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TITLE:

Agrobacterium-Mediated Genetic Transformation, Transgenic Production, and Its Application for the Study of Male Reproductive Development in Rice

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KEYWORDS:

rice, CRISPR-Cas9, *Agrobacterium*, callus, tissue culture, genotype, phenotype, anther, pollen

SUMMARY:

This work describes the use of CRISPR-Cas9 genome editing technology to knockout endogenous gene *OsABCG15* followed by a modified *Agrobacterium*-mediated transformation protocol to produce a stable male-sterile line in rice.

ABSTRACT:

Male sterility is an important agronomic trait for hybrid seed production that is usually characterized by functional defects in male reproductive organs/gametes. Recent advances in CRISPR-Cas9 genome editing technology allow for high editing efficacy and timesaving knockout mutations of endogenous candidate genes at specific sites. Additionally, *Agrobacterium*-mediated genetic transformation of rice is also a key method for gene modification, which has been widely adopted by many public and private laboratories. In this study, we applied CRISPR-Cas9 genome editing tools and successfully generated three male sterile mutant lines by targeted genome editing of *OsABCG15* in a *japonica* cultivar.

We used a modified *Agrobacterium*-mediated rice transformation method that could provide excellent means of genetic emasculation for hybrid seed production in rice. Transgenic plants can be obtained within 2 – 3 months and homozygous transformants were screened by genotyping using PCR amplification and Sanger sequencing. Basic phenotypic characterization of the male sterile homozygous line was performed by microscopic observation of the rice male reproductive organs, pollen viability analysis by iodine potassium iodide (I_2-KI) staining semi-thin cross-sectioning of developing anthers.

INTRODUCTION:

Rice is the most important food crop, particularly in developing countries, and represents a staple food for over half of the world's population. Overall, the demand for rice grain is growing and is projected to increase 50% by 2030 and 100% by 2050[1,2]. Future improvements in rice yield will need to capitalize on diverse molecular and genetic resources that make rice an excellent model for monocotyledonous plant research. These include an efficient transformation system, advanced molecular map, and publicly accessible database of expressed sequence tags, which have been generated over many years[3,4]. One strategy to improve crop yield is hybrid seed production[5], a central element of which is the ability to manipulate male fertility. Understanding the molecular control of male fertility in cereal crops can help to translate key knowledge into practical techniques to improve hybrid seed production and enhance crop productivity[6,7].

Genetic transformation is a key tool for basic research and commercial agriculture since it enables introduction of foreign genes or manipulation of endogenous genes in crop plants, and results in the generation of genetically modified lines. An appropriate transformation protocol can help to accelerate genetic and molecular biology studies for fundamental understanding of gene regulation[8]. In bacteria, genetic transformation takes place naturally; however, in plants, it is performed artificially using molecular biology techniques[9,10]. *Agrobacterium tumefaciens* is a soil-borne, Gram-negative bacterium that causes crown gall disease in plants by transferring T-DNA, a region of its Ti plasmid, into the plant cell via a type IV secretion system[11,12]. In plants, *A. tumefaciens*-mediated transformation is considered a widespread method for gene modification because it leads to stable and low copy number integration of T-DNA into the host genome[13]. Transgenic rice was first generated through *Agrobacterium*-mediated gene transformation in the mid-1990s in the japonica cultivar[14]. Using this protocol, several transgenic lines were obtained within a period of 4 months with a transformation efficiency of 10%–30%. The study indicated that there are two critical steps for the successful transformation: one is the induction of embryogenic callus from mature seeds and another is the addition of acetosyringone, a phenolic compound, to the bacterial culture during co-cultivation, which allows for higher transformation efficiency in plants[14,15]. This protocol has been extensively used with minor alterations in *japonica*[16-19] as well as other cultivars such as *indica*[20-23] and tropical *japonica*[24,25]. Indeed, over 80% of the articles describing rice transformation use *Agrobacterium*-mediated gene transformation as a tool[13]. To date, several genetic

transformation protocols have been developed using rice seed as a starting material for callus induction[16-19]. However, very little is known about young inflorescence as explants for callus production. Overall, it is important to establish a rapid, reproducible, and efficient gene transformation and regeneration protocol for functional genomics and studies on crop improvement.

In recent years, the advancement of CRISPR-Cas9 technology has resulted in a precise genome editing mechanism to understand gene function and deliver agronomically important improvements for plant breeding[26,27]. CRISPR also offers considerable promise for the manipulation of male reproductive development and hybrid production. In this study, we utilized a gene knockout system using CRISPR-Cas9 technology and coupled it to an efficient rice gene transformation protocol using young inflorescences as explants, thereby creating stable male sterile lines for the study of reproductive development.

PROTOCOL:

1. sgRNA-CAS9 plant expression vector construction and *Agrobacterium*-mediated transformation

1.1. Target a male sterile gene *OsABCG15* in rice according to the published literature[28].

1.2. Design sgRNA for the targeted site located between 106–125 bp in the second exon of *OsABCG15* (**Figure 1**).

1.3. Use T4 polynucleotide kinase to synthesize the sgRNA oligos (sgR-OsABCG15-F: 5'TGGCAAGCACATCCTCAAGGGGAT3' and 5'sgR-OsABCG15-R: AAACATCCCCTTGAGGATGTGCTT').

1.4. Use endonuclease BbsI to digest the psgR-Cas9 backbone vector[29].

1.5. Ligate the synthesized sgRNA oligos with linearized psgR-Cas9 backbone using T4 ligase following the manufacturer's protocol.

1.6. Using HindIII/EcoRI, digest the sgRNA cassette from step 1.5 and subclone into the HindIII/EcoRI site of the pCAMBIA1300 binary vector for stable transformation[29].

1.7. Confirm the constructed plasmid by enzyme digestion and sequencing. Later, transform the binary construct into the *Agrobacterium tumefaciens* strain EHA105 and grow them on Kan/Rif selection plates at 28 °C for 2–3 days.

2. Rice genetic transformation and plant tissue culture

2.1. Callus induction and regeneration

NOTE: Use fresh young rice inflorescences as the starting material to induce callus (**Figure 2**).

2.1.1. Collect the young inflorescences from the paddy field or green house at meiosis stage, determined by floret length of 1.6-4.8 mm (**Figure 2A**)[29]. Ensure that the rice inflorescences are covered with leaf sheath. Wipe each inflorescence with a 70% alcohol swab and let it dry before cutting.

2.1.2. Bring the inflorescence to a clean sterile bench. Cut it into small pieces (the smaller the better) with sterilized scissors and then transfer cuttings to a Petri plate containing NBD2 medium (**Figure 2B, Table 1**).

