

Chapter 8

Doubled Haploidy as a Tool for Chimaera Dissolution of TALEN-Induced Mutations in Barley

Maia Gurushidze, Hannes Trautwein, Petra Hoffmeister, Ingrid Otto,
Andrea Müller, and Jochen Kumlehn

Abstract Site-specific genome engineering is a breakthrough technology that facilitates the functional validation of genes and offers versatile novel opportunities of crop improvement. In the approach described in this chapter, the two units of transcription activator-like effector nucleases (TALENs) required for site-directed cleavage activity are separately used to produce homozygous transgenic lines, each carrying only one unit of the TALEN pair. Crossings of these plants result in pairwise combination of both TALEN units, which causes their activation during early zygotic embryogenesis in the hybrid caryopses and facilitates site-directed mutagenesis with unprecedented efficiency in a Triticeae species. As revealed by sequencing of target-specific PCR amplicons, the individual primary mutants are not only heterozygous for the target gene but also chimaeric typically harbouring multiple mutant alleles. We demonstrate the highly efficient production of homozygous mutant lines from the chimaeric primary mutants using haploid microspores as embryogenic founder cells for the generation of doubled haploid plants.

Keywords Customizable endonucleases • Genome engineering • Haploid technology • Pollen embryogenesis • Site-directed mutagenesis

M. Gurushidze • H. Trautwein • P. Hoffmeister • I. Otto • A. Müller • J. Kumlehn (✉)
Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research
(IPK), Corrensstrasse 3, 06466 Seeland/OT, Gatersleben, Germany
e-mail: kumlehn@ipk-gatersleben.de

8.1 Introduction

8.1.1 *Generation of Primary Mutants by Cross-Combination of Parental Lines Carrying Complementary Single TALEN Units*

Transcription activator-like effector nucleases (TALENs) represent a universal tool for site-directed mutagenesis as they are capable of inducing double-strand breaks (DSBs) at user-defined positions in the genome. The processing of a resulting DSB by the host cell's DNA repair machinery can lead to nucleotide deletions or insertions (indels) at the breakage site. TALENs are fusion proteins between a customizable, target-specific DNA binding domain (Moscou and Bogdanove 2009) and the nucleolytic domain of FokI endonuclease, with the former directing the nuclease to the genomic site of choice, while the latter cleaves the double-strand DNA (Christian et al. 2010). However, FokI exerts its activity only in a dimeric format, which is achieved by introducing a pair of TALEN units binding in appropriate distance and orientation to the two strands of the DNA (Bitinaite et al. 1998). Conventionally, the expression cassettes of left and right TALEN units are cloned into a single T-DNA used for plant transformation. As an alternative, two separate T-DNAs can be used in co-transformation approaches.

In barley, immature embryos or embryogenic pollen cultures have been used as recipient material for *Agrobacterium*-mediated transfer of TALEN-coding expression units, and both methods have resulted in moderate efficiency of site-specific mutagenesis (Wendt et al. 2013; Gurushidze et al. 2014). However, somatic mutations occurring in the primary transformants are not necessarily inherited to the next generation (Wendt et al. 2014). The advantage of the approach described in this chapter is that it induces mutations in cells whose genetic make-up is particularly likely to be transmitted to the generative tissues including the female and male gametophytes (i.e. embryo sac and pollen, respectively) of a given plant. To this end, the two TALEN units required for site-directed cleavage activity at the model *gfp* gene were separately used to produce homozygous transgenics, each carrying and expressing only one of these units. Pairs of resultant transgenics that express complementary TALEN units at homozygous state were used for reciprocal crosses. An additional advantage of the approach is that it allows validating the expression of the individual TALEN units in the plants before crossing. The combination of both complementary TALEN units within a hybrid plant results in TALEN activation during early zygotic embryogenesis, which is associated with extremely high efficiency of targeted mutagenesis in cells that genetically contribute to the germ line towards the next generation. As shown by sequencing of the cloned target-specific PCR amplicons, multiple mutant *gfp* alleles were present in each of the analysed seedlings, indicating unprecedented efficiency in site-directed mutagenesis in a Triticeae species. Thus, in the presented approach, there are only two successful transformation events necessary to generate the two lines carrying complementary single TALEN units. Once they are established in homozygous

state, no further transformation is necessary to generate mutants. A huge number of independent mutants can be generated by crossing these pre-validated single TALEN unit lines, which is a particularly valuable option, especially in plants that are not readily transformable.

