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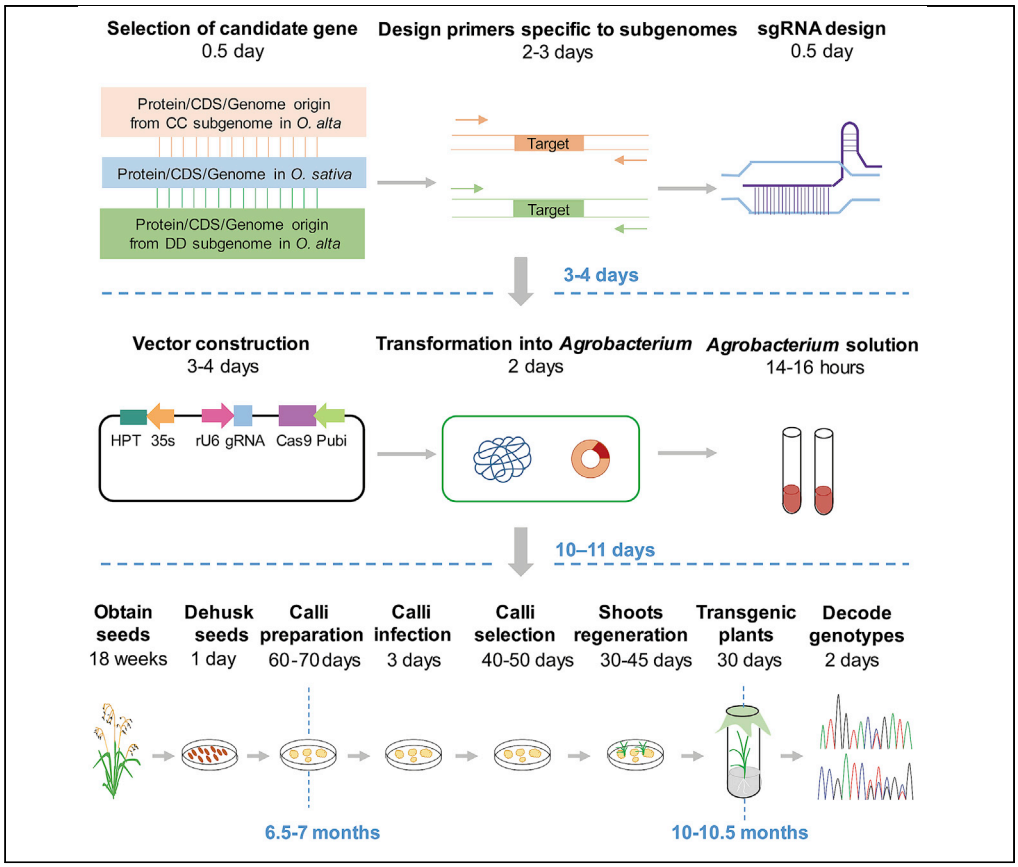
Protocol for genome editing in wild allotetraploid rice *Oryza alta*



We present a protocol for *Agrobacterium*-mediated genetic transformation and genome editing in wild allotetraploid rice *Oryza alta*. We detail steps to induce embryogenic calli from mature seeds and co-cultivate with *Agrobacterium* after infection. We further describe how to select transformed cells that proliferated from infected calli based on hygromycin resistance, resulting in regeneration of transformed plantlets with multiple edited alleles. Edited lines produced *via* this protocol can be used for gene functions studies and *de novo* domestication of *O. alta*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A step-by-step approach for genome editing in allotetraploid rice *Oryza alta*

Detailed steps for *Oryza alta* genetic transformation *via* *Agrobacterium* mediation

Infected calli selection, shoot regeneration, and transgenic plantlet hardening

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Protocol for genome editing in wild allotetraploid rice *Oryza alta*

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SUMMARY

We present a protocol for *Agrobacterium*-mediated genetic transformation and genome editing in wild allotetraploid rice *Oryza alta*. We detail steps to induce embryogenic calli from mature seeds and co-cultivate with *Agrobacterium* after infection. We further describe how to select transformed cells that proliferated from infected calli based on hygromycin resistance, resulting in regeneration of transformed plantlets with multiple edited alleles. Edited lines produced *via* this protocol can be used for gene functions studies and *de novo* domestication of *O. alta*.

For complete details on the use and execution of this protocol, please refer to Yu et al. (2021).

BEFORE YOU BEGIN

Mature embryos (i.e., seeds) are used as explants to support transformation. Researchers must first grow wild allotetraploid rice to obtain enough seeds for callus induction. Due to strong seed shattering in wild allotetraploid rice, we recommend bagging the panicle at the heading stage so that a sufficient number of seeds can be harvested.

Growing *O. alta* to obtain seeds as explants

Timing: 18 weeks (depending on the season)

1. Sprout 100 *O. alta* seeds with 30 mL 1% H_2O_2 overnight to accelerate germination (Figure 1A).
2. Transfer germinated seeds to plastic pots (17 cm*15 cm with 4 seedings per pot) containing a 3:1 mixture of plant nutrient soil and vermiculite and immerse the pots in water.

Growing the seedlings in the greenhouse at 30°C / 20°C (day and night) under a 16 h light and 8 h dark photoperiod (200–500 μmol m^{-2} s^{-1}) (Figure 1B).

Seedlings can grow up to 20 cm in 14 days and then can be transplanted to field (step 3) or greenhouse (step 4).

Note: To break strong dormancy of *O. alta* seeds and avoid uneven germination, we recommend sprouting seeds of *O. alta* with 1% H_2O_2, which is higher than diploid rice cultivars. At the juvenile stage, it is normal that the seedlings are slender and grow slowly.

