

Chapter 7

Site-Directed Mutagenesis in Barley by Expression of TALE Nuclease in Embryogenic Pollen

Maia Gurushidze, Stefan Hiekel, Ingrid Otto, Götz Hensel, and Jochen Kumlehn

Abstract In this chapter, a detailed protocol for the induction of targeted mutations in barley using customized transcription activator-like effector nucleases (TALENs) is provided. As explant for the introduction of TALEN expression constructs, embryogenic pollen cultures are used which consist primarily of haploid cells able to be converted via pollen embryogenesis and genome duplication into fertile doubled haploid plants. Thanks to the haploid nature of these target cells, a mutation induced by TALE nucleases in just one *gfp* allele can be readily detected in vitro and thus genetically fixed primary mutant plants obtained in one generation.

Keywords Customizable endonuclease • Doubled haploids • Embryogenesis • Homozygosity • Microspore • Targeted knockout

7.1 Introduction

7.1.1 Site-Directed Mutagenesis in Plants

The continuously growing demand of the world population for food is a tremendous challenge for modern agriculture, plant breeding, and biotechnology (Griggs et al. 2013). To rapidly produce new plant varieties with improved gene variants and traits, more efficient, flexible, and reliable methods such as site-directed mutagenesis are needed. In addition, these techniques will also have a big impact on basic science by helping to further investigate gene function and regulation.

Site-directed mutagenesis enables researchers to alter the DNA sequence at a predefined location in the genome of a plant cell. Such mutations can be generated by the use of customized endonucleases for target sequence-specific double-strand

M. Gurushidze • S. Hiekel • I. Otto • G. Hensel • J. Kumlehn (✉)
Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)
Gatersleben, Corrensstrasse 3, 06466 Seeland, OT Gatersleben, Germany
e-mail: kumlehn@ipk-gatersleben.de

break (DSB) induction. The repair of DSBs via nonhomologous end-joining is error-prone to some extent and therefore can lead to nucleotide deletions or insertions (indels) at the breakage site (Waterworth et al. 2011). Depending on the type of mutation, this can result in functional modification, complete disruption of gene function, or removal of whole gene clusters from the plant genome.

For application of this technique in model and crop plant species, the following customizable endonuclease platforms with different benefits and drawbacks are available: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided endonucleases (RGENs); the latter derive from a clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) microbial defense system [reviewed in Fichtner et al. (2014)].

TALENs are created by the fusion of a customized TALE DNA-binding domain (DBD) and the FokI restriction-endonuclease domain (FokI-R) (Christian et al. 2010). Primarily, a TALE-DBD from plant pathogenic bacteria *Xanthomonas* sp. consists of a variable number of nearly identical amino acid repeats. Each repeat has a length of 33–35 amino acids and binds preferentially to a certain nucleotide of the target DNA sequence (Boch et al. 2009; Moscou and Bogdanove 2009). Different binding specificity of distinct repeats can be explained by variations in amino acids 12 and 13, which are therefore called repeat variable diresidues (RVDs). The amino acid pairs most commonly used in RVDs of TALE-DBDs are NI (binding to adenine), NG (thymine), NN (guanine), and HD (cytosine). Given the opportunity of rearrangement of these repeats, it is possible to assemble a TALE-DBD for virtually any DNA target sequence of choice.

Such customized TALEN-DBD in combination with the 25 kDa FokI-R domain, with the latter not having any own DNA-binding specificity (Li et al. 1992), TALENs represent a universal tool for DSB induction. However, FokI-R needs to form dimers to be catalytically active (Bitinaite et al. 1998). Therefore, two TALEN subunits binding to the desired target sequence in inverse orientation to each other are required. This obligatory dimerization decreases the probability for off-target mutations but also doubles the effort to produce an effective TALEN. TALEN-coding DNA subunits can be highly efficiently assembled via Golden Gate cloning [e.g., Cermak et al. (2011)], PCR-based methods [e.g., Yang et al. (2013)], or FLASH assembly (Reyon et al. 2012).

TALENs are more cost effective and much easier to handle than zinc-finger nucleases (Beumer et al. 2013). Therefore, TALENs were successfully applied for site-directed mutagenesis in a number of model and crop plants like *Arabidopsis* (Christian et al. 2013), tobacco (Zhang et al. 2013), *Nicotiana benthamiana* (Mahfouz et al. 2011), *Brachypodium distachyon* (Shan et al. 2013), rice (Li et al. 2012), *Brassica oleracea* (Sun et al. 2013), soybean (Haun et al. 2014), wheat (Wang et al. 2014), maize (Liang et al. 2014), and barley (Wendt et al. 2013; Gurushidze et al. 2014).