2.1.3. Incubate the plate in the dark at 26 °C for about 10-14 days to induce callus (**Figure 3A**).

NOTE: Use the newly formed callus for *Agrobacterium* infiltration (step 2.2.7) directly or recondition one more time in fresh NBD2 medium to obtain more callus. Transfer the callus into the new NBD2 medium every 8–10 days.

2.2 Transformation and co-cultivation

2.2.1. Use *A. tumefaciens* bacteria containing the binary plasmid from step 1.6 for transformation. Store the *A. tumefaciens* strains in YEB medium (**Table 1**) with 50% glycerol at -80 °C for further use.

2.2.2. Streak *A. tumefaciens* from a -80 °C glycerol stock to YEB agar medium containing selective antibiotics (**Table 1**) and grow at 25–28 ˚C for 48–72 h, to allow colonies to appear.

2.2.3. Inoculate a single colony from the YEB plate with selective antibiotics to 5 mL of liquid YEB medium containing the same selective antibiotics (**Table 1**), in a 50 mL conical sterile test tube. Shake on an orbital shaker at 250 *x g*, at 25–28 °C until bacteria grow to an OD_{600} of 0.5.

2.2.4. Add 1 mL of bacterial suspension to 100 mL of YEB medium (**Table 1**) with the same selective antibiotics in a 250 mL conical flask and shake on an orbital shaker at 250 *x g*, at 28 °C for 4 h.

2.2.5. Centrifuge at 4,000 *x g* for 10 min at room temperature to collect bacteria. Discard the supernatant.

2.2.6. Resuspend the bacterial pellet with AAM-AS medium (**Table 1**) and dilute the suspension to an OD_{600} = 0.4.

NOTE: The perfect OD of bacterial suspension is critical for efficient transformation. It helps in eliminating excess bacterial growth on the callus.

2.2.7. Collect around 150 healthy growing light-yellow fragile embryogenic calli from step 2.1.3 into a 150 mL sterile flask. Add 50–75 mL bacterial cell suspension from step 2.2.6 into the sterile flask, and then add some 10–25 mL fresh AAM-AS medium to immerse the calli for 10–20 min, shaking occasionally.

2.2.8. Pour out the bacterial suspension from the flask carefully and dry the excess bacterial suspension from the callus using sterile filter paper #1 or tissue paper. Then place them on a Petri dish with NBD-AS medium (**Table 1**) covered with a sterile filter paper #1. Incubate at 25–28 °C in the dark for 3 days and check for bacterial overgrowth.

2.3. Selection for resistant callus

2.3.1. After 3 days of co-cultivation, transfer the calli to a sterile Petri dish with a sterile filter paper.

2.3.2. Air-dry the calli for 2 h on a clean bench. Ensure that the callus is not adhered to the filter paper.

2.3.3. Transfer the calli evenly using sterile tweezer to the primary selection medium NBD2 (with 40 mg/L hygromycin and 250 mg/L timentin) (**Table 1**). Culture calli for 2 weeks at 25–28 °C in the dark.

2.3.4. After 2 weeks, transfer calli evenly to a new plate containing fresh selection medium NBD2 (with 40 mg/L hygromycin and 250 mg/L timentin) (**Table 1**). Culture the calli for another 2 weeks at 25–28 °C in the dark.

2.4. Callus differentiation

2.4.1. Transfer callus to fresh MS Medium (with 25 mg/L hygromycin and 150 mg/L timentin) (**Table 1**). Culture under the light at 25–28 °C for around 2 weeks.

2.4.2. Repeat step 2.4.1 one more time.

2.4.3. Transfer the shoot buds to the new MS medium (with 10 mg/L hygromycin) to proliferate more shoots.

2.5. Root induction

2.5.1. Transfer the new shoots into ½ MS medium (**Table 1**). Culture under the light at 25 °C - 28 °C. The transformed plants produce roots within 2 weeks.

2.5.2. After 1 week, transfer the plants to clean pots covering the plant to prevent dehydration.

3. Genotype identification

3.1. Collect young leaves from newly generated plants for total DNA extraction using the cetyltrimethyl ammonium bromide (CTAB) method[31].

3.2. Using the genomic DNA as template, choose the primers (Hygromycin-F: 5' GATGTTGGCGACCTCGTATT 3' and Hygromycin-R: 5' CGAAGAATCTCGTGCTTTCA 3' located in the Hygromycin region) to perform polymerase chain reaction (PCR) to validate the transgene integration and frequencies (**Figure 4**). The following PCR conditions: 94 °C for 5 min; 36 cycles (94 °C for 30 s; 56 °C for 30 s; 72 °C for 1 min); 72 °C for 7 min. Successful PCR reactions yield a 604 bp amplicon.

3.3. Perform another PCR to amplify the genomic region surrounding the CRISPR target sites using specific primers (OsABCG15-ID-F: 5' GTCTCATTGCTCAACAGTTTCT 3' and OsABCG15-ID-R: 5' TTGGTGTTTAAAACATTGCTAT 3') to identify the genotype of the transformants. The PCR conditions are the same as in step 3.2.

3.4. Use PCR fragments from step 3.3 directly for Sanger sequencing to identify mutations.

4. Observe the basic phenotype of the mutant

4.1. Prepare the I_2-KI solution: dissolve 2 g of KI (potassium iodide) in 10 mL of distilled water and then add 0.2 g of I_2 (resublimed iodine) in it. After mixing, bring the total volume to 100 mL with distilled water.

4.2. Prepare the FAA solution (63% anhydrous ethanol, 5% glacial acetic acid, 5% formaldehyde and 27% water) and mix before using.

4.3. Prepare the 0.5% Toluidine blue staining solution: dissolve 0.5 g of Toluidine blue in 100 mL of distilled water.

4.4. Perform vegetative phenotypic observation, by imaging the whole plants with a digital camera.

4.5. Perform reproductive phenotype observation, remove the palea and lemma from the floret. Take photos of whole anthers under a stereomicroscope.

4.6. Observe the pollen viability by iodine staining.

4.6.1. Pick mature rice spikelets before the flower anthesis, remove the palea and lemma to release the anthers.

4.6.2. Take 6 anthers and place them on a glass slide. Add 1 drop of distilled water, crush the anther well with tweezers to release the pollen grains, and then add 2–3 drops of I_2-KI solution. Cover with the coverslip.

4.6.3. Observe under a low magnification microscope. Pollen grains dyed black show more vigorous viability, no color or yellow-brown dyed are stunted or degenerated.