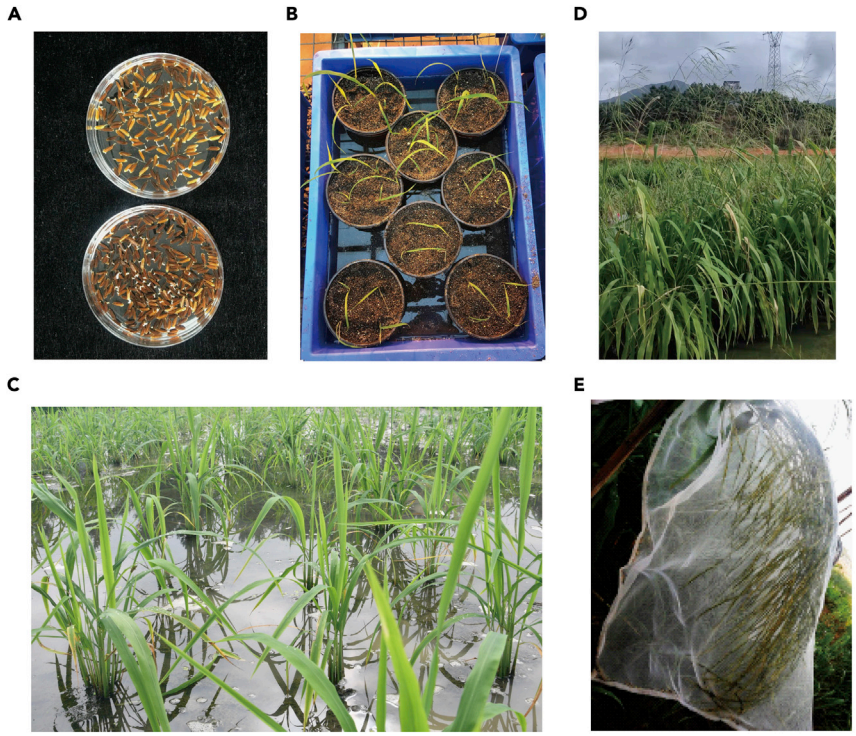

Figure 1. Growing *O. alta* to harvest seeds
(A) Sprouting *O. alta* seeds.
(B) Seedlings in greenhouse.
(C) Transplanted plants in Beijing.
(D) Transplanted plants in Hainan.
(E) Bagging panicles at the heading stage.

3. Transplant the seedlings to the experimental field with normal rice field management and grow for another 4–5 months to harvest seeds (Figures 1C and 1D).

   Note: We recommend transplanting *O. alta* to fields where plants have an adequate growth period due to photoperiod sensitivity.

4. Transfer the seedings from step 2 to bigger plastic pots (30 cm*25 cm with one plant per pot).
   a. After about 3 months, change the photoperiod condition to 10 h light and 14 h dark photoperiod (200–500 μmol m^{-2} s^{-1}) for induction of flowering.
   b. Maintain the temperature between 20°C to 26°C for another 1–2 months to harvest the seeds.
5. Bag the panicle at heading state for seed harvest (Figure 1E).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Agrobacterium tumefaciens strain EHA105	(Meng et al., 2017)	N/A
DH5α Chemically Competent Cell	TransGen Biotech	Cat#CD201
Chemicals, peptides, and recombinant proteins		
2,4-Dichlorophenoxyacetic acid	Sigma	D7299
6-Benzylaminopurine	Sigma	B3408
Acetosyringone	Sigma	D134406

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agar	Sigma	A1296
Agarose	Sigma	Cat#9012-36-6
Ampicillin sodium	PhytoTech Labs	A116
$CaCl_2 \cdot 2H_2O$	Sinopharm	20011160
Carbenicillin	Sigma	P8169
Casein acid hydrolysate	Sigma	C93860
Cetyltrimethylammonium Bromide (CTAB)	Sinopharm	CC3991
$CoCl_2 \cdot 6H_2O$	Sinopharm	10007216
$CuSO_4 \cdot 5H_2O$	Sinopharm	10008218
Dimethyl sulfoxide (DMSO)	Sinopharm	30072418
D-Maltose Monohydrate	PhytoTech Labs	M588
D-Sorbitol	PhytoTech Labs	S744
Ethyl alcohol	Sinopharm	YC-SJ06026
Ethylenediaminetetraacetic Acid Ferric-Sodium Salt	PhytoTech Labs	E676
Glucose	Sigma	V900116
Glycine	Sigma	G8790
H_2O_2	Sinopharm	10011208
H_3BO_3	Sinopharm	10004828
HCl	Sinopharm	Cat#7647-01-0
Hygromycin B (50 mg/mL in PBS)	PhytoTech Labs	H370
Indole-3-butyric acid	Sigma	G7021
K_2HPO_4	Sinopharm	20032118
Kanamycin sulfate	PhytoTech Labs	K378
KCl	Sinopharm	10016308
KH_2PO_4	Sinopharm	10017608
KI	Sinopharm	10017118
KNO_3	Sinopharm	10017218
KOD FX	Toyobo	Cat#KFX-101
KOH	Sinopharm	10017018
L-Glutamic acid	PhytoTech Labs	G399
L-Glutamine	Sigma	G8540
L-Proline	Sigma	P5607
L–Serine	PhytoTech Labs	S807
Mannitol	Sigma	M1902
$MgSO_4 \cdot 7H_2O$	Sinopharm	10013018
2 mM dNTPs	Toyobo	Cat#KFX-101
$MnSO_4 \cdot H_2O$	Macklin	M813649
MS salt	PhytoTech Labs	M524
MS Vitamin Powder	PhytoTech Labs	M533
myo-Inositol	Sigma	I3011
NaCl	Sinopharm	10019308
$Na_2MoO_4 \cdot 2H_2O$	Sinopharm	10019818
1-Naphthylacetic acid	PhytoTech Labs	N600
N6-Furfuryladenine	Sigma	K0753
$(NH_4)_2SO_4$	Sinopharm	10002918
Nicotinic acid	Sigma	N0761
2-(N-Morpholino) ethanesulfonic Acid Monohydrate (MES)	PhytoTech Labs	M825
2×PCR Buffer for KOD FX	Toyobo	Cat#KFX-101
Phytagel	Sigma	P8169
Pluronic F68	PhytoTech Labs	P770
Pyridoxine hydrochloride	Sigma	P8666
Rifampicin	PhytoTech Labs	R501
Sodium hypochlorite	Sinopharm	Cat#7681-52-9
Sucrose	Sinopharm	YC-SJ03064