8.1.2 *Chimaerism Upon TALEN-Induced Targeted Mutagenesis*

As mentioned above, every analysed individual of the hybrid offspring contained multiple mutant *gfp* sequences, i.e. produced TALEN mutants are chimaeric regarding the induced mutations. Virtually, the TALEN pair expressed in each cell could initiate independent mutation events resulting in extremely high diversity of the induced sequence modifications within one individual. Indeed, by sequencing up to 65 individual clones of the PCR amplicons derived from the target gene (*gfp*), as many as 61 different mutations were detected per hybrid seedling. Although chimaerism is a quite often occurring phenomenon in targeted mutagenesis, the proportion of heritable mutations strongly depends on the method. While none of the TALEN-induced mutations proved heritable among the seedlings of primary barley-mutant plants produced *via* immature embryo transformation (Wendt et al. 2014), multiple mutations were inherited within doubled haploid siblings using the method presented in this chapter (*see Note 1*).

8.1.3 *Haploid Technology*

Haploid technology is one of the most powerful biotechnological tools in plant breeding and functional genomic studies. The advantage of haploid plants is that homozygosity can be readily achieved at all loci *via* whole-genome duplication in just one generational step. Nowadays, haploid technology is also exploited to produce instantly homozygous mutants and transgenics (Kumlehn et al. 2006; Gurushidze et al. 2014).

Pollen embryogenesis is a phenomenon that allows generating haploid and doubled haploid plants from immature pollen. In this approach, each doubled haploid individual originates from a unique gametophytic cell, and accordingly, the resulting population of doubled haploid plants represents a collection of meiotically independently recombined but genetically fixed individuals. In this chapter, we describe the utilization of pollen embryogenesis for chimaera dissolution of TALEN-induced mutations in barley.

8.2 Materials

8.2.1 Growth of Parental Lines and Primary Mutant Plants

1. The parental lines used in crossing experiments carrying stably incorporated either left or right TALEN units represent winter-type cultivar 'Igri'. Parental lines were produced as described in the Chap. 7.
2. Grains of the parental lines were germinated on Substrate 2 (Klasmann, Germany), which is a 3:1:2 mixture of garden mulch, sand and white and black peats, respectively.
3. The soil substrate used after vernalization is a mixture of compost, Klasmann Substrate 2 and sand mixed at a ratio of 2:2:1. Osmocote (19 % N, 6 % P and 12 % K) was used as a fertilizer.
4. Petuniensubstrat (Klasmann, Germany) was used for the acclimation of in vitro regenerants to grow on soil substrate.

8.2.2 Stock Solutions and Culture Media

8.2.2.1 Solutions for Isolation, Purification and Induction of Embryogenic Development of Immature Pollen

Carbohydrates

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

Maltose·H₂O (1 M, Sigma grade I, 360.3 g/L): filter sterilized and stored at room temperature.

Maltose·H₂O (1 M, standard quality, 360.3 g/L): filter sterilized and stored at room temperature.

Maltose·H₂O (0.55 M, standard quality, 198.2 g/L): filter sterilized and stored at 4 °C.