4.7. Perform a semi-thin section of rice anther

4.7.1. Fix the rice flowers from different developmental stages into an FAA solution. Vacuum the materials at 4 °C (generally 15 min) to allow the fixative to penetrate to the tissues until the material sinks to the bottom of the collection bottle.

4.7.2. Replace with a new FAA solution the next day. Once the material sinks to the bottom, replace with 70% ethanol and store at 4 °C for long-term use.

4.7.3. Dehydrate the materials with different gradients of ethanol (70%, 80%, 95% each time for 30 min, 100% ethanol for 2 h, 100% ethanol containing a little of Safranin dye for 2 h).

NOTE: Add a little of Safranin dye into the second 100% ethanol to color the anthers for easier sectioning afterwards.

4.7.4. Perform embedding by following the manufacturer's instruction (**Table of Materials**). Then, bake in an oven at 60 °C for 48 h before sectioning.

4.7.5. Section the sample to a thickness of 2 µm using a microtome. Add a drop of water on the glass slide. Then, pick up the thin sections containing anther blocks by forceps and put them onto the surface of the water drop on the slides.

4.7.6. Place the slides on the water bath at 50 °C to completely spread the sections to let it firmly adhere to the slide.

4.7.7. Use 0.5% toluidine blue solution stain the slides for 30 min. Then, rinse with water and dry in the fume hood. Seal with neutral tree glue. Gently place a coverslip on the slide

and dry in the fume hood.

4.7.8. Observe and photograph the sample under a microscope.

REPRESENTATIVE RESULTS:

Demonstrated here is the use of gene editing technology to create a male sterile line for future research by *Agrobacterium*-mediated genetic transformation in rice. To create the male sterile line of *osabcg15*, CRISPR-CAS9-mediated mutagenesis was used for binary vector construction. The sgRNA was driven by the OsU3 promoter, whereas the expression cassette of hSpCas9 was driven by the double 35S promoter, and the middle vector was assembled in a single binary vector *pCAMBIA1300* designed for *Agrobacterium*-mediated stable transformation of rice. A graphical representation of the construct that was used for CRISPR/Cas9-mediated mutagenesis of rice is provided in **Figure 1A**.

Figure 2 and **Figure 3** represent the entire procedure of rice transgene production. The young inflorescences were chosen as explants (**Figure 2**) and induced the friable embryogenic callus (FEC) within 14 days of inoculation on the NBD2 medium (**Figure 3A**). The embryogenic calli were directly used for *Agrobacterium*-mediated transformation (**Figure 3C**) or subculture for proliferation to obtain more calli for further infection (**Figure 3B**). After co-cultivating for 48 h on NBD2-AS medium in dark conditions (**Figure 3D**), the calli were shifted to a second round of selection medium. After 10–14 days, transformed calli showed some small newly formed microcalli on the periphery of the mother calli, while the color of untransformed calli turned into brown and died eventually (**Figure 3E,F**). Later, healthy and creamy colored calli were transferred into the regeneration medium twice. At this stage, transformed calli gradually showed green spots (shoot buds) (**Figure 3G,H**). These newly generated green-spots were cultured in a sterile plastic bottle containing regeneration medium to allow shoot growth (**Figure 3I**), and then shifted (as fresh shoots) into a 1/2 MS medium to induce roots. Later, healthy and vigorous growing shoots with well-developed roots were harvested (**Figure 3J**).

In total, 21 regenerated seedlings were obtained. PCR amplification for the hygromycin region showed that 18 out of 21 can amplify the corresponding band, indicating these lines are transgenic (**Figure 4**), and the corresponding transformation frequency is about 85.71% (**Table 2**). The transformants were genotyped to identify the mutations at the sgRNA target site(s) using primers spanning the target region by PCR amplicon and Sanger sequencing, Among 18 T_0 transgenic plants, eight heterozygous lines and three homozygous lines were CRISPR-Cas9 positive, with corresponding mutation frequencies of 61.11% (**Figure 1B**, **Table 2**).

The homozygous knockout mutants were validated by basic male reproductive organ observation (**Figure 5**). The anthers of *osabcg15* were smaller and paler than those of the wild-type (**Figure 5A,D**), and lacked mature pollen grains (**Figure 5B,E**). Transverse

sectioning microscopy was performed to investigate the anther morphological defects in *osabcg15* compared to the wild type. At stage 10, wild-type microspores became round shaped and vacuolated with dark blue-stained exine (**Figure 5C**). By contrast, *osabcg15* microspores collapsed and degraded, only debris of degenerated microspore exist in the anther locule (**Figure 5F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Construct information for the CRISPR/Cas9 system and targeted mutagenesis of the *OsABCG15*. (**A**) The sgRNA was driven by the OsU3 promoter, whereas the expression cassette of hSpCas9 was driven by the double 35S promoter; the middle vector was assembled in a single binary vector designed for *Agrobacterium*-mediated stable transformation of rice. (**B**) The sequence (5'-AAGCACATCCTCAAGGGGAT-3') located in the second exon of the *OsABCG15* gene was selected as the target site of sgRNA. Three types of homozygous mutation events were generated by CRISPR-Cas9 in *japonica* cultivar 9522 background, and Sanger sequencing chromatograms of CRISPR-Cas9 induced mutation sites are shown. Line $9522^{osabcg15\text{-}1}$ has a single-nucleotide (1-nt) 'A' insertion; line $9522^{osabcg15\text{-}2}$ has a single-nucleotide (1-nt) 'G' insertion; and line $9522^{osabcg15\text{-}3}$ has a single-nucleotide (1-nt) 'G' deletion.

Figure 2: Starting materials for inducing callus. (**A**) Young rice inflorescences at meiosis stage. (**B**) Dissected young inflorescences growing on the NBD2 medium. Bar = 2 cm in (A).

Figure 3: Procedure for producing transgenic lines by *Agrobacterium*-mediated transformation. (**A**) Rice calli generated from the young inflorescences. (**B**) Subculture of calli. (**C**) Incubation of calli with *A. tumefaciens.* (**D**) Co-cultivation of calli with *A. tumefaciens.* (**E**) First selection cycle of transformed calli in the presence of Hygromycin (40 mg/L). (**F**) Second selection cycle of transformed calli in the presence of Hygromycin (40 mg/L). (**G**) First regeneration of transformed calli. (**H**) The second regeneration of transformed calli, noting the green shoot point in the culture plate. (**I**) Shoot induction culture. (**J**) Root induction.

Figure 4: Analysis of transformation frequencies of regenerated rice seedlings. Amplified PCR products that show a 604 bp fragment (1–8, 10–14, 16–20) after 1.5% agarose gel electrophoresis indicate transgenic positive lines. Lines that lack the fragment are untransformed (9, 15, 21), similar to the wild-type (WT) control.