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Thiamine hydrochloride	Sigma	T3902
Tryptone	Sinopharm	69024138
Yeast extract	Sigma	Y1625
$ZnSO_4 \cdot 7H_2O$	Sinopharm	10024018
Critical commercial assays		
EasyPure Plasmid MiniPrep Kit	TransGen	Cat#EM101
Cas9/gRNA Vector Construction Kit	Viewsolid Biotech	Cat#VK005-01
Experimental models: Organisms/strains		
Oryza alta cv. 2007-24	National Nursery of Wild Rice Germplasm_China	Accession No: 2007-24
Deposited data		
Reference genome of *O. alta*	https://ngdc.cncb.ac.cn/bioproject/browse/PRJCA002366	Accession No: PRJCA002366
Supplemental information	Mendeley Data	https://doi.org/10.17632/ks6cvx79xv.1
Oligonucleotides		
Forward oligo DNA for *OaGS3* gRNA target construction	(Yu et al., 2021)	CAGCCGGCAGCGCCCGACCCCTG
Reverse oligo DNA for *OaGS3* gRNA target construction	(Yu et al., 2021)	AACCAGGGGTCGGGCGCTGCCGG
Primer for amplification of *OaGS3* target site in CC subgenome, GS3-CC-F	(Yu et al., 2021)	CCTCCGCCATTTATAATCCA
Primer for amplification of *OaGS3* target site in CC subgenome, GS3-CC-R	(Yu et al., 2021)	TATGCATTCGTGGTTTCAGC
Primer for amplification of *OaGS3* target site in DD subgenome, GS3-DD-F	(Yu et al., 2021)	GCTGCCTTTCCATCATCATT
Primer for amplification of *OaGS3* target site in DD subgenome, GS3-DD-R	(Yu et al., 2021)	ATGTTGGGCCATGCATATTT
Primer for detection of transgene Cas9-F	(Yu et al., 2021)	TGTTCGTTATCCTCTGGGCTG
Primer for detection of transgene Cas9-R	(Yu et al., 2021)	AGTCCGCAAGGTTCTCTCTATG
Primer for detection of transgene HPT-F	(Yu et al., 2021)	ATGAAAAAGCCTGAACTCACCGCGA
Primer for detection of transgene HPT-R	(Yu et al., 2021)	CTATTTCTTTGCCCTCGGACGAGT
Primer for monoclone identification OaGS3-F	N/A	CAGCCGGCAGCGCCCGACCCCTG
Primer for monoclone identification VK005-R	N/A	AAAACCTCACCAAAATACGA
Primer for construct sequencing VK005-seqprimer	N/A	GCCATGAATAGGTCTATGACC
Recombinant DNA		
Cas9/gRNA Vector VK005-01	Viewsolid Biotech	Cat#VK005-01
GS3-Vk005	(Yu et al., 2021)	N/A
Software and Algorithms		
CRISPR-direct	N/A	http://crispr.dbcls.jp/
CRISPR-GE	(Xie et al., 2017)	http://skl.scau.edu.cn/
CRISPR-P 2.0	(Liu et al., 2017)	http://crispr.hzau.edu.cn/CRISPR2/
RNAfold web server	(Lorenz et al., 2011)	http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi
RNAstructure website	(Reuter and Mathews, 2010)	https://rna.urmc.rochester.edu/RNAstructureWeb/
Cas-OFFinder software	(Bae et al., 2014)	http://www.rgenome.net/cas-offinder/
Primer3Input	N/A	https://primer3plus.com/primer3web/primer3web_input.htm
Primer-BLAST	N/A	https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome
Other		
BTX electroporation system	Electro Cell Manipulator®	N/A
Molecular Imager Gel Doc XR+ System with Image Lab Software	Bio-Rad	Cat#1708195
0.22-μm PES filter	Minisart®	Cat#16541-K
NanoDrop 2000 Spectrophotometer	Thermo Scientific	N/A
T100™ Thermal Cycler	Bio-Rad	N/A
Flask	BOMEX	N/A

MATERIALS AND EQUIPMENT

Make stock solutions

Timing: 1–2 days

Kanamycin (Kan, 100 mg/mL)

Dissolve 10 g kanamycin sulfate in 90 mL of distilled water. Add water to a final volume of 100 mL. Sterilize with a 0.22-μm polyethersulfone (PES) filter in clean bench, and store in 1.5-mL aliquots at −20°C for up to 6 months.

