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Minoru Murata *Editor*

Chromosome and Genomic Engineering in Plants

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Cover illustration: Arabidopsis transgenic plants in plate, expressing Ac transposase

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Preface

Transformation or transfection is an indispensable tool in basic and applied studies in biological sciences. In plants, a number of species can be transformed by an Agrobacterium-mediated system, particle bombardment, and/or protoplast fusion. Compared to other organisms, however, these three techniques are uncontrollable with regard to the insertion of exogenous genes or DNA because the insertion into the genome or chromosome is quite random, and multiple-copy insertion frequently occurs. This random and multiple-copy insertion increases the risk of disrupting essential genes. To avoid such risk, gene targeting via homologous recombination is most desirable, as has been shown in yeast and mice. However, the occurrence of homologous recombination is quite limited in plants, except for in some lower plants (i.e., *Physcomitrella patens* and *Chlamydomonas reinhardtii*).

To overcome such difficulties in controlling exogenous DNA insertion, at least two approaches have recently been developed. The first approach is a "plant chromosome vector" system that allows us to introduce desired genes or DNA into target sites on the chromosome vector. Although these systems are not completely established, plant artificial chromosomes, which could be used as platforms for introducing exogenous genes, have been successfully generated in some plant species. This approach requires various techniques, such as telomere DNA-induced chromosome truncation, sequence-specific recombination (i.e., Cre/LoxP), and transposon (i.e., Ac/DS) systems, in addition to knowledge of chromosome functional elements (centromere, telomere, and origin of replication). The second approach is "genome editing," which makes it possible to introduce mutations into any of the genes or DNA that we wish to change. This technique has been used since the discovery of zinc finger nucleases in 1996. To date, more efficient and mature techniques have been developed such as TALEN and CRISPR/Cas9. These two approaches are not independent from each other and can be applied cooperatively. Hence, this volume assembles protocols for chromosome engineering and genome editing that are needed when using the two aforementioned approaches to manipulate chromosomal and genomic DNA in plants. In addition, other related techniques supporting these two approaches are used to accelerate progress in plant chromosome and genome engineering.

Finally, I would like to extend my heartfelt thanks to all of the authors who contributed their excellent and interesting research results to this volume. I am also grateful to the series editor, John Walker, for encouraging me to edit one part of the series, "Methods in Molecular Biology".

Kurashiki, Japan *Minoru Murata*

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Chapter 1

Production of Engineered Minichromosome Vectors via the Introduction of Telomere Sequences

Nathaniel Graham, Nathan Swyers, Jon Cody, Morgan McCaw, Changzeng Zhao, and James A. Birchler

Abstract

Artificial minichromosomes are non-integrating vectors capable of stably maintaining transgenes outside of the main chromosome set. The production of minichromosomes relies on telomere-mediated chromosomal truncation, which involves introducing transgenes and telomere sequences concurrently to the cell to truncate an endogenous chromosomal target. Two methods can be utilized; either the telomere sequences can be incorporated into a binary vector for transformation with *Agrobacterium tumefaciens*, or the telomere sequences can be co-introduced with transgenes during particle bombardment. In this protocol, the methods required to isolate and introduce telomere sequences are presented. Following the methods presented, standard transformation procedures can be followed to produce minichromosome containing plants.

Key words Minichromosome, Chromosomal truncation, Artificial chromosome, Telomere, In-gel ligation, Plant transformation, *Agrobacterium tumefaciens* transformation, Biolistic bombardment

1 Introduction

Artificial minichromosomes are any non-integrating vectors with large DNA carrying capacities that can be stably maintained in sequential generations [1]. Production of minichromosomes is a result of telomere truncation, where telomere sequences are introduced into a chromosome during transformation resulting in the loss of genetic material distal to the insertion point. Engineered minichromosomes must be generated in this way to utilize an endogenous centromere because isolating centromere sequences and re-introducing them into a cell will not work due to the epigenetic nature of centromeres [1]. Telomere-mediated truncation is accomplished through transformation of a plant with a plasmid containing a telomere repeat after the selectable marker and other desired genetic cargo, or by particle bombardment with a plasmid and separate telomere repeat. It is presumed that the

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non-homologous end joining pathway for double strand break repair attaches the plasmid to a double-stranded break in an endogenous chromosome. The presence of the telomere sequence then recruits telomerase, which adds telomere repeats to the end of the plasmid, creating a functional end of the chromosome. B chromosomes in maize and other plants are good targets for creating minichromosomes because they contain no genes essential to the survival of the plant. If an A chromosome is truncated, the event will generally result in the loss of genes, which are essential to the survival of the plant, making recovery of truncation events less likely. Truncating a B chromosome will have little to no effect on the survival of the plant. The use of a B chromosome has the added benefit of having no linkage between the transgene and endogenous genes in the transformed line. Transgenes on an A chromosome may be linked to alleles from the transformable line, which are undesirable in a high yielding commercial line. A suite of transgenes can be carried on a single minichromosome and introgressed into new lines as a single unit, reducing the complexity of breeding programs to stack multiple different transgenes in a single plant. The complexity and duration of introgressing multiple transgenes into a line may possibly be further reduced by transferring minichromosomes through haploid induction and doubling the ploidy of a resultant haploid containing a minichromosome would create a completely homozygous line with minichromosomes [1].

Minichromosomes exist in association with the normal chromosome set and are subject to modification via site-specific recombination technology. Modifications add or remove gene fragments in a targeted manner, enabling continuous concatenation of sequences while recycling a single selection marker. As minichromosome technology develops, this strategy could allow researchers to stack multiple genes or whole biosynthetic pathways on a single location within the genome. This would circumvent limitations associated with current popular genetic engineering methods, such as disruption of endogenous gene function, transgene silencing, linkage drag, and inefficient recovery of multiple transgenes [2].

This system relies on the development of a minichromosome platform, which is used as a target for subsequent modification events. In plants, platforms are produced through telomere-mediated truncation of pre-existing genetic material via *Agrobacterium tumefaciens* transformation or particle bombardment. Each method requires the utilization of a telomere array; however, the mode of delivery and materials used in these processes are slightly different. Agrobacterium transformation requires an advanced cloning strategy to position a telomere fragment near the right border of a T-DNA vector, while particle bombardment simply requires an isolation of the telomere sequence. Preparation of telomere for both cloning and bombardment can be carried out in two different ways, through gel extraction or telomere repeat concatenation via PCR.

Due to the difficulty in manipulating the repetitive sequences of the telomere, it must be moved into a transformation vector via in-gel ligation. After positive clones have been identified they must be screened and sequenced to ensure that the insert is intact and in the correct orientation. Following sequencing of positive colonies, those in the correct orientation should be screened for insert size. The minimum size required for telomere truncation has not yet been determined, but it is thought that the greatest chance of success will come from the use of the largest telomere sequence possible. Interestingly, telomere sequences often form a secondary structure within agarose gels making it difficult to get an accurate size estimate. Performing a Southern hybridization [3] on these agarose gels will show evidence of the full size of the telomere repeat that is present in a clone.

For particle bombardment, telomere DNA conglomerates can be produced using polymerase chain reaction. Differing lengths of telomere DNA are created by the annealing of specific primers to each other resulting in the creation of telomere repeats of varying sizes. The resulting fragments of telomere can be visualized by gel electrophoresis and particular sizes of telomere can be obtained by DNA gel extraction from an agarose gel. The obtained telomere DNA can then be used in particle bombardment with a construct of interest to create a minichromosome. The protocol described in the following section has been adapted from a protocol that labels telomere to make fluorescent probes [4].

Protocol 1: Ligation of Telomere Sequences within Agrobacterium **transformation vectors**

2 Materials

2.1 Materials for In-Gel Ligation into Agrobacterium Competent Vector

1. *Agrobacterium tumefaciens* competent transformation vector (*see* **Note 1**).
2. Plasmid pWY82 (*see* **Note 2**).
3. Oligonucleotide $(TTTAGGG)_{10}$ can be synthesized in either the 5′ or 3′ direction.
4. Luria broth.
5. Spectinomycin.
6. 2xYT Medium.
7. QIAprep Spin Miniprep Kit (Qiagen).
8. Restriction Enzymes compatible with pWY82 and target plasmid.
9. Agarose.
10. DNA Gel Loading Dye (6×).
11. GeneRuler 1 kb DNA Ladder (Life Technologies).
12. Ethidium Bromide.

13. Low Melting Point Agarose.
14. Antarctic phosphatase.
15. T4 DNA Ligase and Ligase Buffer.
16. ElectroMax Stbl4 Cells (Life Technologies).
17. S.O.C Media (Super Optimal Broth with Catabolic Repressor).
18. Agar.
19. Petri Dishes.

2.2 Equipment for In-Gel Ligation into Agrobacterium Competent Vector

1. 30 °C Incubator.
2. 30 °C Shaker.
3. 250 mL baffled culture flasks.
4. Vacuum Concentrator.
5. Nanodrop Spectrophotometer.
6. Gel electrophoresis system.
7. Ultraviolet Transilluminator.

2.3 Recipes for In-Gel Ligation into Agrobacterium Competent Vector

1. *Luria Broth*: For 500 mL dissolve 12.5 g of LB media in 400 ml water. Bring to 500 mL and autoclave for 20 min.
2. *Luria Broth Plates*: For 500 mL, dissolve 12.5 g of LB media and 6 g of agar is 400 mL water. Bring to 500 mL and autoclave for 20 min. Place in 50 °C water bath until completely cooled then add appropriate antibiotics. Gently mix and pour thin layer into petri dishes. Store at 4 °C for up to 1 month.
3. *2xYT Broth*: For 500 mL of culture dissolve 15.5 g of 2× YT in 400 mL water. Bring final volume to 500 mL and autoclave for 20 min.
4. *TAE*: To prepare 1 L of 50× TAE add 242 g trizma base, 14.6 g EDTA, and 57.1 mL of acetic acid to 500 mL of water and dissolve. Bring total volume to 1 L with water.

2.4 Materials for Preparation of Telomere for Particle Bombardment

1. Telomere Primers (*see* **Note 3**):
   (a) Forward Primer-5′ $(TTTAGGG)_{10}$ 3′.
   (b) Reverse Primer-5′ $(CCCTAAA)_{10}$ 3′.
2. LongAmp® Taq DNA Polymerase (New England BioLabs).
3. DNA Gel Loading Dye (6×).
4. GeneRuler 1 kb DNA Ladder (Life Technologies).
5. Ethidium Bromide.
6. Wizard® SV Gel and PCR Clean-Up System (Promega).
7. Nuclease-Free Water.

2.5 Equipment for Preparation of Telomere for Particle Bombardment

1. Gel electrophoresis system.
2. Ultraviolet Transilluminator.

3 Methods

3.1 Insertion of Telomere Through In-Gel Ligation

1. Streak pWY82 onto LB plates containing 100 mg/mL spectinomycin.
2. Place plates into 30 °C incubator for 48 h (*see* **Note 4**).
3. Begin a starter culture by picking a single colony into 3 mL of 2xYT liquid media containing 100 mg/mL spectinomycin and shaking for 48 h at 30 °C.
4. Add 500 μL of starter culture to 125 mL of 2xYT in a baffled culture flask and shake at 250 rpm at 30 °C until the culture reaches an OD600 ~2 (~48 h).
5. Extract culture 4 mL at a time with the QIAprep Spin Miniprep kit (Qiagen) eluting with 50 μL of 50 °C nuclease-free water.
6. Combine each miniprep into one 1.7 mL tube and reduce the volume in a vacuum concentrator until the concentration is ~1 μg/μL when measured with a Nanodrop spectrophotometer.
7. Individually test each restriction enzyme to be used by digesting 1 μg of plasmid following manufacturer's instructions.
8. Add 6× loading dye to each digest after digestion is complete.
9. Load each digest into a 1% (w/v) TAE agarose gel flanked by GeneRuler 1 kb DNA ladder.
10. Run gel until loading dye approaches bottom of gel.
11. Stain gel with 0.5 μg/mL ethidium bromide for 30 min.
12. Visualize gel under UV light to check integrity of plasmid and efficiency of restriction enzymes (*see* **Note 5**) (Fig. 1).
13. Digest 10 μg of pWY82 and 5 μg of the target plasmid with 10 units of each restriction enzyme according to the manufacturer's instructions in a 50 μL total volume.
14. Prepare a 1% (w/v) TAE low melting point agarose gel and allow to solidify for 30 min in a 4 °C cold room (*see* **Note 6**).
15. Pre-chill the 1× TAE for electrophoresis by filling the electrophoresis chamber and allowing to chill in 4 °C cold room.
16. After restriction digest has completed, treat the target plasmid with 5 units of Antarctic phosphatase for 15 min at 37 °C (*see* **Note 7**).
17. Add 15 μL of 6× loading dye to each restriction digest and gently mix.

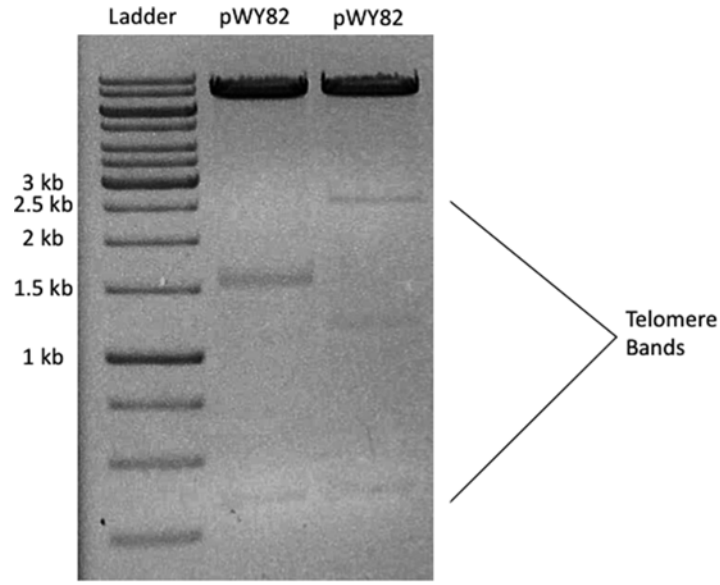

Fig. 1 Example digest of pWY82. pWY82 in both lanes was cut with *Eco*RV and *Hin*dIII though different sized bands can be seen throughout the gel

18. Carefully lower low melting point agarose gel into the electrophoresis chamber (*see* **Note 8**).
19. Load the full restriction digest into gel.
20. Load 6 μL of GeneRule 1 kb DNA ladder into the flanking wells of the gel.
21. Run the gel at 100 V in the cold room until the lower band of the loading dye is at the bottom of the gel.
22. Carefully move the gel to a glass dish and stain with 0.5 μg/mL ethidium bromide for 30 min.
23. Visualize under UV light and estimate the DNA concentration by comparing the intensity of the ladder to sample bands according to manufacturer's instructions.
24. Excise the uppermost telomere band with a fresh scalpel and place into a 1.7 mL tube (Fig. 2).
25. Excise the target plasmid backbone with a fresh scalpel and place into a 1.7 mL tube (Fig. 2).
26. In order to remove salts from the agarose slice add 1 mL of nuclease-free water to each tube and place at 4 °C overnight.
27. Completely remove water from each tube and place in a 70 °C water bath.
28. Flick tubes every minute until gel has completely melted.
29. Once agarose has melted (~5 min), move to a 37 °C water bath.
30. Allow gel fragments to cool to 37 °C, about 5 min.
31. Prepare ligation mixture in a 1.7 mL tube as shown in Table 1.

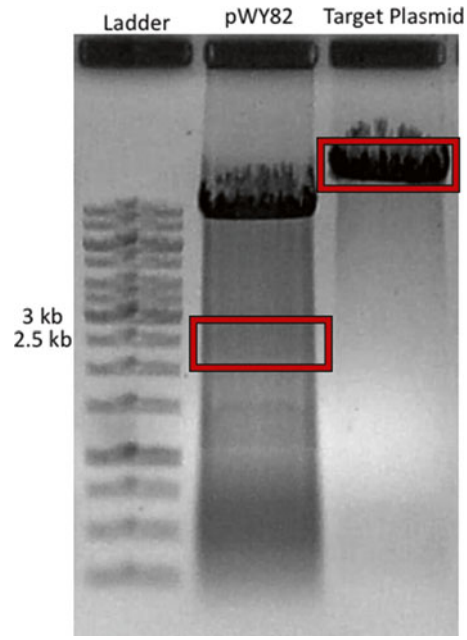

Fig. 2 Example gel used for telomere ligation. The *boxed sections* were removed and used for ligation

Table 1
Ligation mixture components

Ligase buffer	5 μL
Target plasmid gel fragment	100 ng
Ligase	1 μL (400 units)
Telomere gel fragment	To 50 μL

32. Once each component has been added, quickly mix by pipetting before ligation re-solidifies.
    (a) Flick tube until gel is floating in water.
33. Incubate the solidified ligation overnight at room temperature.
34. Remove salts by incubating ligation in 1 mL of nuclease-free water for 15 min.
35. While ligation is dialyzing, begin to thaw Stbl4 cells on ice.
36. Replace water with 50 μL of fresh nuclease-free water and place in a 70 °C water bath.
37. Flick tube every minute until completely melted.
38. Add 2 μL of ligation to 40 μL of Stbl4 cells and electroporate according to manufacturer's instructions.

39. Resuspend cells in 900 μL of S.O.C media and shake in 15 mL culture tubes at 30 °C for 1.5 h at 250 rpm.
40. Plate 100 μL of transformation onto LB plates with appropriate antibiotics.
41. Incubate plates at 30 °C (*see* **Note 9**).
42. Screen colonies via colony PCR or colony hybridization (*see* **Note 10**).
43. Confirm telomere orientation using standard sequencing methods (*see* **Note 11**).

3.1.1 Screening Potential Colonies for Telomere Size

1. Prepare a starter culture by inoculating 3 mL of 2xYT liquid media cultures with the appropriate antibiotics of each colony to be tested, pWY82 for a positive control, and empty target vector as a negative control (*see* **Note 12**).
2. Shake at 250 rpm for 8 h at 30 °C.
3. Inoculate fresh 5 mL 2xYT liquid cultures with appropriate antibiotics and 100 μL of starter cultures.
4. Shake cultures at 250 rpm for 12–18 h at 30 °C.
5. Extract 4 mL of each culture with the QIAprep Spin Miniprep Kit (Qiagen) and elute with 40 μL of 50 °C nuclease-free water.
6. Estimate concentration of each extraction using a nanodrop spectrophotometer.
7. Digest 1 μg of each plasmid extraction with restriction enzymes that flank the telomere insert as closely as possible in a 50 μL reaction volume.
8. Pour a 1% (w/v) 1× TAE agarose gel and insert a comb large enough to contain a 60 μL volume and allow to solidify at room temperature.
9. Add 10 μL of 6× loading dye to each restriction digest once completed.
10. Mix thoroughly by pipetting.
11. Load each digest into agarose gel and run at 100 V in 1× TAE.
12. Stain gel in 0.5 μg/mL ethidium bromide solution for 30 min.
13. Visualize with UV light to confirm successful digest.
14. Transfer to a nitrocellulose membrane by Southern transfer [5].
15. Follow Southern hybridization protocol [5] using the radiolabeled oligonucleotide $(TTTAGGG)_{10}$.
16. Compare the signal to the size standard to estimate the insert size.

3.2 Production of Telomere Fragments via Polymerase Chain Reaction

1. PCR Reaction Assembly (*see* **Note 13**):

   The following PCR reaction (Table 2) has been assembled using LongAmp Taq DNA polymerase from New England Biolabs (volumes listed are per reaction volumes) (*see* **Note 14**):

Table 2
PCR reaction components

Nuclease-free water	12 μL	24 μL
5× LongAmp Taq buffer	4 μL	8 μL
Forward telomere primer	0.5 μL	1 μL
Reverse telomere primer	0.5 μL	1 μL
10 mM DNTPs	2 μL	4 μL
LongAmp Taq polymerase	1 μL	2 μL

2. Use the following thermocycler protocol to perform PCR with the assembled reaction mixtures (Table 3).
3. After completion of thermocycler protocol, gel electrophoresis should be performed. Load the entire volume of each sample into the gel, as DNA gel extraction will be performed following visualization by gel electrophoresis. Example protocol for gel electrophoresis can be found in Sambrook and Russell 2001. Telomere DNA will appear as "smears," such as those visible in Fig. 3.
4. DNA gel extraction is performed using a kit such as Wizard® SV Gel and PCR Clean-Up System. Excise the gel band corresponding to the size of telomere DNA desired. Follow manufacturer's instructions for extraction of DNA from the gel piece (*see* **Note 15**).
5. The resulting DNA from the gel extraction can be used in a cobombardment with a transgene.

3.3 Transgene Delivery into Plants with Telomere Arrays

1. Insert transgene and telomere sequence into target organism following standard transformation protocols (*see* **Note 16**).
2. Screen transgenic plants for minichromosomes using fluorescence in situ hybridization (*see* **Note 17**).

4 Notes

1. This vector should have cloning sites that are compatible with those bordering the telomere sequence in plasmid pWY82.
2. This plasmid contains ~2.6 kb of telomere sequence and can be obtained through AddGene (addgene.org Plasmid# 65721).
3. A working stock of these primers should be prepared at a 1.25 μM/μL concentration.
4. pWY82 is maintained in STBL2 cells to stabilize the repeated sequences. These cells are slow growing and often will not be visible on plates or in culture for 24 h.

Table 3
Thermocycler protocol for PCR with the assembled reaction mixture

98 °C 10 s 55 °C 20 s 72 °C 5 s	Repeat 5×
98 °C 20 sc 55 °C 20 s 72 °C 5 s	Repeat 5×
98 °C 20 s 55 °C 20 s 72 °C 10 s	Repeat 5×
98 °C 20 s 62 °C 20 s 72 °C 30 s	Repeat 5×
72 °C 5 min	
4 °C ∞	

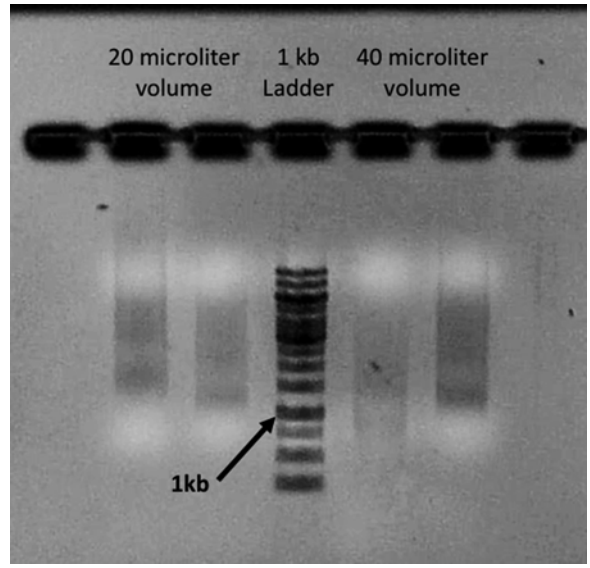

Fig. 3 Telomere PCR example using 20 and 40 μL reaction volumes. The "smears" in the sample lanes are indicative of telomere DNA conglomerates formed of various sizes

5. Telomere sequences are unstable, and often will form secondary structures when run in an agarose gel. In most cases, the telomere will appear as multiple bands, or a smear, in addition to the 2.6 kb full telomere band (Fig. 1).
6. A comb must be used that is large enough to fit 65 μL.
7. If restriction enzymes and Antarctic phosphatase are both purchased from the same manufacturer, it is not usually necessary to clean up the restriction digest between reactions.
8. Caution: low melting point gels must be handled extremely carefully as they are fragile.
9. As cells must be grown at 30 °C, it often takes 48 h for colonies to appear.
10. Though the technique requires radiation safety training, Southern Hybridization [6] is the recommended procedure for screening colonies for telomere. In our experience, hundreds of colonies can be screened at once, and using a labeled oligonucleotide probe of $(TTTAGGG)_{10}$ in either the 5′ or 3′ direction is extremely sensitive.

    Screening via colony PCR has been used in our lab; however, it is not as effective as Southern Hybridization. While most colony PCR procedures will suggest choosing primers that will cause amplification across the inserted DNA, this is not possible with telomere as the complex repeats will disrupt amplification. As a result, the PCR will fail and give false negative results. Consequently, there are two options for colony PCR to detect telomere repeat insertion. First, reliable primers can be used flanking the insertion site and a blank band can be considered a positive insertion. While this method has been successful, it relies on the polymerase failing during amplification. The second option relies on a primer in the target plasmid, and another on the sequence adjacent to the telomere repeats that will also be inserted into the target plasmid during ligation. This method has also been successful; however, because the primers originate in different plasmids, the user is not able to have a positive control.
11. Due to the complexity of the telomere repeats, sequencing will often fail after a few hundred base pairs. While this procedure cannot determine the complete length of inserted telomere, it is helpful to ensure that the ligation was completed in the correct orientation.
12. Prepare in the morning so that full cultures can be started in the evening and allowed to grow overnight.
13. A proofreading Taq polymerase should be used, for example, LongAmp Taq DNA Polymerase from New England Biolabs.

14. Make several reactions so that plenty of DNA can be obtained from DNA gel extraction. If using a different proofreading Taq, follow manufacturer's instructions for volume of buffer, Taq, and DNTPs if required.
15. Performing DNA gel extraction will greatly reduce the amount of DNA in each sample. For this reason, it is recommended that multiple thermocycler reactions are prepared to insure enough DNA is obtained. Nuclease-free water may be used for elution of DNA from the kit's column to ensure no interference with ligation reactions.
16. *Agrobacterium* transformation and particle bombardment are the two methods that have been successfully used to induce telomere-mediated truncation in maize [7, 8]. Particle bombardment can accomplish telomere-mediated truncation by bombarding in a transgene with attached telomere or separately by cobombardment of the transgene with free telomere arrays [7]. The standard protocols for both particle bombardment and *Agrobacterium* transformation are unchanged by inclusion of telomere arrays and should be followed for the organism of interest.
17. Visualization of the genome using fluorescence in situ hybridization (FISH) is useful for finding the general location of chromosomal insertions or truncations [7, 8]. If the inserted transgene is small, a protocol has been established for finding small targets in the maize genome using FISH [9], and should be applicable to other organisms. Minichromosomes can be distinguished from standard transgene inserts as the transgene will be located on the tip of a chromosome arm, and the chromosome is usually distinctly shorter when compared to its homologue.

Acknowledgment

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References

1. Gaeta RT, Masonbrink RE, Krishnaswamy L, Zhao C, Birchler JA (2012) Synthetic chromosome platforms in plants. Annu Rev Plant Biol 63:307–330
2. Yu W, Han F, Birchler JA (2007) Engineered minichromosomes in plants. Curr Opin Biotechnol 18:425–431
3. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
4. Ijdo JW, Wells RA, Baldini A, Reeders ST (1991) Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. Nucleic Acids Res 19:4780
5. Green MR, Sambrook J (2012) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
6. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

7. Gaeta R, Masonbrink R, Zhao C, Sanyal A, Krishnaswamy L, Birchler J (2013) In vivo modification of a maize engineered minichromosome. Chromosoma 122:221–232
8. Yu W, Lamb J, Han F, Birchler J (2006) Telomere-mediated chromosomal truncation in maize. Proc Natl Acad Sci U S A 103: 17331–17336
9. Lamb JC, Danilova T, Bauer MJ, Meyer UM, Holland JJ, Jensen MD, Birchler JA (2007) Single-gene detection and karyotyping using small-target fluorescence in situ hybridization on maize somatic chromosomes. Genetics 175:1047–1058

Chapter 2

Method for Biolistic Site-Specific Integration in Plants Catalyzed by Bxb1 Integrase

Ruyu Li, Zhiguo Han, Lili Hou, Gurminder Kaur, Qian Yin, and David W. Ow

Abstract

Crop improvement is a never ending process. With a transgenesis approach, it is not inconceivable to envision a continuous addition of new transgenes to existing cultivars. Previously, we described a recombinase-directed gene stacking method in tobacco (Hou et al., Mol Plant 7:1756–1765, 2014). Being able to stack DNA to a previous location ensures that the number of genetic loci does not increase with each new round of transgene addition. Whereas the previous demonstration was conducted through polyethylene glycol to mediate uptake of DNA into tobacco protoplasts, we now describe protocols for using biolistic transformation to stack DNA in tobacco and rice.

Key words Site-specific recombination, Gene targeting, Transformation, Recombinase, Transgene expression, Gene stacking

1 Introduction

Future biotech plants will likely harbor multiple transgenic traits as new traits are developed out of research laboratories. There are a number of ways to stack transgenes into a commercial cultivar.

Option 1. Transgenic loci can be combined through conventional breeding of independently transformed lines. This option will increase the number of segregating loci that breeders face for line conversion, i.e., the introgression of transgenic traits from a laboratory line to field cultivars. In moving the transgenes to an elite line, a breeder must obtain a breeding line homozygous not only for the transgenes but also for the elite traits of each elite cultivar. For diploid plants, or allopolyploids that behave as diploid, $(¼)n$ is the probability for assembling the "n" number of independent linkage units (assuming no linkage drag) into a homozygous breeding line (or homozygous parent lines for hybrid seed production). Hence, $(¼)^{10}$, or over a million plants, would be required for the co-assortment of, for example, seven elite traits plus three transgenic loci. With parallel breeding programs on a large number

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of region-specific cultivars and their requisite local field trials, increasing the number of segregating transgenic loci would compound higher cost with longer time for crop improvement through transgenesis.