Figure 5: Basic phenotypic observation of the wild-type (9522) and mutant $9522^{osabcg15\text{-}1}$. (**A**) Wild-type (9522) flower and (**D**) $9522^{osabcg15\text{-}1}$ mutant flower after removal of the lemma and palea. gl, glume; fi, filament; lo, lodicule; pi, pistil; st, stamen. (**B**) I_2-KI staining of mature pollen grains from wild-type (9522) and (**E**) $9522^{osabcg15\text{-}1}$ line. (**C**) Analysis of anther development in the wild-type (9522) and (**F**) $9522^{osabcg15\text{-}1}$ mutant by transverse section

observation. DMsp, Degenerated microspores; E, epidermis; En, endothecium; Msp, microspore; T, tapetum. Bars = 2 mm in **(A)** and **(D)**, 100 µm in **(B)** and **(E)**, 50 µm in **(C)** and **(F)**.

Table 1: Media compositions for rice transformation.

Table 2: Transformation and gene editing efficiency of the mutants of *OsABCG15* generated by CRISPR-Cas9 in the T_0 generation

DISCUSSION:

Artificial genic male sterile mutants are traditionally generated by random physical, chemical, or biological mutagenesis. Although these are powerful techniques, their random nature fails to capitalize on the vast amount of modern genomic knowledge that has the potential to deliver tailored improvements in molecular breeding[32]. The CRISPR-Cas9 system has been widely used in plants due to its simple and affordable means to manipulate and edit DNA[29,33]; it has potential to save time compared to traditional mutagenesis methods.

Here, we developed a convenient *Agrobacterium*-mediated transformation system using young inflorescences as explants for callus induction. There are some steps in the transgene procedure that can be omitted to save time. Using young inflorescences as starting material is a good choice to minimize the overall duration of the callus induction compared with using seeds as explants. In addition, the cell differentiation and regeneration capacity are very strong in the calli obtained from young inflorescences. In this process, subculture of rice calli can be avoided, which eventually saves time as well as reduces the risk of bacterial and fungal contamination. Moreover, timentin was used as an antibiotic instead of carbenicillin, which is more effective in inhibiting bacteria.

The study and the introduced protocol for basic phenotypic observation of male reproductive development in rice is useful for undergraduate and graduate students who are interested in plant reproductive biology. This convenient protocol should be quick to grasp and reproducible for use in subsequent research studies.

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DISCLOSURES:

None.