Ampicillin (Amp, 50 mg/mL)

Dissolve 5 g ampicillin sodium in 90 mL of distilled water. Add water to a final volume of 100 mL. Sterilize with a 0.22-μm PES filter in clean bench, and store in 1.5-mL aliquots at −20°C for up to 6 months.

Rifampicin (Rif, 25 mg/mL)

Dissolve 1 g rifampicin in 40 mL of dimethyl sulfoxide (DMSO), and store in 1.5-mL aliquots at −20°C for up to 6 months.

Acetosyringone (AS, 100 mM)

Dissolve 196.2 mg acetosyringone in 10 mL of dimethyl sulfoxide (DMSO), and store in 1.5-mL aliquots at −20°C for up to 6 months.

KOH (1 M)

Dissolve 5.61 g of KOH in 90 mL of distilled water. Add water to a final volume of 100 mL, and store at room temperature for up to 2 months.

KCl (1 M)

Dissolve 7.46 g of KCl in 90 mL of distilled water. Add water to a final volume of 100 mL, and store at room temperature for up to 2 months.

Carbenicillin (200 mg/mL)

Dissolve 20 g of carbenicillin in 90 mL of distilled water. Add water to a final volume of 100 mL. Sterilize with 0.22-μm PES filter in clean bench, and store in 1.5-mL aliquots at −20°C for up to 6 months.

2,4-Dichlorophenoxyacetic acid (2,4-D, 2 mg/mL)

Dissolve 200 mg of 2,4-Dichlorophenoxyacetic acid in 20 mL 1 M KOH and heat gently with a magnetic stirrer in a microwave. Then replenish final volume to 100 mL with distilled water. Sterilize with 0.22-μm PES filter in clean bench, and store in 1.5-mL aliquots at 4°C for up to 2 months.

N6-Furfuryladenine (kinetin, 1 mg/mL)

Place 25 mg kinetin in a small glass beaker. Add 10 mL 1 M KOH to dissolve. Then replenish final volume to 25 mL with distilled water. Sterilize with 0.22-μm PES filter in clean bench, and store in 1.5-mL aliquots at 4°C for up to 2 months.

6-Benzylaminopurine (6-BA, 2 mg/mL)

Dissolve 200 mg of 6-BA in 10 mL 1 M KOH and heat gently. Then replenish final volume to 100 mL with distilled water. Sterilize with 0.22-μm PES filter in clean bench, and store in 1.5-mL aliquots at 4°C for up to 2 months.

1-Naphthylacetic acid (NAA, 1 mg/mL)

Dissolve 100 mg of NAA in 10 mL 1 M KOH and heat gently. Then replenish final volume to 100 mL with distilled water. Sterilize with 0.22-μm PES filter in clean bench, and store in 1.5-mL aliquots at −20°C for up to 2 months.

Indole-3-butyric acid (IBA, 100 μg/mL)

Dissolve 10 mg IBA in 10 mL 1 M KOH to until completely dissolved. Then replenish final volume to 100 mL with distilled water. Sterilize with 0.22-μm PES filter in clean bench, and store in 1.5-mL aliquots at 4°C for up to 2 months.

MS vitamin (100×, 10.31 mg/mL)

Dissolve 2.578 g MS Vitamin Powder in 250 mL with distilled water, and store at 4°C for up to 1 month.

Macronutrient solution (20×)	
Reagent	Weight/Volume
KNO_3	56.6 g
$(NH_4)_2SO_4$	9.26 g
KH_2PO_4	8.0 g
$MgSO_4 \cdot 7H_2O$	3.7 g
$CaCl_2 \cdot 2H_2O$	8.0 g
Replenish final volume to 1 L, and store at 4°C for up to 1 month.	

Micronutrient solution (100×)	
Reagent	Weight/Volume
$MnSO_4 \cdot H_2O$	7.6 g
H_3BO_3	3.0 g
$ZnSO_4 \cdot 7H_2O$	2.0 g
$Na_2MoO_4 \cdot 2H_2O$	0.25 g
$CuSO_4 \cdot 5H_2O$	0.05 g
$CoCl_2 \cdot 6H_2O$	0.025 g
KI	0.75 g
Replenish final volume to 1 L, and store at 4°C for up to 1 month.	

Ferric Salt (100×)

Dissolve 4 g of Ethylenediaminetetraacetic Acid Ferric-Sodium Salt in 1 L distilled water, and store at 4°C for up to 1 month.

Organic solution (100×)	
Reagent	Weight/Volume
Thiamine hydrochloride	1.1 g
Pyridoxine hydrochloride	0.1 g
Nicotinic acid	0.1 g
Glycine	0.2 g
Replenish final volume to 1 L, and store at 4°C for up to 1 month.	

Make media

Timing: 1–2 days

LB liquid medium	
Reagent	Weight/Volume
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Adjust pH to 7.0. Replenish final volume to 1 L, sterilize at 121°C for 20 min, and store at 4°C for up to 2 months.	

LB solid medium	
Reagent	Weight/Volume
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Adjust pH to 7.0. Replenish final volume to 1 L, sterilize at 121°C for 20 min, and store at 4°C for up to 2 months.	

Calli-Induction Medium (CIM)	
Reagent	Weight/Volume
Macronutrient (20×)	50 mL
Micronutrient (100×)	10 mL
Ferric Salt (100×)	15 mL
Organic (100×)	10 mL
myo-Inositol	500 mg
Casein acid hydrolysate	300 mg
L-Glutamine	500 mg
L-Proline	500 mg
2,4-Dichlorophenoxyacetic acid (2 mg/mL)	1 mL
Sucrose	15 g
D-Maltose Monohydrate	15 g
Phytagel	4 g
Replenish final volume to 1 L with distilled water and adjust pH to 5.8. Sterilize at 121°C for 20 min and cool to 50°C. Then pour 30 mL of the solution per 90 mm sterile petri dish in the clean bench. Seal with plastic wrap after media solidifies and store at 4°C in the dark for up to 2 weeks.	