Option 2. The de novo transformation with a stack of transgenes constructed in vitro can be used to deliver a transgene package to a single segregation unit. For a collection of newly available transgenes, transferring all of them as a molecular stack is a most logical strategy. However, for adding new traits to existing commercial cultivars that already harbor existing transgenic traits, this approach could require including previously introduced transgenes along with the newly added ones, unless the molecular stack can be integrated next to the pre-existing transgenic locus. Reintroducing previously approved traits poses a risk, as they may be subjected to a new round of deregulation due to it being a new integration event.

Option 3. Re-transformation of existing transgenic lines by random integration. Should a commercial line be re-transformed with another transgene, then line conversion can be avoided entirely as the desired traits are already in the elite genotype. A problem with the direct transformation of elite cultivars is that most of them are difficult to transform, requiring greater effort in obtaining the sufficient number of independent transformants for field evaluation. Another problem is that there are also too many locally adapted cultivars that require reliable high frequency transformation protocols. The most serious problem may be that from a regulatory perspective, as each commercial cultivar derived from individual transformation of the same DNA could be construed as an independent event that needs individual deregulation, in contrast to the deregulation of a single integration event that is bred out to numerous field cultivars.

Option 4. Re-transformation of existing transgenic lines by site-specific integration. If DNA can be directed to the same locus as previously placed transgenes, increasing the number of transgenic traits would not increase the number of segregating loci, and would thereby expedite the downstream line conversion process. Directing DNA to integrate at a designated chromosome location can be performed through the use of homologous recombination or recombinase-mediated site-specific integration. In plants, Zn-finger nucleases-mediated insertion of a second gene at a transgenic locus has been reported [1], as has recombinase-directed insertion of a second construct into a pre-existing transgenic locus by Cre-*lox* [2–4], R-*RS* [5], FLP-*FRT* [6], and Bxb1-*att* [7]. Recently, we have described an *in planta* gene stacking method that can integrate new DNA more than a single instance near a previously placed transgenes [8], specifically, with two additional rounds of new DNA stacked to the same genetic locus. In this method, a transgene-containing molecule is inserted into a genomic recombination target using the *Mycobacteriophage* Bxb1-*att* site-specific

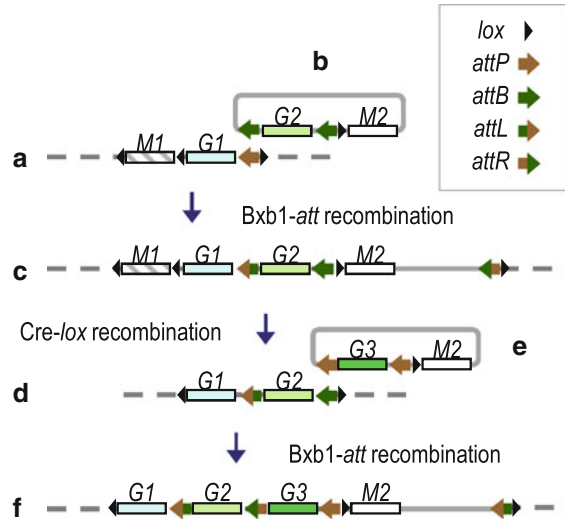

Fig. 1 Strategy for recombinase-mediated gene stacking *in planta* (Ow 2005; Hou et al. 2014). The target locus (**a**) comprises a first selectable marker (*M1*), a first trait gene (*G1*), and an *attP* site. Trait gene 2 (*G2*) circular DNA (**b**) integrates through genomic *attP* x plasmid *attB* recombination at the marker 2 (*M2*)-distal *attB* site to produce the structure in (**c**). Activation of Cre-*lox* recombination deletes unneeded DNA to yield the configuration in (**d**). Trait gene 3 (*G3*) circular DNA (**e**) integrates into the genomic target shown in (**d**) to yield the structure in (**f**). Subsequent stacking steps are analogous

integration system, in which the Bxb1 integrase (recombinase) catalyzes recombination between a 48 bp *attP* and a 38 bp *attB* to generate *attL* and *attR* without other proteins or high-energy cofactors [9]. DNA not needed after transformation was removed by excision by the coliphage P1 Cre recombinase that recombines 34 bp *lox* sites. As shown in Fig. 1, this exemplifies that the stacking process can continue with additional rounds since each integrating molecule brings in a new recombination target for the next round of integration [10].

Although this demonstration proved successful, it was conducted using the polyethylene glycol-mediated DNA uptake method on plant protoplasts, which works efficiently with tobacco but may not be applicable with many other crop plants. In contrast, biolistic transformation has proven widely successful in creating genetically modified commercial crop cultivars for over past decades. Hence, we now describe protocols for Bxb1-mediated gene stacking using the micro-projectile bombardment approach.

For recombinase-mediated site-specific integration in rice, we needed a target site in the rice genome. Through conventional *Agrobacterium*-mediated transformation, we first generated a target line, TS131, that harbors *hpt* (encoding hygromycin phosphotransferase) and *gus* (encoding beta-glucuronidase) flanked

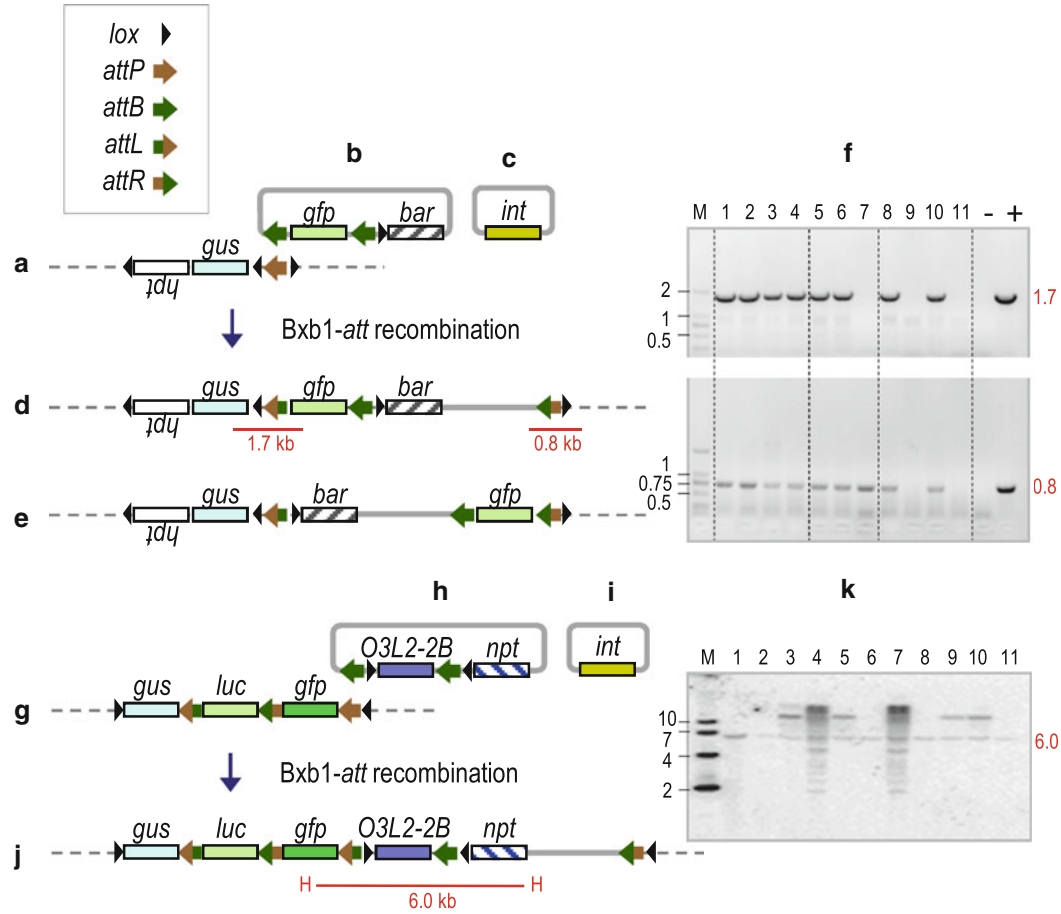

Fig. 2 Bxb1-mediated gene integration in rice and tobacco. The target rice line TS131 (**a**) harbors a single *attP* site for integration by pZH210B (**b**) mediated by transient expression of Bxb1 *int* (integrase gene) from co-bombarded pC35S-BNK (**c**) to yield configuration shown in (**d**) if recombination with the *bar*-distal *attB*, or that in (**e**) if with the *bar*-proximal *attB*. (**f**) Representative PCR of regenerated plants detects integration junctions of configurations shown in (**d**). Plants in lanes 1–4, 5–7 and 8–11 derived from three calluses; M, – and + are lanes for marker, negative (WT) and positive controls, respectively. Sizes of gel bands in kb. (**g**) Tobacco stacked line 23.C.4-9.d8.BC1 (Hou et al. 2014) harbors an *attP* site for the integration by pHL002 (**h**) mediated by the co-introduction of pC35S-BNK (**i**). Recombination with the *npt*-distal *attB* yields the configuration shown in (**j**). Configuration from recombination with the *npt*-proximal *attB* not shown. (**k**) Representative Southern blot of regenerated plants probed with *npt* DNA shows 6 kb band spanning from gfp to npt DNA. All 11 plants shown this band, but only lines 1,2, 6, 8, and 11 show it as the only hybridizing band. Other lines show additional bands to indicate additional copies integrated elsewhere in the genome. M is marker lane. Gene promoters and terminators not shown; all genes transcribe from *left* to *right* except for *hpt* indicated by *upside-down lettering*

by a set of *lox* sites from the Cre-*lox* site-specific recombination system as depicted in Fig. 2a. Downstream of *gus* lies an *attP* site recognized by Bxb1 integrase, followed by a third *lox* site in the opposite orientation. A circular DNA with a Bxb1 *attB* site, such as pZH210B shown in Fig. 2b, can integrate into the target site to create *attL* and *attR* sites after *attP* x *attB* recombination if Bxb1 integrase is produced by co-transformed pC35S-BNK shown in Fig. 2c. Since the molecule

shown in Fig. 2b contains two *attB* sites, it can generate two integrated configurations as shown in Fig. 2d, e. If Cre recombinase is introduced into the genome, the configuration in Fig. 2e would remove all transgenes except for an *attL* flanked by oppositely oriented *lox* sites, whereas the configuration in Fig. 2d would leave behind not only these same sites, but also the integrated *gfp* and an *attB* that could serve as a target for the next round of site-specific gene stacking. The configurations shown in Fig. 2d, e can be detected by the presence of distinct recombination junctions using PCR primers. After co-bombardment of line TS131-derived embryogenic calluses with pZH210B and pC35S-BNK, integrant calluses were screened for configuration shown in Fig. 2d and most plants regenerated from these calluses show the expected PCR junctions (Fig. 2f). Southern blotting can then follow to confirm structure as well as to detect if additional molecules had integrated elsewhere in the genome (data not shown). Below describes the 3–4 months process of callus induction, bombardment, selection, regeneration, and rooting (Fig. 3) of shoots regenerated from the bombardment of embryogenic calluses which takes 3–4 months to obtain integrant plant (Fig. 4).

For tobacco, we had previously described stacking two rounds of transgenes into a tobacco target line [8] that led to creation of line 23.C.4-9.d8.BC1 with the structure depicted in Fig. 2g. Here we describe the protocol for integrating a fourth gene, *OsO3L2-2B* [10], to the molecular stack through use of micro-particle bombardment into leaf explants instead of the previously used polyethylene glycol-mediated transformation of leaf mesophyll protoplasts. PCR analysis (not shown) of regenerated plants detected the expected recombination junctions for the configuration shown in Fig. 2j. Southern blotting (Fig. 2k) of 11 PCR positive integrants shows that 5 of them harbor only the site-specific copy of the introduced DNA (Fig. 2k). Other clones show additional hybridizing bands to suggest additional random copies elsewhere in the genome. A flowchart of the protocol is shown in Fig. 5.

2 Materials

2.1 Rice and Tobacco Target Lines

For rice, conventional *Agrobacterium*-mediated transformation of *Oryza sativa* (subsp. *japonica* cv. Zhonghua 11) yielded TS131, a target line containing a single copy of the target construct shown in Fig. 2a. The target line was greenhouse grown and embryogenic calluses were induced from mature seeds for use in biolistics. For tobacco, seedlings of *Nicotiana tabacum* (cv. Wisconsin 38) line 23.C.4-9.d8.BC1 [8] were germinated and maintained vegetatively in aseptic glass or plastic containers. This line harbors a single copy of three reporter genes *gus*, *luc*, and *gfp* shown in Fig. 2g followed by an *attP* site for the next round of Bxb1 integrase-mediated site-specific integration.

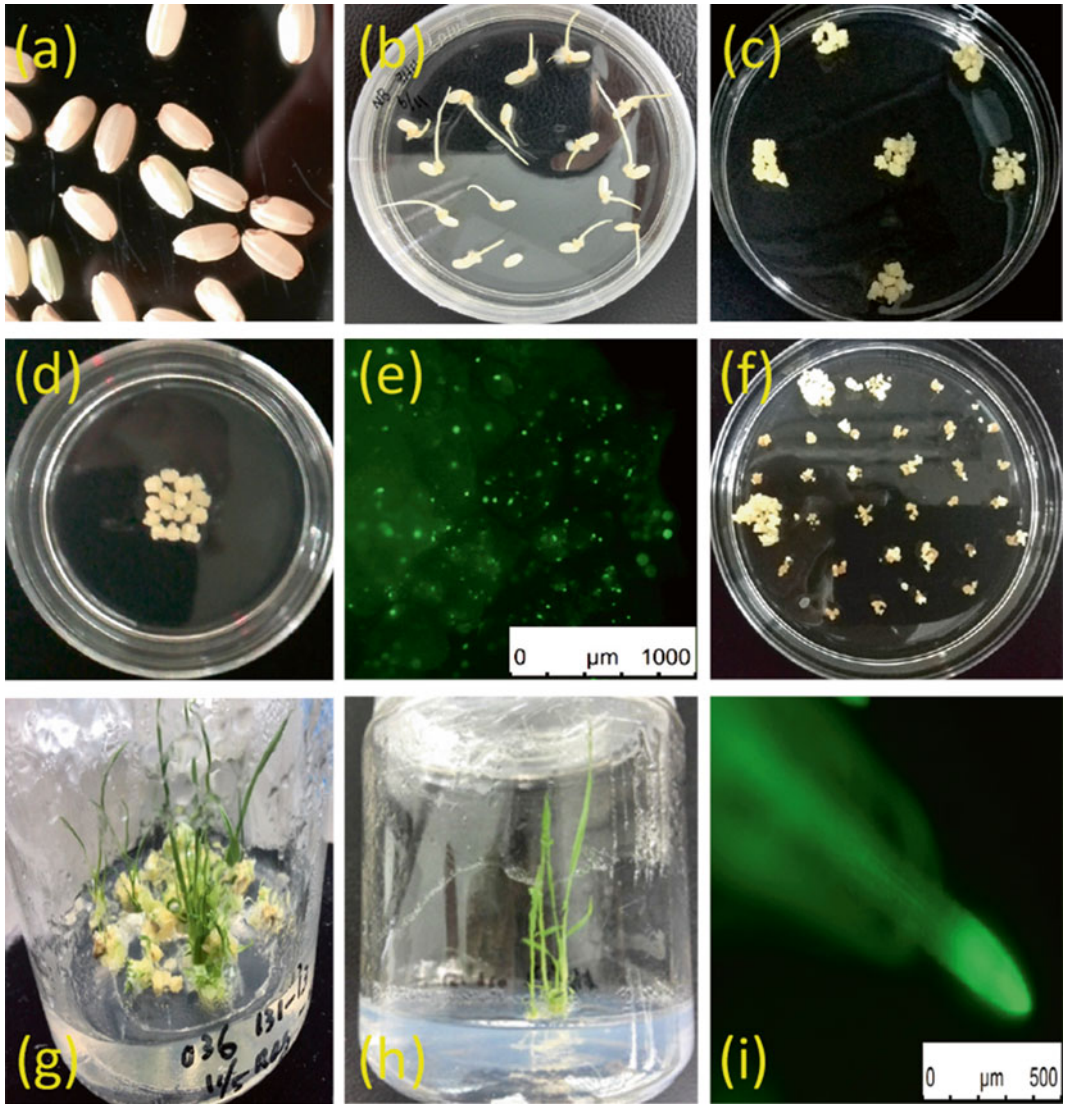

Fig. 3 Bxb1-mediated biolistic transformation in rice. (**a**) Dehusked mature seeds of "Zhonghua 11" target line, (**b**) Calli induction from seeds, (**c**) Two-week subcultured calli, (**d**) Selected calli on osmotic medium before bombardment, (**e**) GFP expression of bombarded calli 18 h after bombardment, (**f**) Calli on the selection medium with bialaphos after third round selection, (**g**) Shoots regenerate from bialaphos-resistant calli, (**h**) Regenerated shoots form root, (**i**) GFP expression in the root of the transgenic plant

2.2 DNA Constructs

For rice, the integrating construct pZH210B (Fig. 2b) comprises *gfp* (encoding green florescent protein) flanked by *attB* sites, a *lox* site, and the plant selectable marker *bar* (encoding bialaphos resistance). For tobacco, the integrating construct pHL002 comprises a trait gene *OsO3L2-2B* [11], flanked by two directly oriented *attB* sites, a set of opposing *lox* sites, and *npt* (encoding neomycin phosphotransferase) for selection (Fig. 2h). For both rice and tobacco, pC35S-BNK [7] carries the Bxb1-integrase gene (*int*) driven by the CaMV 35S RNA promoter (Fig. 2c).

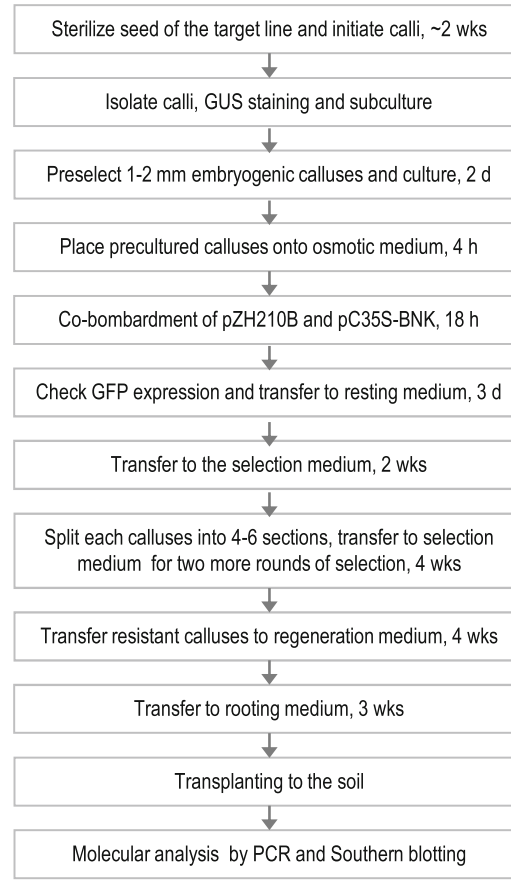

Fig. 4 Flowchart of Bxb1-mediated biolistic transformation of rice

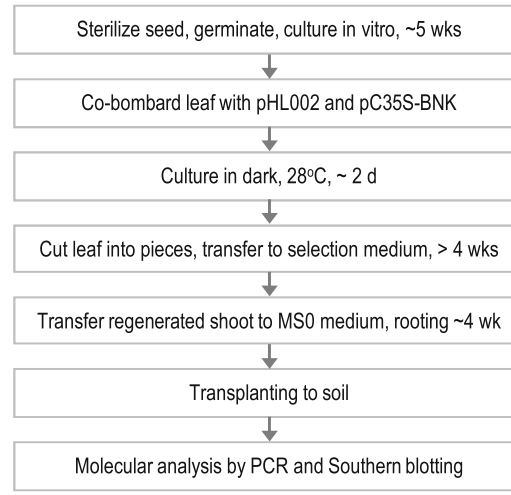

Fig. 5 Flowchart of Bxb1-mediated biolistic transformation of tobacco

2.3 Stock Solutions

1. N6 macro salts (10×): Dissolve 28.3 g KNO_3, 4.63 g $(NH_4)_2SO_4$, 4.0 g KH_2PO_4 and 1.85 g $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, in 800 mL distilled water and adjust the volume to 1000 mL. Store at 4 °C.
2. N6 micro salts (100×): Dissolve 440 mg $MnSO_4{\cdot}4H_2O$, 150 mg $ZnSO_4{\cdot}7H_2O$, 160 mg H_3BO_3, and 83 mg KI in 800 mL distilled water and adjust the volume to 1000 mL. Store at 4 °C.
3. B5 micro salts (100×): Dissolve 1000 mg $MnSO_4{\cdot}4H_2O$, 200 mg $ZnSO_4{\cdot}7H_2O$, 2.5 mg $CuSO_4{\cdot}5H_2O$, 25 mg $Na_2MoO_4{\cdot}2H_2O$, 2.5 mg $CoCl_2{\cdot}6H_2O$, 300 mg H_3BO_3, and 75 mg KI in 800 mL distilled water and adjust the volume to 1000 mL. Store at 4 °C.
4. B5 vitamins (100×): Dissolve 1 g thiamine hydrochloride, 100 mg pyridoxine hydrochloride, 100 mg nicotinic acid, and 10 g myo-inositol in 800 mL distilled water and adjust the volume to 1000 mL. Store at 4 °C.
5. Ethylenediamine-tetraacetic acid-iron (FeEDTA, 100×): Dissolve 2.78 g $FeSO_4{\cdot}7H_2O$ in 400 mL distilled water and add 3.73 g Na_2EDTA. Bring volume up to 1000 mL. Store at 4 °C.
6. MS macro salts (10×): Dissolve 19.0 g KNO_3, 16.5 g NH_4NO_3, 1.7 g KH_2PO_4, 3.7 g $MgSO_4{\cdot}7H_2O$ and 4.4 g $CaCl_2{\cdot}2H_2O$ in 800 mL distilled water and adjust the volume to 1 L. Store at 4 °C.
7. MS micro salts (100×): Dissolve 2.23 g $MnSO_4{\cdot}4H_2O$, 860 mg $ZnSO_4{\cdot}7H_2O$, 620 mg H_3BO_3, 83 mg KI, 2.5 mg 1.66 g $CuSO_4{\cdot}5H_2O$, 25 mg $NaMoO_4{\cdot}2H_2O$, and 2.5 mg $CoCl_2{\cdot}6H_2O$ in 800 mL distilled water and adjust the volume to 1 L. Store at 4 °C.
8. MS vitamins (100×): Dissolve 10 mg thiamine·HCl, 50 mg pyridoxine HCl, 50 mg nicotinic acid, 10 g myo-inositol, and 200 mg glycine in 800 mL distilled water and adjust the volume to 1 L. Store at 4 °C.
9. 2,4-Dichlorophenoxy acetic acid (2,4-D, 1 mg/mL): Add 95% ethanol dropwise to 100 mg 2,4-D until completely dissolved. While stirring quickly, add distilled water stirring to bring volume up to 100 mL. Sterilize by filtration and store at 4 °C.
10. 6-Benzylaminopurine (6BA, 1 mg/mL): Add 1 M HCl dropwise to 100 mg 6BA until completely dissolved. Adjust up to 100 mL with distilled water. Store at 4 °C.
11. Naphthalene acetic acid (NAA, 1 mg/mL): Dissolve 100 mg NAA in 5 mL 1 M KOH, bring up to 100 mL final volume with water. Sterilize with filtration. Store aliquots at −20 °C.
12. Bialaphos (1 mg/mL): Dissolve 250 mg bialaphos (e.g., Goldbio, USA) in 250 mL distilled water and sterilize by filtration. Store aliquots at −20 °C.

13. Phosphinothricin (PPT, 10 mg/mL): Dissolve 200 mg phosphinothricin (e.g., Goldbio,) in 20 mL distilled water and sterilize by filtration. Store aliquots at −20 °C.
14. Kanamycin (40 mg/mL): Dissolve 2 g kanamycin in 50 mL distilled water, filter-sterilize, and store 1 mL aliquots at −20 °C.

2.4 Medium Composition

2.4.1 Rice Media Composition

1. Callus induction medium (NB0): Add 100 mL of 10× N6 macro salts, 10 mL of 100× B5 micro salts and vitamins, 10 mL of 100× FeEDTA, 300 mg/L casamino acids, 500 mg glutamine, 500 mg/L L-proline, 30 g/L sucrose to 800 mL distilled water and bring volume up to 1 L. Adjust pH to 5.8 and add 4 g/L phytagel. Add 3 mg/L 2,4-D after autoclaving.
2. Callus subculture medium (NB1): Same as NB0 except that 2,4-D concentration is 2 mg/L in NB medium.
3. Osmotic medium: NB1 medium plus 46.6 g/L mannitol and 46.6 g/L sorbitol.
4. Selection Medium: Add 100 mL of 10× N6 macro salts, 10 mL of 100× N6 micro salts and vitamins, 10 mL of 100× FeEDTA, 300 mg/L casamino acids, 2.8 g/L L-proline, 30 g/L sucrose to 800 mL distilled water, and make up volume to 1 L. Adjust pH to 5.8 and add 4 g/L gelrite. Add to 2 mg/L bialaphos and 2 mg/L 2,4-D after autoclaving.
5. Regeneration Medium: Add 100 mL of 10× MS macro salts, 10 mL of 100× MS micro salts and vitamins, 10 mL of 100× FeEDTA, 2 g/L casamino acids, 30 g/L sucrose, 30 g/L sorbitol to 800 mL distilled water, and bring volume up to 1 L. Adjust pH to 5.8 and add 4 g/L gelrite. Add 1 mg/L NAA and 2 mg/L 6BA after autoclaving.
6. Rooting medium: Add 100 mL of 10× MS macro salts, 10 mL of 100× MS micro salts and vitamins, 10 mL of 100× FeEDTA, 100 mg/L myo-inositol, 30 g/L sucrose to 800 mL distilled water, and make up volume to 1 L. Adjust pH to 5.8 and add 3 g/L gelrite. Add 2 mg/L PPT after autoclaving.
7. X-Gluc solution: 50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA pH 8.0, 0.1 % (v/v) TritonX-100, 0.5 mg/mL X-Gluc.

2.4.2 Tobacco Medium Composition

1. Basic medium (MS0): Add 100 mL of 10× MS macro salts, 10 mL of 100× MS micro salts and vitamins, 10 mL of 100× FeEDTA, 30 g/L sucrose to 800 mL distilled water, and bring volume up to 1 L. Adjust pH to 5.8 and add 4 g/L phytagel.
2. Shoot induction medium (MS1): MS0 plus 1 mL of 1 mg/mL BA and 0.1 mL of 1 mg/mL NAA (add after autoclaving).
3. Selection medium (MS2): MS1 plus 2 mL of 40 mg/mL kanamycin (add after autoclaving).

2.5 Materials for Bombardment

1. PDS-1000/He particle delivery system from Bio-Rad (Hercules, CA, USA).

2.5.1 Biolistic Gun

1. 1.0 μm gold particles.
2. Macrocarrier holders, macrocarriers, 1100 psi rupture disks and stop screens.

2.5.2 Materials for Gold Particles Preparation (See Note 1)

3. $CaCl_2$ (2.5 M): Dissolve 1.84 g $CaCl_2 \cdot 2H_2O$ in 5 mL distilled water, filter-sterilize, and store aliquots at −20 °C.
4. $MgCl_2$ (2.5 M): Dissolve 1.19 g $MgCl_2$ in 5 mL distilled water, filter-sterilize, and store aliquots at −20 °C.
5. Protamine (3 mg/mL, Sigma, USA): Dissolve 30 mg protamine in 1 mL distilled water. Filter-sterilize and store aliquots at −20 °C. When used for DNA coating, dilute 20 μL stock in 180 μL sterilized distilled water and use 2 μL for DNA coating of each bombardment.
6. Spermidine (0.1 M, Sigma, USA): Pipette 7 μL spermidine into 493 μL distilled water and store at −20 °C. Use within 1 month.
7. 100% ethanol and sterilized distilled water.

3 Methods

3.1 Explants Preparation

3.1.1 Rice Callus Initiation and Proliferation

1. Dehusk the rice seeds (Fig. 3a). Rinse in 75% ethanol by vigorously shaking the tube for 1 min. Discard the ethanol, add 7.5% hypochlorite, and place on a shaker at 200 rpm for 30 min. Discard the hypochlorite and rinse the seeds five times with sterile distilled water until water is clear. Pour off water and place the seeds onto sterilized tissue paper inside clean bench and dry 2 h.
2. Place 15 seeds in each petri dish containing the callus induction medium. Wrap the plates with vent tape and incubate in the dark at 28 °C (*see* **Note 2**).
3. Confirm *gus* expression by GUS staining the germinated shoot (Fig. 3b, *see* **Note 3**).
4. In 2–3 weeks, yellowish calluses (Fig. 3b) are induced on the scutellum of the mature seed. Discard the young shoot and scutellum. Isolate the yellowish calluses and subculture them to callus subculture medium (NB1) for proliferation (Fig. 3c) (*see* **Note 4**).