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Figure 1

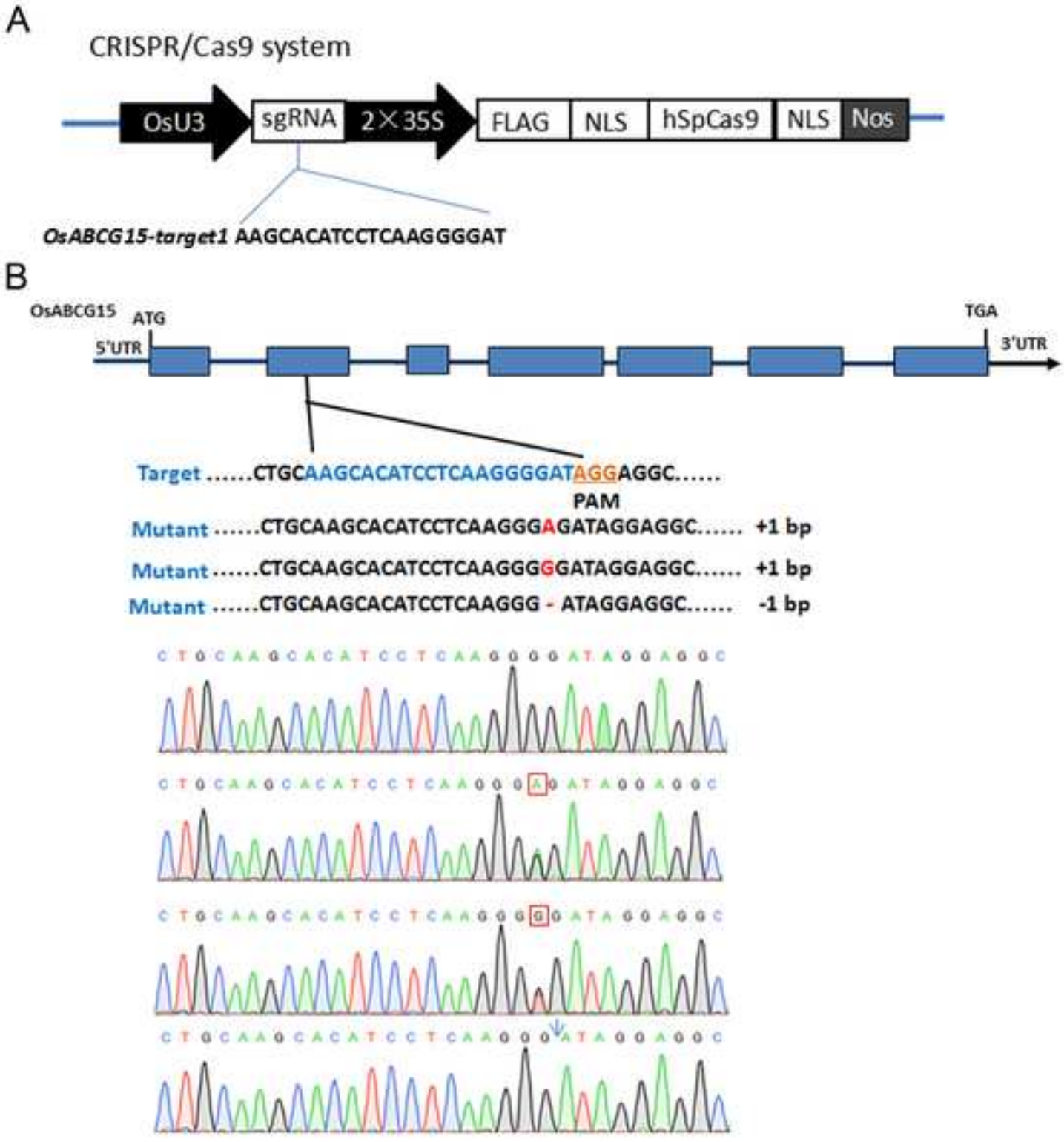

Figure 2

Click here to access/download;Figure;Figure 2.psd

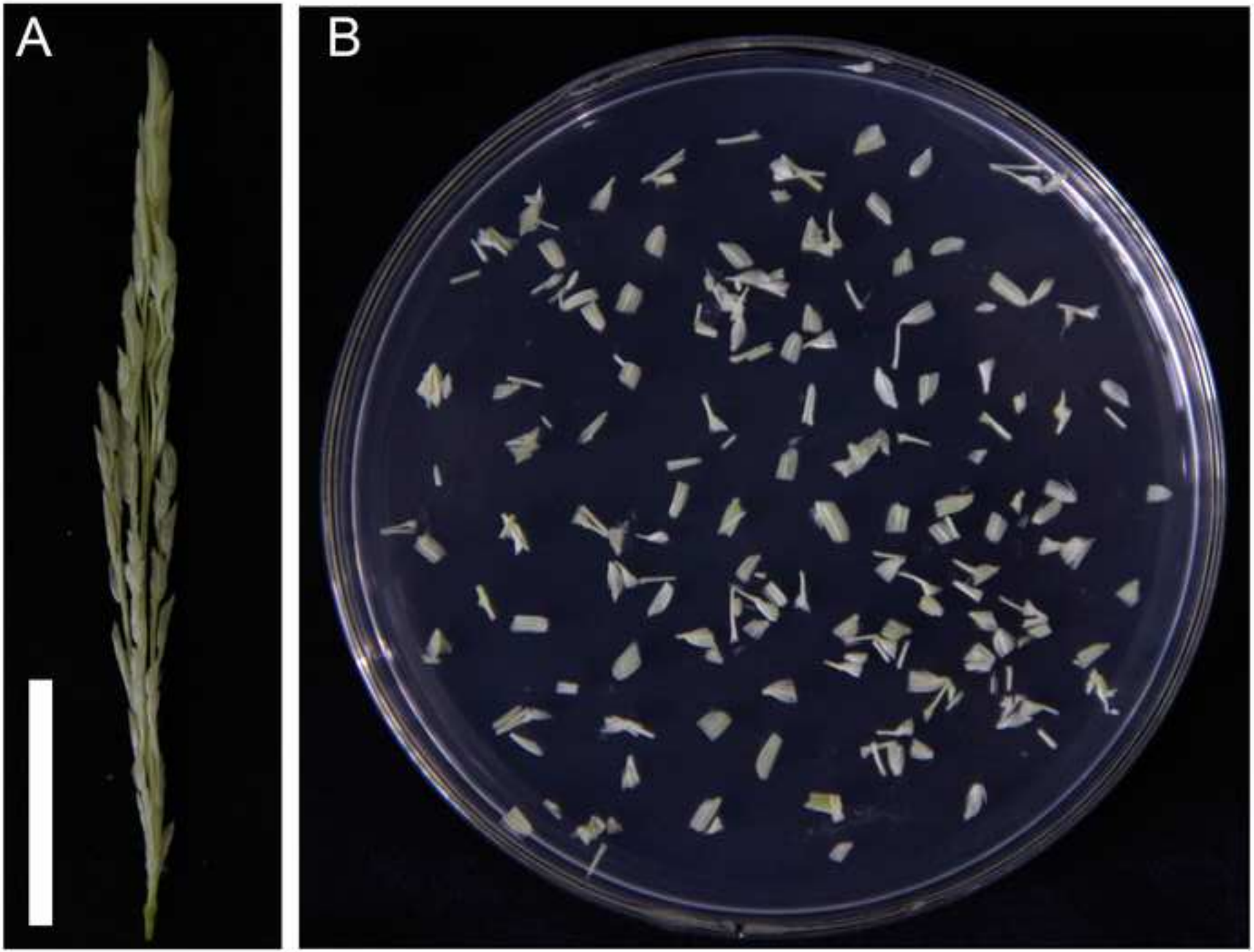

Figure 3

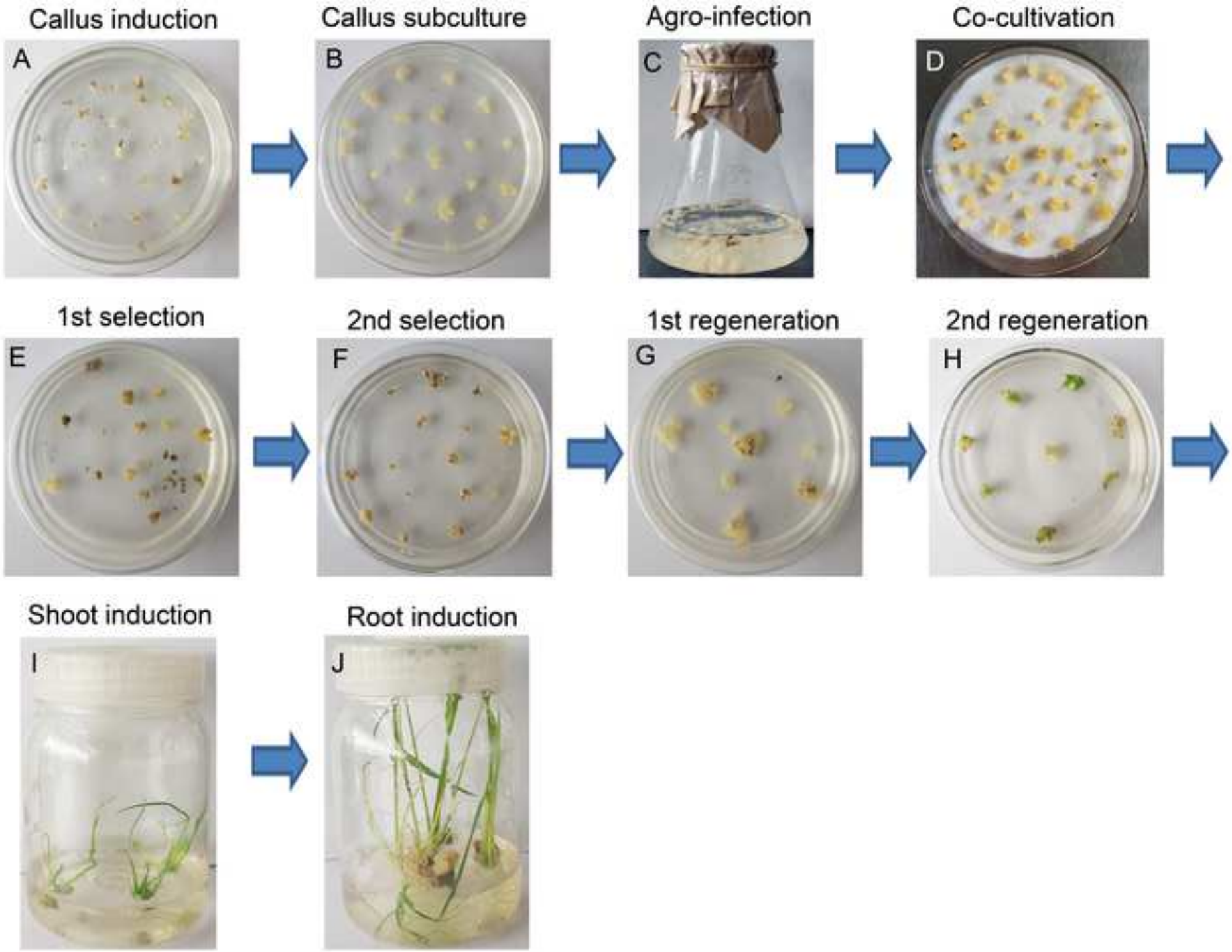

Figure 4

Click here to access/download;Figure;Figure 4.psd

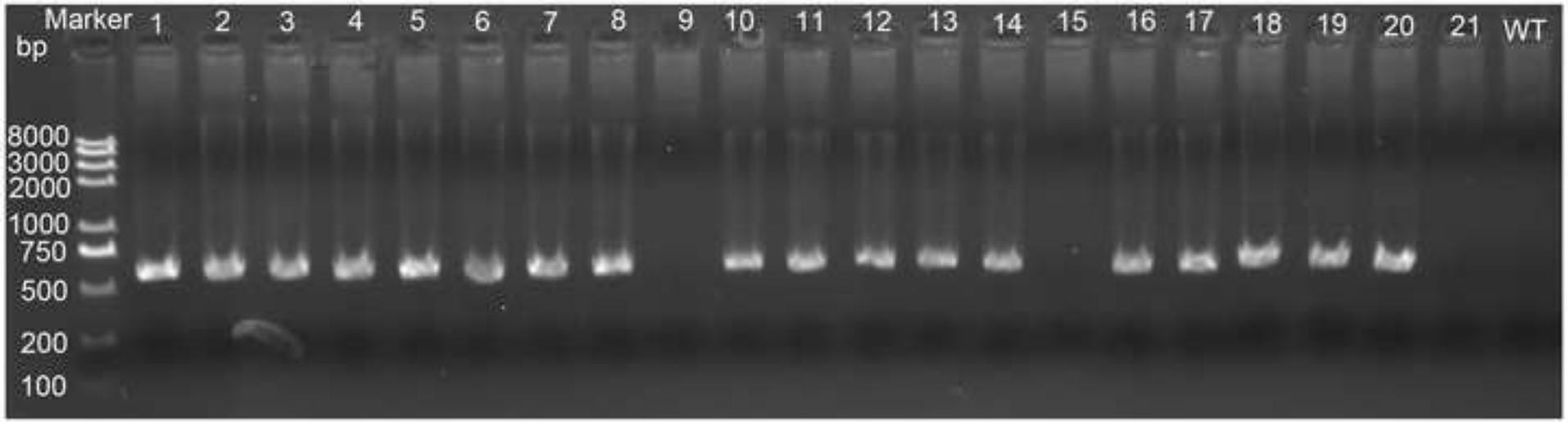

Figure 5

Click here to access/download;Figure;Figure 5.psd

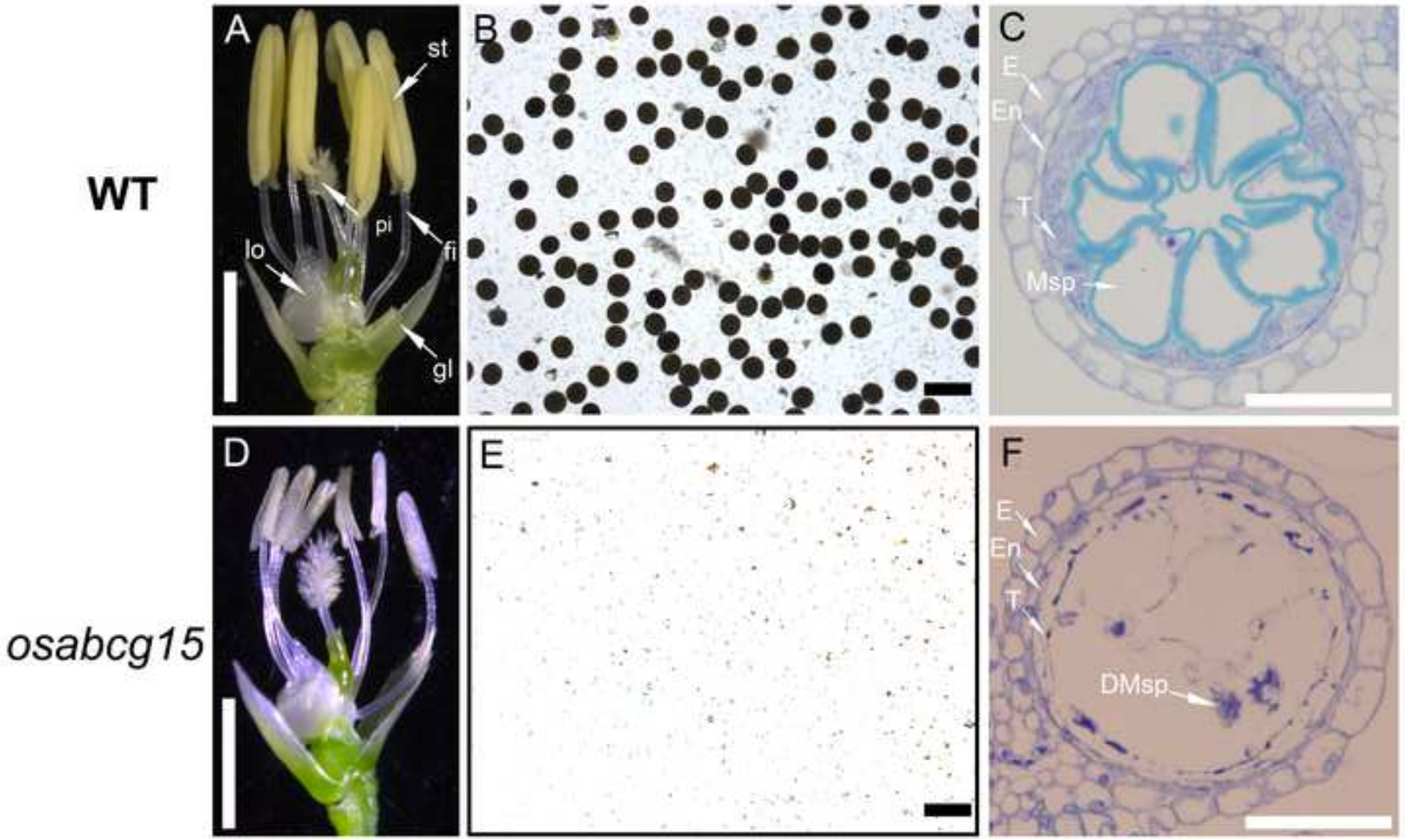

Table 1

NBD2 medium Callus inducing	YEB medium Agrobacerium cultivation	AAM-AS medium Agrobacterium inflitration	NBD-AS medium Co-cultivation	Slection medium NBD2 Selection for resistant callus
20x macroelement I, 50 mL/L 40x macroelement II, 25 mL/L 40x macroelement III, 25 mL/L 100x B5 microelement I, 10 mL/L 1000x B5 microelement II, 1 mL/L 100x B5 vitamin I, 10 mL/L 100x B5 vitamin II, 10mL/L 100x 2,4-D, 10 mL/L 200x Fe, 5 mL/L Sucrose, 30 g/L Inositol, 0.1 g/L Casein acid hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-Glutamine, 0.5g/L	Beaf extract, 5 g/L Peptone, 5 g/L Yeast extract, 1 g/L Sucrose, 5 g/L $MgSO_4 \cdot 7H_2O$, 0.24 g/L	10x AA macroelement, 100 mL/L 100x AA microelement I, 10 mL/L 1000x AA microelement II, 1 mL/L 10x AA, 100 mL/L 200x Fe, 5 mL/L 100x MS vitamin, 10 mL/L KCL, 2.94 g/L Casein acid hydrolysate, 0.5 g/L Sucrose, 68.5 g/L Glucose, 36 g/L Inositol, 0.1 g/L	20x macroelement I, 50 mL/L 40x macroelement