7.1.2 *Haploid Technology*

The development of immature barley pollen at the vacuolated microspore or early bicellular stage can be switched from the normal process of pollen maturation to an embryogenic pathway (Sunderland et al. 1979). The embryogenic structures formed can be regenerated into haploid and doubled haploid, instantly homozygous plants. As each doubled haploid individual originates from a different gametophytic cell produced via meiotic recombination, a population of doubled haploid plants represents a collection of genetically unique and fixed individuals. For this reason, haploid technology has become a valuable tool in functional genomics as well as in modern plant breeding. The genetic transformation of such haploid target cells followed by whole genome duplication opened up the unique opportunity to immediately generate plants homozygous with regards to the integrated transgene (Kumlehn et al. 2006). Similarly, the ability to mutagenize haploid cells in a targeted manner via expression of customized endonucleases (in the present case TALENs) permits the generation of instantly homozygous mutants, which makes this approach highly advantageous compared to previously published methods of gene targeting.

7.2 Materials

7.2.1 *Donor Plants*

1. The protocol is optimized for the winter type cultivar “Igri” released by Saatucht Ackermann (Irlbach, Germany) in 1976. The target chosen in the example demonstrated here is the reporter gene encoding green fluorescent protein (GFP), since this provides a simple bioassay for knockout events. Accordingly, the transgenic “Igri” lines PV 89 and BPI 09 were used, which each carry a single copy of *gfp* in homozygous state.
2. The germination substrate is a 3:1:2 mixture of garden mulch/sand/white and black peat (Substrate 2, Klasmann, Germany).
3. The soil substrate used after vernalization is a 2:2:1 mixture of compost/Klasmann Substrate 2 and sand. Osmocote is used as a fertilizer containing 19 % N, 6 % P, and 12 % K.
4. *Petuniensubstrat* (Klasmann, Germany) is used for the acclimation of in vitro regenerants to grow on soil substrate. After vernalization, the substrate is refreshed as described above.

7.2.2 Stock Solutions and Culture Media

7.2.2.1 Stock Solutions

1. Major mineral salts:

K macro (20×): 1.6 g/L NH_4NO_3 , 40.4 g/L KNO_3 , 6.8 g/L KH_2PO_4 , 8.8 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.9 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

K4N macro (20×): 6.4 g/L NH_4NO_3 , 72.8 g/L KNO_3 , 6.8 g/L KH_2PO_4 , 8.8 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.9 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

2. Trace mineral salts:

K micro (1000×): 8.4 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 3.1 g/L H_3BO_3 , 7.2 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 120 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 25 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 24 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 170 mg/L KI.

3. Individual mineral salts:

Ferric sodium ethylenediaminetetraacetate (NaFeEDTA; 75 mM, 27.5 g/L): filter-sterilized and stored at 4 °C.

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1 M, 147 g/L): filter-sterilized and stored at 4 °C.

KH_2PO_4 (1 M, 136 g/L): filter-sterilized and stored at room temperature.

K_2HPO_4 (1 M, 174 g/L): filter-sterilized and stored at room temperature.

Phosphate buffer (1 M, pH 5.0): 97.5 % 1 M KH_2PO_4 , 2.5 % 1 M K_2HPO_4 , filter-sterilized and stored at 4 °C.

Phosphate buffer (1 M, pH 5.9): 90 % 1 M KH_2PO_4 , 10 % 1 M K_2HPO_4 , filter-sterilized and stored at 4 °C.

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (10 mM, 2.5 g/L): filter-sterilized and stored at 4 °C.

4. Carbohydrates:

Mannitol (0.4 M, 72.9 g/L): autoclaved and stored at 4 °C.

Maltose·H₂O (1 M, Sigma Grade I, 360 g/L): filter-sterilized and stored at room temperature.

Maltose·H₂O (1 M, standard quality, 360 g/L): filter-sterilized and stored at room temperature.

Maltose·H₂O (0.55 M, standard quality, 198 g/L): filter-sterilized and stored at 4 °C.