3.1.2 Tobacco Seed Sterilization and Plant Maintenance

1. Sterilize tobacco seeds in 1.5 mL eppendorf tube with 3.75% sodium hypochlorite.
2. Disperse seeds well on the MS0 medium until seeds germinated, transfer plantlets over 2 cm to an aseptic glass or plastic containment for further growth. It takes ~5 or more weeks to obtain sufficient leaves for bombardment (Fig. 6a).

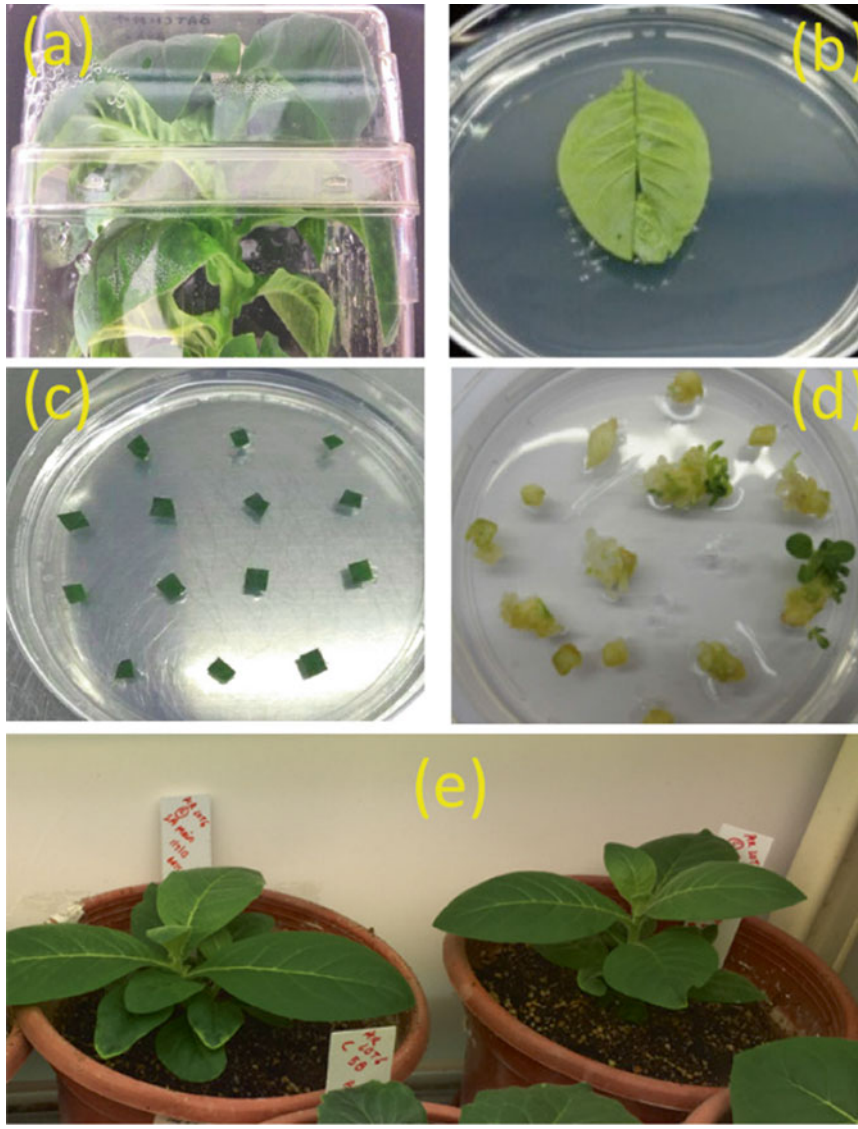

Fig. 6 Bxb1-mediated biolistic transformation in tobacco (**a**) In vitro maintained plants, (**b**) Selected leaf without midrib ready for bombardment, (**c**) Sliced bombarded leaf disc was placed on the selection medium, (**d**) Shoot regeneration after 8-week selection, (**e**) The transgenic grew in soil

3.2 Bxb1-Mediated Biolistic Transformation

3.2.1 DNA Preparation

1. Inoculate 1–2 mL of freshly growing *E. coli* containing the desired plasmid in a 500 mL flask containing LB medium with appropriate antibiotic selection (e.g., kanamycin or ampicillin). Place it at 37 °C with 250 rpm shaking overnight.
2. Use Qiagen Endofree Plasmid Mega Kit for DNA extraction.
3. Measure DNA concentration by Nanodrop. Run DNA in a gel to make sure most (over 90%) extracted DNA is in the supercoiled form (*see* **Note 5**). Aliquot DNA and store at −20 °C.

3.2.2 Gold Particle Preparation

Wash Gold and Make Aliquots

1. Weigh 30 mg 1.0 μm gold particles and transfer to a sterile, 1.5 mL microcentrifuge tube.
2. Add 500 μL 100% ethanol, vortex, centrifuge at 9400×*g* (10,000 rpm) for 30 s, remove ethanol.
3. Add 200 μL sterilized distilled water and vortex, repeat two more times, final volume should be 600 μL. Centrifuge at 10,000 rpm for 30 s, pipette off water (*see* **Note 6**).
4. Repeat **step 3**. Add 600 μL sterilized distilled water. Make 20 aliquots (1.5 mg/30 μL) and store at −20 °C until use. Each aliquot is for ten bombardments. For tobacco, add 500 μL sterilized distilled water to make ten aliquots of 3 mg/50 μL. Each aliquot is for also ten bombardments.

Coating the Gold with DNA

1. Sterilize macrocarrier holders, macrocarriers, 1100 psi rupture disk and stop screen in 70% ethanol 30 min followed by 100% ethanol 30 min. Take out and let air dry.
2. Take one gold aliquot prepared above, sequentially add DNA of 10 μg pZH210B, 10 μg pC35S-BNK, 20 μL 3 mg/L protamine, and 50 μL 2.5 M $MgCl_2$, vortex gently, and place on ice alternately for 3 min (*see* **Note 7**). For tobacco, add 4 μg pHL002, 4 μg pC35S-BNK, 20 μL 0.1 M spermidine, and 50 μL 2.5 M $CaCl_2 \cdot 2H_2O$.
3. Centrifuge at 10,000 rpm for 30 s and remove supernatant.
4. Add 200 μL 100% ethanol, vortex gently, repeat **step 2**.
5. Add 120 μL cold 100% ethanol. Vortex gently to let coated gold particles fully suspend (*see* **Note 8**).
6. Pipette 10 μL suspensions and evenly load on the center of each macrocarrier until dry.

3.2.3 Performing Bombardment (See Note 9)

1. For rice, select vigorous growing calluses (1–2 mm) two days prior to the bombardment and transfer them to the subculture medium. For tobacco, select top leaves from aseptically grown plantlets for bombardment. Put a piece of filter paper on top of MS0 medium in a petri dish. Place one leaf with adaxial side up on the filter paper and cut off the midrib. Transfer the cut leaf to the center of MS1 medium (Fig. 6b) (*see* **Note 10**).
2. For rice, prior to bombardment, transfer 15 pieces of pre-cultured calluses to the center of a petri dish containing the osmotic medium for 4 h (Fig. 3d). For tobacco, the osmotic treatment is not necessary.
3. Load 1100 psi rupture disk (*see* **Note 11**).
4. Assemble the macrocarrier launch. Lay in place a stopping screen followed by an inverted, pre-loaded macrocarrier holder.
5. Slide the launch assembly into place. Set the gap distance as 6 mm.

6. Slide a stop screen onto the shelf directly below the launch assembly.
7. Place opened petri dish containing the selected calluses (rice) or the leaf explants (tobacco) at 6 or 9-cm distance from the stopping screen.
8. Close the vacuum chamber and turn on vacuum. When the pressure reaches 28 in. of Hg, release the fire button.
9. Vent the chamber, remove the plate and seal it. Replace the rupture disk, macrocarrier and stopping screen for the next bombardment.
10. Repeat **steps 3–9** for each plate of bombardment. For tobacco, two bombardments per plate are applied.
11. Leave the bombarded calluses on osmotic medium in the dark at 28 °C for 18 h. For tobacco, the bombarded leaf explants are left in the dark at 28 °C for 2 days.

3.3 Resting and Selection

Check if GFP is being produced from transient expression of the introduced DNA (Fig. 3e). Transfer the calluses onto the resting medium without breaking the calluses and let culture in the dark at 28 °C for 3 days. Transfer the calluses to selection medium and let culture in the dark at 28 °C for 2 weeks. Split each callus into 4–6 small sections and place them onto selection medium for another two rounds of selection. Each round takes 2 weeks (Fig. 3f). For tobacco, slice the bombarded leaf into 7 × 7 mm squares and place them on the MS2 medium with adaxial side up (Fig. 6c). Seal the plate with vent tape and culture it at 28 °C with a 16 h light/8 h dark cycle. Subculture the leaf explants every 4 weeks (Fig. 6d), *see* **Note 12**).

3.4 Regeneration and Rooting

For rice, pick the actively growing calluses and transfer them to regeneration medium to culture at 28 °C with a 16 h light/8 h dark cycle. After 2–3 weeks, shoots should regenerate from calluses (Fig. 3g). When the sizes of the shoots are over 2 cm, place each shoot onto rooting medium (Fig. 3h). When a plantlet has five to six leaves, transplant it to soil. For tobacco, transfer the resistant regenerated shoots to MS0 medium to establish roots. Transfer plantlets with 4–5 leaves to soil (Fig. 6e) (*see* **Note 13**).

3.5 GUS Staining and GFP Observation

Immerse tissue with X-Gluc solution and incubate at 37 °C for 12–16 h to develop the blue color.

3.5.1 GUS Staining

3.5.2 GFP Expression

The fluorescence of GFP is visualized using a fluorescence inverted microscope (e.g., Leica, DMI6000B, Germany). The wavelength for excitation filter ranges from 440 to 520 nm, and a long pass filter of 510 nm was chosen for the barrier filter (*see* **Note 14**).

3.6 PCR Analysis

Isolation of DNA as described [12]. The DNA pellet is dried in the hood and dissolved in 100 μL ddH_2O. DNA quality is checked by gel electrophoresis. PCR is conducted using 2× Taq Master Mix (Microanalysis, USA). Sequence is confirmed by sequencing the amplified band.

3.7 Southern Blot Analysis

Genomic DNA (10 μg) cleaved with restriction enzyme is transferred to Hybond-N+ membrane (GE Healthcare, Buckinghamshire, UK) by 10× SSC using Model 785 Vacuum Blotter (Bio-Rad, CA, USA). [α-^{32}P] dCTP labeled DNA with Amersham Rediprime II Random Prime Labelling System (GE Healthcare, Buckinghamshire, UK) is used as hybridization probe. Hybridization and washing methods is according to established protocols [13]. After washing, the membrane is exposed to a phosphor screen for 5–12 h and scanned on Typhoon FLA 9500 (IP: 635 nm, PMT: 500 V, Pixel size 200 μm).

4 Notes

1. Gold particles, macrocarrier holders, macrocarriers, 1100 psi rupture disk and stop screen are all supplied by Bio-Rad.
2. While inoculating seed onto induction medium, be careful not to let embryo side face down on the medium in case that the emerging shoot lifts the seed away from the medium. We place the embryo side facing the side.
3. Using a hemizygote of the target line makes analysis of the precise integration easier, as there is only one target for integration. GUS staining can be used to eliminate non-target line-derived plants. Young shoots emerged from the seeds for callus induction can be used for GUS staining.
4. Rice embryogenic calluses emerge from the first isolated embryogenic calluses as a bunch of scattered small clones. It takes a month to establish the culture. To lessen lack of regeneration and/or somaclonal variation, it is better to reinitiate callus induction after 6 month of subculture.
5. It is also feasible to use other method to extract DNA. Just make sure DNA concentration is above 1 μg/μL. Keep DNA as pure as possible. It is necessary to check DNA quality by gel electrophoresis for supercoiled form.
6. If 600 μL of water is added at one time, the quality of gold suspension is not as good, whereas repeated addition of 200 μL water followed by vortexing works better.
7. For co-bombardment, the amount of each plasmid has to be tested on different molar ratios of each construct. The optimum ratio could be determined by GFP transient expression. Also coating DNA is one of the key steps for biolistic transformation,

and it may be necessary to test type and concentration of cations and polyamines.

8. Well suspended particles determine the uniformity of bombardment. Aside from vortexing, sometimes we scrape the tube 2–3 min to make sure that the particles are well dispersed.
9. To understand each element and operation safety, it is better to read through gene gun manual from Bio-Rad (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1652249.pdf).
10. The quality of calluses is one of the important factors for transformation. Usually calluses are selected from 2 to 3-week subcultures. The callus should be yellowish, in sizes of 1–2 mm. We select more calluses than the numbers planned for bombardment. After a 2-day preculture, those calluses that grow faster and are bigger are chosen for bombardment. For tobacco, the length and width of the leaves should be no more than 4 cm to ensure that the leaf stays within the bombarded area.
11. Optimum pressure and distance need to be tested for your own experiment.
12. Optimum selection pressure has to be tested for different medium or cultivar. For tobacco, After 5–12 weeks, leaf explants may turn yellow, as if bleached, or may regenerate green shoots (Fig. 6d).
13. To insure independent transformation events, it is better to use clones from different leaf explants as plants from the same explant may be vegetative clones.
14. Be cautious to choose a long pass filter of 510 nm for observing GFP florescence in rice calluses. Otherwise, the autofluorescence of calluses interferes with the GFP expression.

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References

1. Ainley WM, Sastry-Dent L, Welter ME, Murray MG, Zeitler B, Amora R, Corbin DR, Miles RR, Arnold NL, Strange TL et al (2013) Trait stacking via targeted genome editing. Plant Biotechnol J 11:1126–1134
2. Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites place in the plant genome. Plant J 7:649–659
3. Vergunst AC, Jansen LET, Hooykaas PJJ (1998) Site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* mediated by Cre recombinase. Nucleic Acids Res 26:2729–2734

4. Srivastava V, Ow DW (2002) Biolistic mediated site-specific integration in rice. Mol Breed 8:345–350
5. Nanto K, Yamada-Watanabe K, Ebinuma H (2005) *Agrobacterium*-mediated RMCE approach for gene replacement. Plant Biotechnol J 3:203–214
6. Li Z, Moon BP, Xing A, Liu ZB, McCardell RP, Damude HG, Falco SC (2010) Stacking multiple transgenes at a selected genomic site via repeated recombinase-mediated DNA cassette exchanges. Plant Physiol 154: 622–631
7. Yau YY, Wang Y, Thomson JG, Ow DW (2011) Method for Bxb1-mediated site-specific integration *in planta*. Methods Mol Biol 701:147–166
8. Hou LL, Yau YY, Wei JJ, Han ZG, Dong ZC, Ow DW (2014) An open-source system for *in planta* gene stacking by Bxb1 and Cre recombinases. Mol Plant 7:1756–1765
9. Ghosh P, Kim A, Hatfull GF (2003) The orientation of *mycobacteriophage* Bxb1 integration is solely dependent on the central dinucleotide of *attP* and *attB*. Mol Cell 12:1101–1111
10. Ow DW (2005) Transgene management via multiple site-specific recombination systems. In Vitro Cell Dev Biol-Plant 41:213–219
11. Wang C, Guo W, Ye S, Wei P, Ow DW (2016) Reduction of Cd in rice through expression of *OXS3*-like gene fragments. Mol Plant 9: 301–304
12. Lu YJ, Zheng KL (1992) A simple method for isolation of rice DNA. Chin J Rice Sci 6:47–48
13. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Chapter 3

Protocol for In Vitro Stacked Molecules Compatible with In Vivo Recombinase-Mediated Gene Stacking

Weiqiang Chen and David W. Ow

Abstract

Previously, we described a method for a recombinase-directed stacking of new DNA to an existing transgenic locus. Here, we describe how we can similarly stack DNA molecules in vitro and that the in vitro derived gene stack can be incorporated into an *Agrobacterium* transformation vector by in vitro recombination. After transfer to the chromosome by Agroinfection, the transgenic locus harbors a new target site that can be used for the subsequent in vivo stacking of new DNA. Alternatively, the in vitro derived gene stack has the potential to be integrated directly into the plant genome in vivo at a preexisting chromosomal target. Being able to stack DNA in vitro as well as in vivo, and with compatibility between the two systems, brings new flexibility for using the recombinase-mediated approach for transgene stacking.

Key words Site-specific recombination, Bxb1, *phi*C31, Cre-*lox*, Gene transfer, Transgenesis

1 Introduction

Plant genetic transformation has achieved great progress the past three decades [1]. In 2013, genetically modified crops was cultivated in 175.2 million hectares worldwide (www.isaaa.org) taking up ~11.5 % of global arable land. Although most GM crops contain only herbicide or insect resistance genes, the trend in future transgenesis is the development of stacked varieties with multiple transgenic traits, including output traits with enhanced consumer appeal [2]. There are a number of ways to stack transgenes into a commercial cultivar and a major concern has been on how one can keep the number of transgenic loci to a minimum, as more transgenic loci will decrease the probability of being able to reassert all of the desirable alleles into an elite genotype.

One approach that has been touted as a way to maintain a single transgenic locus in the host genome is commonly referred as in vitro stacking. In this approach, de novo transformation is conducted with a stack of transgenes constructed in vitro, which can be used to confine transgenes to a single segregation unit.

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This calls for constructing a linked set of transgenes in vitro for a single genomic insertion event. For a collection of newly available transgenes, transferring all of them as a molecular stack is a most logical strategy. For example, the metabolic engineering of complex pathways often requires the cotransfer of multiple transgenes. However, for adding new traits to existing transgenic cultivars, this approach could require including previously introduced transgenes along with the newly added ones, unless the molecular stack can be integrated next to the preexisting transgenic locus. Reintroducing previously approved traits may be a risk, as they may be subjected to a new round of deregulation due to it being a new integration event. The ideal situation would be if an in vitro gene stack can be directed to integrate next to previously placed transgenes, giving it both the flexibility of adding from one to many genes at a time, and the ability to maintain a single segregating transgenic locus.

Currently, there are several popular methods for the in vitro assembly of large DNA molecules. These include sequence and ligation-independent cloning [3], Gateway cloning [4–6], and a Cre-*lox* based in vitro gene assembly method [7]. While each of the above methods is suitable for creating linked transgene constructs for plant transformation, they lack the provision for in vivo integration of the molecular stack near previously introduced transgenes. Here, we describe a method for in vitro multigene assembly with use of three site-specific recombination systems. The *phi*C31-*att* system from *Streptomyces* temperate phage *phi*C31 [8] is used to cointegrate separate DNA molecules, the Cre-*lox* system from coliphage P1 [9] is used to excise unnecessary DNA, and the Bxb1-*att* system from *Mycobacterium smegmati* bacteriophage Bxb1 [10] is reserved for inserting the molecular stack into a designated target.

As with other methods described above, the molecular stack can be inserted into an *Agrobacterium* vector for transformation into a host genome. The integrated molecule would harbor a Bxb1 *attP* or *attB* site to permit additional in vivo (including *in planta*) stacking of new DNA to the locus. The unique feature of the method described here is the option for using the Bxb1-*att* system to integrate the molecular stack in vivo into an existing genomic target site [11], and with the integrated molecule harboring a Bxb1 *attP* or *attB* site to permit additional in vivo stacking of new DNA to the locus. This system is hence compatible with the *in planta* gene stacking system described previously [12–14]. Inserting the molecular stack into a preexisting transgenic locus would maintain the minimal number of segregating loci to ease the downstream line conversion process and expedite crop improvement *via* transgenesis.

2 Materials

2.1 Strains

1. *E. coli* BL21(DE3) competent cells: Genotype, *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5* [15].
2. *E. coli* DH5α competent cell: Genotype, *F– endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF) U169 hsdR17(rK– mK+), λ–* [16].

2.2 Antibiotics and Mediums

1. 50 mg/mL kanamycin: dissolve 0.5 g kanamycin in 10 mL double distilled water, filter sterilized with 0.2 μm filter, aliquot 0.5 mL per tube, stored at –20 °C.
2. 34 mg/mL chloramphenicol: dissolve 0.34 g ampicillin in 10 mL absolute ethanol, filter sterilized using 0.2 μm filter, aliquot 0.5 mL per tube, stored at –20 °C.
3. 100 mg/mL ampicillin: dissolve 1 g ampicillin in 10 mL double distilled water, filter sterilized using 0.2 μm filter, aliquot 0.5 mL per tube, stored at –20 °C.
4. 10 mg/mL phosphinothricin (Basta): Dissolve 200 mg phosphinothricin in 20 mL distilled water. Filter sterilize through 0.2 μm filter, aliquots and store at –20 °C.
5. LB medium: dissolve 2 g tryptone, 2 g NaCl and 1 g yeast extract in 200 mL distilled water (for solid medium 3 g agar was added), autoclaved. Liquid medium stored at 4 °C, antibiotics added to the solid medium before pouring into petri dish (*see* **Note 1**). Plates stored at 4 °C.
6. MS medium: Add 4.3 g MS salt, 10 g sucrose and 0.5 g MES to a 1.5 L beaker, add 800 mL ddH_2O and adjust to pH 5.6 (*see* **Note 2**), add 5 g agar and bring up to 1 L. Autoclaved, add Basta to MS medium (~50 °C) to the final concentration of 10 mg/L.

2.3 Solutions

1. PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5, autoclaved and store at 4 °C.
2. Recombination buffer: 20 mM Tris–HCl, pH 7.5, 30 mM NaCl, 10 mM EDTA, 10 mM spermidine, 10 mM DTT, 0.1 mg/mL BSA. Filter sterilize and store at –20 °C (*see* **Note 3**).
3. 50 mg/mL isopropyl-β-d-1-thiogalactopyranoside (IPTG): dissolve 1 g IPTG in 20 mL ddH_2O, filter sterilized, aliquot and store at –20 °C.

2.4 Enzymes, Recombinant DNA

1. Phusion® High-Fidelity DNA Polymerases. Store at –20 °C.
2. 2× Taq Master Mix. Store at –20 °C.
3. Restriction enzymes. Store at –20 °C.

4. T4 DNA ligase. Store at −20 °C.
5. T4 polynucleotide kinase. Store at −20 °C.
6. DNA polymerase Large (Klenow) Fragment. Store at −20 °C.
7. pMD18-T simple vector (Takara, Japan). Store at −20 °C.
8. pACYCDuet-1. Store at −20 °C.
9. Plasmid DNA isolation kit. Store at room temperature.
10. DNA purification kit. Store at room temperature.

3 Methods

The previously described *in planta* gene stacking strategy consists of reiterations of the Bxb1-*att* recombination reaction to integrate a circular molecule into the chromosomal target, and the Cre-*lox* recombination reaction to excise selectable markers and unneeded plasmid backbone [13]. To add in vitro stacking into the existing *in planta* stacking scheme, we incorporated the use of a second *attB* x *attP* type of recombination system, the *phi*C31-*att* system [8], to link circular DNAs in vitro, while reserving the Bxb1-*att* system for the *in planta* integration reaction [13]. Both the Bxb1-*att* and the phiC31-*att* systems are similar in that a single polypeptide integrase catalyzes *attB* x *attP* recombination to yield *attL* and *attR* that no longer recombine with each other in the absence of an excisionase. In contrast, the product sites from Cre-*lox* recombination are the same as the substrate sites, and the recombination reaction is freely reversible.

The molecular strategy of in vitro gene stacking is illustrated in Fig. 1. The acceptor vector, molecule A, contains the bacterial selectable marker *cat* for resistance to chloramphenicol, the plant selectable marker *bar* for resistance to bialaphos, and a gene1 exemplified by *gus* encoding β-glucuronidase. The *gus* fragment is flanked by a Bxb1 *attB* site on one end, and on the other end a *phi*C31 *attP* site followed by a *lox* site. The first donor vector, molecule B, comprises of the bacterial selectable marker *amp* for resistance to ampicillin, two *phi*C31 *attB* sites flanking a gene2 exemplified by *luc* encoding firefly luciferase, followed by a Bxb1 *attB* site and a *lox* site. Integrase *phi*C31 promotes recombination between corresponding *attB* and *attP* to yield two integration products depending on which *attB* sites in vector B is used. The cointegration products are retrieved from *E. coli* resistant to both ampicillin and chloramphenicol. Although different plasmids with the same replication origin can coexist within *E. coli* as plasmid–plasmid incompatibility is not strictly observed [17], a significant percentage (25–50%) of clones are expected to harbor recombinant cointegrates. Of the two types of cointegration products, the one shown as molecule AB (cointegration using *luc*-upstream

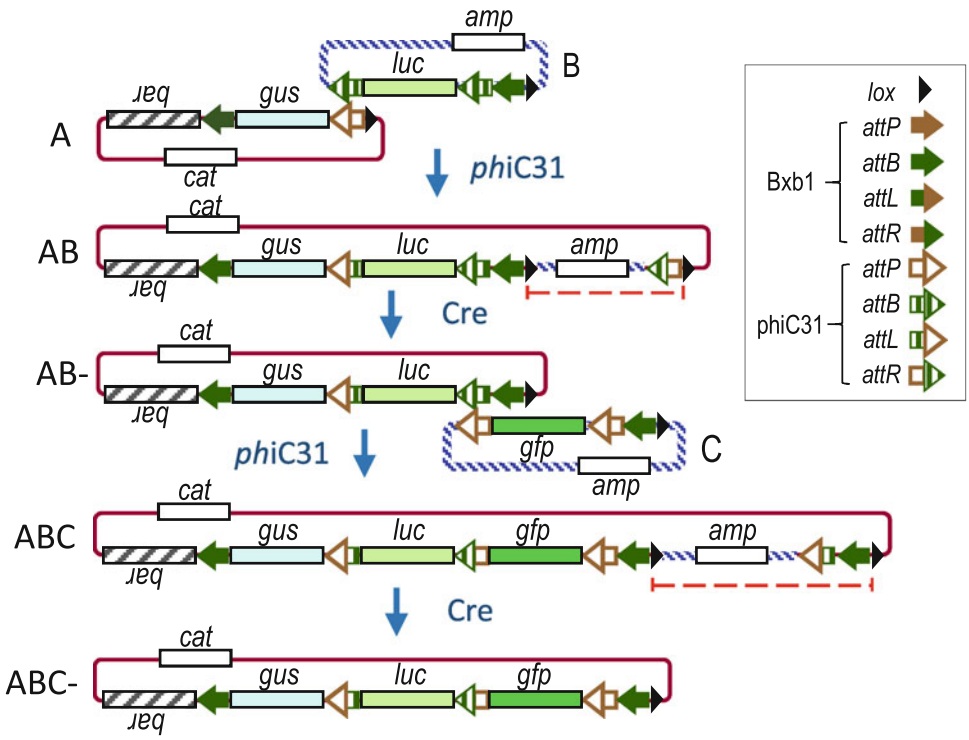

Fig. 1 Scheme of recombinase-mediated in vitro gene stacking compatible with in vivo gene stacking system. *phi*C31 integrase promotes in vitro *attP* (A) × *attB* (B, upstream of *luc*) to yield molecule AB. Cre recombinase deletes the unneeded DNA between directly placed *lox* sites to produce AB–. C integrates into AB– to generate ABC. Cre-*lox* in vitro reaction deletes undesired DNA in ABC to yield ABC–. Subsequent in vitro gene stacking is conducted as described for *gfp* and *luc*, with alternate use of *phi*C31 *attB* or *phi*C31 *attP* bearing vector. Genes described in text, promoters and terminators not shown, *upside down lettering* indicates transcription from right to left

*phi*C31 *attB*) in Fig. 1 is advanced for further gene stacking. Molecule AB is then converted to molecule AB– by Cre recombinase in vitro, and the resulting product transformed into *E. coli* screening for a phenotype that is resistant to chloramphenicol, but sensitive to ampicillin.

To stack gene3 to molecule AB–, molecule C is used, in which the structure resembles molecule B except that gene3, exemplified by *gfp* encoding enhanced green fluorescent protein is flanked by *phi*C31 *attP* rather than *attB* sites. Through the same in vitro *phi*C31 integrase catalyzed reaction as for creating molecule AB, C integrates into AB– to yield two possible cointegration products that confer resistance to both antibiotics. The molecule chosen to advance further is shown as molecule ABC from recombination with the *gfp*-upstream *attP* site. As before, recombination by Cre recombinase in vitro resolves the structure to that shown as molecule ABC–, and recovered after transformation into *E. coli*. Subsequent stacking steps are reiterations of the previous steps.