MG/L Medium	
Reagent	Weight/Volume
Tryptone	5 g
Yeast Extract	2.5 g
NaCl	5 g
Mannitol	5 g
$MgSO_4 \cdot 7H_2O$	100 mg
K_2HPO_4	250 mg
L-Glutamic acid	1.2 g
Sucrose	15 g
Agar	15 g
Replenish final volume to 1 L with distilled water and adjust pH to 7.2. Sterilize at 121°C for 20 min and cool to 50°C. Then pour 30 mL of the solution into 90 mm sterile petri dish in the clean bench. Seal with plastic wrap after media solidifies and store at 4°C for up to 1 month.	

Note: Liquid MG/L Medium with all the above reagents except agar.

Infection Medium	
Reagent	Weight/Volume
MS Salts (M524)	2.15 g
Organic (100×)	20 mL

(*Continued on next page*)

Continued	
Reagent	Weight/Volume
myo-inositol	2 g
Casein acid hydrolysate	2 g
L-Glutamine	2 g
Glutamic acid	1 g
L-Proline	800 mg
L–Serine	500 mg
Sucrose	68.5 g
Glucose	36 g
MES	1 g
2,4-Dichlorophenoxyacetic acid (2 mg/mL)	1 mL
Replenish final volume to 1 L with distilled water and adjust pH to 5.2. Sterilize with 0.2 μm-PES filter in clean bench and store at 4°C in the dark for up to 2 weeks. Add 1 mL of 100 mM AS before use.	

Selection Medium	
Reagent	Weight/Volume
CIM as mentioned above	
Replenish final volume to 1 L with distilled water and adjust pH to 5.8 Sterilize at 121°C for 20 min	
Cool to 50°C, and then add the following components	
Carbenicillin	2 mL (200 mg/mL)
Hygromycin B	1 mL (50 mg/mL)
Pour 30 mL of the solution into 90 mm sterile petri dish in the clean bench. Seal with plastic wrap after media solidifies and store at 4°C in the dark for up to 1 week.	

Regeneration Medium	
Reagent	Weight/Volume
Macronutrient (20×)	50 mL
Micronutrient (100×)	10 mL
Ferric Salt (100×)	15 mL
Organic (100×)	10 mL
myo-inositol	2 g
Casein acid hydrolysate	600 mg
L-Glutamine	500 mg
L-Proline	500 mg
L–Serine	500 mg
D-Maltose	30 g
Sorbitol	30 g
Phytagel	4 g
Replenish final volume to 1 L with distilled water and adjust pH to 5.8 Sterilize at 121°C for 20 min	
Cool to 50°C, and then add the following components	
N6-Furfuryladenine (Kinetin, 1 mg/mL)	0.5 mL
6-Benzylaminopurine (2 mg/mL)	1 mL
1-Naphthylacetic acid (1 mg/mL)	0.2 mL
Hygromycin B (50 mg/mL)	0.6 mL
Pour 30 mL of the solution into a 90 mm sterile petri dish in the clean bench. Let cool and solidify and store at 4°C in the dark for up to 1 week.	

Rooting Medium	
Reagent	Weight/Volume
MS Salts	433 mg
MS Vitamin (100×)	15 mL (10.31 mg/mL)
Casein acid hydrolysate	300 mg
L-Glutamine	500 mg
L-Proline	500 mg
Sucrose	20 g
Agar	5 g
Replenish the volume to 1 L with distilled water and adjust pH to 5.8 Sterilize at 121°C for 20 min	
Cool to 50°C, and then add the following components	
Indole-3-butyric acid	0.1 mL (100 μg/mL)
Hygromycin B	1 mL (50 mg/mL)
Pour 50 mL of the solution into a sterile glass culture bottle in the clean bench. Seal with parafilm after cooling and solidifying and store at 4°C in the dark for up to 1 week.	

STEP-BY-STEP METHOD DETAILS

The first transgenic rice plants were reported more than 30 years ago (Shimamoto et al., 1989). Since 1994, *Agrobacterium*-mediated transformation has become the most popular method for rice transformation, and various optimizations have been made for efficient and simplified transformation (Wu and Sui, 2019). However, the regeneration and transformation of allotetraploid wild rice still remains a challenge (Shimizu-Sato et al., 2020).

Calli induction

Timing: 2–3 months (depending on the subculture cycle)

This section describes calli induction of mature seeds.

1. Remove the coats of full mature seeds by hand (Methods video S1).

   Note: The seeds should be fully filled, healthy and pathogen-free.

2. Sterilize the surface of the seeds with 75% ethanol by vigorously hand-shaking for 2 min. Pour out ethanol and then rinse seeds with sterile water (Methods video S1).
3. Sterilize the seeds in a flask with 50 mL of a bleach solution (sodium hypochlorite with 2.5% active chlorine) on a rotary shaker at 200 rpm for 30 min at room temperature (Methods video S1).
4. Move the seeds to the clean bench (Methods video S1).
5. Rinse the seeds with sterile water, repeat five times and dry the seeds on sterile filter paper for five minutes (Methods video S1).
6. Place the 8–10 sterilized seeds on a petri dish, with the embryos facing towards fresh callus induction medium and incubate in dark at 28°C for 20 days (Figure 2A and Methods video S1).
7. Select the embryogenic calli that have started to form the scutella and remove the shoots or shoot-like structures (Figure 2B).
   a. To obtain sufficient embryogenic calli for infection and maintain vitality, subculture the embryogenic calli on fresh CIM for several times.
   b. Replace CIM for every 7–10 days (Figures 2C and 2D).

