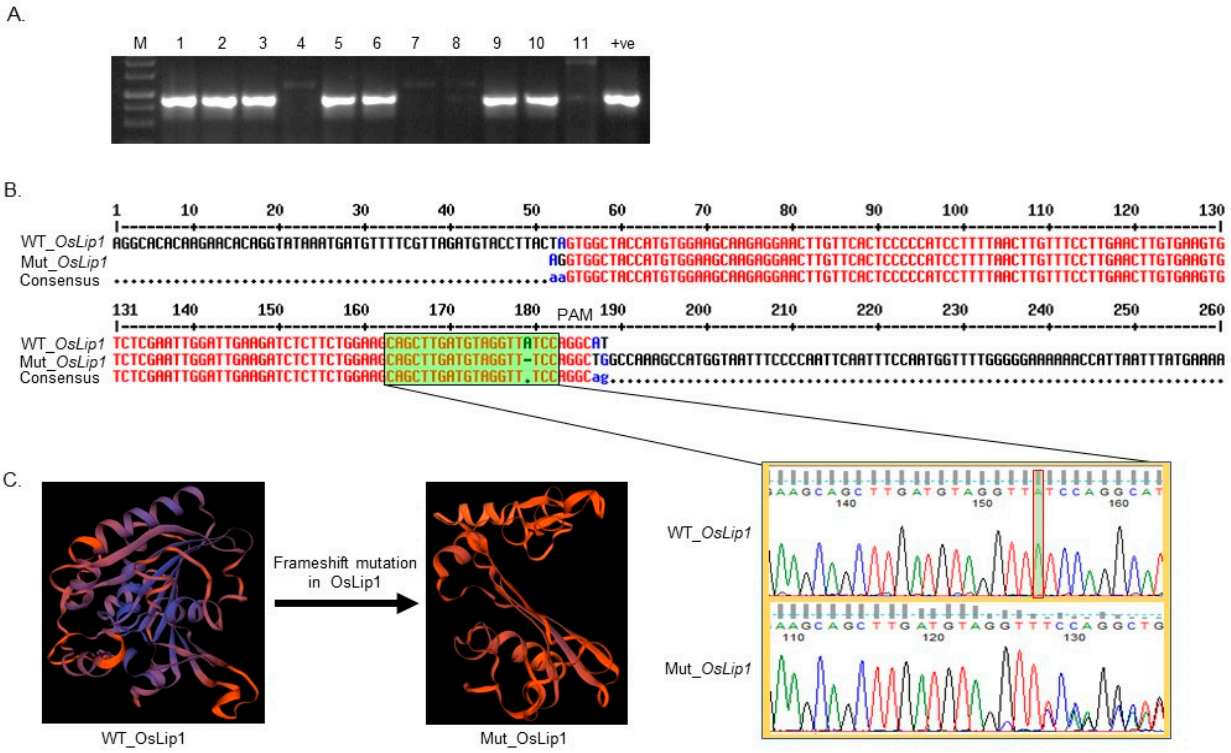

Figure 2. Schematic representation of CRISPR/Cas9-mediated targeted mutagenesis in the *OsLip1* (LOC_Os11g43510) gene. (**A**) Identification of transgenic rice plants carrying the *Cas9* transgene, with a sgRNA targeting the *OsLip1* gene. Genotyping was performed using the *OsLip1*-specific sequencing primers. (**B**) Sequence alignment and sequencing chromatogram at the target site of *OsLip1* in the edited plants (T0). The vertical box indicates the deletion mutation (Mut) of adenosine (**A**) in the *OsLip1* gene. (**C**) Frameshift mutation distorted the overall protein structure of OsLip1. WT; wild-type.