For example, the stacking of gene4 would be analogous to the *phi*C31 integrase, Cre recombinase sequential reactions leading to molecule AB–, while the stacking of gene5 would be analogous to the two reactions yielding molecule ABC–. In essence, gene stacking in vitro proceeds through the alternative use of *phi*C31 *attB* and *attP* site-bearing vectors, and without a theoretical limit to the number of genes stacked by this process.

3.1 Preparation of Recombinase E. coli Cell Extracts

1. *E. coli* BL21(DE3) harboring recombinase expression vectors are selected on kanamycin supplemented LB plates and a single colony is cultured overnight at 37 °C in 5 mL LB medium supplemented with 50 μg/mL kanamycin.
2. 0.5 mL of the overnight culture are used to inoculate 50 mL fresh LB medium supplemented with 50 μg/mL kanamycin and at 15 °C until OD_{600} of 0.3–0.4 (*see* **Note 4**).
3. IPTG (isopropyl-β-d-1-thiogalactopyranoside) is added to the cell culture at a final concentration of 0.1 mM, and the culture further incubated at 15 °C for up to 20 h.
4. Cells are harvested by centrifugation at 1,500×*g* (4,000 rpm) for 30 min, the pellet resuspended in 10 mL cold PBS, and the cells were sonicated on ice water mixture (*see* **Note 5**).
5. Lysate is centrifuged at 13,500×*g* (12,000 rpm) 4 °C for 30 min and the supernatant (cell extracts) collected to aliquot into 20 μL per tube for storage at −80 °C.

3.2 Recombinant DNA

Standard methods were used throughout. All plasmids use the origin of replication derived from pBR322 [18], except pZH36D that used ColE1 and the wild host range replication origin from pVS1 plasmids in *E. coli* and *Agrobacterium*, respectively [19]. *E. coli* DH5α was used throughout for recovery of recombinant molecules.

1. *phi*C31, Bxb1 *int*, and *cre* coding sequences were PCR amplified and inserted into pET28b to produce respective recombinase expression vector.
2. Molecule A: A *phi*C31 *attP-lox* fragment from pYWSP3 [11] was PCR amplified and inserted between *Pme*I and *Hin*dIII sites of pC35SCreB (Zhiguo Han, unpublished) to yield pDT01. *P35S* (CaMV 35S RNA promoter)-*gus-nos3′* (nopaline synthase gene terminator) fragment was isolated from pCAMBIA1301 by PCR and inserted between *Hin*dIII and *Sac*I of pDT01 to generate pDT02. *P35S* in pDT02 was replaced by *Pc* (commelina yellow mottle virus promoter) from pYWP72 [11] by PCR to generate pDT03. The *cat* (chloramphenicol resistant) gene was amplified from pACYCDuet-1 (NOVAGEN) and inserted between *Spe*I and *Pvu*I sites of pYWJTSB2 [11] to yield pCat, then the DNA fragment consisting of CaMV 35S terminator-*bar-P35S-Pc-gus-nos3′-phiC31*

attP-lox was isolated from pDT03 and inserted between *Not*I and *Spe*I sites of pCat to yield pDT04. A synthetic Bxb1 *attB* site was inserted into the pDT04 *Eco*RI site to produce pDT05. Finally, the *lox* site of pDT05 between the *Pst*I and *Hin*dIII was replaced by another synthetic *lox* sequence of opposite orientation to produce molecule A.

3. Molecule B: The *phi*C31 *attB-lox* fragment was amplified from pYWJTSB2 and inserted into pMD18-T simple vector (TAKARA). The resulting phiBB′-lox-T was cleaved with *Sac*II between *phi*C31 *attB* and *lox*, and a Bxb1 *attB* synthetic sequence inserted to generate phiBB′-Bxb1BB′-lox-T. The *phi*C31 *attB*-Bxb1 *attB-lox* fragment was cut out and ligated into the backbone of pYWJTSB2 using *Spe*I and *Sac*I to yield pDD01. DNA fragment consisting of *phi*C31 *attB-Pd35S* (CaMV 35S RNA promoter with duplicated enhancers)-*luc* (firefly luciferase)-*ocs*3′ (octopine synthase terminator) was cloned into pDD01 as two PCR fragments: a *Bam*HI to *Sac*I fragment to yield pDD02, then a *Sac*I to *Hin*dIII fragment to generate pDD03. However, sequencing result revealed an extra *phi*C31 *attP* residing upstream of *P35S*, thus the extra site was removed by *Not*I and *Pac*I cleavage followed by blunt end ligation to yield pDD04. The orientation of the *lox* in pDD04 was changed by replacing it with a new synthetic *lox* site with *Kpn*I and *Spe*I overhangs to yield molecule B.
4. Molecule C: DNA fragment consisting of *phi*C31 *attP-lox* was amplified from pYWSP3 and ligated into pMD18-T simple vector through TA coning to yield vector phiPP′-lox-T. A synthetic *Bxb1 attB* was inserted into the PstI site between *phi*C31 *attB* and *lox* of phiPP′-lox-T to yield phiPP′-Bxb1BB′-lox-T. DNA fragment consisting of *phi*C31 *attP*-Bxb1 *attB-lox* was isolated from phiPP′-Bxb1BB′-lox and inserted between *Sac*I and *Spe*I sites of pYWJTSB2 to generate pDD05. DNA fragment consisting of *phi*C31 *attP*-sugarcane Bacilliform virus promoter (*Ps*)-green fluorescent protein (*gfp*)-*nos* terminator DNA was amplified from pYWSP3 and inserted between *Sac*I and *Eco*RI sites to yield pDD06. The Bxb1 *attB-lox* sequence in the pDD06 was replaced with another synthetic Bxb1 *attB*-AscI-*lox* sequence, where *lox* is in the opposite orientation to generate molecule C.
5. Molecule D: A *Hin*dIII-*luc*-XbaI fragment from molecule B was replaced by a *Hin*dIII-*DsRed-nos* terminator-*Xba*I fragment from pBluKSP (Yongqing Li, this lab) to generate molecule D.
6. Molecule E: The *gfp* ORF in molecule C was replaced by yfp [20] using *Xma*I and *Not*I to produce molecule E.

7. pZH36D: Constructed by in vitro Cre-*lox* recombination of pZH36 (Zhiguo Han, unpublished) to delete DNA between the directly oriented *lox* sites.

3.3 Example of In Vitro Gene Stacking of Five Genes

3.3.1 First Round Stacking In Vitro

1. Add ~1 μg each of Molecules A and B, 5 μL 10× recombination buffer and 15 μL active *phi*C31 integrase cell extracts into a 1.5 mL tube, bring volume up to 50 μL with sterilized ddH_2O, mix well and incubate the reaction at 30 °C for 30 min.
2. Terminate reaction by heat inactivation at 75 °C for 10 min.
3. DNA is retrieved using DNA purification kit and resuspended in 30 μL sterile ddH_2O.
4. 2 μL retrieved DNA is transformed into 50 μL *E. coli* DH5α competent cells and selected on chloramphenicol and ampicillin supplemented LB plates.
5. 20 single clones is selected, each into 10 μL sterilized ddH_2O for 30 cycles of colony PCR in 20 μL reaction volume containing 10 μL 2× Taq Master Mix, 1 μL colony template, 10 μM each primers and sterile ddH_2O.
6. PCR product is separated on 1% agarose gel. Of the 20 colonies resistant to chloramphenicol and ampicillin, four clones (20%) showed PCR products amplified by primer sets *b*+*d* and *a*+*c* consistent with molecule AB structure, two clones showed PCR products amplified by primer *b*+*d2* and *a2*+*c*, consistent with structure AB′ (Fig. 2a, b) (*see* **Note 6**).
7. *Hin*dIII restriction analysis shows that molecule A cleaves into fragments of 0.9, 2.5, and 5.0 kb, while molecule B is linearized to a 6.8 kb fragment. Molecule AB or AB′, however, is expected to yield 0.9, 1.5, 2.5, and 10.2 kb, or 0.9, 2.5, 4.5, and 7.2 kb fragments, respectively. The four putative AB clones, as well as the 2 putative AB′ clones yielded the expected restriction pattern (Fig. 2c).
8. To remove *lox*-flanked DNA, 15 μL Cre crude extract is added to 1.5 μg of molecule AB DNA in 50 μL of recombination buffer for 30 min at 30 °C.
9. Reaction terminated by 75 °C for 10 min, and DNA is retrieved for *E. coli* transformation.
10. 2 μL of Cre-treated DNA is used to transform 50 μL of *E. coli* DH5α competent cells. Colonies selected on chloramphenicol supplemented LB plates.
11. Single colonies are tested for resistance to chloramphenicol but sensitive to ampicillin by replicate plating and candidate clones PCR analyzed in a 20 μL reaction volume containing 1 μL *E. coli* culture, 10 μM each primer, 10 μL 2× Taq Master Mix and sterile ddH_2O. In the test shown in Fig. 2, 8 of 36 chloramphenicol resistant colonies were ampicillin sensitive. Primer set *y*+*z* amplified the

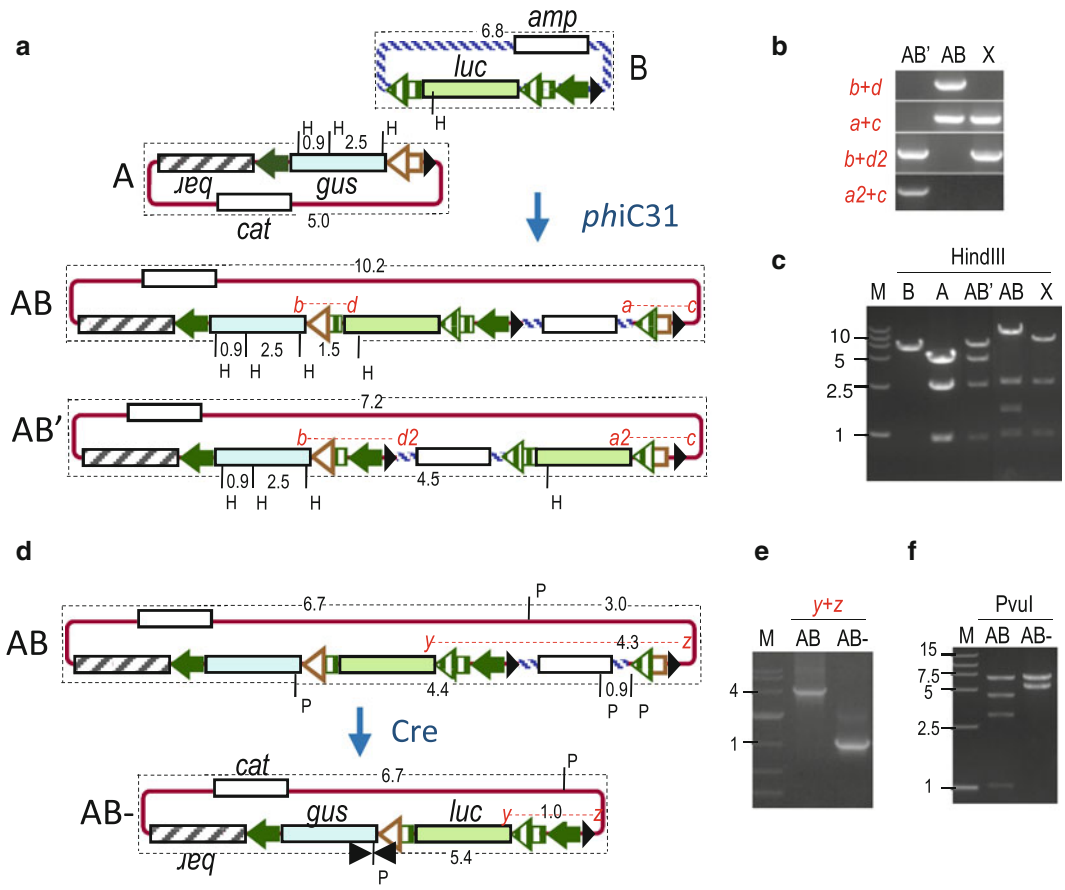

Fig. 2 First cycle cointegration and removal of unneeded DNA. (**a**) Not to scale depiction of recombination between *phi*C31 *attB* (B) and *attP* (A) to yield AB if *luc*– upstream *attB* were used, or AB′ if *luc*– downstream *attB* were used. (**b**) PCR and (**c**) HindIII (H) analysis of representative cointegrate clones. (**d**) Deletion between directly oriented *lox* sites to produce AB–. (**e**) PCR and (**f**) PvuI (P) analysis of representative chloramphenicol resistant but ampicillin sensitive clones. PCR fragments shown in *red*, restriction fragment sizes in kb. Lane M is marker lane. Lane X clone gave unexpected PCR pattern. Genes and symbols as in Fig. 1

predicted 1.0 kb PCR product in all eight clones, consistent with the expected deletion junction (Fig. 2d, e).

12. Clones containing expected PCR bands are grown for plasmid DNA isolation and restriction analysis by *Pvu*I. Molecule AB– yielded the expected two bands of 5.4 and 6.7 kb rather than four bands of 0.9, 3.0, 4.4, and 6.7 kb as in progenitor molecule AB (Fig. 2f).

3.3.2 Following Three Rounds of In Vitro Gene Stacking

1. In the second round of gene stacking, integrase *E. coli* extracts containing *phi*C31 integrase promoted the *attB* (AB–) x *attP* (C) reaction to yield molecule ABC (if *gfp*-upstream *attP* is used) or molecule ABC′ (if *gfp*-downstream *attP* is used). Out

of 20 chloramphenicol and ampicillin resistant colonies, five clones (25%) gave PCR products with primer sets *e*+*h* and *g*+*f* consistent with molecule ABC structure, while another five clones (25%) show PCR products with primer sets *g2*+*f* and *e*+*h2* corresponding to molecule ABC′ structure (Fig. 3b). When cleaved by *Sph*I, molecule AB– yielded 5.0 and 7.1 kb bands, while molecule C was linearized to 6.2 kb. In contrast, molecule ABC or ABC′ is expected to show 2.5 kb, 7.1 kb, and 8.7 kb, or 5.5 kb, 5.7 kb, and 7.1 kb, respectively. All five putative ABC clones and all five putative ABC′ clones generated the predicted restriction pattern (Fig. 3c).

To generate molecule ABC–, a representative molecule ABC was incubated with Cre recombinase extracts before transformation into *E. coli*. Of 36 single colonies screened, two were

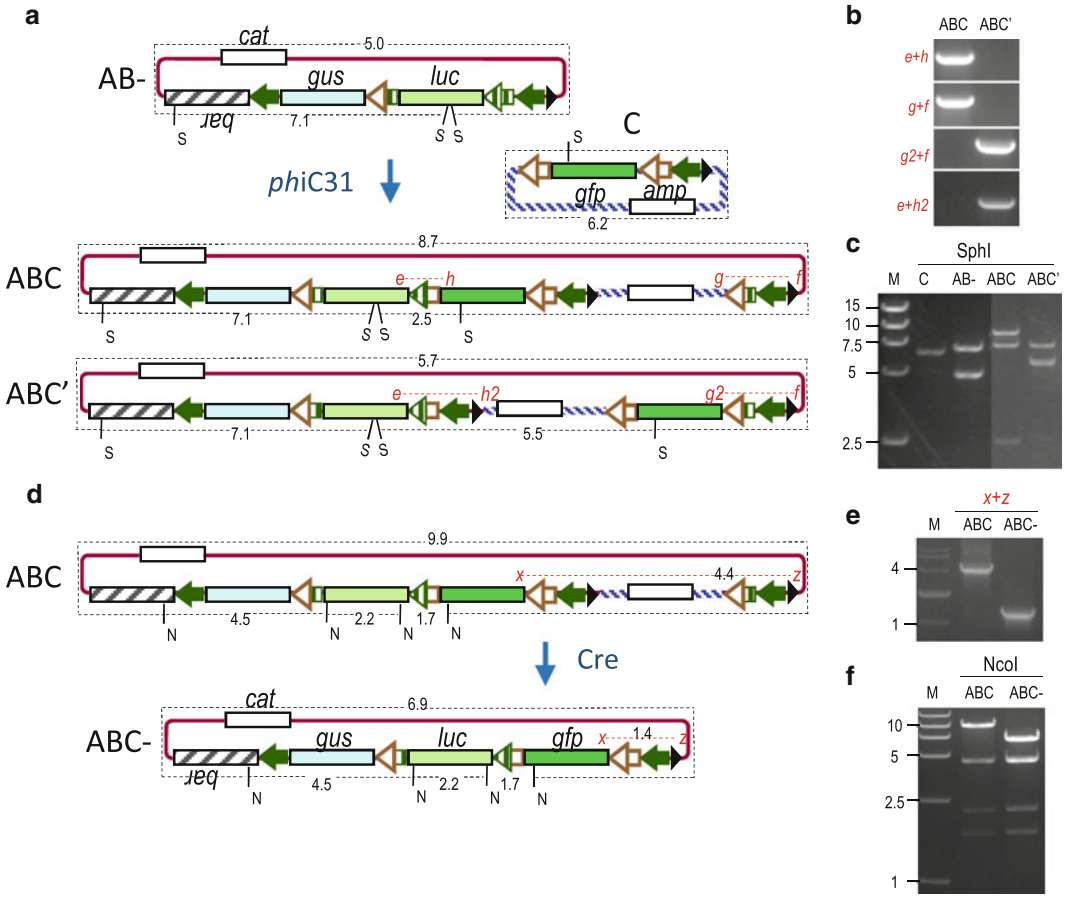

Fig. 3 Second cycle cointegration and removal of unneeded DNA. (**a**) Not to scale depiction of recombination between molecules AB– and C to yield ABC or ABC′. (**b**) PCR and (**c**) SphI (S) analysis of representative cointegrate clones. (**d**) Deletion between directly oriented *lox* sites to produce ABC–. (**e**) PCR and (**f**) NcoI (N) analysis of representative chloramphenicol resistant but ampicillin sensitive deletion clones. PCR fragments shown in *red*, restriction fragment sizes in kb. Lane M is marker lane. Genes and symbols as in Fig. 1

chloramphenicol resistant but ampicillin sensitive. Primer sets *x*+*z* amplified the predicted 1.4 kb deletion junction in the two clones (Fig. 3d, e). Cleavage with *Nco*I was consistent with PCR data (Fig. 3f). As expected, molecule ABC was cleaved into fragments of 1.7, 2.2, 4.5, and 9.9 kb, whereas molecule ABC– yielded 1.7, 2.2, 4.5, and 6.9 kb fragments.

2. In the third round of stacking, in which a plasmid containing *DsRed* was added to molecule ABC–, 2 out of 20 chloramphenicol and ampicillin resistant clones (10%) showed PCR bands amplified by primer sets *i*+*j* and *a*+*c*, consistent with the ABCD structure (Fig. 4a, b). Two anomalous clones were positive for primer sets *a*+*c*, but negative for primer sets *i*+*j* (lane X). As for the ABCD′ structure, we did not design primers to detect this configuration. When treated with SalI, molecule ABCD showed the expected fragments of 1.3, 1.7, 3.0, 3.5, and 11.8 kb (Fig. 4c), in contrast to molecule ABC– that showed fragments of 1.3, 3.5, and 10.5 kb, and molecule D with fragments of 1.7 and 4.4 kb.

   As above, a representative molecule ABCD was subjected to the in vitro Cre-*lox* reaction prior to transformation into *E. coli*. Of 36 chloramphenicol resistant colonies screened, four were ampicillin sensitive. The deletion junction was detected in all four clones when amplified by primer sets *w*+*z* (Fig. 4d). When cleaved with SalI, molecule ABCD yielded 1.3, 1.7, 3.0, 3.5, and 11.8 kb bands, whereas molecule ABCD– showed all bands but the 3.0 fragment, confirming the Cre-*lox* deletion (Fig. 4e).

3. The fourth round stacking was perfumed as for the second round stacking. Nine out of 20 chloramphenicol and ampicillin colonies show the PCR product when amplified by primer sets *p*+*h* and *g*+*f*, consistent with the molecule ABCDE structure (Fig. 5a, b). *Sal*I cleavage pattern for all nine clones met expectations of a 1.3, 3.0, 3.5, 4.8, and 11.8 kb bands (Fig. 5c).

   Prior to transformation into E. *coli*, representative molecule ABCDE was incubated with Cre recombiase cell extracts. Three out of 36 colonies were chloramphenicol resistant but ampicillin sensitive. Two of the three showed the 1.4 kb deletion junction amplified by primer sets *x*+*z* which is consistent with ABCDE– structure (Fig. 5a, d). SalI endonuclease cleavage of these two clones confirmed the correct molecular structure with 1.3, 3.5, 4.8, and 11.8 kb bands (Fig. 5e).

3.3.3 Gene Stack Integrated into Agrobacterium Gene Transfer Vector

If the aim is to conduct *Agrobacterium*-mediated transformation of the in vitro stacked genes, the entire molecule can be recombined into an *Agrobacterium* binary vector through Bxb1-mediated recombination in vitro. One such acceptor binary vector is shown

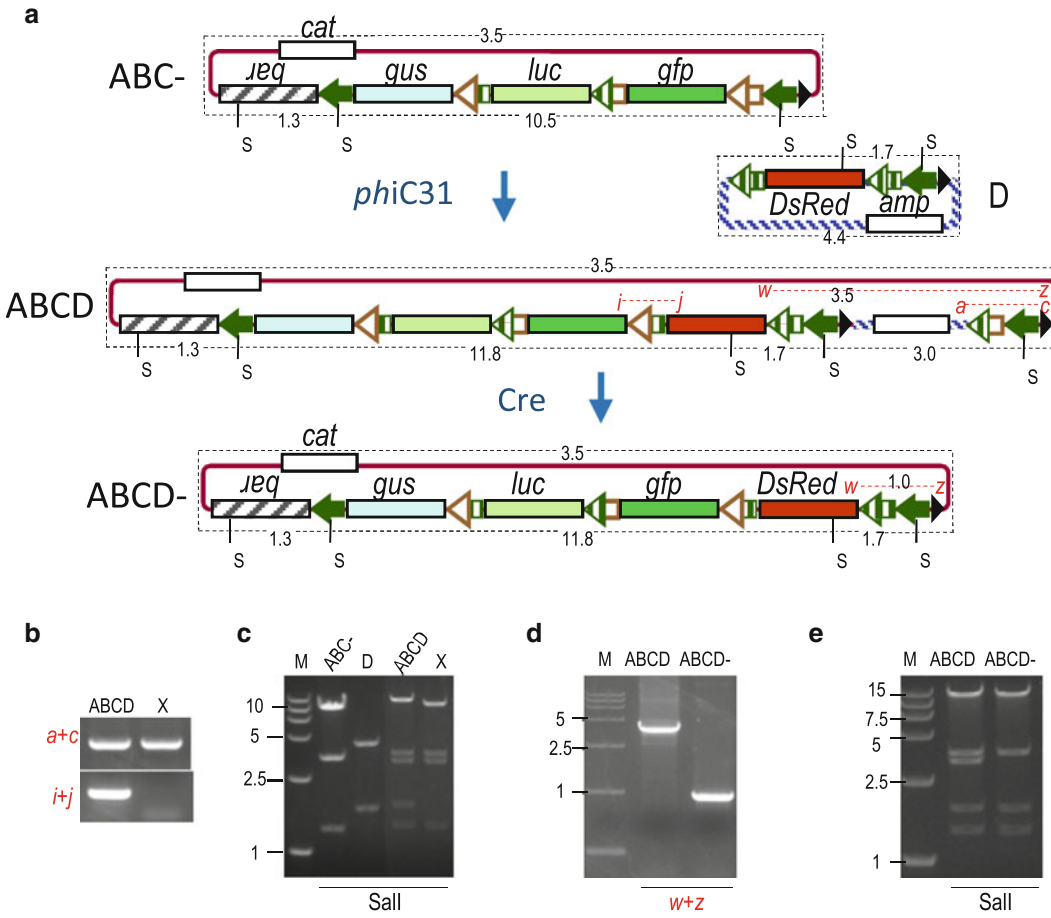

Fig. 4 Third cycle cointegration and removal unwanted DNA. (**a**) Not to scale depiction of recombination between molecules ABC– and D to yield ABCD (or ABCD′, not shown), followed by deletion to generate ABCD–. PCR and SalI (S) analysis of representative cointegrate clones (**b** and **c**, respectively) and deletion clones (**d** and **e**, respectively). PCR fragments shown in *red*, restriction fragment sizes in kb. Lane M is marker lane. Lane X clone gave unexpected PCR pattern. Genes and symbols as in Fig. 1

as in Fig. 6a, in which pZH36D contains the bacterial selectable marker *kan* for resistance to kanamycin, and a Bxb1 *attP* site to recombine with one of the two Bxb1 *attB* sites in pABCDE–. Recombination with the *gus* proximal Bxb1 *attP* site would yield the configuration shown as pStack in Fig. 6a. The structure of pStack shows that while *bar* can be used as a plant selectable marker, its subsequent *in planta* excision of the DNA fragment comprising of *cat*, *bar*, and other vector sequences should be possible upon introduction of Cre recombinase [21, 22]. Since the entire transgenic segment is flanked by *RS2* recombination sites from the CinH-*RS2* recombination system [23], future strategies can be devised to remove the entire transgenic locus if so desired [24]. Finally, the integration of pStack provides a genomic Bxb1 *attB* site to serve as the target for subsequent gene stacking *in planta* by an *attP* site-bearing vector.

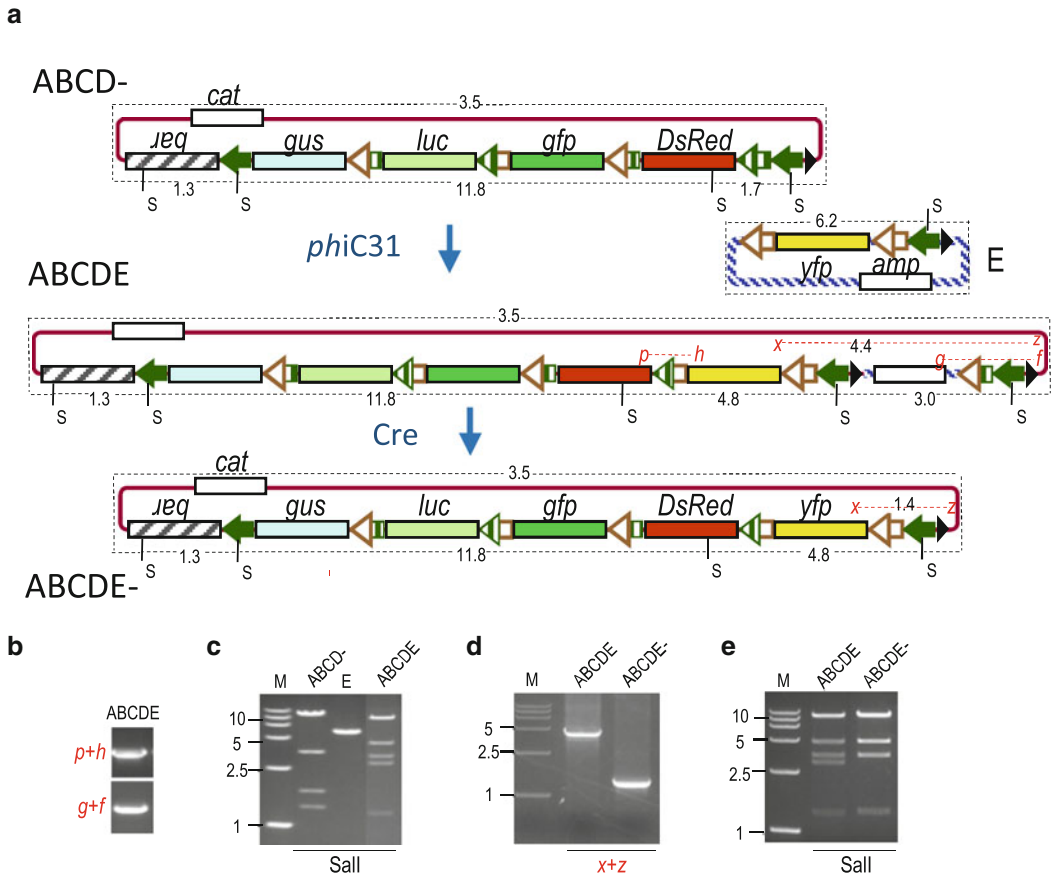

Fig. 5 Fourth cycle cointegration and removal unwanted DNA. (**a**) Not to scale depiction of recombination between molecules ABCD– and E to yield ABCDE (or ABCDE′, not shown), followed by deletion to generate ABCDE–. PCR and SalI (S) analysis of representative cointegrate clones (**b** and **c**, respectively) and deletion clones (**d** and **e**, respectively). PCR fragments shown in *red*, restriction fragment sizes in kb. Lane M is marker lane. Genes and symbols as in Fig. 1

1. Add ~1 μg each of molecule ABCDE– and pZH36D, and 15 μL Bxb1 cell extract to a final volume of 50 μL recombination buffer and incubate at 30 °C for 30 min.
2. Heat stop reaction at 75 °C for 10 min, ethanol precipitate the DNA to resuspend in 30 μL sterile TE buffer.
3. Transform 2 μL DNA to 50 μL *E. coli* DH5α competent cell and selected for chloramphenicol and kanamycin resistant clones.
4. Select 20 colonies into 10 μL sterile ddH_2O for 30 cycles of colony PCR (20 μL reaction volume containing 10 μL 2× Taq Master Mix, 1 μL colony template, 10 μM each primers and sterile ddH_2O). Of 20 kanamycin and chloramphenicol resistant colonies screened, five (20%) clones showed the PCR products amplified by primer sets *o*+*r* and *q*+*p1* (Fig. 6b), predicted from the pStack structure.