Mineral salts

NaFeEDTA (75 mM, 27.5 g/L): filter sterilized and stored at 4 °C

CaCl₂ (1 M, 147 g/L CaCl₂·2H₂O): filter sterilized and stored at 4 °C

CuSO₄·5H₂O (10 mM, 2.5 g/L): filter sterilized and stored at 4 °C

NH₄Cl (1 M, 53.5 g/L): filter sterilized and stored at 4 °C

K macro minerals (20×): 1.6 g/L NH₄NO₃, 40.4 g/L KNO₃, 6.8 g/L KH₂PO₄, 8.8 g/L CaCl₂·2H₂O and 4.9 g/L MgSO₄·7H₂O; filter sterilized and stored at 4 °C

K4 N macro minerals (20×): 6.4 g/L NH₄NO₃, 72.8 g/L KNO₃, 6.8 g/L KH₂PO₄, 8.8 g/L CaCl₂·2H₂O and 4.9 g/L MgSO₄·7H₂O; filter sterilized and stored at 4 °C

K micro minerals (1000×): 8.4 g/L MnSO₄·H₂O, 3.1 g/L H₃BO₃, 7.2 g/L ZnSO₄·7H₂O, 120 mg/L Na₂MoO₄·2H₂O, 25 mg/L CuSO₄·5H₂O, 24 mg/L CoCl₂·6H₂O and 170 mg/L KI; filter sterilized and stored at 4 °C

Organic supplements

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 and 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 and 10 mg/L thiamine-HCl; filter sterilized and stored at -20°C .

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 .

6-Benzylaminopurine (BAP, 1 mM): 225 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 .

Morpholinoethanesulfonic acid monohydrate (MES, 1 M, 213.2 g/L, pH = 5.0, pH = 5.5 and pH = 5.9): dissolved in 40 ml H₂O, the pH adjusted with KOH (few pellets), stored at room temperature overnight, and the pH readjusted with either KOH or HCl, made up to the final volume, filter-sterilized and stored at 4°C .

Gelling agent

Phytigel (2×, 6 g/L): suspended in cold water of double-distilled quality (at best 1.5 g Phytigel per 250 ml unit), autoclaved and stored at room temperature.

8.2.2.2 Nutrient Media for Embryogenic Pollen Culture and Plant Regeneration

1. Starvation medium barley (SMB): 1 ml/L CaCl₂, 1 ml/L NH₄Cl, 2 ml/L MES pH 5.5 and 400 ml/L mannitol stocks; filter-sterilized and stored at 4°C .
2. 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 .
3. Solid barley pollen culture (KBP4P) medium: for a final volume of 0.5 L KBP4P, appropriate amounts of 50 ml/L K macro minerals, 1 ml/L K micro minerals, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, standard quality), 1 ml/L BAP and 12 ml/L L-glutamine stocks were mixed 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).
4. Regeneration (K4NB) medium: for a final volume of 0.5 L K4NB, appropriate amounts of 50 ml/L K4N macro minerals, 1 ml/L K micro minerals, 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 and 0.49 ml/L CuSO₄ stocks were mixed 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).

8.2.3 Materials for the Isolation of Embryogenic Pollen Culture and Plant Regeneration

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 Petri dishes with lid (10 cm in diameter).
5. Divided Petri dishes with two sections and lid (10 cm in diameter).
6. Sterile screw cap polypropylene centrifuge tubes (round bottom 12 ml and skirted conical 50 ml tubes).
7. Magenta boxes (autoclaved).
8. Nylon mesh (100 µm grid, autoclaved).
9. Haemocytometer (type Rosenthal).
10. Filter paper discs (7 cm diameter, ash-free, autoclaved).
11. Sterile filter-stopped tips (1 ml) for standard pipettes.