   Note: The selection of embryogenic calli from non-embryogenic calli is essential at this step. The embryogenic calli are identifiable as solid granular structures with smooth surfaces and creamy/yellow color rather than filamentous and gelatinous. The subculturing process can

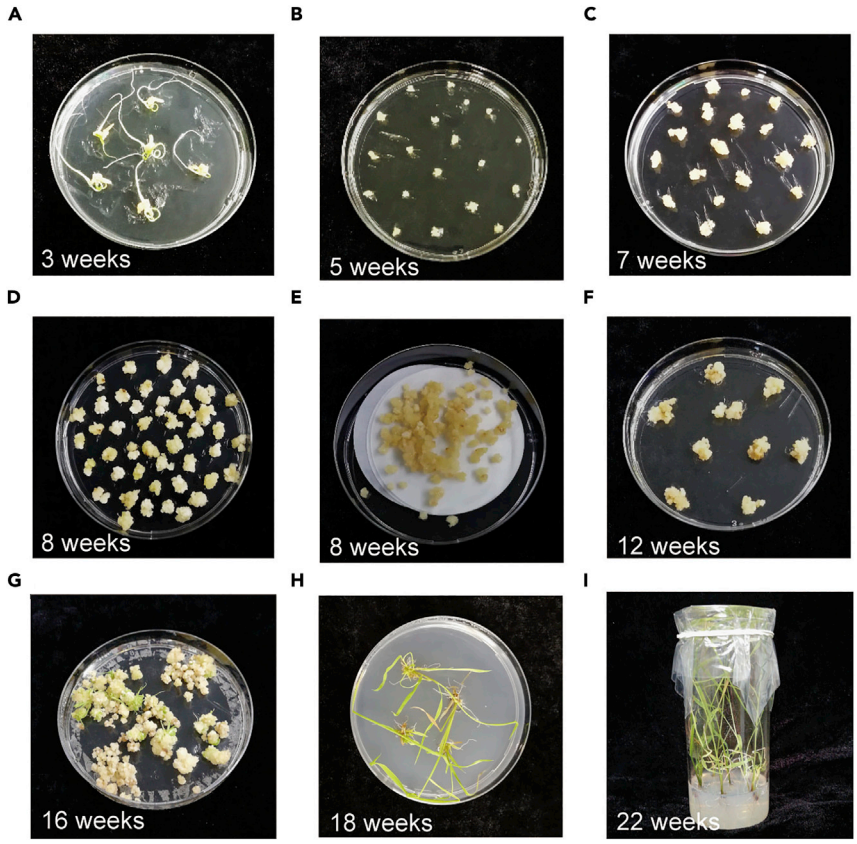

Figure 2. *Agrobacterium*-mediated transformation of allotetraploid rice
(A) Calli induction of mature seeds, 20 days after induction.
(B) Two weeks later, yellow, compact calli were subcultured on fresh CIM.
(C) Propagated calli.
(D) The calli after three cycles of subculture.
(E) *Agrobacterium*-infected rice calli on sterile filter paper in clean bench.
(F) Healthy, yellow and resistant calli in fresh selection medium.
(G) Visible green spots from resistant calli on regeneration medium.
(H) Two weeks later, the plantlets with formed shoot transferred to new rooting medium plates for root inducing.
(I) Seedlings after transferring to rooting medium for hardening.

be repeated several times until sufficient calli are obtained, but should be performed for less than six months from initiation of cultures to ensure vitality.

Design sgRNA in *O. alta*

Timing: 2–3 days

This section describes sgRNA design.

The reference genome of *O. alta* have been deposited in the Genome Sequence Archive in BIG Data Center under accession number: PRJCA002366 (https://ngdc.cncb.ac.cn/bioproject/browse/PRJCA002366) (Yu et al., 2021).

8. Obtain the sequences of gene of interest: https://ngdc.cncb.ac.cn/bioproject/browse/PRJCA002366.

Identify 20 nt target sequence within an exon of the gene of interest.

Note: When Cas9 is employed, the target sequence must contain the PAM sequence 5′-NGG-3′.

Note: Example websites for gRNA design include CRISPR-direct: http://crispr.dbcls.jp/, CRISPR-GE: http://skl.scau.edu.cn/ (Xie et al., 2017), and CRISPR-P 2.0: http://crispr.hzau.edu.cn/CRISPR2/ (Liu et al., 2017).

If multiple-recessive mutants are desired, align the two homeologous sequences of subgenomes CC and DD for the gene of interest, and find identify conserved regions.

9. Analyze the predicted secondary structures of the sgRNA sequence, including target site and gRNA scaffold to avoid TTTTs and target-scaffold pairing.

Note: Example websites include RNAfold web server: http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (Lorenz et al., 2011), and RNAstructure website: https://rna.urmc.rochester.edu/RNAstructureWeb/ (Reuter and Mathews, 2010).

10. Predict off-target edits by Cas-OFFinder software: http://www.rgenome.net/cas-offinder/ (Bae et al., 2014) and minimize sgRNA-dependent off-target activity by sgRNA selection.
11. Design primers specific to target regions on two subgenomes for amplifying the fragments including the target sites.

Note: Example websites for primer design include Primer3Input: https://primer3plus.com/primer3web/primer3web_input.htm, and Primer-BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome.

Note: Editing efficiency is largely dependent on perfect matching between the sgRNA and target sequence. The target sites should be sequenced first to confirm as genomic variations exist among different lines of *O. alta*.