3.3.2. Overexpression of *OsGolS*

The key role of GolS is to catalyze the transfer of the galactosyl moiety from UDP galactose to myo-inositol, to generate galactinol as a product. The galactinol synthesized through the action of GolS is vital for the synthesis of other members of raffinose family oligosaccharides (RFOs). Hence, GolS is known to have a key regulatory role in RFO biosynthesis [30–32]. These RFOs, along with galactinol and raffinose, are widely known for their role in mitigating abiotic stress tolerance in diverse crops like *Arabidopsis*, chickpea, and *Vitis vinifera*, to name a few. Studies have suggested the role of galactinol and raffinose in scavenging stress-induced reactive oxygen species (ROS) [30,33–35]. However, studies related to the role of *OsGolS* in stress tolerance are rather limited. Therefore, in this study, we have assessed *GolS* overexpression lines for their salt-stress tolerance potential. For the generation of *OsGolS* overexpression rice transgenic lines, we transformed the rice calli with a transformation construct containing the rice *GolS* encoding gene, under the control of a constitutive promoter. Among the putative transformants, nearly 68% of the plants (11 out of 16 randomly selected specimens) exhibited PCR amplification for the hygromycin-resistant gene, *hptII* (Figure 3A). To determine the transcript abundance of transgenes, we performed semi-quantitative PCR analysis of five positive transformants, wherein two transgenic rice lines (line 1 and 2) showed higher transcript abundance of *OsGolS*, compared to the wild-type control (Figure 3B).