8.2.4 Materials for the Molecular Analyses of Pollen-Derived Plants

1. Liquid nitrogen.
1. Safe-lock tubes (1.5 and 2 ml).
2. Phenol (equilibrated, stabilized) to chloroform to isoamylalcohol (25:24:1) (AppliChem, Darmstadt, Germany).
3. Isopropyl alcohol (≥99.8 %).
4. Ethanol (70 %).
5. Taq DNA polymerase with respective PCR buffer.
6. MgCl₂ (25 mM).
7. Primers for amplification of target gene *gfp* (forward: GH-GFP-R2 TACGGCAAGCTGACCCTGAA; reverse: GH-GFP-F1 GGTCACGAACTCCAGCAGGA).
8. QIAquick PCR purification kit (QIAGEN, Hilden, Germany).
9. Cloning pGEM-T vector kit (Promega, Madison, WI).
10. Agarose gel (0.8 %); freshly prepared.
11. Gel electrophoresis system (Bio-Rad, Munich, Germany).

8.2.5 *Materials for 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 three drops of Tween per 100 ml.

8.3 Methods

8.3.1 *Growth of Primary Mutant Plants*

1. T1 grains of homozygous transgenic plants of cv. 'Igri' expressing single TALEN units were germinated (*see Note 2*) on germination substrate in a chamber providing 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. Vernalization of the seedlings was performed at 4 °C under an 8 h photoperiod for 8 weeks.
3. The seedlings were then transferred to 18 cm diameter pots, fertilized by providing 15 g Osmocote and grown under controlled conditions as described in **Step 1** of this section.

8.3.2 *Crossing of Pairs of Complementary Single TALEN Plants and Analysis of Hybrid Plants*

Left and right TALEN unit-carrying homozygous barley lines were grown under the conditions described above (Sect. 8.3.1). Crossing partners were chosen in such a way that the resulting hybrids possess both TALEN units. After reciprocal crossing of the selected TALEN carrying lines, the hybrid plants were analysed as follows: the target sequence (*gfp*) was amplified and cloned into pGEM-T vector. The DNA was isolated from various numbers of positive clones and sequenced to gain preliminary information about mutation frequencies.

8.3.3 *Spike Preparation*

Spikes were harvested when the awns had just emerged from the sheath of the flag leaf, boots were surface-sterilized by spraying with 70 % ethanol, and the flag leaf sheath was removed under aseptic conditions. Dissected spikes were placed in one section of a divided Petri dish (10 cm in diameter), whereas the other section was supplied with sterile water to provide saturated humidity in the dish's airspace. The dishes were sealed and placed at 4 °C for 2 weeks in the fridge.

8.3.4 *Isolation, Purification and Inductive Treatment of Immature Pollen*

1. Ten to fifteen pretreated spikes were chopped into ca. 1 cm pieces and macerated in a blender in the presence of 20 ml 0.4 M mannitol. Two bursts of 15 s each were performed in a Waring blender set on 'low' speed (*see Note 3*).
2. The macerate was filtered through a 100 µm mesh into a Magenta box. The blender was rinsed with 10 ml of 0.4 M mannitol, which was also passed through the mesh.
3. The debris remaining on the mesh was squeezed gently with tweezers transferred back to the blender and re-macerated twice for 10 s in another 10 ml of 0.4 M mannitol. The macerate was again filtered through the mesh, and the blender was rinsed again with 10 ml of 0.4 M mannitol. The latter was then also passed through the mesh into a Magenta box.
4. The material collected in the Magenta box was transferred to the 50 ml centrifuge tube, and the Magenta box flushed with 5 ml 0.4 M mannitol, which was also added to the same tube. The suspension was centrifuged ($100 \times g$, 10 min, 4 °C).
5. The supernatant was discarded, and the pellet was re-suspended in 3 ml 0.55 M maltose in a round-bottomed 12 ml tube. The centrifuge tube was flushed with 2 ml 0.4 M mannitol, which was poured carefully over the top of the 0.55 M maltose suspension, so that two distinct liquid layers were formed.
6. The tube containing these two layers was subjected to density gradient centrifugation in swing-out baskets ($100 \times g$, 10 min, 4 °C) (*see Note 4*). After the centrifugation step, the immature pollen accumulated in the interphase. The interphase was carefully withdrawn by pipetting, transferred to a fresh 50 ml tube to which 10 ml 0.4 M mannitol was added and resuspended with gentle shaking. 100 µL aliquot of the material was placed into a haemocytometer cell to estimate the population density. Meanwhile the remaining microspores were pelleted by centrifugation ($100 \times g$, 10 min, 4 °C). After centrifugation, the tube was left in the centrifuge for ca. 5 min before the supernatant was withdrawn to allow settling down of the floating pollen.