II, 25 mL/L 40x macroelement III, 25 mL/L 100x B5 microelement I, 10 mL/L 1000x B5 microelement II, 1 mL/L 100x B5 vitamin I, 10 mL/L 100x B5 vitamin II, 10mL/L 100x2,4-D, 10 mL/L 200x Fe, 5 mL/L Casein acid hydrolysate, 1g/L inositol, 0.1g/L sucrose, 30g/L glucose, 10g/L	20x macroelement I, 50 mL/L 40x macroelement II, 25 mL/L 40x macroelement III, 25 mL/L 100x B5 microelement I, 10 mL/L 1000x B5 microelement II, 1 mL/L 100x B5 vitamin I, 10 mL/L 100x B5 vitamin II, 10mL/L 100x 2,4-D, 10 mL/L 200x Fe, 5 mL/L Sucrose, 30 g/L Inositol, 0.1 g/L Casein acid hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-Glutamine, 0.5g/L
pH to 5.8 with NaOH Add phytagel, 3 g/L	pH to 5.8 with NaOH Add agar 15 g/L for solid medium	pH to 5.2 with NaOH	pH to 5.2 with NaOH Add phytagel, 3 g/L	pH to 5.8 with NaOH Add phytagel, 3 g/L
Autoclave at 121 °C 20 mins	Autoclave at 121 °C 20 mins add 1000×Kanamycine, 1 mL/L 1000× Rifampicin, 1mL/L (before use)	Autoclave at 115 °C 20 mins add AS Stock 0.4 mL to final concentration at 200 µM/L (before use)	Autoclave at 115 °C 20 mins add AS Stock 0.2 mL/L to final concentratin at 100 µM/L (before use)	Autoclave at 121 °C 20 mins add Hygromycin Stock, 0.8 mL/L to final concentration at 40 mg/L Timentin Stock, 1.25 mL/L to final concentration at 250 mg/L (before use)

Stock	Concentration	Solvent
macroelement I Stock (20x)	KNO_3, 56.6 g/L $(NH_4)_2SO_4$, 9.26 g/L KH_2PO_4, 8 g/L	dH_2O