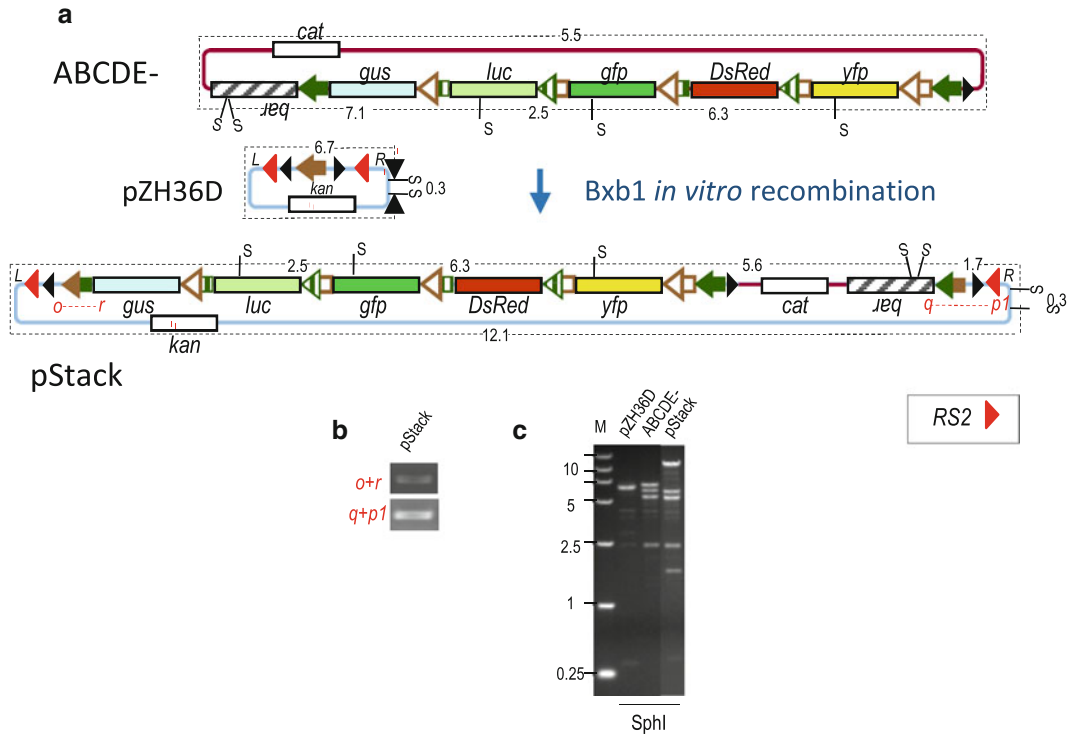

Fig. 6 Transfer of stacked genes to *Agrobacterium* vector. (**a**) Not to scale depiction of recombination between molecules ABCDE– and pZH36D to yield pStack, with *bar* for plant selection (pStack′ configuration not shown). (**b**) PCR and (**c**) SphI (S) analysis of representative cointegrate clones. PCR fragments shown in *red*, restriction fragment sizes in kb. Lane M is marker lane. Genes and symbols as in Fig. 1 except for *RS2* sites

5. Candidate pStack clones are cultured in 5 mL chloramphenicol and kanamycin supplemented LB for plasmid extraction.
6. Plasmid DNA cleaved with SphI detected the expected fragments of 0.3, 1.7, 2.5, 5.6, 6.3, and 12.1 kb (Fig. 6c). Four clones that failed to show the PCR products from primer sets *o+r* and *q+p1* were found by the *Sph*I cleavage pattern to be consistent with the pStack′ structure (configuration not shown) derived from recombination with the *yfp* proximal *attB* site of ABCDE–.

4 Notes

1. Solid medium should not too hot before adding antibiotics (100 μg/mL ampicillin, 50 μg/mL kanamycin, or 34 μg/mL chloramphenicol).
2. If pH was not adjusted, the medium would not solidify.

3. When thaw the frozen recombination buffer, there will be white floccule, this does not affect the function of recombinase.
4. Since Cre produced in abundance has adverse effects on *E. coli*, cells contain *cre* grow slower, and the yield of Cre recombinase is lower than Bxb1 and *phi*C31 recombinases.
5. During sonication, cell suspension should not get too hot as high temperature inactivates recombinases.
6. During the first cycle of in vitro gene stacking of *gus* and *luc* plasmids, two clones showed the PCR product amplified by primer sets *a*+*c* and *b*+*d2* (Fig. 2b, lane X), and the *Hin*dIII cleavage pattern indicates that they were neither the AB nor AB′ configuration (Fig. 2c, lane X). Plasmid DNA in these two clones had a smaller size than AB or AB′. A similar phenomenon was also observed during the third cycle of stacking (Fig. 4b, c). Since the appearance of smaller molecules was observed only in odd number cycles, with the difference between the odd and even number cycles in the donor molecule having two *phi*C31 *attB* sites, we considered the possibility of intramolecular recombination between the two *phi*C31 *attB* sequences. We incubated molecule B with *phi*C31 cell extracts and separated the resulting DNA by gel electrophoresis and observed two new faint bands (Fig. 7a). b1 and b2 were separately transformed into *E. coli* and selecting for ampicillin resistance, but only b1 yielded transformants. Plasmids from eight b1 clones cleaved by SpeI all showed the smaller size molecule compared to molecule B (Fig. 7b). Sequencing near the SpeI site revealed deletion of *luc* as shown in Fig. 7c. The *phi*C31 integrase is not known to recombine two *phi*C31 *attB* sites, but the *phi*C31 recombinase cell extract used for in vitro recombination was derived from *E. coli* BL21(DE3), commercially available (New England Biolabs) for routine protein expression but is proficient for homologous recombination. Indeed, when molecule B (Fig. 7d) was treated with *E. coli* BL21(DE3) crude extract, recombination between two *phi*C31 *attB* sites was observed. Interesting, we had not observed this when the donor molecule contained two *phi*C31 *attP* sites. Given the active homologous recombination between *attB* sites in vitro, future preparation of recombinase extracts would best be prepared from homologous recombination-deficient *E. coli*.

Acknowledgments

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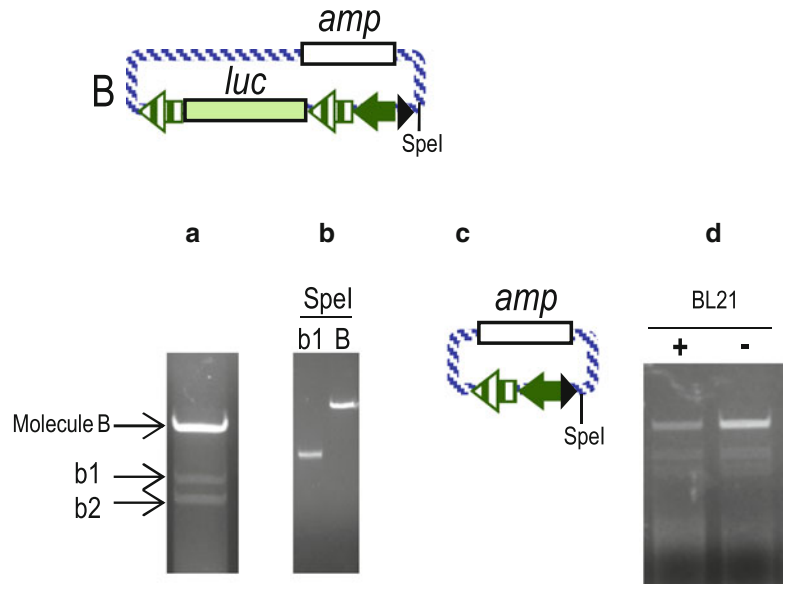

Fig. 7 Homologous recombination between intramolecular *phi*C31 *attB* sites. (**a**) Molecule B incubated with cell extract with *phi*C31 expressing plasmid produced bands b1 and b2 that were gel purified to transform *E. coli*. Only b1 yielded transformants. (**b**) Comparison of cleavage pattern between molecule B and b1 plasmid DNA treated with SpeI. (**c**) Deduced configuration of b1. (**d**) Molecule B incubated with *E. coli* BL21(DE3) cell extracts with (+) or without (–) *phi*C31 expressing plasmid. Genes and symbols as in Fig. 1

References

1. Vain P (2007) Thirty years of plant transformation technology development. Plant Biotechnol J 5:221–229
2. Que Q, Chilton M-DM, de Fontes CM, He C, Nuccio M, Zhu T, Wu Y, Chen JS, Shi L (2010) Trait stacking in transgenic crops: challenges and opportunities. GM Crops 1:220–229
3. Li MZ, Elledge SJ (2007) Harnessing homologous recombination *in vitro* to generate recombinant DNA via SLIC. Nat Methods 4:251–256
4. Chen QJ, Zhou HM, Chen J, Wang XC (2006) A Gateway-based platform for multigene plant transformation. Plant Mol Biol 62:927–936
5. Hartley JL (2000) DNA Cloning using *in vitro* site-specific recombination. Genome Res 10:1788–1795
6. Sasaki Y, Sone T, Yoshida S, Yahata K, Hotta J, Chesnut JD, Honda T, Imamoto F (2004) Evidence for high specificity and efficiency of multiple recombination signals in mixed DNA cloning by the Multisite Gateway system. J Biotechnol 107:233–243
7. Lin L, Liu YG, Xu X, Li B (2003) Efficient linking and transfer of multiple genes by a multigene assembly and transformation vector system. Proc Natl Acad Sci U S A 100:5962–5967
8. Thorpe HM, Smith MCM (1998) *In vitro* site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc Natl Acad Sci U S A 95:5505–5510
9. Abremski K, Hoess R (1984) Bacteriophage P1 site-specific recombination. purification and properties of the Cre recombinase protein. J Biol Chem 259:1509–1514
10. Kim AI, Ghosh P, Aaron MA, Bibb LA, Jain S, Hatfull GF (2003) Mycobacteriophage Bxb1 integrates into the *Mycobacterium smegmatis groEL1* gene. Mol Microbiol 50:463–473
11. Yau YY, Wang Y, Thomson JG, Ow DW (2011) Method for Bxb1-mediated site-specific integration in planta. Methods Mol Biol 701:147–166
12. Ow DW (2005) Transgene management *via* multiple site-specific recombination systems. In Vitro Cell Dev Biol-Plant 41:213–219

13. Hou LL, Yau YY, Wei JJ, Han ZG, Dong ZC, Ow DW (2014) An open-source system for in planta gene stacking by Bxb1 and Cre recombinases. Mol Plant 7:1756–1765
14. Li RY, Han ZG, Hou LL, Kaur G, Ow DW (2015) Method for biolistic site-specific integration in plants catalyzed by Bxb1 integrase. Methods Mol Biol. This volume
15. Studier FW, Moffatt BA (1986) Use of bacteriophage-T7 RNA-polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189:113–130
16. Grant SGN, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into Escherichia-Coli methylation-restriction mutants. Proc Natl Acad Sci U S A 87:4645–4649
17. Velappan N, Sblattero D, Chasteen L, Pavlik P, Bradbury ARM (2007) Plasmid incompatibility: more compatible than previously thought? Protein Eng Des Sel 20:309–313
18. Sutcliffe JG (1979) Complete nucleotide-sequence of the *Escherichia-Coli* plasmid-pBR322. Cold Spring Harb Symp 43:77–90
19. Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25:989–994
20. Ormo M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ (1996) Crystal structure of the *Aequorea victoria* green fluorescent protein. Science 273: 1392–1395
21. Dale EC, Ow DW (1991) Gene-Transfer with subsequent removal of the selection gene from the host genome. Proc Natl Acad Sci U S A 88:10558–10562
22. Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. Mol Gen Genet 234:49–59
23. Kholodii G (2001) The shuffling function of resolvases. Gene 239:121–130
24. Moon HS, Abercrombie LL, Eda S, Blanvillain R, Thomson JG, Ow DW, Stewart CN (2011) Transgene excision in pollen using a codon optimized serine resolvase CinH-RS2 site-specific recombination system. Plant Mol Biol 75:621–631

Chapter 4

Generation and Analysis of Transposon *Ac/Ds*-Induced Chromosomal Rearrangements in Rice Plants

Yuan Hu Xuan, Thomas Peterson, and Chang-deok Han

Abstract

Closely-located transposable elements (TEs) have been known to induce chromosomal breakage and rearrangements via alternative transposition. To study genome rearrangements in rice, an *Ac/Ds* system has been employed. This system comprises an immobile *Ac* element expressed under the control of CaMV 35S promoter, and a modified *Ds* element. A starter line carried *Ac* and a single copy of *Ds* at the *OsRLG5* (*Oryza sativa receptor-like gene 5*). To enhance the transpositional activity, seed-derived calli were cultured and regenerated into plants. Among 270 lines regenerated from the starter, one line was selected that contained a pair of inversely-oriented *Ds* elements at the *OsRLG5* (*Oryza sativa receptor-like gene 5*). The selected line was again subjected to tissue culture to obtain a regenerant population. Among 300 regenerated plants, 107 (36%) contained chromosomal rearrangements including deletions, duplications, and inversions of various sizes. From 34 plants, transposition mechanisms leading to such genomic rearrangements were analyzed. The rearrangements were induced by sister chromatid transposition (SCT), homologous recombination (HR), and single chromatid transposition (SLCT). Among them, 22 events (65%) were found to be transmitted to the next generation. These results demonstrate a great potential of tissue culture regeneration and the *Ac/Ds* system in understanding alternative transposition mechanisms and in developing chromosome engineering in plants.

Key words Rice, *Ac/Ds* transposable elements, Alternative transposition, Chromosomal rearrangements, Tissue culture

1 Introduction

A classical maize transposable element family, *Activator* (*Ac*)/*Dissociation* (*Ds*), transposes by a cut-and-paste mechanism [1, 2] and has been widely used for gene tagging in heterologous plants including rice [3, 4]. Also, it has been well recognized that transposable elements (TEs) can induce chromosomal breakage and rearrangements via alternative transposition when TEs are closely located nearby each other. During alternative transposition reaction, transposases recognize the 5′ and 3′ termini of different TEs and subsequently generate deletions, duplications, inversions, and translocations. Alternative transposition events of *Ac/Ds* and

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their consequent chromosomal rearrangements have been extensively characterized in maize, Arabidopsis, and rice [5–7]. This chapter describes isolation of a line carrying a pair of closely located *Ds* elements and analysis of chromosomal rearrangements induced by transpositional activities of a pair of inversely-oriented *Ds* elements in rice.

We previously generated a population of insertional mutants by using the *Ac/Ds* system [3]. T-DNA carrying an immobile *Ac* element was transformed into rice plants. In the T-DNA construct, *Ac* cDNA was driven by CaMV 35S promoter. Another T-DNA construct harbored a gene trap *Ds* element in which a GUS coding region and a *BAR* gene were inserted (Fig. 1). Extensive analysis with these *Ac/Ds* lines of rice revealed that tissue culture-mediated regeneration not only reduced methylation levels at the termini of *Ds* [8] but also greatly enhanced *Ds* transposition activities [9]. Regeneration of plants from seed-derived calli led to rapid generation of a large population of *Ds* insertional mutants [9]. Among the *Ds* population, one line carried a single copy of *Ds* inserted in the promoter region of the *OsRLG5* (*Receptor-Like kinase Gene 5*) gene. *OsRLG5* is 1 of 36 homologous genes encoding similar receptor-like kinases clustered on the short arm of rice chromosome 1. To obtain lines carrying a pair of closely located *Ds* elements in the *cis* configuration at the *OsRLG5* locus, *OsRLG5::Ds* seeds were subjected to another round of tissue culture regeneration. To identify plants carrying a pair of closely located *Ds* elements, Southern blot analysis was performed using genomic DNA from 270 regenerated plants. Genomic DNA was digested with *Sac*I restriction enzyme and hybridized with an *OsRLG5*-specific probe. *Sac*I does not cut within the *Ds* element but recognizes sites 2.6 kb upstream and 13.7 kb downstream of the original *Ds* insertion site in the *OsRLG5* locus, which should generate a 22.2 kb *Sac*I fragment in the progenitor *OsRLG5::Ds* line (Fig. 2a). Out of

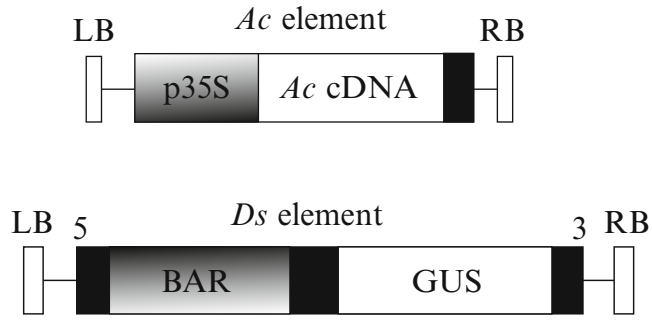

Fig. 1 Structures of the *Ac* and *Ds* T-DNA vectors. Expression of *Ac* cDNA is driven by the CaMV 35S promoter in the *Ac* T-DNA vector. A *BAR* selection marker and GUS reporter gene were inserted inside the *Ds* T-DNA vector. LB and RB indicate left and right border, respectively. "5" and "3" indicate *Ds* termini

270 plants, 2 produced a 28.1 kb *Sac*I fragment. PCR analysis revealed that one line (*OsRLG5-161*) contained one additional *Ds* element that was located 1.1 kb distal to the original *Ds* element in an inverse orientation (Fig. 2b). In summary, using tissue-culture regeneration system, a pair of nearby *Ds* elements inserted in inverse orientation was obtained from a single copy *Ds* insertion site at a frequency of 0.7%.

Previous studies showed that pairs of inversely oriented *Ac/Ds* elements could undergo sister chromatid transposition (SCT) in plants [5, 10–12]. Two transposable elements on different sister chromatids are involved in SCT. The 5′ and 3′ termini of transposons on different sister chromatids are reinserted into a target site on one of two sister chromatids, which leads to diverse chromosomal rearrangements including deletion and duplication (Fig. 3). Lines containing chromosomes derived from SCT events could carry inversion of intertransposon segments (ITS). The inversion can be detected by primer sets 1 + 3 or 2 + 4 that recognized *Ds1* and ITS or *Ds2* and ITS, respectively (Table 1, Fig. 3). The primers were oriented in the same direction. Among 300 regenerated plants from seed-derived calli of *OsRLG5-161*, 61 generated PCR products with primers 1 + 3 while 13 gave PCR products with primers 2 + 4 (Table 1). Therefore, among the total population, 24.7% of plants contained chromosomal rearrangements derived from SCT.

Wang and Kunze proposed that competence for transposition might be determined by strand-specific methylation patterns. Immediately following replication, two daughter *Ds* elements are hemimethylated on opposite DNA strands; one element will have coding-strand methylation, while the other has noncoding-strand

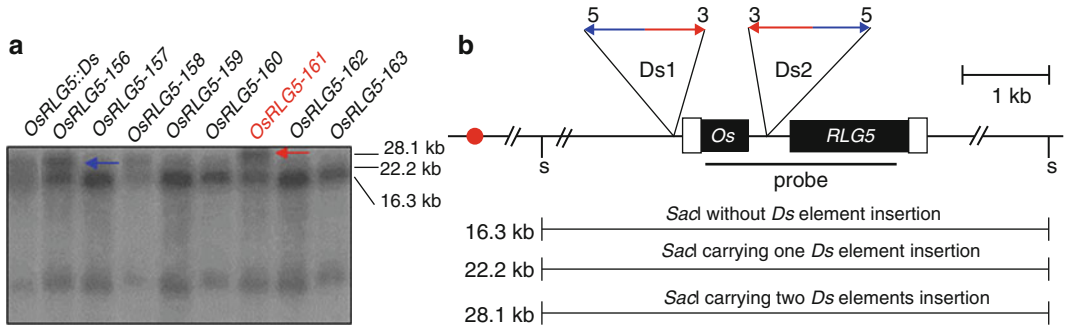

Fig. 2 Selection of plants containing two *Ds* elements at the *OsRLG5* locus. (**a**) Southern blot hybridization to identify lines containing two copies of *Ds* inserted in the *OsRLG5* locus. *Sac*I-digested DNA samples from the indicated lines were hybridized with a probe from the *OsRLG5* gene. The 16.3 kb band represents the reference *OsRLG5* allele lacking *Ds*. The original *OsRLG5::Ds* allele produced a 22.2 kb fragment (*blue arrow*), while the *OsRLG5-161* allele has a 28.1 kb *Sac*I fragment (*red arrow*). (**b**) The *OsRLG5-161* allele carries two *Ds* elements in the inverted orientation and separated by a distance of 1.1 kb. *White* and *black boxes* indicate UTRs and exons of *OsRLG5*, respectively. *Short vertical arrows* indicated by "S" are *Sac*I restriction sites. *Horizontal lines* indicate the sizes of restriction fragments generated by *Sac*I digestion. The *red oval* indicates a centromere

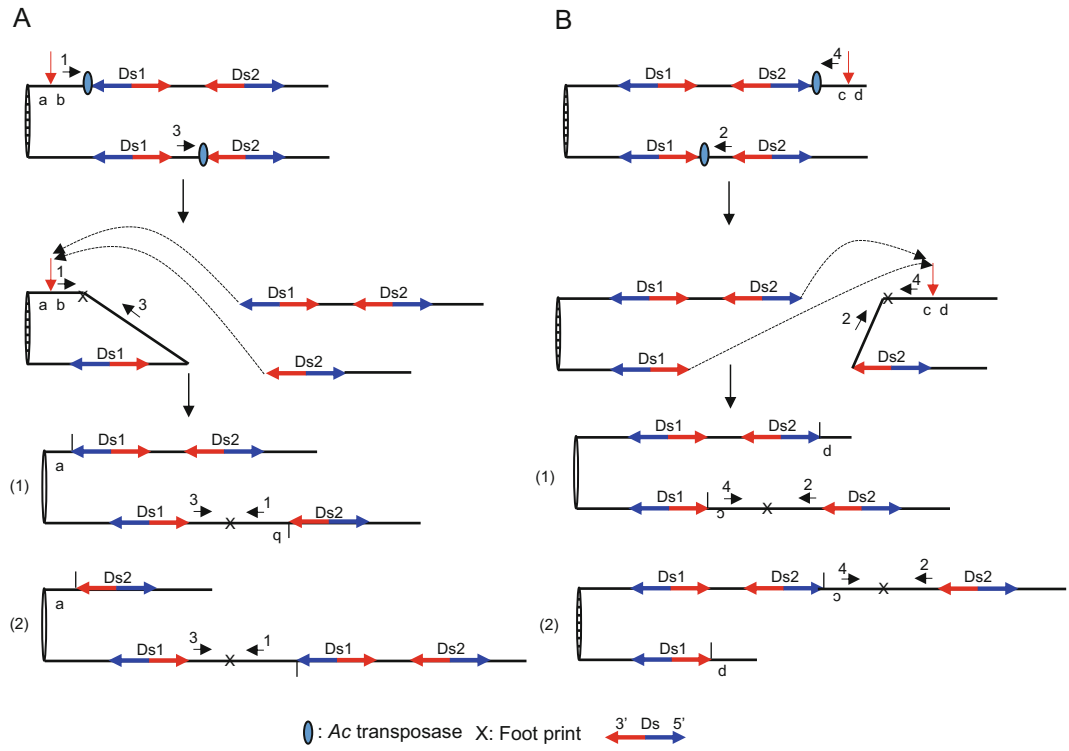

Fig. 3 Models for SCT. (**a**) SCT with proximal target site is depicted in two steps. In all the diagrams, sister chromatids are attached at the centromere (*left*). Transposases cut the 5′ end of *Ds1* on the upper chromatid and the 3′ end of *Ds2* on the lower chromatid, as indicated by the *blue oval* in the *top* diagram. The two ends are then reinserted into a new target site between *a* and *b*, as shown by the *dotted arrows* in the second diagram. A *red vertical arrow* indicates the new target site. Part (1) of the third diagram shows that insertion of the 5′ end of *Ds1* next to *a* and the 3′ end of *Ds2* next to *b* generates one chromatid (*upper*) containing a flanking proximal deletion and two copies of *Ds*, and a second chromatid (*lower*) containing an inverted duplication and two copies of *Ds*. Note both outcomes result in inversion of sequences flanking *Ds*. Part (2) of the fourth diagram shows that insertion of the 3′ end of *Ds2* next to *a* and the 5′ end of *Ds1* next to *b* generates one chromatid (*upper*) containing a flanking proximal deletion and a single copy of *Ds*, and a second chromatid (*lower*) containing an inverted duplication and three copies of *Ds*. (**b**) *Ac* transposase cuts at the 5′ end of *Ds2* on the upper chromatid and the 3′ end of *Ds1* on the lower chromatid. The transposition target site between c and d is indicated by the *red vertical arrow*. Parts (1) and (2) indicate two possible outcomes depending on the orientation of 5′ and 3′ *Ds* termini upon insertion: (1) Insertion of the 5′ end of *Ds2* next to *d* and the 3′ end of *Ds1* next to *c* generates one chromatid (*upper*) containing a flanking proximal deletion and two copies of *Ds*, and a second chromatid (*lower*) containing an inverted duplication and two copies of *Ds*. (2) Insertion of the 5′ end of *Ds2* next to *c* and the 3′ end of *Ds1* next to *d* generates one chromatid (*lower*) containing a flanking proximal deletion and a single copy of *Ds*, and a second chromatid (*upper*) containing an inverted duplication and three copies of *Ds*. *Blue* and *red arrows* indicate the 5′ and 3′ directions of *Ds* elements, respectively. *X* indicates a footprint. Primers used to detect rearrangements were shown as *horizontal arrows with numbers*

methylation [13]. In vitro binding assays have shown that *Ac* transposase binds more strongly to *Ds* ends with coding-strand methylation than to ones with noncoding-strand methylation [14]. This implies that the transposition competent termini are located in different sister chromatids. Therefore, the 5′ and 3′ termini of

Table 1
Primer sequences

Primers	Sequences
Primer 1	5′-CTTATTCCAGAAGACTTG-3′
Primer 2	5′-AGATACCAGTAGCTAAAG-3′
Primer 3	5′-ATCAACGAGTGCCTAATG-3′
Primer 4	5′-CTTGGTTCTATTCCATTG-3′

two different *Ds* elements on the same chromatid might be not competent for alternative transposition. However, methylation is reduced at the *Ds* termini during regeneration [8], which raises the possibility that two different *Ds* elements on the same chromatid may be able to participate in alternative transposition. Single chromatid transposition (SLCT) events takes place when *Ac* transposase recognizes the 5′ and 3′ termini of two different *Ds* elements on the same chromatid. SLCT generates inversions and deletions on the same chromatid [15]. The first DNA rearrangement expected during SLCT is inversion of ITS, which can be detected by primer sets 1 + 3 or 2 + 4 that recognized *Ds1* and ITS or *Ds2* and ITS, respectively (Table 1, Fig. 4). The primers are oriented in the same direction. Based on the PCR data, among the 300 regenerated plants tested, eight (2.7%) induced SLCT events. One plant carried inversion while seven plants contained deletions.

If two inversely-oriented *Ds* elements are aligned and paired, homologous recombination (HR) may occur between the *Ds* elements, resulting in inversion of the ITS. To detect HR, primer sets 1 + 3 or 2 + 4 that recognized *Ds1* and ITS or *Ds2* and ITS, respectively (Fig. 5), were utilized for PCR. The primers were oriented in the same direction. In 33 plants (11%), HR was detected. In conclusion, tissue culture regeneration induced exceptionally high frequency of chromosomal rearrangements and diverse alternative transposition mechanisms were able to be identified in *OsRLG5-161* derivatives.

SCT and SLCT generate segmental deletions by reinsertion of *Ds* termini to either the proximal or distal regions of *OsRLG5*. To detect deletions in the proximal and distal regions, a series of primers that were derived from highly conserved regions of the *OsRLG* gene cluster were utilized. These primers could be paired with primers specific to *OsRLG5* or to *Ds* termini. PCR with these primer combinations enabled the detection of deletion breakpoints in the lines carrying ITS inversion. As described above, plants carrying ITS inversions could be identified by PCR using primer pairs 1 + 3 or 2 + 4 (Table 1; Figs. 3 and 4). Using this strategy, 28 deletions (9.3%) were identified from the regenerated population of *OsRLG5-161*. Deletions ranged in size from 184 bp to 520 kb. All the deletions originated from the *OsRLG5* locus.