5. Complex organics:

KM organics (100×, Sigma K-3129): 2 mg/L *p*-aminobenzoic acid, 200 mg/L L-ascorbic acid, 1 mg/L D-biotin, 100 mg/L D-calcium pantothenate, 2 mg/L cyanocobalamin, 40 mg/L folic acid, 10 g/L myo-inositol, 100 mg/L nicotinamide, 100 mg/L pyridoxine·HCl, 1 mg/L retinol, 20 mg/L riboflavin, 100 mg/L thiamine·HCl, stored at -20 °C.

Gamborg B5 organics (1000×): 100 mg/L myo-inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine·HCl, 10 mg/L thiamine·HCl, filter-sterilized and stored at -20 °C.

6. Growth regulators:

2,4-Dichlorophenoxyacetic acid (2,4-D, 1 mM): 221 mg/L dissolved in few drops of 50 % ethanol by heating gently, made up to the final volume with hot H₂O, filter-sterilized and stored at 4 °C.

6-Benzylaminopurine (BAP, 1 mM): 224 mg/L dissolved in 30 ml hot H₂O and 3–5 drops 1 M NaOH, made up to the final volume, filter-sterilized and stored at 4 °C.

7. Selective agents:

Bialaphos (41 mg/ml): filter-sterilized and stored at –20 °C.

Spectinomycin (100 mg/ml): filter-sterilized and stored at –20 °C.

Tetracycline (10 mg/ml): dissolved in few drops of 50 % ethanol, filter-sterilized and stored at –20 °C.

Timentin (150 mg/ml): filter-sterilized and stored at –20 °C.

8. Gelling agent:

Phytagel (2×, 6 g/L): suspended in cold water (at best 1.5 g Phytagel per 250 ml unit), autoclaved and stored at room temperature.

9. Other working solutions:

Acetosyringone (1 M, 196 mg/ml): dissolved in dimethyl sulfoxide (DMSO) and stored at –20 °C.

Ethanol (70 %): stored at room temperature.

Glycerol (15 % w/v): autoclaved and stored at room temperature.

L-glutamine (37 mg/ml): dissolved by adding a few drops of 0.1 M KOH and heating in a water bath, filter-sterilized and stored at –20 °C.

Morpholinoethanesulfonic acid (MES, 212.2 g/L, pH 5.0, pH 5.5, and pH 5.9): dissolved in 40 ml water, the pH adjusted with KOH (few pellets), stored at room temperature overnight, and the pH readjusted with either 1 M KOH or 1 M HCl, made up to the required final volume, filter-sterilized and stored at 4 °C (*see Note 1*).

7.2.2.2 Medium for *Agrobacterium Tumefaciens*

CPY medium for *A. tumefaciens* strain LBA4404: 0.1 % (w/v) yeast extract, 0.5 % (w/v) pancreatic peptone, 0.5 % (w/v) sucrose, 2 mg/L MgSO₄·7H₂O (pH 7); add 1.2 % w/v Bacto Agar to produce solid medium.

7.2.2.3 Media for Plant Cell Culture

1. Barley pollen culture (KBP) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 12 ml/L L-glutamine, 4 ml/L BAP, and 250 ml/L maltose (1 M, Sigma Grade I) stocks, pH adjusted to 5.9 and stored at 4 °C.

2. Coculture (CK) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, Sigma Grade I), 2 ml/L BAP,

- 0.5 ml/L acetosyringone, 10 ml/L MES (pH 5.9), and 50 ml/L phosphate buffer (pH 5.9) stocks, stored at 4 °C.
3. AgroStop (ASt) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, Sigma Grade D), 10 ml/L MES (pH 5.0), 25 ml/L phosphate buffer (pH 5.0), 7 ml/L CaCl₂, 4 ml/L L-glutamine, 10 ml/L 2,4-D, 2 ml/L BAP, 0.5 ml/L acetosyringone, 750 µL/L bialaphos, and 1.3 ml/L Timentin stocks, stored at 4 °C.
 4. Selection medium (S3): 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, standard quality), 10 ml/L MES (pH 5.5), 12 ml/L L-glutamine, 1 ml/L BAP, 750 µL/L bialaphos, and 1.3 ml/L Timentin stocks, stored at 4 °C.
 5. Solid barley pollen culture (KBP4PT) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, standard quality), 1 ml/L BAP, 12 ml/L L-glutamine, 750 µL/L bialaphos, and 1.3 ml/L Timentin stocks; appropriate amounts for a final volume of 0.5 L KBP4PT made up to an intermediate volume of 250 ml (double concentrated), adjusted to pH 5.9, heated to about 40 °C, and then mixed (1:1) with 250 ml Phytigel stock (melted by heating).
 6. Regeneration (K4NBT) medium: 50 ml/L K4N macro, 1 ml/L K Micro, 0.75 ml/L NaFeEDTA, 1 ml/L B5 organics, 4 ml/L L-glutamine, 100 ml/L maltose (1 M, standard quality), 1 ml/L BAP, 0.49 ml/L CuSO₄, 100 µL/L bialaphos, and 1.3 ml/L Timentin stocks; appropriate amounts for a final volume of 0.5 L K4NBT made up to an intermediate volume of 250 ml (doubled concentrated), adjusted to pH 5.9, heated to about 40 °C, and then mixed (1:1) with 250 ml Phytigel stock (melted by heating).