macroelement II Stock (40x)	$CaCl_2 \cdot 2H_2O$, 6.64 g/L	dH_2O
macroelement III Stock (40x)	$MgSO_4 \cdot 7H_2O$, 7.4 g/L	dH_2O
B5 microelement I Stock (100x)	$MnSO_4 \cdot H_2O$, 0.781 g/L $ZnSO_4 \cdot 7H_2O$, 0.2 g/L H_3BO_3, 0.3 g/L KI, 0.075 g/L	dH_2O
B5 microelement II Stock (1000x)	$Na_2MoO_4 \cdot 2H_2O$, 0.25 g/L $CuSO_4 \cdot 5H_2O$, 0.25 g/L $CoCl_2 \cdot 6H_2O$, 0.25 g/L	dH_2O
B5 vitamin I Stock (100x)	Aneurine hydrochloride, 1 g/L Vitamin B6, 0.1 g/L Nicotinic acid, 0.1 g/L	dH_2O
B5 vitamin II Stock (100x)	glycine, 0.2 g/L	dH_2O
2,4-D Stock (100x)	2, 4-Dichlorophenoxyacetic Acid, 0.2 g/L	dH_2O
Fe Stock (200x)	EDTA-$Na_2 \cdot 2H_2O$, 7.4 g/L $FeSO_4 \cdot 7H_2O$, 5.56 g/L	dH_2O
10× AA macroelement	KH_2PO_4, 1.7 g/L $MgSO_4 \cdot 7H_2O$, 3.7 g/L $CaCl_2 \cdot 2H_2O$, 4.4 g/L	dH_2O
100× AA microelement I	$MnSO_4 \cdot H_2O$, 1.69 g/L $ZnSO_4 \cdot 7H_2O$, 0.86 g/L H_3BO_3, 0.62 g/L KI, 0.083 g/L	dH_2O
1000× AA microelement II	$CuSO_4 \cdot 5H_2O$, 0.025 g/L $NaMoO_4 \cdot 2H_2O$, 0.25 g/L $CoCl_2 \cdot 6H_2O$, 0.025 g/L	dH_2O
10× AA	Glutamine, 8.77 g/L aspartic acid, 2.66 g/L arginine, 2.88 g/L glycine, 0.75 g/L	dH_2O
100×MS vitamin	Nicotinic acid, 0.05 g/L aneurine hydrochloride, 0.1 g/L Vitamin B6, 0.05 g/L	dH_2O
AS Stock	Acetosyringone, 0.5 M/L	DMSO
Hygromycin Stock	50 mg/ml	dH_2O
Timentin Stock	200 mg/ml	dH_2O
1000×Kanamycine	50 mg/ml	dH_2O
1000× Rifampicin	50 mg/ml	Methanol