Alternatives: Overlap between the site of DNA cleavage of Cas9 and a restriction recognition sequence allows for detection of putative edits by PCR/RE (Shan et al., 2014). PCR products specific to the two subgenomes should be used to detect the mutations. In order to increase the likelihood of successful editing, preliminary evaluations of sgRNA efficiency could be carried out in protoplasts first (Lin et al., 2018; Nadakuduti et al., 2019).

Vector construction

Timing: 3–4 days

This section describes vector construction of genome editing through CRISPR/Cas9.

12. Synthesize forward and reverse oligo DNAs. The chimeric oligo DNAs comprise the 20-bp target sequence and a 3-bp overhangs specific for the gRNA cloning vector VK005 (Figure S1A). The oligo DNAs are designed as below:

Target-sense: 5′-CAGNNNNNNNNNNNNNNNNNNN (gRNA sense)-3′

Target-antisense: 5′-AACNNNNNNNNNNNNNNNNNN (gRNA antisense)-3′

For example, for *OaGS3* target (5′-CCGGCAGCGCCCGACCCCTG-3′) (Yu et al., 2021), oligo DNAs are designed as:

OaGS3 gRNA-F: 5′-CAGCCGGCAGCGCCCGACCCCTG-3′

OaGS3 gRNA-R: 5′-AACCAGGGGTCGGGCGCTGCCGG-3′

13. Dilute 5 μL of forward and 5 μL reverse oligo DNAs (10 μM) into 15 μL distilled water in PCR tube, and centrifuge the mix after blending.
14. Anneal the oligonucleotides to form the oligoduplex in a T100™ Thermal Cycler. The program is below.

PCR cycling parameters

Steps	Temperature	Time	Cycles
Denaturation	95°C	3 min	1
Annealing	95°C–20°C	0.5°C lower every 10 s	150
Hold	20°C	forever	

Alternatives: This step can also be performed as follows. Boil the oligonucleotides at 95°C for 3 min and leave at room temperature (20°C–25°C) for 30 min to anneal the oligonucleotides.

15. Ligate the annealed gRNA oligoduplex into the Cas9/gRNA vector according to the manufacturer's instructions of Cas9/gRNA Vector Construction Kit: http://www.v-solid.com/product_detail/106.html.

Ligation reaction set up

Reagent	Volume (μL)
Cas9/gRNA vector	1
Oligoduplex	1
Solution 1 in kit	1
Solution 2 in kit	1
H_2O	6
Total	10

16. Transform 10 μL of the ligation reaction into *E. coli* DH5α Competent Cells. Plate the cells onto LB agar containing kanamycin (100 μg/mL), and incubate at 37°C for 16 h.
17. Select monoclone to amplify assembled expression cassettes with two flanking primers (Figure S1B), and culture the positive clone into LB liquid medium containing kanamycin at 37°C for 16 h (Table 1).
18. Extract correct plasmid DNA with an *EasyPure* Plasmid MiniPrep Kit and sequence for correct construction.

Transform the binary vector into *A. tumefaciens*

Timing: 3–4 days

This section describes transforming the binary vector into *A. tumefaciens* through electrotransformation.

Table 1. Primers used for monoclone identification

Primer	Sequence
OaGS3-F	CAGCCGGCAGCGCCCGACCCCTG
VK005-R	AAAACCTCACCAAAATACGA
VK005-seqprimer	GCCATGAATAGGTCTATGACC

19. Pre-wash 2 mm electroporation cuvettes using 75% ethyl alcohol and ddH_2O and dry in a clean bench.
20. Add 50 ng correct plasmid into EHA105 competent cells.
21. Transfer the competent cells into electroporation cuvettes and pulse an electric shock at 1.8 kV.
22. Recover EHA105 competent cells in MG/L liquid medium without antibiotic at 28°C for 45–60 min.
23. Plate the cells onto MG/L solid medium containing kanamycin and rifampicin at 28°C for 2 days.
24. Pick a monoclone and culture in 3–4 mL liquid MG/L liquid medium containing kanamycin and rifampicin and acetosyringone at 28°C for 14–16 h with vigorous shaking until the OD_{600} reaches 0.6–0.8.

Note: Keep EHA105 competent cells on ice and operate gently.

Agrobacterium-mediated genetic transformation

Timing: 3 days

This section describes *agrobacterium*-mediated genetic transformation.

25. Collect *Agrobacterium* cells containing constructs by centrifugation at 800 g, 4°C for 5–10 min.
26. Resuspend the *Agrobacterium* in cooled fresh infection medium containing 100 μM acetosyringone and 0.1% Pluronic F68 and adjust OD_{600} at 0.5 (Methods video S2).
27. Incubate the resuspension solution on ice for 10–20 min (Methods video S2).
28. Place the calli in sterile conical flasks (Figure 2E and Methods video S2) on ice for 10 min prior to the infection.
29. Pour the suspension into the flask immersing the calli for infection and then gently shake the flask at 40 rpm for 10 min (Methods video S2).
30. Place the infected calli on sterile filter paper in the dishes and co-cultivate in the dark at 22°C for 3 days (Methods video S2).

Selection of infected calli, shoot regeneration and transgenic plantlet hardening

Timing: 3–4 months

This section describes plant regeneration.

31. Transfer the calli to fresh selection medium and culture in the dark at 28°C for 7 days.
32. Subculture the hygromycin B resistant and actively growing calli on fresh selection medium every 10 days for three or four cycles under the same condition (Figure 2F and Methods video S3).
33. Transfer resistant calli to regeneration medium with 5 pieces on each dish and culture at 28°C in dark for 1 week (Methods video S3).
34. Culture the calli under 16-h light and 8-h dark photoperiod for 3 weeks at 50–100 μmol m^{-2} s^{-1} light intensity.