7. The pollen was resuspended in SMB with a density of 200,000 microspores/ml; aliquots of 1 ml pollen suspension were transferred into 3.5 cm Petri dishes. The dishes were sealed and held in the dark at 25 °C for 48 h to induce embryogenic competence.

8.3.5 *Regeneration of Pollen-Derived Plants*

1. After the inductive treatment, the SMB was withdrawn through a 1 ml pipette tip (see Note 5). The medium was then replaced with 1 ml KBP medium, and the sealed plates were further held at 25 °C in the dark for another 2 weeks.
2. Then, the formed embryogenic calli were transferred to an ash-free filter disc placed over solid KBP4P medium in a 10 cm Petri dish. After sealing, these dishes were held in the dark at 25 °C for 2 weeks.
3. Calli that reached more than 1 mm in diameter can be transferred into a 10 cm Petri plate containing K4NB medium, while those which have not yet reached this size can be kept for another week on KBP4P before being transferred to regeneration medium. The sealed K4NB plates were held in the dark at 25 °C for 1 week and then transferred into the light.
4. After 3 weeks, the calli and any emerging regenerants were transferred to tissue culture boxes containing K4NB and subcultured twice at 3-week intervals.
5. Regenerated plants with roots were 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 was 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.
6. After 2 weeks, the hood was removed and the tray left uncovered for a further week. The plantlets were then vernalized for 8 weeks at 4 °C under an 8 h photoperiod.
7. After vernalization, the plants were grown under the conditions described for the donor plants.

8.3.6 *Analysis of Pollen-Derived Plants*

8.3.6.1 Ploidy Determination

During the vernalization period, flow cytometry of leaf tissue was used to determine the ploidy level as follows:

1. For the isolation of nuclei, a leaf sample was supplied with ice-cold staining buffer (CyStain UV Ploidy staining solution, Partec, Münster, Germany), and the leaf tissue was disintegrated gently with a wire brush.

2. The resulting suspension was then filtered through a nylon mesh filter (CellTrics, 30 μm mesh diameter, Partec).
3. The filtered cell suspensions of the regenerants and of controls from caryopsis-derived wild-type seedlings were analysed by a Ploidy Analyser PA I (Partec) following the manufacturer's instructions.

8.3.6.2 DNA Isolation, PCR and Sequencing

1. A 200–400 mg sample of fresh leaf tissue was snap-frozen in liquid nitrogen.
2. DNA was isolated using the method of Pallotta et al. (2000) (see **Note 6**).
3. The target *gfp* gene was amplified by PCR reaction using 50–100 ng genomic DNA as template. PCRs were performed in reaction volume of 40 μl .
4. The *gfp* amplicons were subjected to purification and sequencing. Purification was performed using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.
5. The PCR products of the transgenic individuals showing chromatograms with multiple peaks in the TALEN target site, which indicated the presence of multiple *gfp* alleles among the amplicons, were cloned into pGEM-T vector. After blue-white selection, plasmid DNA was isolated from individual positive clones and sequenced.
6. Sequencing chromatograms were analysed and aligned to the wild-type sequence to see if there were differences (indels/mutations) in the targeted region.