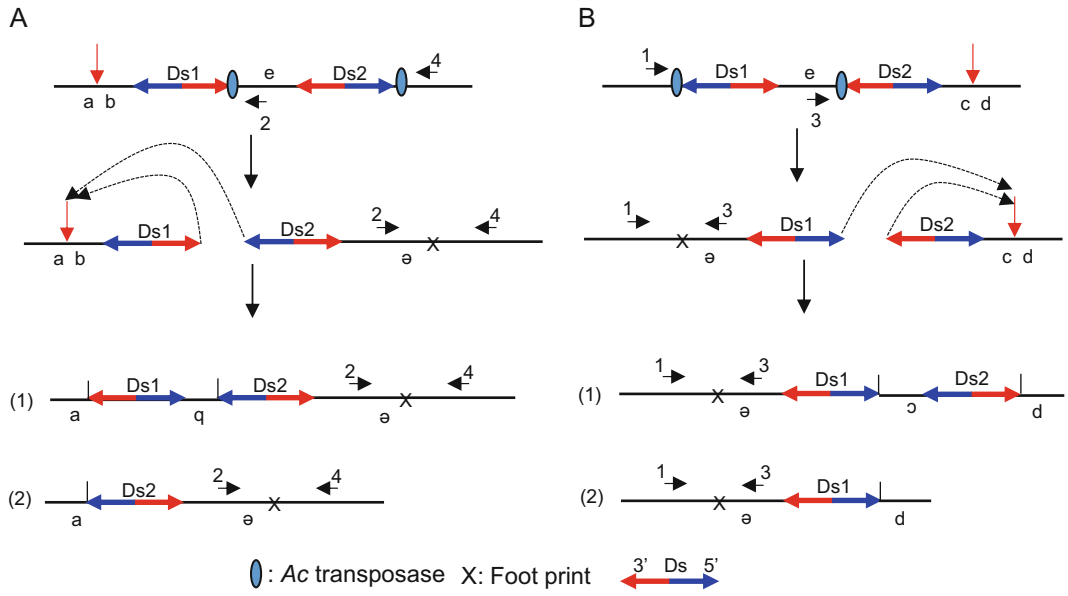

Fig. 4 Models for SLCT. (**a**) The inversion/deletion process derived from SLCT is depicted in three steps. Transposases (*blue ovals* in *top diagram*) cut the 3′ end of *Ds1* and the 5′ end of *Ds2*. The 3′ and 5′ termini of *Ds1* and *Ds2*, respectively, are reinserted into the proximal region with respect to the original *Ds* sites, as shown by the *dotted arrows* and *red vertical arrow* in the second diagram. Consequently, the fragment from the reinsertion site on the 3′ end of *Ds1* was inverted and was joined to the 5′ end of *Ds2*. Reinsertion of the 3′ and 5′ termini of *Ds1* and *Ds2*, respectively, at the target site between *a* and *b* leads to two configurations, as shown in parts (1) and (2) in the diagram. Part (1) shows inversions of fragment carrying *b* located between 5′ end of *Ds1* and the 5′ end of *Ds2*, and fragment *e* was inverted. Part (2) indicates deletion, including fragments carrying *b* and *Ds1*. (**b**) Transposases (*blue ovals*) cut the 5′ end of *Ds1* and the 3′ end of *Ds2*. The 5′ and 3′ termini of *Ds1* and *Ds2*, respectively, are reinserted into the distal region with respect to the original *Ds* sites, as shown by the *dotted arrows* and *red vertical arrow* in the second diagram. Consequently, the fragment from the reinsertion site on the 5′ end of *Ds1* was inverted and was joined to the 3′ end of *Ds2*. Reinsertion of the 5′ and 3′ termini of *Ds1* and *Ds2*, respectively, at the target site between *c* and *d* leads to two configurations, as shown in parts (1) and (2) in the diagram. Part (1) shows inversions of fragment carrying *c* located between *d* and the 5′ end of *Ds2*, and fragment *e* was inverted. Part (2) indicates deletion, including fragments carrying *c* and *Ds2*. *Blue* and *red arrows* indicate the 5′ and 3′ directions of *Ds* elements, respectively. *X* indicates a footprint. Primers used to detect rearrangements were shown as *horizontal arrows with numbers*

In the rice genome, 29% of the predicted genes are reported to be organized in clustered gene families The Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). It is challenging to determine the biological roles of these gene clusters due to their functional redundancy. Point or insertional mutations are often not sufficient to define the functions of clustered gene families. Therefore, generation of overlapping deletions should be a powerful strategy to explore the biological functions of clustered genes. In our study, SCT and SLCT were able to generate deletions of diverse sizes ranging from 184 bp up to 520 kb. Some large deletions were unable to be maintained as homozygotes. However, we identified two homozygous mutants that contained 85 and 124 kb proximal deletions.

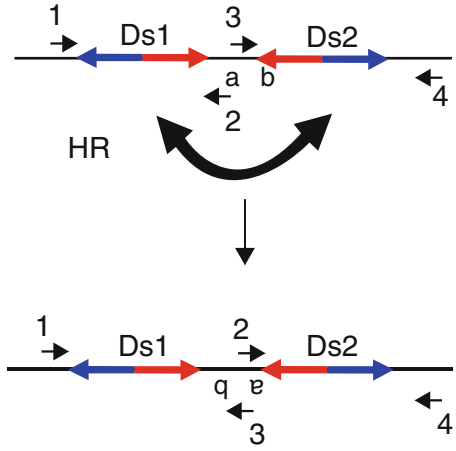

Fig. 5 Inversion induced by HR. The inversion process derived from HR between *Ds* elements is depicted in two steps. In the first diagram, the intertransposon segment (ITS) is denoted by "a" and "b." Inversion of the ITS ("a" and "b") is shown in the second diagram. Primers used to detect rearrangements were shown as *horizontal arrows with numbers*

These deletion mutants showed a leaf necrosis phenotype [16]. In fact, the genes of the *OsRLG* cluster are highly homologous to a wheat putative rust resistance kinase gene *Lr10* (*Leaf rust resistance 10*) [17]. The necrotic leaf phenotypes exhibited by the *Ds*-mediated deletion lines suggest the possibility that the *RLK* cluster might perform a similar pathogen-related function as the *Lr10* homolog does.

In summary, tissue culture regeneration not only activates standard *Ac/Ds* transposition but also increases the frequency of alternative transposition, which facilitates the production of serial deletion mutants in a relatively short period. The *Ac/Ds* system provides an excellent tool for exploring the functions of clustered gene families that are prevalent in rice genomes.

2 Materials

2.1 Tissue Culture for Regeneration of Plants

To produce plantlets from seed-derived calli, sequential incubations are performed using four types of tissue culture media [8, 18]: NB medium for callus induction, N6-7-CH medium for Pre-Regeneration, N6S3-CH-I medium for Regeneration I (*see* **Note 1**), and N6S3-CH-II medium for Regeneration II. Culture media are best when they have been prepared and left at room temperature (RT) for 2 days before use. Culture media can also be used if they are kept at 4 °C for up to 2 months.

1. Seeds: dry mature *OsRLG5:Ds* and *OsRLG5-161* seeds; sterilization solution: 1.2% sodium hypochlorite, one or two drops of TritonX-100.

2. NB medium: 5% (v/v) N6 macro-elements 20× stock (for 1 L 20× stock: 56.6 g KNO_3, 9.26 g $(NH_4)_2SO_4$, 8 g KH_2PO_4, 1.8 g $MgSO_4$, 3.3 g $CaCl_2{\cdot}2H_2O$), 1% (v/v) B5 micro-elements 100× stock (for 1 L 100× stock: 1 g $MnSO_4{\cdot}4H_2O$, 200 mg $ZnSO_4{\cdot}7H_2O$, 75 mg KI, 25 mg $NaMoO_4{\cdot}2H_2O$, 300 mg H_3BO_3, 2.5 mg $CuSO_4{\cdot}H_2O$, 2.5 mg $CoCl_2{\cdot}6H_2O$), 0.1% (v/v) B5 vitamin 1000× stock (for 1 mL 1000× stock: 100 mg myo-inositol, 1 mg nicotine acid, 1 mg pyridoxine·HCl, 10 mg thiamine·HCl), 0.11 mM Fe-EDTA in MS medium, 9 μM 2,4-D, 87.6 mM sucrose, 4.3 mM L-proline, 3.4 mM L-glutamine, and 0.03% (v/v) casein enzymatic hydrolysate. Adjust pH to 5.8 and add 2.5 g/L phytagel.
3. N6-7-CH medium: 5% (v/v) N6 macro-elements 20× stock (for 1 L 20× stock: 56.6 g KNO_3, 9.26 g $(NH_4)_2SO_4$, 8 g KH_2PO_4, 1.8 g $MgSO_4$, 3.3 g $CaCl_2{\cdot}2H_2O$), 1% (v/v) B5 micro-elements 100× stock (for 1 L 100× stock: 1 g $MnSO_4{\cdot}4H_2O$, 200 mg $ZnSO_4{\cdot}7H_2O$, 75 mg KI, 25 mg $NaMoO_4{\cdot}2H_2O$, 300 mg H_3BO_3, 2.5 mg $CuSO_4{\cdot}H_2O$, 2.5 mg $CoCl_2{\cdot}6H_2O$), 0.1% (v/v) B5 vitamin 1000× stock (for 1 mL 1000× stock: 100 mg myo-inositol, 1 mg nicotine acid, 1 mg pyridoxine·HCl, 10 mg thiamine·HCl), 0.11 mM Fe-EDTA in MS medium, 9 μM 2,4-D, 61.6 mM sucrose, 16.5 mM sorbitol, and 0.2% (v/v) casein enzymatic hydrolysate. Adjust pH to 5.8 and add 2.5 g/L phytagel. After autoclaving, add 0.5 mg/L 6-benzyladenine.
4. N6S3-CH-I medium: 5% (v/v) N6 macro-elements 20× stock (for 1 L 20× stock: 56.6 g KNO_3, 9.26 g $(NH_4)_2SO_4$, 8 g KH_2PO_4, 1.8 g $MgSO_4$, 3.3 g $CaCl_2{\cdot}2H_2O$), 1% (v/v) B5 micro-elements 100× stock (for 1 L 100× stock: 1 g $MnSO_4{\cdot}4H_2O$, 200 mg $ZnSO_4{\cdot}7H_2O$, 75 mg KI, 25 mg $NaMoO_4{\cdot}2H_2O$, 300 mg H_3BO_3, 2.5 mg $CuSO_4{\cdot}H_2O$, 2.5 mg $CoCl_2{\cdot}6H_2O$), 0.1% (v/v) B5 vitamin 1000× stock (for 1 mL 1000× stock: 100 mg myo-inositol, 1 mg nicotine acid, 1 mg pyridoxine·HCl, 10 mg thiamine·HCl), 0.11 mM Fe-EDTA in MS medium, 9 μM 2,4-D, amino acids (amino acids for 1 L medium: 877 mg glutamine, 228 mg arginine, 75 mg glycine, 266 mg aspartic acid), 61.6 mM sucrose, 16.5 mM sorbitol, and 0.2% (v/v) casein enzymatic hydrolysate. Adjust pH to 5.8 and add 5 g/L phytagel. After autoclaving, add 1 mg/L naphthalene acetic acid and 5 mg/L kinetin.
5. N6S3-CH-II medium: 5% (v/v) N6 macro-elements 20× stock (for 1 L 20× stock: 56.6 g KNO_3, 9.26 g $(NH_4)_2SO_4$, 8 g KH_2PO_4, 1.8 g $MgSO_4$, 3.3 g $CaCl_2{\cdot}2H_2O$), 1% (v/v) B5 micro-elements 100× stock (for 1 L 100× stock: 1 g $MnSO_4{\cdot}4H_2O$, 200 mg $ZnSO_4{\cdot}7H_2O$, 75 mg KI, 25 mg $NaMoO_4{\cdot}2H_2O$, 300 mg H_3BO_3, 2.5 mg $CuSO_4{\cdot}H_2O$, 2.5 mg

$CoCl_2 \cdot 6H_2O$), 0.1% (v/v) B5 vitamin 1000× stock (for 1 mL 1000× stock: 100 mg myo-inositol, 1 mg nicotine acid, 1 mg pyridoxine·HCl, 10 mg thiamine·HCl), 0.11 mM Fe-EDTA in MS medium, 9 μM 2,4-D, amino acids (amino acids for 1 L medium: 877 mg glutamine, 228 mg arginine, 75 mg glycine, 266 mg aspartic acid), 87.6 mM sucrose, 16.5 mM sorbitol, and 0.2% (v/v) casein enzymatic hydrolysate. Adjust pH to 5.8 and add 5 g/L phytagel. After autoclaving, add 0.5 mg/L naphthalene acetic acid and 2 mg/L kinetin.

2.2 Genomic DNA Extraction

1. Lysis buffer: 350 mM NaCl, 100 mM Tris–HCl (pH 7.6), 7 M urea, 50 mM EDTA, 2% SDS.
2. Phenol:chloroform:isoamyl alcohol, 25:24:1.

   Phenol: analytical quality phenol is melted at 60 °C and mixed with 1 g 8-hydroxyquinoline and 0.1 M Tris–HCl, pH 8.0. The solution is mixed by shaking and maintained at 4 °C until the two phases are completely separated. The supernatant is discarded. Neutralization is repeated several times with 1 volume of 0.1 M Tris–HCl (pH 8.0) until the pH of the phenol is close to 7.0.
3. Isopropanol.
4. 70% Ethanol.
5. TE: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 7.5).
6. 10 g/L RNase.

2.3 Primers for PCR Reactions

The primers used to analyze the rearrangement events are listed in Table 1.

2.4 Southern Blot Analysis

1. Restriction enzyme: *Sac*I, *Eco*RI.
2. Denaturation solution: 1.5 M NaCl, 0.5 M NaOH.
3. Neutralization solution: 1 M Tris–HCl (pH 8.0), 1.5 M NaCl.
4. Nylon membrane.
5. Prehybridization solution: 6× SSC, 5× Denhardts solution, 0.5% SDS, 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.1 mg/mL salmon sperm DNA, 5% dextran sulfate.
6. Probe synthesis: 1× A buffer (90 mM HEPES (pH 6.6), 10 mM $MgCl_2$), 1× B buffer (2 mM DTT, 3 mM dNPT (-dATP), 0.2 mM BSA).
7. Isotope: ^{32}P-dATP.
8. Washing solution I: 2× SSC, 0.1% SDS.
9. Washing solution II: 0.2× SSC, 0.1% SDS.
10. Detection agent: 1× developer, 1× fixer, X-ray film.

3 Methods

3.1 Tissue Culture for Transgenic Plants

1. Dry mature seeds are shaken in the sterilization solution for 30–40 min at RT.
2. Seeds are washed with autoclaved dH_2O 15–20 times every 2 min (*see* **Note 2**).
3. Seeds are dried on paper towels to remove water, and then dried for 30 min inside a clean-bench hood.
4. Sterilized seeds are sown on NB media for callus induction, which takes 4 weeks in the dark.
5. Calli are transferred onto N6-7-CH medium for 10 days in the dark (*see* **Note 3**).
6. After 10 days of preregeneration, calli are transferred to N6S3-CH-I medium and maintained for 1 month under a 16/8 h light/dark cycle (*see* **Note 4**).
7. After green spots are well developed, calli are transferred onto N6S3-CH-II medium and maintained for an additional 1 month under a 16/8 h light/dark cycle.
8. Before being transferred to the greenhouse, regenerated plants are transplanted into bottles that contain half-strength MS media (*see* **Note 5**).

3.2 Preparation to Grow Rice Plants in Paddy Field

Daily high and low temperatures in a greenhouse at Gyeongsang National University were typically 30 °C during the day and 20 °C at night. For vegetative growth, the light/dark cycle in the greenhouse was 14/10 h. To induce flowering, the light period was reduced to 10 h. The following procedures were used for seed germination and seedling culture before transplantation into the outdoor paddy fields:

1. Seedling trays are filled with soil and watered until completely wet.
2. Seeds are sown in rows and covered with 5-mm-deep dry soil.
3. Seedling trays are covered with dark plastic films and maintained at about 30 °C until coleoptiles emerge 10–15 mm long from the surface.
4. Trays are transferred to wet beds and covered with white light cotton fabrics until coleoptiles turn completely green.
5. Young seedlings are nurtured to 10–15 cm with occasional watering (never being flooded).
6. Trays are moved to seedling beds in the paddy field and submerged to less than one-third of the plant height.
7. Seedlings are grown until the three- or four-leaf stage before being transplanted.

3.3 Genomic DNA Extraction

1. A leaf piece about 5 cm long is frozen in liquid nitrogen and ground with a mortar and pestle (glass pestle or machine) into powder.
2. 750 μL of lysis buffer are added and vortexed well, and the solution is maintained at 37 °C for 20 min with shaking.
3. An equal volume of PCI (phenol:chloroform:isoamyl alcohol) is added to the sample solution and vortexed vigorously.
4. The upper (aqueous) phase is saved after a 10-min centrifugation.
5. PCI extraction is repeated one or two times.
6. Either 0.6 or the same volume of isopropanol is added to the supernatant and kept at RT or −20 °C for 10 min.
7. DNA is pelleted by 15 min centrifugation.
8. DNA pellets are washed with 75% EtOH, centrifuged for 5 min, and the EtOH is discarded.
9. Air-dried DNA pellets are dissolved with 30–50 μL TE-RNase and kept at RT overnight or at 37 °C for 1 h.

3.4 PCR Reactions

Taq DNA polymerase and dNTPs were used for PCR with 500 ng of genomic DNA as a template. Annealing temperatures in the range of 55–58 °C were used, depending on primer sequences. A typical reaction consisted of an initial denaturation at 95 °C for 3 min, followed by 33 cycles of denaturation for 30 s, annealing for 30 s, and extension at 72 or 65 °C for 30–90 s, followed by a final extension at 72 °C for 7 min.

3.5 Southern Blot Analysis

1. 5 μg genomic DNA was digested with *Sac*I or *Eco*RI and separated on 0.8% agarose gel for 12–16 h at 20 V.
2. The gel was stained with EtBr for 10 min, and destained with sterilized water for 15 min.
3. After being photographed, the gel was denatured in a denaturation solution for 40 min.
4. The gel was rinsed with water, and then the gel was neutralized with a neutralization solution for 40 min.
5. The gel was overlaid with a Nylon membrane, being careful to avoid bubbles, in a tank containing 10× SSC solution. Paper towels and a light weight were placed on top of the membrane to enhance fluid movement and DNA transfer.
6. After 18–24 h, the damp membrane was UV-cross linked for 2 min, rinsed in distilled water for 1 min, and then air dried.
7. The dried membrane was put into a prehybridization bag, prehybridization solution was added, and the bag kept at 65 °C for 3 h.

8. ^{32}P-dATP-labeled probe DNA was made using template DNA fragment, random primers, and Klenow DNA polymerase for 1 h.
9. Synthesized probe DNA was added into prehybridization bag and hybridized with membrane at 65 °C for 12–14 h.
10. The membrane was washed with washing solution I for 30 min, and then washed with washing solution II for 5–10 min at 65 °C.
11. The membrane was exposed to X-ray film for 1–3 days at –80 °C.

4 Notes

1. For plant regeneration, N6S3-CH-I medium should contain 5 g/L of phytagel. If less phytagel is used, plates will be overly moist, preventing further development of green calli.
2. Air-drying of seeds after surface sterilization may help prevent cross-contamination from seeds that are not completely sterilized. When treating with sodium hypochlorite solutions, sterilization efficiency can be enhanced by treating aliquots of 100 seeds in separate vessels.
3. Usually, calli were grown on N6-7-CH for 10 days before transfer to N6S3-CH-I. However, sometimes calli failed to generate green spots on N6S3-CH-I medium, even though the calli appeared to be healthy. In such cases, the incubation time on N6-7-CH medium can be extended up to 14–15 days, which helps to increase the regeneration frequency on N6S3-CH-I.
4. It is recommended that calli of less than 1 mm in diameter be transferred to regeneration medium. This may reduce the chance of producing multiple plants carrying the same *Ds* insertion event from the same calli.
5. Two or 3 days before transfer to soil, an appropriate amount of water is added to the bottles, which greatly enhances survival of plantlets on soil.

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References

1. Gorbunova V, Levy AA (2000) Analysis of extrachromosomal *Ac/Ds* transposable elements. Genetics 155:349–359
2. Gorbunova V, Levy AA (1997) Circularized *Ac/Ds* transposons: formation, structure and fate. Genetics 145:1161–1169
3. Chin HG, Choe MS, Lee SH, Park SH, Koo JC, Kim NY et al (1999) Molecular analysis of rice plants harboring an *Ac/Ds* transposable element-mediated gene trapping system. Plant J 19:615–623
4. Izawa T, Ohnishi T, Nakano T, Ishida N, Enoki H, Hashimoto H et al (1997) Transposon tagging in rice. Plant Mol Biol 35:219–229
5. Weil CF, Wessler SR (1993) Molecular evidence that chromosome breakage by *Ds* elements is caused by aberrant transposition. Plant Cell 5:515–522
6. Krishnaswamy L, Zhang J, Peterson T (2008) Reversed end *Ds* element: a novel tool for chromosome engineering in Arabidopsis. Plant Mol Biol 68:399–411
7. Yu C, Han F, Zhang J, Birchler J, Peterson TA (2012) Transgenic system for generation of transposon *Ac/Ds*-induced chromosome rearrangements in rice. Theor Appl Genet 125:1449–1462
8. Ki CM, Je BI, Piao HL, Par SJ, Kim MJ, Park SH et al (2002) Reprogramming of the activity of the *activator/dissociation* transposon family during plant regeneration in rice. Mol Cells 14:231–237
9. Kim CM, Piao HL, Park SJ, Chon NS, Je BI, Sun B et al (2004) Rapid, large-scale generation of *Ds* transposant lines and analysis of the *Ds* insertion sites in rice. Plant J 39:252–263
10. Zhang J, Peterson TA (1999) Genome rearrangements by nonlinear transposons in maize. Genetics 153:1403–1410
11. Zhang J, Peterson TA (2005) Segmental deletion series generated by sister-chromatid transposition of *Ac* transposable elements in maize. Genetics 171:333–344
12. English JJ, Harrison K, Jones J (1995) Aberrant transpositions of maize double *Ds*-like elements usually involve *Ds* ends on sister chromatids. Plant Cell 7:1235–1247
13. Wang L, Heinlein M, Kunze R (1996) Methylation pattern of *Activator* transposase binding sites in maize endosperm. Plant Cell 8:747–758
14. Ros F, Kunze R (2001) Regulation of *activator/dissociation* transposition by replication and DNA methylation. Genetics 157:1723–1733
15. Xuan YH, Piao HL, Je BI, Park SJ, Park SH, Huang J et al (2011) Transposon *Ac/Ds*-induced chromosomal rearrangements at the rice *OsRLG5* locus. Nucleic Acids Res 39:e149
16. Xuan YH, Zhang J, Peterson T, Han CD (2012) *Ac/Ds*-induced chromosomal rearrangements in rice genomes. Mob Genet Elem 2:67–71
17. Feuillet C, Schachermayr G, Keller B (1997) Molecular cloning of a new receptor-like kinase gene encoded at the *Lr10* disease resistance locus of wheat. Plant J 11:45–52
18. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Chapter 5

One-Step Generation of Chromosomal Rearrangements in Rice

Minoru Murata, Asaka Kanatani, and Kazunari Kashihara

Abstract

The combination of the DNA sequence-specific recombination system Cre/*LoxP* and the DNA transposon system *Activator* (*Ac*)/*Dissociation* (*Ds*) has been used for insertional and deletional mutagenesis, as well as for the generation of artificial ring chromosomes in model plants such as Arabidopsis and tobacco. However, it takes a long time to complete this process, even in Arabidopsis. To overcome this issue, a new binary vector, pDLHC, has been developed to induce chromosomal rearrangements for a short time in rice. pDLHC has been found to be effective in the induction of deletions between two *LoxP*s in the T2 generation of "Nihon bare" expressing *Ac* TPase. pDLHC has potential for the efficient generation of various types of chromosomal rearrangements including deletions, inversions, translocations and artificial ring chromosomes in plants, and the detailed protocol for rice is described here.

Key words Cre/*LoxP*, *Oryza sativa*, *Ac*/*Ds* transposons, Chromosome rearrangements

1 Introduction

The combination of the DNA sequence-specific recombination system Cre/*LoxP* and the DNA transposon system *Activator* (*Ac*)/*Dissociation* (*Ds*) was originally developed for insertional and deletional mutagenesis in tobacco [1] and in Arabidopsis [2, 3]. These techniques are quite useful for manipulating genomes and chromosomes and were recently applied to generate artificial ring chromosomes in Arabidopsis [4]. However, it takes a considerably long time to complete this process, even in *Arabidopsis thaliana*. The protocol consisted mainly of three steps: (1) the production of transgenic plants carrying a single copy or a few copies of T-DNA from pDs-Lox [2] or its equivalent binary vectors; (2) the introduction of the *Ac* TPase gene into the plants selected at step 1 by crossing, inducing the transposition of the Ds-*LoxP*-Ds (DLD) cassette and selecting for hygromycin resistance; and (3) the induction of recombination between the two *LoxP* sites on the same chromosome by introducing the Cre gene into the hygromycin-resistant plants. The

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first step requires at least 3 months to generate the possible candidates, and it takes 3 more months to obtain the F1 seeds following the cross between the selected transgenic plants and the *Ac*-derived transposase (TPase)-expressing plants.

To overcome this issue and accelerate this process, we have developed a simpler method for inducing chromosomal rearrangements: deletions, inversions, translocations and/or artificial ring chromosomes. This protocol consists of two steps: (1) the production of transgenic plants carrying a single copy or a few copies of T-DNA from a newly developed binary vector (Fig. 1); and (2) the introduction of the *Ac* TPase gene into the plants selected in step 1 by crossing, which would induce the transposition of the DLD cassette. This transposition results in the fusion of a promoter and a Cre recombinase gene. Subsequently, the expressed Cre recombinase is expected to induce recombination between the two *LoxP* sites, resulting in fusion between a promoter and the hygromycin-resistance gene. If the transformation at step 1 is performed directly in the plants expressing *Ac* TPase, the introduction of the *Ac* TPase gene by crossing could be skipped, meaning that only one step, "transformation of the *Ac* TPase-expressing plants," is required for chromosomal rearrangements induced by recombination between two *LoxP*s. The seeds obtained in the final step are designed to express hygromycin resistance if recombination occurs between two *LoxP* sites.

This one-step generation of chromosomal changes would be applicable in various types of plant species, but in this chapter, the application in rice plants is described in detail.

2 Materials

2.1 Plant Materials

1. Seeds of *Oryza sativa* cv. Nipponbare expressing the maize-derived *Ac* TPase gene [5], a gift from Dr. Kurata (Natl. Inst. Genet., Mishima, Japan).

2.2 Agrobacterium Strain and the T-DNA Vector

(A) The use of *A. tumefaciens* EHA105 is preferred for rice transformation.