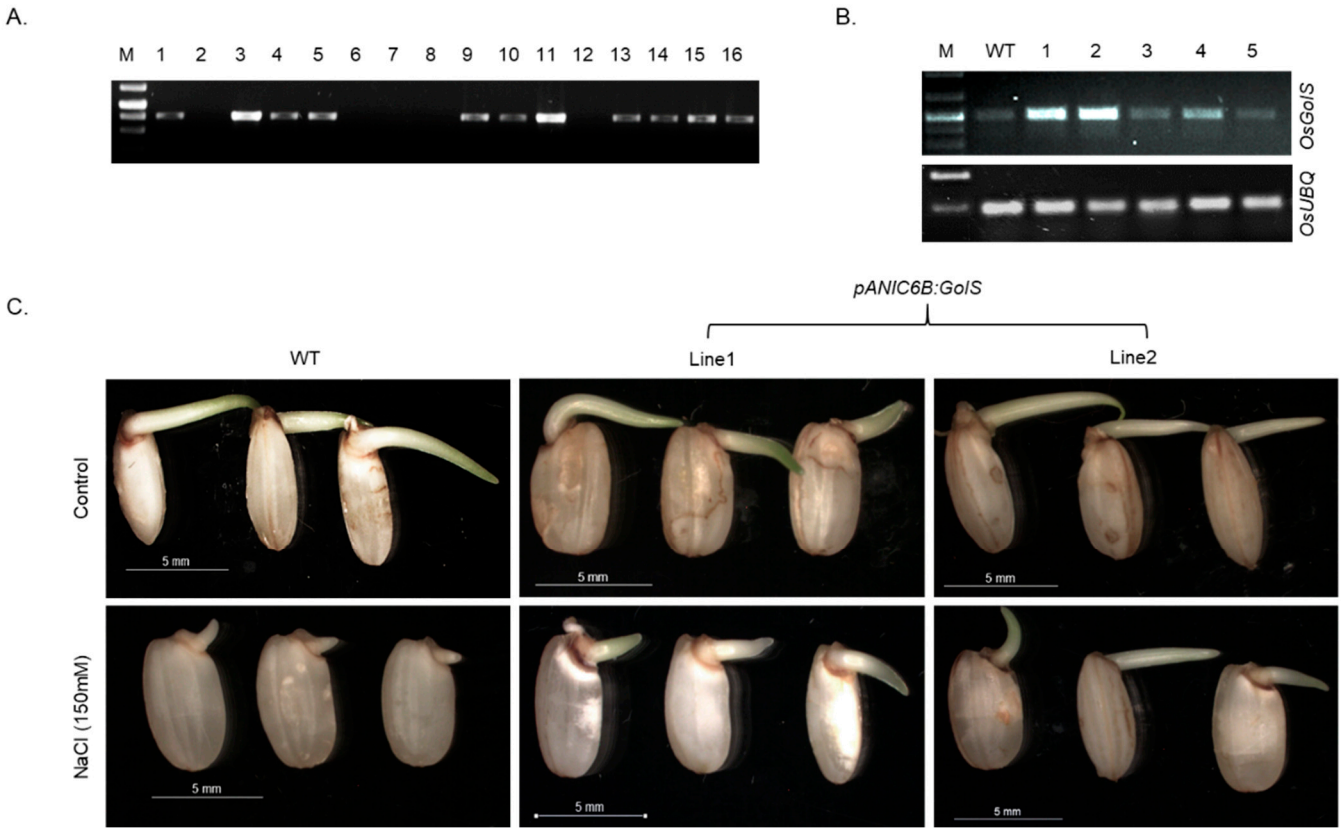

Figure 3. Molecular analysis of *OsGolS* overexpression lines. (**A**) Screening of positive transformation events via amplification of the *hptII* gene from the genomic DNA of putative transformants. (**B**) Semi-quantitative RT-PCR analysis of selected positive transformants for *OsGolS* and the rice *ubiquitin* (*OsUBQ)* gene, WT; wild-type plant. (**C**) Germination test of WT and transgenic lines (OE-1 and OE-2) under salinity stress.

3.4. GolS Overexpression Confers Improved Seed Germination under Salt Stress

As *GolS* is known for its role in the abiotic stress response [30,32,33], we examined the effect of salt stress on rice transgenic lines overexpressing *GolS*. As shown in Figure 3C, when seeds from two independent lines of *OsGolS* transgenic plants, along with WT, were subjected to salt stress (150 mM NaCl), the germination of non-transgenic lines was significantly affected. The seeds of the *GolS* overexpressing line exhibited faster germination, while non-transgenic seeds showed delayed germination after salinity stress exposure. The unstressed seeds from transgenic and WT plants did not display a significant difference in germination percentages (Figure 3C). All three independent lines with *OsGolS* overexpression showed improved germination under salinity stress, which clearly revealed that the over-accumulation of *GolS* is implicated in the mitigation of salt stress.

3.5. Detailed Protocol, with Critical Points Highlighted for Troubleshooting

(A) Dehusking the Seeds

- Dehusk the wild-type rice seeds (*Taipei-309*) either manually or using a palm-dehusker (***manual dehusking is preferred, to reduce the embryo damage and, thus, maximize the callus induction per plate***).

(B) Seed Sterilization

- Immerse the dehusked rice seeds (~350 seeds per construct) in Milli-Q or distilled water for one hour.
- Rinse the seeds 2–4 times with autoclaved Milli-Q water by vigorously shaking the Falcon tube (50 mL) containing the seeds.

- Discard the liquid and sterilize the seeds using 20–30mL of 70% ethanol, by continuously shaking the tubes for 2 min.
- Wash the seeds twice with autoclaved Milli-Q water.
- Sterilize the washed seeds, using 25–30 mL of 4% sodium hypochlorite solution containing ~50 µL of Tween-20, by gently shaking for 20 min (***avoid long exposure to hypochlorite as it may inhibit the callus induction***).
- Wash the seeds with autoclaved Milli-Q water until the froth disappears and the chlorine smell disappears (***around 10–12 washes***).
- Air-dry the washed seeds properly by keeping them on sterilized blotting paper.

(C) Seed Inoculation/Plating

- Using sterilized forceps, gently pick up the dried seeds and place them on CIM media plates (15–20 seeds per plate (90 mm diameter) at an even distance apart, to avoid crossing).
- Incubate the plated seeds at 28 °C in the dark for 7–8 days to induce callus generation.

(D) Subculturing the Calli

- Gently separate the calli from the radicle and seed remnants using sterilized forceps and a scalpel and place them on CIM plates (20–22 calli per plate).
- Incubate the plates in the dark at 28 °C for 3–4 days (sub-culturing of calli enhances plant regeneration in the latter stages).

(E) *Agrobacterium*-mediated Transformation

(i) *Agrobacterium* culture preparation

- Primary culture—inoculate 5 mL of LB broth (containing selective antibiotics) with a single isolated colony of the *Agrobacterium* strain and incubate at 28 °C for 20–24 h.
- Secondary culture—inoculate 0.5% inoculum from the primary culture in 100 mL of LB broth (containing selective antibiotics and 100 µM acetosyringone) and incubate at 28 °C until the OD 600 of the culture attains OD = 0.6 to 0.8 absorbance.