7.2.3 Materials for the Isolation of Embryogenic Pollen

1. Refrigerated centrifuge equipped with swing-out baskets
2. Waring blender (heat sterilizable)
3. Sterile Petri dishes with lid (3.5 cm in diameter)
4. Sterile screw-cap polypropylene centrifuge tubes (round bottom 12 ml and skirted conical 50 ml tubes)
5. Magenta boxes (autoclaved)
6. Nylon mesh (100 µm grid, autoclaved)
7. Hemocytometer (type Rosenthal)
8. Filter paper disks (7 cm diameter, ash-free, autoclaved)
9. Sterile filter-stopped tips (1 ml) for standard pipettes

7.2.4 *Materials for Agrobacterium-Mediated Transformation*

1. Temperature-controlled incubators equipped with a rotary shaker
2. Spectrophotometer
3. Erlenmeyer flasks (100 ml) with chicane
4. Sterile screw-cap polypropylene cryotubes (1.5 ml)
5. Sterile screw-cap polycarbonate round-bottomed tubes (12 ml)

7.2.5 *Materials for the Analysis of Transgenic Plants*

7.2.5.1 **Ploidy Determination and Colchicine-Induced Whole Genome Duplication**

1. Flow cytometer, e.g., Ploidy Analyser I (Partec, Münster, Germany)
2. Nylon-mesh filters, e.g., CellTrics (30 µm mesh diameter, Partec)
3. Staining buffer, e.g., CyStain UV Ploidy Staining Solution, Partec)
4. Wire brush
5. Sterile screw-cap polypropylene centrifuge conical tubes (50 ml)
6. 0.1 % w/v colchicine, supplied with 3 drops of Tween 20 per 100 ml

7.2.5.2 **Molecular Analyses**

DNA Isolation, PCR, and DNA Gel Blot Analysis

1. Liquid nitrogen
2. Safe-lock tubes (1.5 and 2 ml)
3. Phenol (equilibrated, stabilized)/chloroform/isoamyl alcohol 25:24:1 (AppliChem, Darmstadt, Germany)
4. Isopropyl alcohol (≥99.8 %)
5. Ethanol (70 %)
6. Taq DNA polymerase with respective PCR buffer
7. MgCl₂ (25 mM)
8. Primers for amplification of TALEN constructs, target, and selectable marker genes:
 - (a) *FokI*-R (forward, FokI-F1 ATCGAGATCGCCCGGAACAGCACC; reverse: FokI-R1 ATCATCTCGCCGCCGATCAGGAGC)
 - (b) Discrimination of the left and right TALEN units (forward: ubi-F1 TTCCGCAGACGGGATCGATCTAGG; reverse: TALEN-R2 TGGCGGCTTGGCGCGTGACAG)
 - (c) Target gene *gfp* (forward: GH-GFP-R2 TACGGCAAGCTGACCCTGAA; reverse: GH-GFP-F1 GGTCACGAACCTCCAGCAGGA)

(d) Selectable marker gene *Bar* (forward: GH-Bar-F1
GGTCTGCACCATCGTCAACC; reverse: GH-Bar-R1
TACCGGCAGGCTGAAGTCCA)

9. QIAquick PCR purification kit (Qiagen, Hilden, Germany)
10. Cloning pGEM-T vector kit (Promega, Madison, WI)
11. Agarose gel (0.8 %), freshly prepared
12. Gel electrophoresis system (Bio-Rad, Munich, Germany)
13. Positively charged nylon membrane (Hybond N+; Roche, Mannheim, Germany)
14. DIG probe synthesis kit (Roche, Mannheim, Germany)
15. DIG system for filter hybridization (Roche, Mannheim, Germany)