2.2.1 Agrobacterium Strain

2.2.2 Binary Vector

A T-DNA vector, pDLHC (Fig. 1), was constructed for the one-tep induction of chromosomal rearrangements in rice; it contains the hygromycin-resistant gene (*HYG*), Cre recombinase gene (*CRE*), in which an intron has been inserted into the coding sequence from the first intron of the castor bean catalase gene [6] (*see* **Note 1**), and the terminal repeats of the maize *Dissociation* (*Ds*) element. The backbone of this construct was the binary

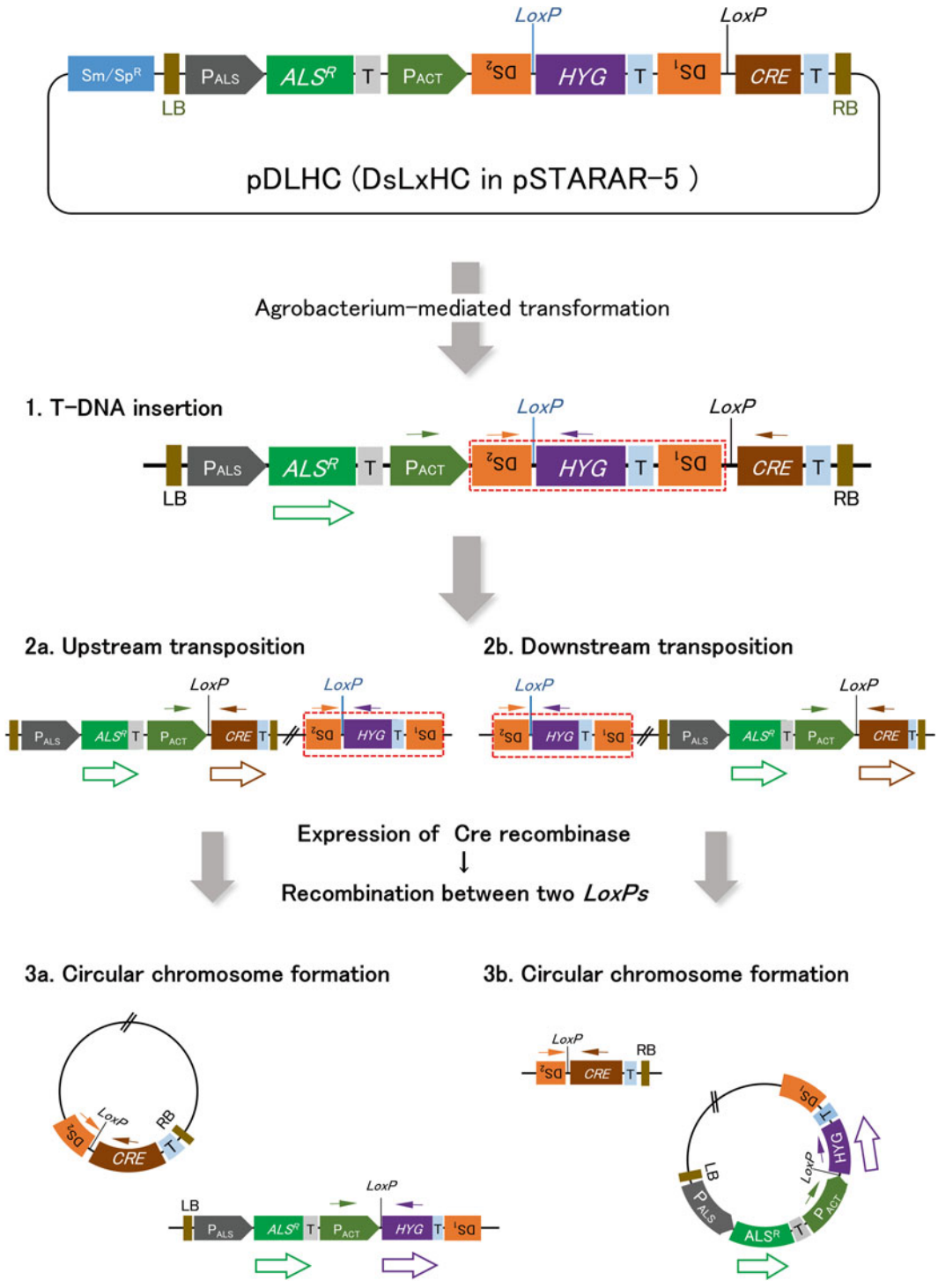

Fig. 1 Schematic representation of the binary vector pDLHC and an outline of the process from T-DNA integration to Cre-mediated recombination resulting in circular DNA formation. *ALSR* rice acetolactate synthase gene with two SNPs, *HYG* hygromycin-resistance gene, *CRE* Cre recombinase gene with an intron, *PALS* rice ALS promoter, *PACT* rice actin promoter, *T* terminator, *DS1* and *DS2* terminal repeats of the maize *Dissociation* element, *LB* and *RB* left border and right border sequences, respectively; and *red dotted box*: mobile DLD cassette. *Open arrows* indicate the gene expression of *ALSR* (*green*), *CRE* (*brown*), and *HYG* (*purple*). *Small arrows* indicate the primers for PCR analysis: PactH-F (*moss green*), Cre-R3 (*brown*), and R-HYG (*purple*)

vector pSTARA-R5 [7], which contains the mutated acetolactate synthase (ALS) gene, including the promoter and terminator for conferring resistance to ALS-inhibiting herbicides (*see* **Note 2**). This construct also contains a rice actin promoter (Pact) (*see* **Note 3**). Terminal inverted repeats (Ds1 and Ds2) were amplified from pED204 [1] and *CRE* from pCIP54 [8]. These constructs were gifts from Drs. Ow (Univ. of California, Berkley, USA) and Ghislain (Intern. Potato Center, Peru), respectively.

2.2.3 Stock Solutions

1. 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.2 mg/ml). Store at 4 °C.
2. Alpha-naphthalene acetic acid (NAA) (0.2 mg/ml). Store at 4 °C.
3. Kinetin (0.2 mg/ml). Store at 4 °C.
4. Acetosyringone (1-(4-hydroxy-3,5-dimethoxyphenyl) ethanone) (10 mg/ml). Dissolve in DMSO and store at 4 °C in the dark.
5. Spectinomycin (100 mg/ml). Filter sterilize and store at −20 °C.
6. Hygromycin B (50 mg/ml). Filter sterilize and store at −20 °C.
7. Bispyribac sodium (0.25 mM). Filter sterilize and store at −20 °C.
8. Meropenem hydrate (Meropen) (25 mg/ml). Filter sterilize and store at −20 °C.
9. Vancomycin (100 mg/ml). Filter sterilize and store at −20 °C.
10. D-(+)-Glucose solution (10% w/v). Autoclave before use.

2.2.4 Media for Callus Formation

1. N6D medium: Add 4.0 g/l Chu (N_6) basal salt mixture (e.g., Sigma-Aldrich); 1 ml of N6 vitamin (1000×) containing 2 mg/ml Glycine, 0.5 mg/ml Nicotinic acid, 0.5 mg/ml Pyridoxine-HCl, 1 mg/ml Thiamine-HCl, and 500 mg/ml myo-Inositol; 300 mg/l Casamino acids; 30 g/l Sucrose; 2.9 g/l (25 mM) Proline; and 10 ml of 2,4-D stock solution (final conc. 2 mg/l) to 700 ml of Milli-Q water. Adjust the pH to 5.8 with 2 N KOH and add Milli-Q water up to 1 l with 4 g/l Gelrite. Autoclave and cool to approximately 60 °C. For the selection of transformants, add an antibiotic solution, 1 ml of 25 mg/l Meropenem hydrate and 1 ml of 0.25 mM bispyribac sodium.
2. 2N6-AS medium: Add 4.0 g/l Chu (N6) basal salt mixture (e.g., Sigma-Aldrich); 1 ml of N6 vitamin (1000×) containing 2 mg/ml Glycine, 0.5 mg/ml Nicotinic acid, 0.5 mg/ml Pyridoxine-HCl, 1 mg/ml Thiamine-HCl and 500 mg/ml myo-Inositol; 300 mg/l Casamino acids, 30 g/l Sucrose; and 10 ml of f 2,4-D stock solution (final conc. 2 mg/l) to 700 ml Milli-Q water. Adjust the pH to 5.2 with 2 N KOH and add Milli-Q water up to 0.9 l with 4 g/l Gelrite. Autoclave and

cool to approx. 60 °C. Then, add 100 ml of Glucose solution and 100 μl of Acetosyringone (final conc. 10 mg/l).

2.2.5 Agrobacterium Culture

1. AB medium: To 700 ml of Milli-Q water in a 1-l beaker, add 3 g/l K_2HPO_4, 1.3 g/l $NaH_2PO_42H_2O$, 1 g/l NH_4Cl, 0.15 g/l KCl, 10 mg/l $CaCl_22H_2O$, and 2.5 mg/l $FeSO_47H_2O$. Adjust the pH to 7.2 with 0.2 N KOH and add Milli-Q water up to 0.9 l with 15 g of Bacto agar. Autoclave and cool to approx. 60 °C; then, add 100 ml of 5 % (w/v) glucose solution, 1.2 ml of 1 M $MgSO_47H_2O$, and 1 ml of 100 mg/ml spectinomycin.
2. AAM medium: To 700 ml of Milli-Q water, add 0.3 g of L-Aspartic acid and 1 ml each of AA1 (75 mg of KI, 2.5 mg of $CoCl_26H_2O$, 2.5 mg of $CuSO_45H_2O$, 25 mg of $Na_2MoO_42H_2O$, 200 mg of $ZnSO_44H_2O$, 1 g of $MnSO_46H_2O$/100 ml), AA2 (15 g of $CaCl_22H_2O$/100 ml), AA3 (25 g of $MgSO_47H_2O$/100 ml), AA4 (4 g of Fe-EDTA/100 ml), AA5 (15 g of $NaH_2PO_42H_2O$/100 ml) and AA6 (10 mg of Nicotinic acid, 10 mg of Pyridoxine-HCl, 100 mg of Thiamine-HCl, and 1 g of myo-Inositol/10 ml), 10 ml of AA7 (1.77 g of L-Arginine and 75 mg of Glycine/100 ml), and 20 ml of AA8 (15 g of KCl/100 ml). Add 0.5 g of Casamino acids, 68.5 g of Sucrose and 0.9 g of L-Glutamine. Adjust the pH to 5.2 with 0.2 N KOH and add Milli-Q water up to 0.9 l. After autoclaving, cool to RT and add 100 ml of 36 % Glucose solution (final conc. 3.6 % (w/v)). Dispense approx. 80 ml into 100-ml Erlenmeyer flasks. Store at 4 °C.

2.2.6 Regeneration Media

1. MS-R medium: Add 4.4 g/l MS basal salt mixture (e.g., Sigma-Aldrich); 1 ml of MS vitamin (1000×) containing 2 mg/ml Glycine, 0.5 mg/ml Nicotinic acid, 0.5 mg/ml Pyridoxine-HCl, 0.1 mg/ml Thiamine-HCl and 500 mg/ml myo-Inositol; 2 g/l Casamino acids; 30 g/l Sucrose; 30 g/l Sorbitol; 10 μl of NAA stock solution (final conc. 2 μg/l); and 10 ml of Kinetin (final conc. 2 mg/l) to 700 ml of Milli-Q water. Adjust the pH to 5.8 with 1 N HCl and add Milli-Q water up to 1 l with 4 g/l Gelrite. Autoclave and cool to approximately 60 °C. To select transformants, add 1 ml of 25 mg/l Meropenem hydrate solution and 1 ml of 0.25 mM bispyribac-sodium solution (the final conc. 0.25 μM).
2. MS-free medium: Add 4.4 g/l MS basal salt mixture (e.g., Sigma-Aldrich); 1 ml of MS vitamin (1000×) containing 2 mg/ml Glycine, 0.5 mg/ml Nicotinic acid, 0.5 mg/ml Pyridoxine-HCl, 0.1 mg/ml Thiamine-HCl and 500 mg/ml myo-Inositol; and 30 g/l Sucrose to 700 ml of Milli-Q water. Adjust the pH to 5.8 with 2 N KOH and add Milli-Q water up to 1 l with 4 g/l Gelrite. Autoclave and cool to approx. 60 °C.

Then, add 1 ml of 0.25 mM bispyribac-sodium solution (final conc. 0.25 μM).

2.3 PCR Reagents and a Restriction Enzyme

1. Taq polymerase (e.g., Ex-Taq polymerase, Takara Biol., Japan).
2. 10× dNTP.
3. Reaction buffer.
4. *Hin*dIII.

2.4 Cytological Reagents

1. 8-Oxyquinoline (e.g., Sigma-Aldrich) (2 mM). Dissolve first in a small amount of ethanol and add Milli-Q water to adjust the concentration. Store at 4 °C (up to 1 year).
2. Fixative: 3 vol. of ethanol and 1 vol. of acetic acid. Store at −20 °C (up to 1 month).
3. Enzyme mix solution for chromosome preparation: 2% (w/v) cellulase Onozuka RS (Kinki Yakult, Japan) and 20% (v/v) Pectinase (Sigma-Aldrich). Dissolve in Milli-Q water, filter sterilize and store at −20 °C (up to 1 year).
4. 2-(4-Aminophenyl)-1H-indole-6-carboxamidine (DAPI) (1 mg/ml). Dissolve in Milli-Q water, filter sterilize and store at −20 °C in the dark (up to 1 year).
5. Antifade mounting medium (e.g., VectorShield, Cat. No. H-1000).
6. Giemsa solution (Merck-Millipore, Cat. No. 109204).
7. 1/15 M phosphate buffer (pH 6.8) (e.g., Merck-Millipore, Cat. No. 111374 for tablets).

3 Methods

3.1 Agrobacterium-Mediated Transformation [9, 10]

3.1.1 Callus Induction

1. Remove hulls from mature seeds of rice using a chaff grinder and put 100 of the seeds into a 50-ml Falcon tube.
2. Add 30 ml of 70% (v/v) ethanol into the tube and mix gently for 30 min at RT.
3. Discard ethanol and add 20 ml of the sterilized solution (50% (v/v) sodium hypochlorite solution with one drop of Tween 20) that was made just before **step 1**.
4. Sterilize the seeds in the tube for 15 min at RT with gentle inversion.
5. Discard the sterilized solution and add another 20 ml of the sterilized solution.
6. Following removal of the solution, rinse the seeds with 40 ml of sterile Milli-Q water for 5 min and repeat this step at least five times.

7. Transfer the seeds to sterile disposable plates with a couple of sterile filter papers to remove the surface water.
8. Place the ten seeds on a plastic disposable plate with N6D media and seal the cover with surgical tape.
9. Incubate the plates at 32 °C for 3 weeks under continuous light.
10. Select yellowish and stiff calli (2- to 3-mm diameter) and transfer them to new N6D plates with a pair of sterilized forceps.
11. Incubate at 32 °C for 3 days under continuous light.

3.1.2 Agrobacterium Infection

1. Spread Agrobacterium harboring the binary vector pDLHC on an AB plate, seal with surgical tape, and incubate at 28 °C for 3 days.
2. Transfer the Agrobacterium that grew on the plate into a 50-ml sterile Falcon tube containing 40 ml of AAM liquid media with 10 mg/l Acetosyringone and suspend well with a disposable pipette.
3. Pour the Agrobacterium suspension onto a sterile plate (80-mm diameter) and put 100–200 calli after 3 days of incubation (from **step 11** of Subheading 3.1.1) on the plate.
4. Incubate for 2 min at RT with gentle agitation.
5. Transfer the calli onto sterilized paper towels to remove excess Agrobacterium.
6. Transfer approximately 100 calli to a 2N6AS plate that has been overlaid with sterile wet filter paper with 1 ml of AAM medium and seal with surgical tape.
7. Incubate the plates at 28 °C in the dark for 3 days.
8. Rinse the calli that were infected with *Agrobacterium* with 40 ml of sterile Milli-Q water in a 50-ml Falcon tube, with occasional inversions. Repeat at least five times.
9. Rinse the calli with 40 ml of the solution containing 50 mg/l Meropenem hydrate and 100 mg/l vancomycin with occasional inversions. Repeat three times.
10. Place 20–30 calli on the Gelrite-solidified N6D media containing 0.25 μM bispyribac sodium and incubate at 32 °C for 2 weeks under continuous light.
11. Transfer the calli to the same selection media and incubate at 32 °C for 2 more weeks.

3.1.3 Regeneration of Transformants

1. Transfer calli (from **step 12** of Subheading 3.1.2) to plates containing MS-R media (10 calli/plate) and incubate at 30 °C for 2–3 weeks under continuous light.
2. Transfer calli showing some green spots to the new MS-R plates and incubate at 30 °C for 1–2 weeks under continuous light.

3. Transfer regenerated plants to MS-free media to stimulate root development.
4. Transfer the plants to pots with soil when the plant height reaches approx. 7 cm.

3.2 Analysis of T_1 Transgenic Plants

3.2.1 PCR Analysis of Marker Genes

1. Extract genomic DNA from leaves of 1- to 2-month-old putative transgenic plants (T_1) that regenerated from calli using a commercially available plant DNA isolation kit (e.g., DNeasy Plant Mini Kit, Qiagen).
2. Perform PCR using the DNA above as a template and a primer set (ALS-F: 5′-TCATCCTATCCCACCGACAT-3′ and ALS-R: 5′-CCCAATAAGATCGACCGAAG-3′) to confirm the involvement of the mutated ALS gene (abbreviated as *ALSR*) that confers resistance to the herbicide bispyribac sodium. The PCR conditions are 94 °C for 2 min, followed by 30–35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 3 min.
3. Digest the PCR samples with the restriction enzyme *Hin*dIII by adding one-half (5 μl) of each PCR sample to a 0.5-ml microtube containing 15 ml of 10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT (pH 7.9), along with 10 units of *Hin*dIII. Incubate the tubes at 37 °C from at least 2 h to overnight.
4. Estimate the size of the PCR fragments by 0.8 % (w/v) agarose electrophoresis and stain with ethidium bromide. Because the wild-type ALS gene (*ALS*) has one *Hin*dIII site, two fragments (1.24 and 1.36 kb in length) are expected to appear. The *ALSR* transgene contains two single nucleotide polymorphisms (SNPs), one of which corresponds to the *Hin*dIII site; thus, no-*Hin*dIII digestion results in a single band of 2.6 kb in length. This cleaved amplified polymorphism (CAP) makes it possible to differentiate the transgenic plants (*ALS/ALS/ALSR*) with three bands from the nontransgenic wild-type plants (*ALS/ALS*) with two bands.
5. Similarly, perform PCR using the same template DNA and a set of primers (PactH-F: 5′-AAGAGGGGAAAAGGGCACTA-3′, and Cre-R3: 5′-CATGTTTAGCTGGCCCAAAT-3′) to estimate the structures of the inserted T-DNA. The PCR conditions are as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 2 min and 72 °C for 45 s, with final extension at 72 °C for 3 min. Approximately 2.9-kb bands indicate that almost all of the components are included in the inserted T-DNA (Fig. 2a). If not, some truncations would occur during T-DNA integration.

6. Transplant the transgenic plants that are confirmed by CAPS to pots with soil and allow to grow to maturity in a closed green house or growth chamber at 30 °C under continuous light.
7. Collect the selfed seeds and store at 4 °C under dry conditions.

3.2.2 T-DNA Mapping

Because launching the Ds cassette does not usually occur in the T_1 generation, the original T-DNA insertion sites could be mapped by Thermal asymmetric interlaced PCR (TAIL-PCR) with the extracted DNA (**step 1** of Subheading 3.3.1) as templates, using primers that are designed especially for pDLHC (Table 1). The PCR conditions that are used are described in Chapter 17 by Fujimoto et al.

3.3 Analysis of T_2 Transgenic Plants

3.3.1 PCR Analysis

1. Germinate the selfed T_2 seeds that were obtained in the previous section on filter paper moistened with Milli-Q water (or 0.25 μM bispyribac sodium, optional). Because the resistance gene segregates in the T_2 generation, three-fourths of the seeds are expected to be resistant to the herbicide if the T-DNA has been inserted into one locus and if the gene is not silenced.

Table 1
TAIL-PCR primers for determination of the T-DNA insertion sites of pDLHC

Primers	Sequence (5′ → 3′)
Nested primers for RB-flanking sequences	
R5-RB1	TGGCGGGTAAACCTAAGAGAAAA
R5-RB2	ATCGGATATTTAAAAGGGCGTGA
R5-RB3	ATCCGTTCGTCCATTTGTATGTG
Nested primers for LB-flanking sequences	
R5-LB1	GCGTCAATTTGTTTACACCACAA
R5-LB2	ACCACAATATATCCTGCCACCAG
R5-LB3	GGCACAAAATCACCACTCGATAC
Arbitrary primers	
AD1-1	TGWGNAGSANCASAGA
AD2-1	NGTCGASWGANAWGAA
AD2-2	AGWGNAGWANCAWAGG
AD3	WGTGNAGWANCANAGA
AD5	STTGNTASTNCTNTGC
AD7	NTCGASTWTSGWGTT
AD17	TCNGSATWTGSWTGT

(N: A, C, G, or T; S: C, or G; W: A or T)

2. Cut one or two leaves from 1-month-old plants that were grown at 30 °C and extract DNA as described above (**step 1** of Subheading 3.2).
3. Perform PCR using the same primer set (ALS-F and ALS-R) as described in **step 2** of Subheading 3.2 to determine the segregation of the resistance gene (*ALSR*) and susceptible gene (*ALS*). Following *Hin*dIII digestion, select the plants showing three bands on electrophoresis, the genotypes of which are *ALS/ALS/ALSR/ALSR* and *ALS/ALS/ALSR/–*. In contrast, one-fourth of the plants (genotype *ALS/ALS/–/–*), will be sensitive to the herbicide, showing only two bands after *Hin*dIII digestion.
4. To detect DLD cassette launch, perform PCR using the same primer set (PactH-F and Cre-R3) as described in **step 5** of Subheading 3.2.1. The 2.9-kb band suggests simple insertion of T-DNA into the chromosome as described above, whereas a 0.6-kb band indicates the DLD cassette launch (Fig. 2a) (*see* **Note 4**).
5. The DLD cassette excision results in a fusion between a promoter (Pact) and the *Cre* gene, which would potentially express Cre recombinase. To determine the occurrence of the subsequent site-specific recombination between two *LoxPs* via Cre recombinase, perform PCR using the same DNA samples from **step 1** as templates and the primer set Ds2-In1 (5′-CATCGGATGTATGGCAGCATTTCACCAAG-3′) and Cre-R3 (5′-CATGTTTAGCTGGCCCAAAT-3′). If recombination occurred, approximately 0.7-kb bands would appear (Fig. 2b). The PCR conditions are the same as before (94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 3 min) (*see* **Note 5**).
6. To determine the stability of circular DNA, perform PCR with the primers PactH-F (5′-AAGAGGGGAAAAGGGCACTA-3′) and R-HYG (5′-GATGTTGGCGACCTCGTATT-3′). The original T-DNA insertion shows 1.4-kb bands, whereas 1.0-kb bands are expected to appear if the circular DNA is retained in the cells (Fig. 2c) (*see* **Note 5**).

3.3.2 Cytological Analysis

1. Various types of chromosomal rearrangements are expected to be induced by the method described here. The final assessment of the types of chromosomal rearrangements that were induced should be made using fluorescence microscopy analysis of the chromosomes.
2. To investigate the chromosome constitutions of transgenic rice plants, collect roots (1–2 cm) from germinating T2 or T3 seeds into a microcentrifuge tube containing 2 mM 8-oxyquinoline and incubate the tubes at RT for 2–4 h. The

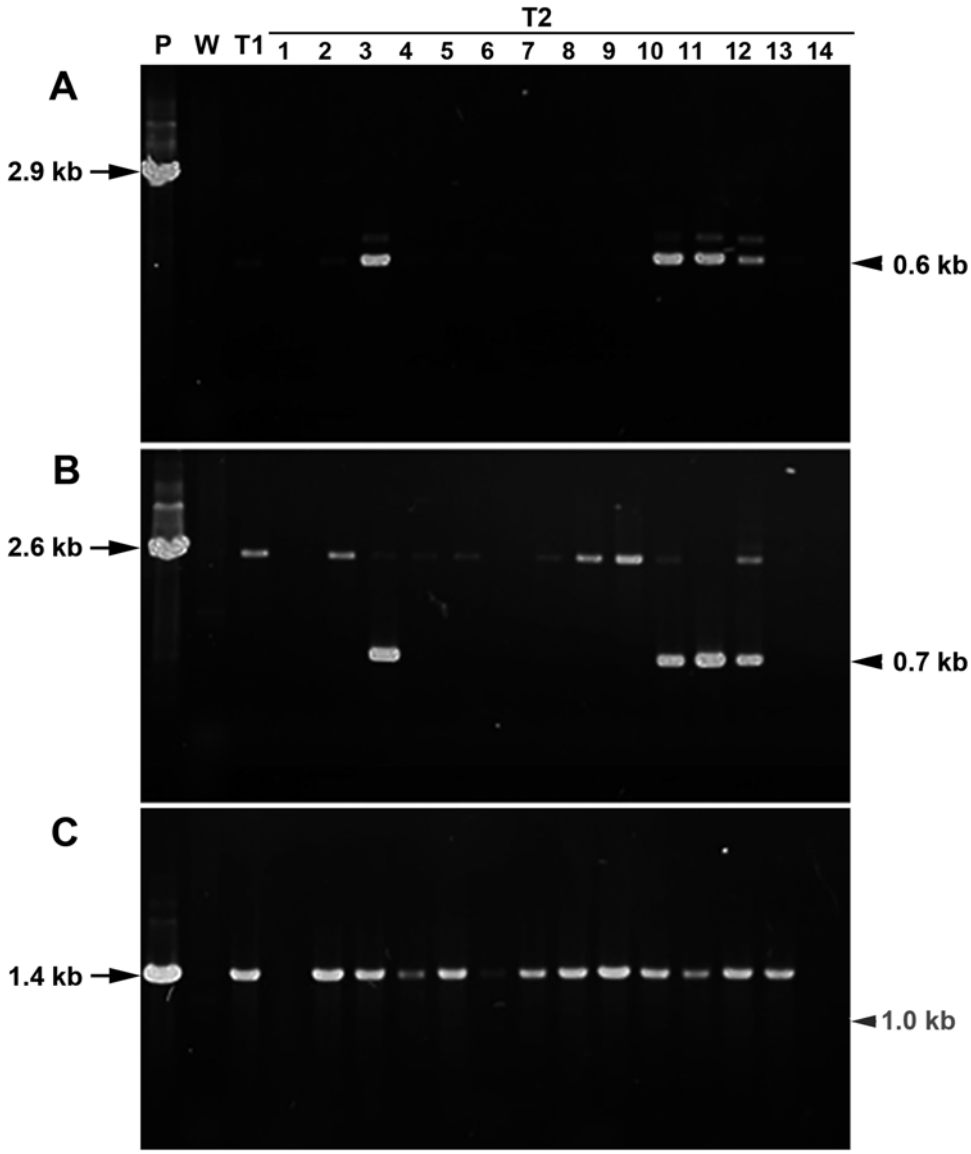

Fig. 2 PCR analyses for a transgenic rice plant (T1) and its offspring (T2 1–14). The primers that were used were as follows: PactF and Cre-R (**a**), DS2-In1 and Cre-R (**b**), and PactF and R-HYG (**a**). *P* pDLHC, *W* nontransgenic Nipponbare (WT). *Arrows* indicate bands that were expected from the original T-DNA. *Arrowheads* indicate 0.6-kb bands that were expected from DLD cassette transposition (**a**) and 0.7-kb bands that were expected from DLD cassette transposition and subsequent recombination between two *LoxPs* (**b**). Only 1.4-kb bands appeared, indicating that no stable circular DNA was retained (**c**)

roots from each germinating seed should be collected separately with clean and fine forceps.

3. Remove 8-oxyquinoline with a pipette and add 1 ml of the fixative (3 vol. of ethanol and 1 vol. of acetic acid) that has been stored at −20 °C or freshly made.

4. Incubate the tubes for at least 2 h at room temp (approx. 25 °C) and store at –20 °C until further use.
5. Discard the fixative and rinse the roots with 1 ml of Milli-Q water at least three times in the microcentrifuge tubes with occasional inversions.
6. Add 0.5 ml of the enzyme mix solution into the tube immediately after removing the final water rinse and incubate the tubes at 37 °C for 1 h.
7. Transfer the treated roots carefully using a pair of forceps onto a glass slide. Rinse with a few drops of Milli-Q water and remove the water with filter paper. Repeat at least three times.
8. Cut the root tips (0.5–1.0 mm) with a razor blade and remove the remaining part of the roots.
9. Add a few drops of the fixative to the root tips and squeeze the meristematic tissues with a fine needle. Add more drops of the fixative to avoid drying.
10. Air-dry or flame-dry the slides with an alcohol lamp.
11. Mount the slides with anti-fading solution containing 1 μg/ml DAPI (*see* **Note 6**).
12. Find the chromosomes under a fluorescence microscope equipped with a filter set for UV-excitation and blue-emission and capture the image with a CCD camera (Fig. 3).

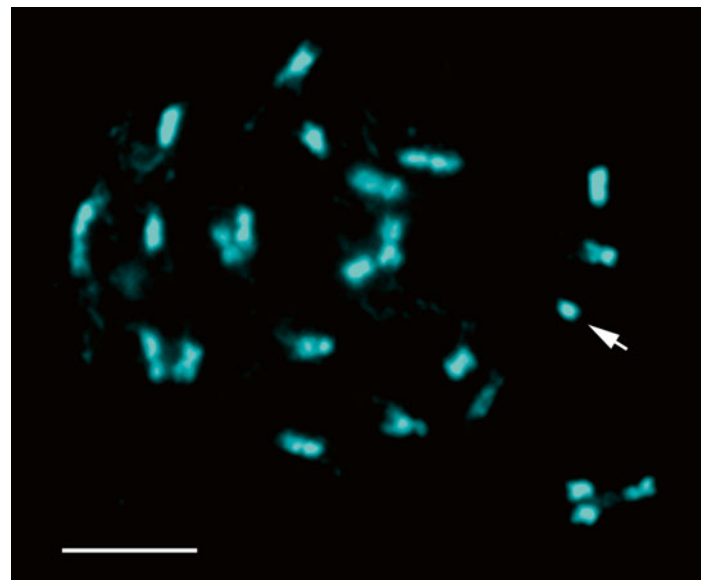

Fig. 3 A DAPI-stained mitotic metaphase cell of transgenic rice (T2). An *arrow* indicates a truncated chromosome originating on chromosome 10

4 Notes

1. To avoid the expression of the *Cre* gene in *E. coli* and *Agrobacterium*, an intron(s) should be inserted into the gene.
2. For *A. thaliana*, pSTARA having ALS with two SNPs is available (Kumiai Chemicals).
3. For dicot plants, the actin promoter should be replaced with another promoter such as the CaMV 35S promoter.
4. The appearance of 0.6-kb bands indicates DLD cassette excision but does not necessarily confirm reintegration.
5. Depending on the direction of the transposition (upward or downward) and on the method of reinsertion (simple or reverse) of the DLD cassette, different types of chromosomal rearrangements are induced. The reverse insertion on the same chromosome leads to two *LoxP*s having opposite directions, which leads to an inversion via Cre recombinase. In contrast, recombination between two *LoxP*s having the same direction on the same chromosome results in the formation of a circular DNA. If no centromere sequences are involved in the DNA molecule, it will vanish soon from the cell, resulting in the deletion of the corresponding DNA sequence. This event might be detected directly on mitotic chromosomes (Fig. 3). If centromere sequences are involved, however, the circular DNA molecule could behave as a ring chromosome.
6. Alternatively, stain the slides with 2% (v/v) Giemsa solution diluted with 1/15 M phosphate buffer (pH 6.8) at RT for 5–10 min, briefly rinse with Milli-Q water, and air-dry. Add a drop of phosphate buffer and cover with a coverslip to observe the chromosomes under light microscopy.