8.3.6.3 Mutant Comparison

The target *gfp* gene of haploids and doubled haploids, derived from hybrid plants' pollen cultures, was amplified and sequenced. *gfp* was amplified and individual clones sequenced also from the pollen donor hybrid plants (see **Note 7**). The number and types of the mutations carried by doubled haploid mutants were recorded and compared with the mutations present within the parental (pollen donor) chimaeric hybrid plants. For example, 58 clones were sequenced for the pollen donor plant #39/4, and all the 58 sequences proved to be mutated (Fig. 8.1). From this donor, 10 doubled haploids were then produced. Whereas in three of those, it was not possible to amplify *gfp* (see **Note 8**), amplification and sequencing were successful in the remaining seven DH plants, five of which carried mutant sequences, and all of these five mutations proved to be unique (Fig. 8.2) (see **Note 9**). As expected, doubled haploid mutants possessed their mutated allele in homozygous state.

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GFP-WT      CACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-1   CACCCCTGGTGAACCGCATCGAGCTGAAGG----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-2   CACCCCTGGTGAACCGCATCGAGCTGAAGG----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-3   CACCCCTGGTGAACCGCATCGAGCTGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-4   CACCCCTGGTGAACCGCATCGAGCTGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-5   CACCCCTGGTGAACCGCATCGAGCTGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-6   CACCCCTGGTGAACCGCATCGAGCTGAAG-----CITCAAGGAGGACGGCAACATCCTGGGGCACAGCTGGAGT
39/4_c1-7   CACCCCTGGTGAACCGCATCGAGCTGAAGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-8   CACCCCTGGTGAACCGCATCGAGCTGAAGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-9   CACCCCTGGTGAACCGCATCGAGCTGAAGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-10  CACCCCTGG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-11  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----CACAAAGCTGGAGT
39/4_c1-12  CACCCCTGGTGAACCGCATCGAGCTGAAG-----T
39/4_c1-13  CACCCCTGGTGAACCGCATCGAGCTGAAG-----T
39/4_c1-14  CACCCCTGGTGAACCGCATCGAGCTGAAG-----T
39/4_c1-15  CACCCCTGGTGAACCGCATCGAGCTGAAG-----T
39/4_c1-16  CACCCCTGGTGAACCGCATCGAGCTGAAG-----TACAACACTACAAGGAGT
39/4_c1-17  CACCCCTGGTGAACCGCATCGAGCTGAAG-----TACAACACTACAAGGAGT
39/4_c1-18  CACCCCTGGTGAACCGCATCGAGCTGAAG-----TACAACACTACAAGGAGT
39/4_c1-19  CACCCCTGGTGAACCGCATCGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-20  CACCCCTGGTGAACCGCATCGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-21  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-22  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-23  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-24  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-25  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-26  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-27  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-28  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-29  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-30  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-31  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-32  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-33  CACCCCTGG-----CGCACCATCTTCTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-34  CACCCCTGG-----CGCACCATCTTCTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-35  CACCCCTGG-----CGCACCATCTTCTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-36  CACCCCTGGTGAACCGCATCGA-----CGTTGTGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-37  CACCCCTGGTGAACCGCATCGAGCTGAAG-----TACAAGCTGGAGT
39/4_c1-38  CACCCCTGGTGAACCGCATCGAGCTGAAGGG---ACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-39  CACCCCTGGTGAACCGCATCGAGCTGAAGGG---ACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-40  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----ACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-41  CACCCCTGGTGAACCGCATCG-----GCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-42  CACCCCTGGTGAACCGCATCG-----AGCTGAAGGGCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-43  CACCCCTGGTGAACCGCATCGAGCTGAAGGGC---GACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-44  CACCCCTGGTGAACCGCATCGAGCTGAAGGGC---GACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-45  CACCCCTGGTGAACCG-----GAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-46  CACCCCTGGTGAACCGCATCGAGCTGAAGGGC---AACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-47  CACCCCTGG-----GGCAAGCTGGAGT
39/4_c1-48  C-----TTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-49  CACCCCTGGTGAACCGCATCGAGCTGAAGGG-----ACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-50  CACCCCTGGTGAACCGCATCGAGCTG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-51  CACCCCTGGTGAACCGCATCGAGCTGAAGGG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-52  CACCCCTGGTGAACCGCATCGAGCTGAAGGG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-53  CACCCCTGGTGAACCGCATCGAGCTGAAGGG-----AATCCTGGGGCACAAAGCTGGAGT
39/4_c1-54  CACCCCTGGTGAACCGCATCGAGCTGAAGGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-55  CCGCCTGCAAGGTCGACCATATGGGAGAGCTCCCAACCGCTTGGATGCATAGCTTGGATTTCTATAGTGTACACTAA
39/4_c1-56  CCGCCTGCAAGGTCGACCATATGGGAGAGCTCCCAACCGCTTGGATGCATAGCTTGGATTTCTATAGTGTACACTAA
39/4_c1-57  CACCCCTGGTGAAGTTTGAGGCGACACCTGGTGAACCGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAA
39/4_c1-58  CACCCCTGGTGAACCGCATCAAGTCCGCCATGCCGAAGCTACGTCAGGAGCGCCACCATCTTCTTCAAGGAGCAGG
    