Green spots and new shoots should be formed on the calli in two and three weeks, respectively (Figures 2G and 2H and Methods video S3).

One transformed line from a single piece of callus is to be identified as an individual transgenic event.

35. Transfer the regenerated shoots to rooting medium and culture at 28°C for four weeks until the shoots grow up to 10 cm (Figure 2I and Methods video S3).

Note: The plants that are truly hygromycin resistant will develop roots in the rooting medium, which are longer and whiter than those which are not resistant.

36. Harden the plants of transformants by opening the lid of medium.
37. Wash the plants with warm water and re-harden the plants to greenhouse conditions.
38. Transplant the plants to soil in the greenhouse or field.

Mutation detection and genotyping

Timing: 2–3 days

This section describes genotyping of transformants.

39. Extract genomic DNA from leaves of transformants using CTAB method (Allen et al., 2006).
40. Confirm positive transformants with Cas9 primers or hygromycin B primers (sequences in key resources table).
41. Amplify the targeted region of positive transformants by PCR with two subgenome specific primer pairs.
42. Sequence the PCR product and decode the genotypes.

Note: For single-gene editing, Sanger sequencing could be used and if more than two genotypes are present in sequencing data, an additional 10–20 clones should be sequenced. A higher proportion of chimeras were observed from transformed seedlings in allotetraploid rice. Genotypes of chimeras can be quickly decoded with next generation sequencing, such as Hi-Tom (Liu et al., 2019).

EXPECTED OUTCOMES

Multiple genome edited mutants can be produced in CCDD allotetraploid rice. Here, we tested different vectors and in these cases the transformation efficiency was 78%–90%, the regeneration efficiency was 38%–42%, and the editing efficiency was 11%–97.5% (Yu et al., 2021). Highly efficient genome editing was observed.

LIMITATIONS

The transformation protocol may be not necessarily applicable for all genotypes of allotetraploid rice.

The harvest of seeds requires bagging the panicle due to seed shattering, therefore increasing the workload.

TROUBLESHOOTING

Problem 1

The mature seeds are used as explant for calli induction. The seed is often contaminated by fungus or bacterium (steps 6 and 7).

Potential solution

The harvested seeds should be dry as soon as possible and store in a dry and cool place. Select the healthy, full filled and pathogen-free seeds. Observe the plate every day in 4–7 days after inducing, and place uncontaminated seeds into a fresh medium once contamination occurs.

Problem 2

Relatively low transformation efficiency and regeneration ability of *O. alta* by *Agrobacterium*-mediated genetic transformation system (steps 34 and 35).

Potential solution

Different genotypes of *O. alta* show variable transformation efficiencies. The transformation system could be optimized based on genotype of interest, by altering parameters including but not limited to media composition, explant type, culture conditions, and infection reagents.

Problem 3

Callus contamination after infection (steps 30 and 31).

Potential solution

Do not grow the *Agrobacterium* beyond an OD_{600} of 1.0. Dry the infected calli during co-cultivation before selection. Use freshly prepared medium and increase the concentration of carbenicillin appropriately in the first selection medium. Re-transform the *Agrobacterium* and ensure strict sterile operation.

Problem 4

Edited progenies were not obtained due to the low efficiency of the selected target (steps 40–42).

Potential solution

Design the target in strict accordance with the design principles mentioned above (steps 8–10) and evaluate the editing efficiency of the target in rice protoplasts before transforming rice calli. If the editing efficiency is extremely low, reselect the target. We also recommend multiple batches of genetic transformation to increase the probability of obtaining edited progenies.

Problem 5

The yield and quality of DNA by CTAB may be low since there are much polyphenol and polysaccharide in allotetraploid leaf (step 39).

Potential solution

Young, healthy and developing leaves make an ideal choice as they can yield good quality of DNA. Polysaccharides interferer DNA isolation as they are hard to remove, use of high concentration of NaCl (1.4 M) reduce polysaccharide contamination. Antioxidants such as 0.2% β-mercaptoethanol can destroy the organization of protein and the polymerization of polysaccharides, which are beneficial for DNA isolation. In addition, adding 1% polyvinyl pyrollidone (PVP) in CTAB buffer is an effective solution to reduce polyphenol contamination.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiangbing Meng (xbmeng@genetics.ac.cn).

Materials availability

Wild allotetraploid rice seeds used in this study are from the National Nursery of Wild Rice Germplasm in Nanning and Guangzhou, China. Cas9/gRNA Vector VK005-01 can be available from Viewsolid Biotech (Cat#VK005-01). Plasmids generated in this study are available from the lead contact

with a Materials Transfer Agreement. And supplemental information was uploaded to Mendeley Data: https://doi.org/10.17632/ks6cvx79xv.1.

Data and code availability

This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101789.

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AUTHOR CONTRIBUTIONS

J.Z., X.M., P.Z., and H.Y. performed the experiments. J.Z., X.M., P.Z., H.Y., and J.L. analyzed the data. X.M. and J.L. designed the experiments. J.L. conceived the project. J.Z., P.Z., H.Y., X.M., and J.L. wrote the manuscript. All authors have read, edited, and approved the content of the manuscript.

DECLARATION OF INTERESTS

The authors have a patent application associated with this protocol (Method for gene transfer into *O. alta* using *Agrobacterium bacterium*, and methods for production of transgenic plants with the edited target genes).

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