RNA Isolation and Reverse Transcriptase Reaction

1. TRIzol reagent (Invitrogen, Life Technologies, Darmstadt, Germany)
2. DNase I treatment kit (DNA-free kit, Ambion, Life Technologies, Darmstadt, Germany)
3. iScript Select cDNA synthesis kit (Bio-Rad, Munich, Germany)
4. Oligo (dT)₂₀ primers (Bio-Rad, Munich, Germany)

7.3 Methods

7.3.1 Vector Construction and Bacterial Strains

1. Design the gene-specific TALEN sequence pair using software tool, *TAL Effector Nucleotide Targeter 2.0* (<https://tale-nt.cac.cornell.edu/>).
2. Evaluate the potential off-target cleavage sites in the barley genome (The International Barley Genome Sequencing Consortium, 2012) using the kmasker tool (<http://webblast.ipk-gatersleben.de/kmasker/>) with standard parameters.
3. Customized TALEN units can be purchased from Invitrogen (Paris, France) and delivered in standard vectors.
4. Clone the left and right TALEN units under the control of maize *ubiquitin 1* promoter (Christensen and Quail 1996) and the *A. tumefaciens nopaline synthase* terminator using *SpeI/HindIII* cloning sites into pUbi-ABM destination vector (DNA Cloning Service, Hamburg, Germany).
5. Synthetic sequences encoding the Simian Virus S40 (SV40) Nuclear Localization Signal (NLS) and NLS-HA (SV40 NLS with an added hemagglutinin tag) are digested with *AscI/Eco47III* and ligated such that the NLS becomes attached to the N-terminus of pUbi-TALEN-Left and NLS-HA to the N-terminus of pUbi-TALEN-Right.

6. Each TALEN expression cassette is then introduced between the *Sfi*I cloning sites of the binary vector p7d35S, which harbors *Bar*, a gene for bialaphos resistance, driven by the cauliflower mosaic virus doubled enhanced 35S (CaMV d35S) promoter.
7. Binary vectors carrying left and right TALEN expression units are then separately introduced via electroporation into *A. tumefaciens* strain LBA4404/pSB1, which carries the disarmed Ti plasmid pAL4404 and the hypervirulence-conferring vector pSB1. Finally, two strains of *A. tumefaciens* LBA4404/pSB1 are obtained, one carrying binary vector with left and the other binary vector with right TALEN expression units.

7.3.2 Growth of Donor Plants

The quality of the isolated pollen cultures is strongly dependent on the conditions of the donor plants. Therefore, it is of high importance to keep the optimized conditions as described:

1. Germinate cv. “Igri” grains in a tray filled with germination substrate in a chamber providing a 12 h photoperiod ($136 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) with temperature regime of 14/12 °C for light/dark phases for 2 weeks.
2. Vernalize the seedlings for 8 weeks at 4 °C under an 8 h photoperiod.
3. After transfer to 18 cm diameter pots, fertilize the plants by providing 15 g Osmocote, and then return to the conditions described in step #1 under Sect. 7.3.2.
4. At the tiller elongation stage, transfer the pots to a glasshouse maintained at 18/16 °C with a minimum of 16 h photoperiod ($170 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density).

7.3.3 Isolation of Immature Pollen

7.3.3.1 Spike Pretreatment

Harvest spikes within the boots when the awns have just emerged from the sheath of the flag leaf, surface-sterilize boots by spraying with 70 % ethanol, remove the flag leaf sheath under aseptic conditions, and place the dissected spikes on a 10 cm diameter Petri dish (five spikes per dish) containing two moistened, 7 cm filter paper disks. After sealing, the plates are held at 4 °C for 4–5 weeks in the dark.

7.3.3.2 Isolation, Purification, and Pre-cultivation of Immature Pollen

All materials coming into contact with the explant need to be sterile and precooled to 4 °C, and all solutions should be kept on ice during the pollen isolation and

purification procedures. Liquid transfer is most effectively carried out using a battery-operated pipette with filter-stopped 5 or 10 ml pipette tips.