```

Fig. 8.1 Alignment of the various cloned *gfp* mutant sequences of the hybrid plant #39/4. The underlined sequences represent TALEN-binding sites in wild-type *gfp*. Deletions are indicated with *dashes*. Note that long insertions in addition to deletions are present in the last four sequences

```

GFP-WT      CTGGTGAACCGCATCGAGCTGAAGGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   WT
39/4_DH-1   CTGGTGAACCGCATCGAGCTGAAGGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   WT
39/4_DH-2   CTGGTGAACCGCATCGAGCTGAAGGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   WT
39/4_DH-3   CTGGTGAACCGCATCGAGCTGAAGG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -15
39/4_DH-4   CTGGTGAA-----CTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -24
39/4_DH-5   CTGGTGAACCGCATCGA-----CTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -15
39/4_DH-6   CTGGTGAACCGCATCGAGCTGAAGG-----CTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -109
39/4_DH-7   CTGGTGAACCGCATCGAGCTGAAGGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT   +1

```

Fig. 8.2 *gfp* alleles found in the pollen-derived homozygous doubled haploids produced from the hybrid plant #39/4 are aligned with the wild type *gfp* sequence

8.4 Notes

- Five distinct mutations were inherited among 10 doubled haploid siblings in the example presented in this chapter.
- Cultivar ‘Igrī’ lines expressing either the left or right TALEN units were produced by transformation of immature pollen cultures using the method described in Chap. 7. For more details about the TALEN vectors, see Gurushidze et al. (2014).
- All materials coming into contact with the explant need to be sterile and pre-cooled to 4 °C, and all solutions should be kept on ice during the pollen isolation and purification procedure. Liquid transfer is most effectively carried out using a battery-operated pipette with filter-stopped 5 or 10 ml tips.
- At this step, it is critical that the two layers do not become mixed. For that reason, the centrifugation step is performed with the acceleration and deceleration forces of the centrifuge being reduced to 30 %.
- The exchange of SMB to KBP medium is to be done with particular care to avoid taking up embryogenic pollen. To this end, the pipette should be slightly pressed with its tip to the bottom of the dish, so as to control the uptake of medium, while the cultivated pollen is collecting around the tip without being taken up.
- For molecular analyses (DNA 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.
- Ten to 65 clones were analysed per hybrid plant. Mutant alleles represented 62.5 to 100 % of the individually cloned sequences.
- Large deletions including at least one primer-binding site might be the most plausible reason for failed amplification.
- Among the five different mutations found in pollen-derived 10 doubled haploid siblings, 2 were already detected among the 58 mutations of their hybrid mother plant (39/4), while 3 mutations were ‘new’, i.e. not present among the 58 previously sequenced clones.

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