MS medium Callus differentiation culture	1/2 MS medium Root induce cultivation
MS, 4.4g/L Casein acid hydrolysate, 500 mg/L 6-BA, 2 mg/L KT, 0.5 mg/L NAA, 0.5 mg/L Sucrose, 30 g/L D- sorbitol, 15 g/L	MS, 2.2g/L Sucrose, 30g/L
pH to 5.8 with NaOH Add phytagel, 3 g/L	pH to 5.8 with NaOH Add phytagel, 1.75 g/L
Autoclave at 121 °C 20 mins add Hygromycin Stock, 0.5 mL/L to final concentration at 25 mg/L Timentin Stock, 0.75 mL/L to final concentration at 150 mg/L (before use)	Autoclave at 121 °C 20 mins

Table 2: Transformation and gene editing efficiency of the mutants of OsABCG15 that generated by C

Target gene	Number of T_0 plants	Number of transgenic positive plants	Transformation efficiency (%)	Number of homozygous plants	Number of heterozygous plants
OsABCG15	21	18	85.71	3	8

:RISPR-Cas9 in T0 generation
Mutation rate (%)
61.11

Name of Material/ Equipment	Company	Catalog Number
1-Naphthaleneacetic acid	Sigma-Aldrich	N0640
2，4-Dichlorophenoxyacetic Acid	Sigma-Aldrich	D7299
6-Benzylaminopurine (6-BA)	Sigma-Aldrich	B3408
Acetosyringone	Sigma-Aldrich	D134406
Agar	Sinopharm Chemical Reagent Co., Ltd	10000561
Ammonium sulfate	Sinopharm Chemical Reagent Co., Ltd	10002918
Aneurine hydrochloride	Sigma-Aldrich	T4625
Anhydrous ethanol	Sinopharm Chemical Reagent Co., Ltd	10009218
Bacteriological peptone	Sangon Biotech	A100636
Beef extract	Sangon Biotech	A600114
Boric acid	Sinopharm Chemical Reagent Co., Ltd	10004808
Calcium chloride dihydrate	Sinopharm Chemical Reagent Co., Ltd	20011160
Casein acid hydrolysate	Beijing XMJ Scientific Co., Ltd	C184
Cobalt(Ⅱ) chloride hexahydrate	Sinopharm Chemical Reagent Co., Ltd	10007216
Copper(Ⅱ) sulfate pentahydrate	Sinopharm Chemical Reagent Co., Ltd	10008218
D(+)-Glucose anhydrous	Sinopharm Chemical Reagent Co., Ltd	63005518
D-sorbitol	Sinopharm Chemical Reagent Co., Ltd	63011037
EDTA, Disodium Salt, Dihydrate	Sigma-Aldrich	E5134
EOS Digital SLR and Compact System Cameras	Canon	EOS 700D
Formaldehyde	Sinopharm Chemical Reagent Co., Ltd	10010018
Fully Automated Rotary Microtome	Leica Biosystems	Leica RM 2265
Glacial acetic acid	Sinopharm Chemical Reagent Co., Ltd	10000208
Glycine	Sinopharm Chemical Reagent Co., Ltd	62011516
Hygromycin	Beijing XMJ Scientific Co., Ltd	H370
Inositol	Sinopharm Chemical Reagent Co., Ltd	63007738
Iodine	Sinopharm Chemical Reagent Co., Ltd	10011517
Iron(Ⅱ) sulfate heptahydrate	Sinopharm Chemical Reagent Co., Ltd	10012116
Kanamycine	Beijing XMJ Scientific Co., Ltd	K378
Kinetin	Sigma-Aldrich	K0753
L-Arginine	Sinopharm Chemical Reagent Co., Ltd	62004034
L-Aspartic acid	Sinopharm Chemical Reagent Co., Ltd	62004736

L-Glutamine	Beijing XMJ Scientific Co., Ltd	G229
L-proline	Beijing XMJ Scientific Co., Ltd	P698
Magnesium sulfate heptahydrate	Sinopharm Chemical Reagent Co., Ltd	10013018
Manganese sulfate monohydrate	Sinopharm Chemical Reagent Co., Ltd	10013418
Microscopes	NIKON	Eclipse 80i
MS	Phytotech	M519
Nicotinic acid	Sigma-Aldrich	N0765
Phytagel	Sigma-Aldrich	P8169
Potassium chloride	Sinopharm Chemical Reagent Co., Ltd	10016308
Potassium dihydrogen phosphate	Sinopharm Chemical Reagent Co., Ltd	10017608
Potassium iodide	Sinopharm Chemical Reagent Co., Ltd	10017160
Potassium nitrate	Sinopharm Chemical Reagent Co., Ltd	1001721933
Pyridoxine Hydrochloride (B6)	Sigma-Aldrich	47862
Rifampicin	Beijing XMJ Scientific Co., Ltd	R501
Sodium hydroxide	Sinopharm Chemical Reagent Co., Ltd	10019718
Sodium molybdate dihydrate	Sinopharm Chemical Reagent Co., Ltd	10019816
Stereo microscopes	Leica Microsystems	Leica M205 A
Sucrose	Sinopharm Chemical Reagent Co., Ltd	10021418
Technovit embedding Kits 7100	Heraeus Teknovi, Germany	14653
Timentin	Beijing XMJ Scientific Co., Ltd	T869
Toluidine Blue O	Sigma-Aldrich	T3260
Water bath for paraffin sections	Leica Biosystems	Leica HI1210
Yeast extract	Sangon Biotech	A515245
Zinc sulfate heptahydrate	Sinopharm Chemical Reagent Co., Ltd	10024018

Comments/Description

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18th June, 2020

Dear Editor

We appreciated very much for your effort and time dealing with our Manuscript entitled "Agrobacterium-Mediated Genetic Transformation, Transgenic Production and Its Application for the Study of Male Reproductive Development in Rice" (JoVE61665). We have revised the manuscript according to your comments. We believe that we have addressed most of the concerns. Briefly, we have made following changes:

1. We have addressed most of the comments marked in the manuscript.

3. We have invited other Professors to proofread the manuscript thoroughly.

3. We have prepared two version of the revised manuscript, a clear version and a version with yellow highlighted for film later.

I believe that we have addressed most of the comments. Hopefully this revision will meet the requirement of the Journal. I look forward to hearing from you soon.

With Sincerely Regards,

Dawei Xu, Ph. D

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