1. Ten to fifteen pretreated spikes are chopped into ca. 1 cm fragments and macerated in a blender in the presence of 20 ml 0.4 M mannitol. Usually, two bursts of 15 s each are performed in a Waring blender set on “low” speed.
2. The macerate is filtered through a 100 μ m mesh, which is placed on top of a Magenta box. The blender is flushed with 10 ml of 0.4 M mannitol, which is also passed through the mesh.
3. The debris remaining on the mesh is squeezed gently with tweezers, then returned to the blender for re-maceration (twice at 10 s) in another 10 ml of 0.4 M mannitol, and the macerate passed through the mesh, which is followed by flushing the blender again with 10 ml of 0.4 M mannitol. The latter is also passed through the mesh.
4. The material filtered through the mesh and collected in the Magenta box is transferred to the 50 ml centrifuge tube, and the Magenta box flushed with 5 ml 0.4 M mannitol, which is also added to the same tube. The suspension is centrifuged ($100 \times g$, 10 min, 4 °C).
5. The pellet is resuspended in 3 ml 0.55 M maltose in a round-bottomed 12 ml tube. The centrifuge tube is flushed with 2 ml 0.4 M mannitol, which is poured carefully over the top of the 0.55 M maltose suspension, thereby forming two distinct liquid layers.
6. The tube containing these two layers is subjected to density gradient centrifugation in swing-out baskets ($100 \times g$, 10 min, 4 °C) with the centrifuge set to give slow acceleration and deceleration so that the two layers do not become mixed. After the centrifugation step, the immature pollen is accumulated in the interphase. The interphase is withdrawn by pipetting, transferred to a fresh 50 ml tube to which 10 ml 0.4 M mannitol is added, and resuspended with gentle shaking. 100 μ L aliquot of the material is placed into a hemocytometer cell to estimate the population density. Meanwhile, the remaining microspores are pelleted by centrifugation ($100 \times g$, 10 min, 4 °C). Before the supernatant is withdrawn, the tube is left at stand for ca. 5 min to allow still-floating pollen to settle down.
7. Aliquots of 1 ml pollen suspension are transferred into 3.5 cm Petri dishes. The dishes are sealed with Parafilm or Nescofilm and held in the dark at 25 °C for 7–8 days prior to cocultivation with *A. tumefaciens*.

7.3.4 *Agrobacterium-Mediated Gene Transfer to Embryogenic Pollen*

7.3.4.1 Preparation of *A. tumefaciens* Stocks

1. As TALENs function as pairs, the left and right TALEN units are subcloned in separate binary vectors, from which two respective clones of *A. tumefaciens*

- strain LBA4404/pSB1 are produced. After electroporation with the selected binary vector, *Agrobacterium* is streaked out onto solidified CPY medium supplemented with 0.5 ml/L of tetracycline stock and 2.5 ml/L of spectinomycin stock and incubated at 28 °C for 48 h (see **Note 2**). Single colonies are sampled using a sterile toothpick and grown overnight with shaking at 180 rpm (28 °C) in a tube containing 3 ml liquid CPY supplemented with the respective antibiotics.
2. Plasmid DNA is isolated and the integrity of the binary vector is confirmed by restriction digest of plasmid DNA.
 3. A fresh culture is initiated by adding 20 µL overnight culture to 5 ml fresh CPY medium containing antibiotics and incubating for a further 24 h as described above until an OD₅₅₀ of approximately 2 has been reached.
 4. Positive clones are grown in 10 ml liquid CPY containing antibiotics for another 24 h. The resulting culture is split into two 12 ml round-bottomed tubes and centrifuged (3.220 × g, 12 min, at room temperature).
 5. The pellets are vortexed each in 2.5 ml fresh CPY without antibiotics, and the suspensions are unified in one of the tubes. The cell density is adjusted by the addition of an appropriate volume of medium.
 6. The cells are then mixed in an equal volume of 15 % w/v autoclaved glycerol, and 0.5 ml aliquots are transferred into 1.5 ml cryotubes, left for 1 h at room temperature, and then stored at –80 °C.