(ii) Callus Infection and Transformation

- Pellet down the secondary culture by centrifugation at 2000× *g* for 15 min.
- Resuspend the *Agrobacterium* pellet in 100 mL of CIM broth, containing 200 µM acetosyringone.
- Using flame-sterilized forceps, transfer the 3–4-day-old subcultured calli in the *Agrobacterium* suspension and keep them at 28 °C in an incubator-shaker for about 30 min at 100 rpm.
- Decant the suspension and air-dry the calli on sterilized blotting paper.

(iii) Co-cultivation

- Place a sterilized round Whatman filter paper on solidified CCM.
- Gently transfer the *Agrobacterium*-inoculated calli to CCM.
- Incubate the plates in the plant growth chamber at 28 °C for 36 h.

(F) Sterilization and Selection

(i) Sterilization/washing

- Transfer the infected calli to a sterile conical flask and wash with sterile water up to six times to remove excess *Agrobacterium* growth.
- Transfer the calli to a fresh sterile conical flask and add approx. 100 mL of sterile water, supplemented with 250 mg each of Carbenicillin and cefotaxime. Keep the flask in an incubator-shaker at 120 rpm for 15–20 min in the dark.
- Repeat the previous step eight times, using fresh sterile water supplemented with antibiotics.

- Finally, air-dry the washed calli on sterilized blotting paper.

(ii) Culturing on Selection Medium

- Transfer the air-dried calli on solidified SM plates (15–20 calli per plate).
- Incubate the plates at 28 °C for 8 days.
- Sub-culture the calli on fresh SM plates two more times at 8-day intervals.

(G) Regeneration

(i) Shoot generation

- After the calli have gone through three rounds of selection, transfer only the microcalli or secondary calli on RGM plates (15–20 calli per plate).
- Incubate the plates at 28 °C for 8 days in the dark for the first regeneration sub-culture.
- After 8 days, sub-culture the calli on the fresh RGM plates and keep under a 16/8-h day/night photoperiod at 28 °C for 8 days.
- Repeat the sub-culturing of calli on fresh RGM every 8 days, until shoots emerge.
- Transfer the shoots of 1–2 cm height to the fresh RGM in phyto jars and keep on sub-culturing every 7 days, until the shoots are 4–5 cm long.

(ii) Root generation

- Transfer the shoots to the RM in the phyto jar for the generation of roots and keep under a 16/8-h day/night photoperiod at 28 °C, until the roots have grown up to 2–3 cm in length.

(H) Hardening

- Gently take out the plant from the RM (without damaging the root) and wash the root 2–3 times with autoclaved Milli-Q water to remove the solidified media.
- Transfer the plant to an autoclaved test tube containing 1× Yoshida solution (sufficient to dip the root part) and keep under a 16/8-h day/night photoperiod at 28 °C for 5–6 days (Keep a check on the plant every day and change the media when contaminated).
- Transfer the plants to pots containing soil (supplemented with NPK or compost). Initially, flood the pots with water and add 1× Yoshida solution in alternate weeks until the seeds are set.

4. Conclusions

To achieve crop improvement, genetic transformation plays an instrumental role in obtaining a genetically engineered transgenic or genome-edited crop with desirable traits. Despite significant efforts, the genetic transformation of rice remains a challenging process since its reproducibility varies greatly. Here, in this study, we presented an efficient protocol for the genetic transformation of rice to develop stable overexpression and genome-edited lines. We also highlighted the critical steps and precautions to be taken while performing the rice transformation. In order to assess the efficacy of the optimized protocol, we genetically transformed rice for *GolS* overexpression and the CRISPR/Cas9-mediated editing of *OsLip1*. Overall, the comprehensive step-by-step methodology for rice transformation is intuitive to use and effective for developing transgenic and genome-edited plants that can be used for crop improvement.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12010179/s1, Table S1: Primer used for the cloning of genes and molecular analysis of transformants.

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