7.3.4.2 Cocultivation of Embryogenic Pollen Cultures and *A. tumefaciens*

1. A 0.5 ml glycerol stock of a strain LBA4404/pSB1 carrying an appropriate binary vector is added to 10 ml CPY medium containing 2.5 µL spectinomycin stock and incubated at 28 °C for 24 h with shaking (180 rpm).
2. The liquid cultures of each bacterial strain are split into two 12 ml round-bottomed tubes, which are centrifuged (3220 × g, 12 min, room temperature). The pellets are vortexed each in 2.5 ml CK medium and then transferred to a 100 ml Erlenmeyer flask, which is held at 28 °C for 1–3 h with shaking (100 rpm).
3. The KBP medium used to pre-cultivate the pollen is withdrawn through a 1 ml pipette tip. This procedure is done with particular care to avoid taking up any embryogenic pollen. Then, 1 ml of CK medium is added. It is important to minimize the time between the removal of the KBP medium and the addition of the CK medium, because microspores are rapidly damaged if they are dried.
4. One out of every ten dishes containing embryogenic pollen is left untouched to later provide a supply of feeder cells to support embryogenic development of the pollen cocultured with *A. tumefaciens*.
5. The *A. tumefaciens* cultures are diluted tenfold in water and the measured OD₅₅₀ used to determine the concentration of cfu present.
6. Each 1 ml of embryogenic pollen culture is challenged with 2.5×10^7 cfu. Immediately prior to the inoculation, the two *Agrobacterium* clones (one

carrying the left and the other the right TALEN unit) are mixed in a 1:1 ratio to obtain a final cell density of 2.5×10^7 per ml.

7.3.5 *Regeneration of Transgenics*

1. After 48 h of cocultivation, the CK medium is withdrawn as described above; the pollen is rinsed in 0.4 ml ASt medium, taking care to remove the bacterial mucilage from the wall and bottom of the dish with a pipette tip (*see Note 3*). The medium is then replaced with a fresh aliquot of 1.1 ml ASt, to which 100 μ L of non-cocultivated embryogenic pollen is added as a feeder. The sealed dishes are held in the dark at 25 °C for 1 week with gentle shaking (75 rpm).
2. The ASt medium is replaced by 1.1 ml S3 medium (taking up any embryogenic pollen should be avoided); no washing step is required. The sealed dishes are held in the dark at 25 °C for 1 week with shaking (65 rpm), after which the used S3 medium is withdrawn and refreshed by a new aliquot of the same medium and the dishes incubated for another week.
3. Two weeks after cocultivation, the formed microcalli are transferred to an ash-free filter disk placed over solid KBP4PT medium in a 10 cm Petri dish. After sealing, these dishes are held in the dark at 25 °C for 2 weeks.
4. Calli that reached more than 1 mm in diameter can be transferred into a 10 cm Petri plate containing K4NBT medium, while those which have not yet reached this size can be kept for another week on KBP4PT before being transferred to regeneration medium. The sealed K4NBT plates are held in the dark at 25 °C for 1 week and then transferred into the light.
5. After 3 weeks, the calli and any emerging regenerants are transferred to tissue culture boxes containing K4NBT and subcultured twice at 3 week intervals.
6. Regenerated plants are transferred to 6 cm diameter pots filled with soil substrate (Klasmann Petuniensubstrat) and placed in a tray covered by a transparent plastic hood to maintain a high-humidity environment. The tray is held in a chamber providing a 12 h photoperiod ($136 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) and a temperature regime of 14/12 °C for light/dark phases, respectively.
7. After 1 week, the hood is removed and the tray left uncovered for a further week. The plantlets are then vernalized for 8 weeks at 4 °C under an 8 h photoperiod.
8. After vernalization, the plants are grown under the conditions described for the donor plants.

7.3.6 Analysis of Putative Transgenic Plants

7.3.6.1 Ploidy Determination and Colchicine-Induced Whole Genome Duplication

1. During the vernalization period, flow cytometry of leaf tissue is used to determine the ploidy level.
2. For the isolation of nuclei, a leaf sample is supplied with ice-cold staining buffer (CyStain UV Ploidy Staining Solution, Partec, Münster, Germany), and the leaf tissue is disintegrated gently with a wire brush.
3. The resulting suspension is then filtered through a nylon-mesh filter (CellTrics, 30 µm mesh diameter, Partec).
4. The filtered cell suspensions of the regenerants and of controls from seed-derived wild type plantlets are analyzed by a Ploidy Analyser PA I (Partec) following the manufacturer's instructions.
5. Haploids identified are exposed to a colchicine treatment after the vernalization period as follows: the soil is washed from the roots, and the tillers and roots are trimmed to a length of about 5 cm and 3 cm, respectively. Each plant is placed in a 50 ml polypropylene conical tube containing 0.1 % w/v colchicine solution. The level of the liquid is adjusted to reach the base of the shoots. The tubes are capped and then held in the light for 6 h at 21 °C.
6. The plants are removed from the colchicine, and the roots carefully rinsed in water before being transferred into a 9 cm diameter pot filled with Petuniensubstrat. The pots are placed in a tray with a plastic hood as described above.
7. More than 80 % of plantlets survive the colchicine treatment, and out of the survivors over 90 % produce grain, which indicates that diploidization of germ line cells has taken place (*see Note 4*).

7.3.6.2 Molecular Analyses

DNA Isolation, PCR, and DNA Gel Blot Analysis

1. A 200–400 mg sample of fresh leaf tissue is snap-frozen in liquid nitrogen.
2. DNA is isolated using the method of Palotta et al. (2000) (*see Note 5*).
3. PCR assays of chosen genes are performed using 50–100 ng genomic DNA as template. In this particular example, four different PCR reactions for the following templates are performed: *FokI*-R nuclease domain, left and right TALEN units giving amplicons of different sizes allowing for differentiation, selectable marker gene (*Bar*), and target *gfp* gene. All PCRs are performed in reaction volume of 20 µl with the exception of *gfp* PCR assay, where reaction volume was set to 40 µl.
4. The *gfp* amplicons are subjected to purification and sequencing. Purification is performed using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany)

according to the manufacturer's instructions. Sequencing chromatograms are analyzed and aligned to the wild type sequence to see if there are differences (indels/mutations) in the targeted region.

5. The PCR products of the transgenic individuals showing chromatograms with multiple peaks after the TALEN target site, which might indicate that multiple mutated *gfp* alleles are present among the amplicons, are cloned into pGEM-T. After blue-white selection, plasmid DNA is isolated from the positive clones and sequenced.
6. In order to determine the DNA copy number of the integrated transgene, DNA gel blot is used. For that purpose, 25 µg aliquot of genomic DNA is digested overnight with suitable restriction enzymes (see **Note 6**). In case of the *gfp*-specific TALEN constructs, either *HindIII* or *SspI* is used. Digested DNA is electrophoresed through a 0.8 % agarose gel and transferred onto a positively charged nylon membrane.
7. The nylon membrane is then hybridized with a DIG-labeled probe (*gfp*, *FokI-R*, or *Bar*) generated from a PCR using a DIG-probe synthesis kit and the relevant primer pairs. Signal is then visualized using a DIG System for Filter Hybridization according to the manufacturer's instructions.

RNA Isolation and Reverse Transcriptase Reaction

In order to check if the integrated DNA copies are also transcribed, RNA is isolated from positive transgenic plants and reverse transcription is performed using *FokI-R* primers (see **Note 7**).

1. Leaf material is snap-frozen, ground by means of a Retsch mill, and 100 g powdered leaf material is used for total RNA isolation using TRIzol following the manufacturer's protocol.
2. The isolated total RNA is treated with DNase I, and then the cDNA first strand is synthesized using an iScript Select cDNA synthesis kit in the presence of oligo (dT)₂₀ primers. The resulting cDNA is used as template for subsequent PCRs based on the *FokI-R* domain specific primer pair (see **Note 8**).

7.4 Notes

1. A separate stock for each pH value (pH 5.0, pH 5.5, and pH 5.9) is required.
2. The disarmed Ti-plasmid pAL4404 present in LBA4404 and also the binary vectors used harbor the same spectinomycin resistance (*aadA*) gene. Consequently, a higher than standard concentration of spectinomycin is required (250 mg/L) to ensure the maintenance of the binary vectors. Tetracycline is used here in addition to select for *Agrobacterium* cells that carry the pSB1 plasmid which renders this LBA4404 derivative hypervirulent.

3. The bacterial mucilage after removal from the wall and bottom of the dish requires to be left inside the dish, as it contains much of the embryogenic pollen.
4. As haploid plants are sterile, only the plants that underwent genome doubling set grains.
5. For molecular analyses (DNA and RNA isolation, PCR) of the transgenics, it is recommended to use filter tips because false positives are often detected due to cross contamination of samples, particularly because of the frequent use of standard marker and/or selection genes.
6. The restriction enzyme(s) used to digest the genomic DNA for gel blot analyses should cut the DNA within the inserted T-DNA but should not cleave within the gene that is used for the hybridization. In addition, the enzyme should frequently cut the genomic DNA of the investigated species, as fragments larger than 10 kb can be difficult to detect.
7. While working with RNA, it is extremely important to keep all equipment and materials RNase-free. Use freshly autoclaved equipment and DEPC-treated solutions.
8. DNase I treatment, cDNA first-strand synthesis, and follow-up PCR are performed according to manufacturer's instructions provided with the kit.

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