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References

1. Medberry SL, Dale E, Qin M, Ow DW (1995) Intra-chromosomal rearrangements generated by Cre-lox site-specific recombination. Nucleic Acids Res 23:485–490
2. Woody ST, Austin-Phillips S, Amasino RM, Krysan PJ (2007) The WiscDsLox T-DNA collection: an arabidopsis community resource generated by using an improved high-throughput T-DNA sequencing pipeline. J Plant Res 120:157–165
3. Zhang S, Raina S, Li H, Li J, Dec E, Ma H, Huang H, Fedoroff NV (2003) Resources for targeted insertional and deletional mutagenesis in *Arabidopsis*. Plant Mol Biol 53:133–150
4. Murata M, Shibata F, Hironaka A, Kashihara K, Fujimoto S, Yokota E, Nagaki K (2013)

Generation of an artificial ring chromosome in *Arabidopsis* by Cre/*LoxP*-mediated recombination. Plant J 74:363–371

5. Ito Y, Eiguchi M, Kurata N (2004) Establishment of an enhancer trap system with Ds and GUS for functional genomics in rice. Mol Genet Genomics 271:639–650
6. Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a beta-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol 31:805–813
7. Okuzaki A, Shimizu T, Kaku K, Kawai K, Toriyama K (2007) A novel mutated acetolactate synthase gene conferring specific resistance to pyrimidinyl carboxy herbicides in rice. Plant Mol Biol 64:219–224
8. Cuellar W, Gaudin A, Solorzano D, Casas A, Nopo L, Chudalayandi P, Medrano G, Kreuze J, Ghislain M (2006) Self-excision of the antibiotic resistance gene nptII using a heat inducible Cre-*loxP* system from transgenic potato. Plant Mol Biol 62:71–82
9. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282
10. Masumoto C, Miyao M (2009) *Agrobacterium*-mediated transformation of rice (in Japanese). Teionkagaku 67:641–647

Chapter 6

Genome Elimination by Tailswap CenH3: In Vivo Haploid Production in *Arabidopsis thaliana*

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Abstract

Artificial production of haploids is one of the important sought-after goals of plant breeding and crop improvement programs. Conventionally, haploid plants are generated by in vitro (tissue) culture of haploid plant gametophytes, pollen (male), and embryo sac (female). Here, we describe a facile, nontissue culture-based in vivo method of haploid production through seeds in the model plant, *Arabidopsis thaliana*. This method involves simple crossing of any desired genotype of interest to a haploid-inducing strain (GFP-tailswap) to directly obtain haploid F1 seeds. The described protocol can be practiced by anyone with basic experience in growing *A. thaliana* plants and will be of interest to Arabidopsis research community.

Key words Haploid, GFP-tailswap, Arabidopsis, CenH3, Centromeres, SeedGFP-HI, Genome elimination, In vivo haploid, Seeds

1 Introduction

Haploid plants constitute an invaluable tool for the rapid generation of homozygous pure lines via doubled haploids (DH) [1, 2] enabling rapid varietal and hybrid cultivar development [3]. Haploids greatly reduce the breeding time required for development of inbred lines that are used as parents for developing hybrids [4, 5]. In addition, haploids can also be exploited as a tool in basic research to expedite genetic analysis [6]. Importance of haploids in plant breeding can be appreciated by a vast number of patents filed/granted for methods developed to produce and use haploids in plants [2, 7].

Haploid plants can be produced either by in vitro (tissue culture based) or in vivo approaches [2]. Following the pioneering observation of Guha and Maheshwari (1964) [8], anther (microspore) culture-based in vitro methods of haploid production have been routinely employed for haploid production in several crop species. Despite successful early attempts, model plant *Arabidopsis thaliana* has proven to be recalcitrant for large-scale production of haploids through conventional in vitro approaches [9–12]. Ravi and Chan (2010) described a

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new method of in vivo haploid production through seeds based on manipulation centromeres by the centromere-specific histone H3 variant (CenH3) in *A. thaliana* [13]. Using this method, it is now possible to generate a large-scale population of haploids in a relatively short span of time [14]. Further, this method is genotype independent unlike in vitro methods and thus can be used to generate haploids not only from any *A. thaliana* and its hybrids but also from any cross compatible sister species such as *A. suecica* [13].

Subsequent to the discovery of *CenH3*-mediated in vivo haploid production, successful attempts have been made to improve the existing transgenic haploid inducer (HI) strain and also to develop nontransgenic haploid inducers in *A. thaliana*. Transgenic haploid inducers are developed in *cenh3-1* null mutant background where in the wild-type (WT) CenH3 function is restored by the expression of altered CenH3 variants. The altered CenH3 variant can be: a heterologous *CenH3* from closely related or distant species that show polymorphisms in CenH3 protein sequence [15], or a simple translational fusion of *A. thaliana* native CenH3 with marker tags such GFP (GFP-CenH3) or a chimeric CenH3 consisting of histone H3.3 N-terminal tail domain fused with C-terminal CenH3 histone fold domain (GFP-tailswap) [13, 16]. The nontransgenic haploid inducer comprises a suite of point mutant alleles of WT *CenH3* possibly induced by chemical (EMS—ethyl methane sulfonate) mutagenesis [17]. When such *cenh3* point mutants are crossed to WT *CenH3* plants they produce haploid progeny at varying frequencies depending on the point mutation.

Among these haploid-inducing strains, transgenic GFP-tailswap strain gives highest frequency of haploids (a minimum of 40% and above depending on the genotype of accessions). In addition, to facilitate preselection of haploid seeds, an improved GFP-tailswap strain called Seed GFP haploid inducer (SeedGFP-HI) has been developed [6]. Here, we describe a stepwise protocol for in vivo haploid seed production employing GFP-tailswap/SeedGFP-HI strains. The below described method complements an earlier published protocol elaborating the use of GFP-tailswap for producing haploids [18].

2 Materials

1. *Arabidopsis* growth chamber/Green house.
2. Murashige and Skoog (MS) medium [19] (*see* **Note 1**):

   To prepare 1 l of 1× MS solution use the following recipe. The amount can be scaled up or down depending upon the requirement. Use distilled (millipore) water free of any contaminating salts to make both the stock and working solutions.

   Store the stock solutions at 4 °C (cold room or refrigerator) for long term storage.

(a) 4 mM $CaCl_2$: 4 ml from 1 M stock (147 g $CaCl_2 \cdot 2H_2O$/l).

(b) 1.5 mM $MgSO_4$: 1.5 ml from 1 M stock (246.47 g $MgSO_4 \cdot 7H_2O$/l).

(c) 1.25 mM KH_2PO_4 pH 5.6: 1.25 ml from 1 M stock (136.09 g/l).

(d) 18.8 mM KNO_3: 18.8 ml from 1 M stock (101.1 g/l).

(e) 20.6 mM NH_4NO_3: 20.06 ml from 1 M stock (80.04 g/l).

(f) 20 mM Fe-EDTA: 2.5 ml from 25× stock solution. (Dissolve 2.5 g of $FeSO_4 \cdot 7H_2O$ in 400 ml of water in a beaker/flask. Add 3.36 g of Na-EDTA. Heat the solution to boil. Stir the solution for 30 min while cooling using a magnetic stirrer. Make up the volume to 450 ml with distilled water. Store the solution in dark brown bottles.)

(g) 1000× Minor nutrients: 1 ml (100 ml of the stock solution is prepared as follows).

- 70 mM H_3BO_3: 7 ml 1 M stock (3.09 g/50 ml), needs to be heated.
- 14 mM $MnCl_2$: 1.4 ml 1 M stock (9.9 g $MnCl_2 \cdot 4H_2O$/50 ml).
- 0.5 mM $CuSO_4$: 5 ml 10 mM stock (0.125 g $CuSO_4 \cdot 5H_2O$/50 ml).
- 1 mM $ZnSO_4$: 1 ml 100 mM stock (1.44 g $ZnSO_4 \cdot 7H_2O$/50 ml).
- 0.2 mM $NaMoO_4$: 2 ml 10 mM stock (0.215 g $NaMoO_4 \cdot 2H_2O$/50 ml).
- 10 mM NaCl: 1 ml 1 M stock (2.92 g/50 ml).
- 0.01 mM $CoCl_2$: 0.1 ml 10 mM stock (0.12 g/50 ml).

3. Agar media for germination *A. thaliana* seeds:

   Prepare 1× MS solution followed by the addition of 2% sucrose (*see* **Note 2**). Adjust the pH 5.6–5.7 using a pH meter. Add agar (0.7%) and autoclave the media (*see* **Note 3**).

4. CTAB buffer for DNA extraction: 100 ml.

   (a) CTAB (hexadecyl trimethyl-ammonium bromide): 2 g.

   (b) 1 M Tris buffer pH 8.0: 10 ml.

   (c) 0.5 M EDTA pH 8.0: 4.0 ml.

   (d) 5 M NaCl: 28.0 ml.

   (e) Sterile distilled water: 40.0 ml

   (f) PVP (polyvinyl pyrrolidone (vinylpyrrolidone homopolymer) (MW 40,000)): 1 g.

5. Soil medium for growth of *A. thaliana* plants.

Prepare soil medium for growing plants by mixing equal proportions of soilrite:perlite (soilrite-c):exfoliated vermiculite:coco peat (*see* **Note 4**).

6. *A. thaliana* (Col-0) GFP-tailswap plants (*cenh3-1/cenh3-1*; *GFP-tailswap/GFP-tailswap*) (Arabidopsis Biological Resource Center (ABRC) Accession No: CS66982).
7. Petri plates to prepare MS agar plates.
8. Magnetic Stirrer.
9. pH meter.
10. Bleach (5% sodium hypochlorite solution).
11. Pots, trays, plastic cover/saran wrap.
12. Weighing Balance.
13. Parafilm.
14. Autoclave/pressure cooker.
15. Fine tweezers (Dumont No. 5).
16. Stereo dissection (fluorescence) microscope (fluorescence attachment is optional if going for preselection of haploid seeds).
17. Upright fluorescence microscope (optional).
18. Microfuge tubes.
19. Glass slides, cover slips.
20. Laminar flow hood.
21. Fine opthalmic scissors.
22. Desiccator.
23. Gel electrophoresis unit.
24. Thermocycler (PCR).
25. Incubator (37 °C).
26. Fine Camel hair (painting) brush.
27. Liquid nitrogen/bullet blender/micropestle (use one depending on availability).
28. Colchicine (optional).
29. Silwet (optional).
30. Pipette pump to pipette acids.
31. *Eco*RV restriction enzyme and buffer.
32. Primers for genotyping.
33. Desktop centrifuge.
34. Ethanol.
35. Concentrated Hydrochloric acid.
36. Chloroform:isoamyl alcohol (24:1).
37. TE (Tris EDTA) buffer pH (8.0): 10 mM Tris–HCl, 1 mM EDTA.

3 Methods

3.1 Growing of *A. thaliana* Plants in Soil Medium [20]

1. Fill in the soil mix in pots with perforated bottom and soak them till saturation in 1× MS nutrient solution.
2. Sow the seeds (typically six seeds/pot of 11 × 11 cm dimension) of desired genotype in the soil medium with uniform spacing between them.
3. Cover the individual pots with saran wrap or the tray with plastic dome and keep it at 4 °C for at least 3 days for cold stratification.
4. After cold treatment, transfer the pots to plant growth chamber. Remove any condensed water droplets from the inner cover of the saran wrap/plastic dome.
5. Remove the saran wrap cover/plastic dome from pots once true leaves emerge and expand from the germinating seedlings. If left covered for longer time, growth of seedlings may be affected by inducing premature flowering and early senescence.
6. Regularly water the pots with distilled water. A month after sowing, supplement with half a dose of 1× MS nutrient solution/nitrogen fertilizers to boost robust growth of plants (*see* **Note 5**).

3.2 Plant Growth Conditions

1. 20 ± 3 °C with a relative humidity (RH) ~75–80%.
2. Long day photoperiod: 16 h light followed by 8 h dark conditions.
3. Light intensity of 130–150 μE/m^2/s achieved by use of fluorescence and incandescent lights.

3.3 Growing, Identification, and Maintenance of Haploid-Inducing GFP-tailswap Plants

GFP-tailswap plants that are used for haploid induction crosses are homozygous for *cenh3-1* null mutation and are complemented by ectopically inserted *GFP-tailswap* transgene (*cenh3-1/cenh3-1*; *GFP-tailswap/GFP-tailswap*).

1. GFP-tailswap plants are predominantly male sterile but some plants do set seeds at later stages of growth (*see* **Note 6**). Harvest these self-pollinated seeds to grow homozygous GFP-tailswap plants for crossing.
2. If you cannot collect enough selfed seeds from GFP-tailswap plants, the mutant can also be maintained in a heterozygous condition (*CenH3/cenh3-1*; *GFP-tailswap/GFP-tailswap*).
3. Sow the ABRC accession CS66982 (*see* **Note 7**) on to soil medium and let the plants to grow for 2 weeks.
4. Identify homozygous *GFP-tailswap* segregants from rest of the siblings by their lettuce-like rosette vegetative phenotype (Fig. 1a) (*see* **Note 8**).
5. Retain the GFP-tailswap plants for crossing and remove other unwanted segregants if not required (*see* **Note 9**).

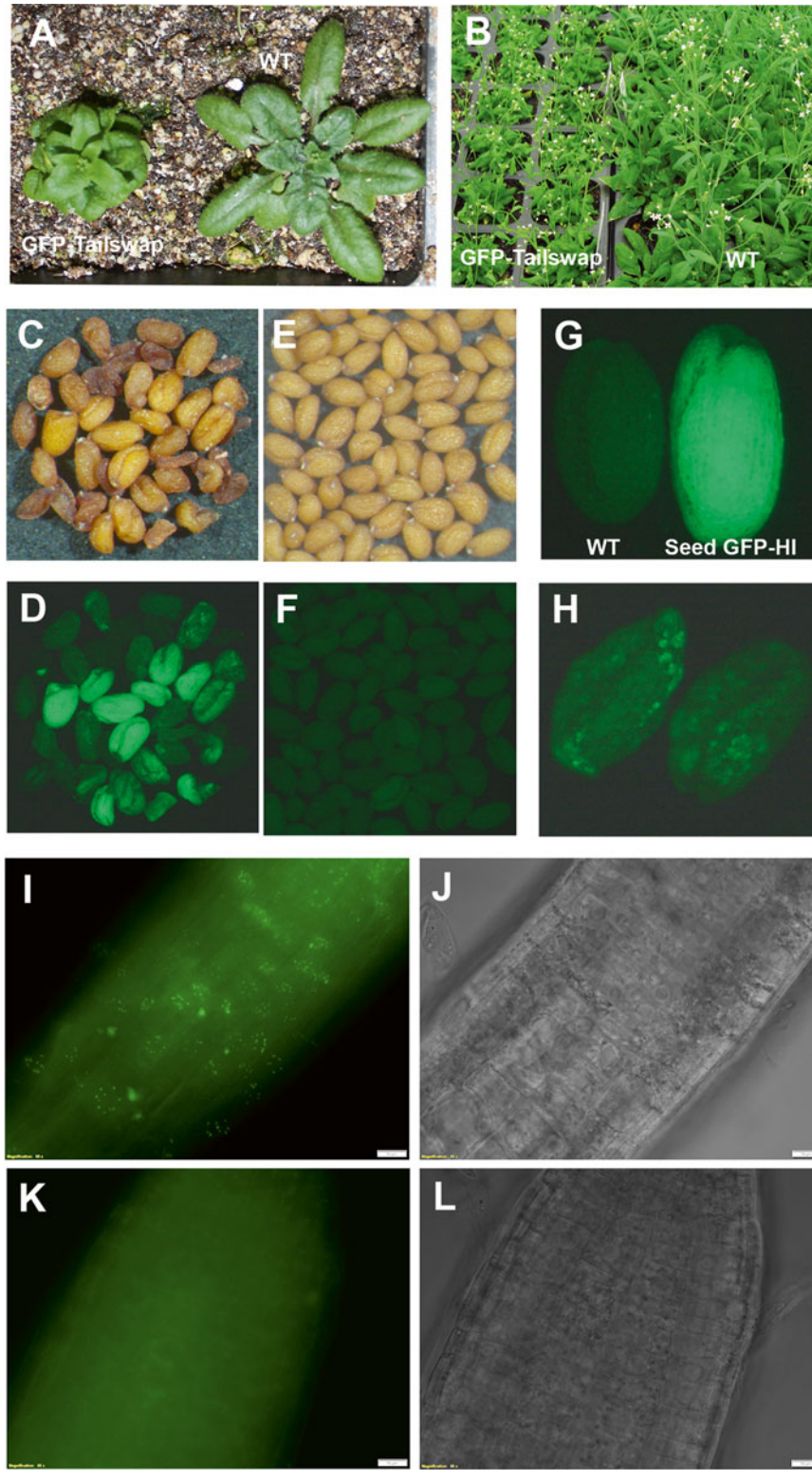

Fig. 1 Identification of GFP-tailswap plants and preselection of haploid F1 seeds and seedlings. (**a**) GFP-tailswap plants can be phenotypically distinguished from WT plants by a lettuce-like compact, stunted growth phenotype (image shown is photographed ~25–30 days postgermination). (**b**) Representative image of selfed progeny from GFP-tailswap and WT plants at flowering showing the phenotypic difference between them. GFP-tailswap plants are sterile and stunted compared to WT plants. (**c**) Bright field image of F1 seeds from SeedGFP-HI × WT cross

6. Upon flowering, observe that GFP-tailswap is stunted in growth compared to WT plants and also note that the inflorescence is sterile showing nonelongated pistils (Fig. 1b).

3.4 Crossing GFP-tailswap as Female Parent to Produce Paternal Haploids

1. Sow GFP-tailswap and other desired genotype from which haploids need to be produced. GFP-tailswap plants generally flowers a month after sowing. Hence plan sowing your genotype of interest considering the time it takes to flower so as to synchronize flowering with GFP-tailswap plants for crossing. Crosses can be initiated as soon as the GFP-tailswap plants starts flowering.
2. Select healthy GFP-tailswap plants with robust inflorescence containing many flowers. Avoid choosing weak and aneuploid looking GFP-tailswap plants.
3. GFP-tailswap plants can be used either as male or female parent in a cross (*see* **Note 10**). However, to achieve high frequency of haploid seeds always use GFP-tailswap as female parent.
4. Gently dust the pollen from the desired plant onto the pistil of open flowers of GFP-tailswap plants (*see* **Note 11**). No emasculation is required for crossing (*see* **Note 6**).
5. Monitor the cross for next few days. If the cross is successful, the pollinated pistil will turn pink the following day and gradually start to elongate (*see* **Note 12**).
6. In the event of unsuccessful pollination, pollinate the same flower the next day or later as long as stigma is receptive (*see* **Note 13**). If not, gently remove the unpollinated flowers from the inflorescence using fine scissors and continue crossing using fresh flowers.
7. Continue with crossing up to a maximum of 2 weeks/until desired number of crosses are made (*see* **Note 14**). Avoid pollination without emasculation after 2–3 weeks of flowering (*see* **Note 15**).
8. Once crossing is complete, decapitate the inflorescence leaving only the crossed siliques. Also remove other inflorescence side shoots that are not used for crossing (*see* **Note 16**).

Fig. 1 (continued) as viewed under stereo dissection microscope. Note the presence of aborted seeds that are *dark brown*, shriveled, lacking embryo along with plump, *uniform brown* colored viable seeds. (**d**) Same seeds as in **c** when viewed under stereo dissection fluorescence microscope using GFP channel. Note three different category of seeds: those showing uniform bright GFP fluorescence, those showing mottled GFP fluorescence, and those not showing GFP fluorescence. (**e**) Bright field image of WT × WT crossed seeds showing uniform brown plump viable seeds. (**f**) Same seeds in **e** when viewed in GFP channel do not show any green fluorescence except for red auto fluorescence. (**g**) Single seed from WT and SeedGFP-HI showing the presence and absence of GFP fluorescence in embryo respectively. (**h**) Two mottled GFP seeds showing distorted GFP fluorescence in the endosperm and not in the embryo. These seeds give rise to haploid progeny. (**i**) Excised root from a GFP-tailswap × WT cross hybrid progeny viewed under upright fluorescence microscope in GFP channel showing distinct punctate green foci marking the centromeres of chromosomes in the nuclei of root tissue. (**j**) Bright field image of the root shown in (**i**). (**k**) Excised root from a GFP-tailswap × WT cross haploid progeny when examined under GFP channel don't show the GFP label at the centromeres except for autofluorescence (**l**) Bright field image of the root shown in (**k**)

9. Allow the developing siliques to reach harvest maturity (typically 15–20 days after crossing) (*see* **Note 17**).
10. Collect the seeds/siliques carefully without losing any seeds to spontaneous shattering.
11. Examine the seeds under a dissection microscope to observe for a mixture of dead and viable seeds (Fig. 1c) (*see* **Note 18**).
12. Count the numbers in each category silique wise, if the aim is to obtain data on percentage of viable and dead seeds, else pool all seeds in a microfuge tube (*see* **Note 19**).
13. Store the harvested seeds at room temperature in a desiccator for a week or two. While in desiccator, keep the microfuge cap open to avoid condensation of moisture inside the tubes. To prevent spillage and contamination of seeds, secure the mouth of the microfuge tube with parafilm. Perforate the parafilm by poking it with fine needle to allow for evaporation of moisture from the seeds.

3.5 Preselection of Haploid Seeds/ Seedlings

Haploid seeds can be preselected before sowing with the use of GFP-tailswap variant strain, the SeedGFP-HI strain for haploid induction crosses (*see* **Note 20**). Preselection can be done immediately after harvesting the seeds with the aid of a fluorescence stereo dissection microscope (*see* **Note 21**). If you are not using SeedGFP-HI for crosses skip to **step 3** below.

1. Place the harvested F1 seeds under a fluorescence dissection microscope and examine the seeds for GFP fluorescence. The seeds can be broadly segregated into three categories: seeds showing uniform GFP fluorescence, seeds showing mottled GFP fluorescence, and seeds without any GFP fluorescence (Fig. 1c–f).
2. Carefully segregate out the seeds that show uniform GFP fluorescence (Fig. 1g) using a fine camel hair (painting) brush (*see* **Note 22**). The mottled GFP seeds (Fig. 1h) and nonfluorescent seeds are mostly enriched for viable haploid seeds along with nonviable seeds (*see* ref. 6 for more details).
3. The preselected mottled GFP seeds/the entire F1 seed lot (if preselection is not done) can be directly sown on to soil media for germination and establishment. However, we recommend germinating seeds in MS agar plates before transplanting to soil medium (*see* **Note 23**).
4. Surface sterilize the seeds before sowing them in MS agar plates following the gas sterilization method described below.

3.5.1 Surface Sterilization of Seeds

For surface sterilization of *A. thaliana* seeds, we follow vapor phase sterilization protocol standardized by Clough and Bent, University of Illinois at Urbana-Champaign (http://www.plantpath.wisc.edu/fac/afb/vapster.html). This method obviates the use of liquid-based sterilization methods that requires soaking of seeds in mercuric chloride/bleach/ethanol.

1. Place the seeds in a 1.5 ml microfuge tubes filled to a quarter of the tube. The lesser the quantity of seeds, better the surface sterilization. If required, split the bulk quantity of seeds into multiple microfuge tubes.
2. Label the tubes with indelible marker pen. Many times, the label ink from permanent markers may get erased upon exposure to chlorine gas. Secure the label by covering it with a transparent cellophane tape.
3. Place the rack with labeled tubes containing seeds in a tightly sealed desiccator unit. Make sure to keep the cap of microfuge tube open to ensure proper exposure of seeds to chlorine gas.
4. Measure approximately 100 ml of bleach in a 250 ml beaker and place it in a desiccator containing the seeds.
5. Carefully pipette out 3–5 ml of Conc. HCl using a pipette pump into the beaker containing bleach and immediately seal the desiccator. This step should be done in a fume hood as exposure to chlorine gas and Conc. HCl are toxic. Care should be taken not to pipette out the acid using mouth or pipette man as Conc. HCl is corrosive.
6. Leave the setup undisturbed for 3–4 h allowing sterilization by chlorine gas. After 3 h, remove the lid of the desiccator and let the chlorine gas escape through fume duct. Longer exposure of the seeds to the gas (>5 h) may decrease the viability of seeds.
7. Plate the surface sterilized seeds in MS agar media (*see* **Note 24**). Avoid clumping of seeds while spreading on MS agar plate. Do this step in a laminar flow hood to prevent microbial contamination in MS plates.
8. Incubate the plates/direct sown seeds in soil media in a cold room or refrigerator at 4 °C for 3–4 days.
9. After cold stratification, transfer the plates to a growth cabinet to promote seed germination. Condensed water droplets, if any, on the top of the plates should be removed by opening the plate in a laminar flow hood. This prevents the spread of microbial contamination.
10. Let the seedlings to grow till four leaf stage. This is an ideal stage to transplant the seedlings to soil media for further establishment. Always grow a pot WT parent used for crosses as a control.

3.5.2 Root-Based Prescreening for Haploid Seedlings

If the haploid seeds are selected using fluorescence stereomicroscope, this step is not required. This step is required only if you have used GFP-tailswap without seed GFP marker parent for haploid induction crosses. Before transplanting the seedlings to soil medium, root tissue-based prescreening can be done for selecting haploid seedlings with aid of an upright fluorescence microscope (not fluorescence dissection microscope) (*see* **Note 25**). On the other hand, if you prefer to grow all the seedlings skip to **step 9** (*see* **Note 26**).

1. Label each seedling and gently excise a root tip (main root or lateral root) using fine scissors and transfer it a glass slide with 50% glycerol or water.
2. Arrange the excised root tips in parallel such that several root samples can be examined simultaneously in a single glass slide.
3. Gently place the coverslip above the aligned root tissues. Avoid formation of air bubbles while placing the cover slip. If there are bubbles, gently remove them without disturbing the aligned roots.
4. Examine the slide under an upright fluorescent microscope at 20× to 100× magnification for the presence or absence of GFP fluorescence at the centromeres (Fig. 1i–l).
5. Mark the roots that do not show GFP fluorescence (*see* **Note 25**) at the centromeres and identify the corresponding seedlings, which can now be transplanted to soil media (Fig. 1k).
6. Discard all those seedlings that show GFP signal in centromeres as they are either diploids or aneuploids (Fig. 1i). In case of ambiguity, do not discard those seedlings. Transplant them to soil. Haploids, if any, can be identified at a later stage of growth.
7. In the event of no preselection either at seed and seedling stage, all seedlings can be transplanted to soil (*see* **Note 26**). After transplantation, cover the pots with saran wrap and place them in plant growth cabinet for its establishment.
8. Remove the saran wrap after ensuring successful establishment of the seedlings in the soil media (typically after 5–7 days posttransfer).

3.6 Identification of Haploid Plants

1. Ten days posttransfer of seedlings, screen for plants that resemble WT parent used in the cross, which are one half to less than one third the size of WT plants (Fig. 2a). They are most likely to be haploids (*see* **Note 27**). Use the diploid WT plants of same age as control for size comparison.
2. Depending upon the nature of chromosome deletion/duplication aneuploids display diverse morphological phenotypes (Fig. 2b, c) mostly reflected in leaf color, size, shape, and vegetative growth (*see* ref. 21 for more details). Identify and remove them leaving only the haploid plants. In case of any ambiguity allow the seedlings to grow till reproductive stage.

Fig. 2 (continued) GFP-tailswap resulting from self-pollination, *A* Aneuploid hybrid resulting from chromosome missegregation, *D* Diploid hybrid. Aneuploids will show diverse phenotypes depending upon the number of chromosomes. (**d**) Gel image showing the dCAPS genotyping of haploids. Haploid progeny from GFP-tailswap × WT cross will show WT polymorphism whereas aneuploid and diploid hybrid sibling would show heterozygous pattern. Shown polymorphism is resolved in a 3% agarose gel ran for an hour. (**e**) Two-month-old haploid L*er* plant showing sterile nonelongated pistils and profused flowering even after 2 months. Wild-type diploid L*er* of similar age cease to flower. (**f**) Decapitated inflorescence head from a haploid ($2n=5$), diploid ($2n=10$), and tetraploid ($2n=20$) *A. thaliana* showing increase in floral organ size with increase in number of chromosomes

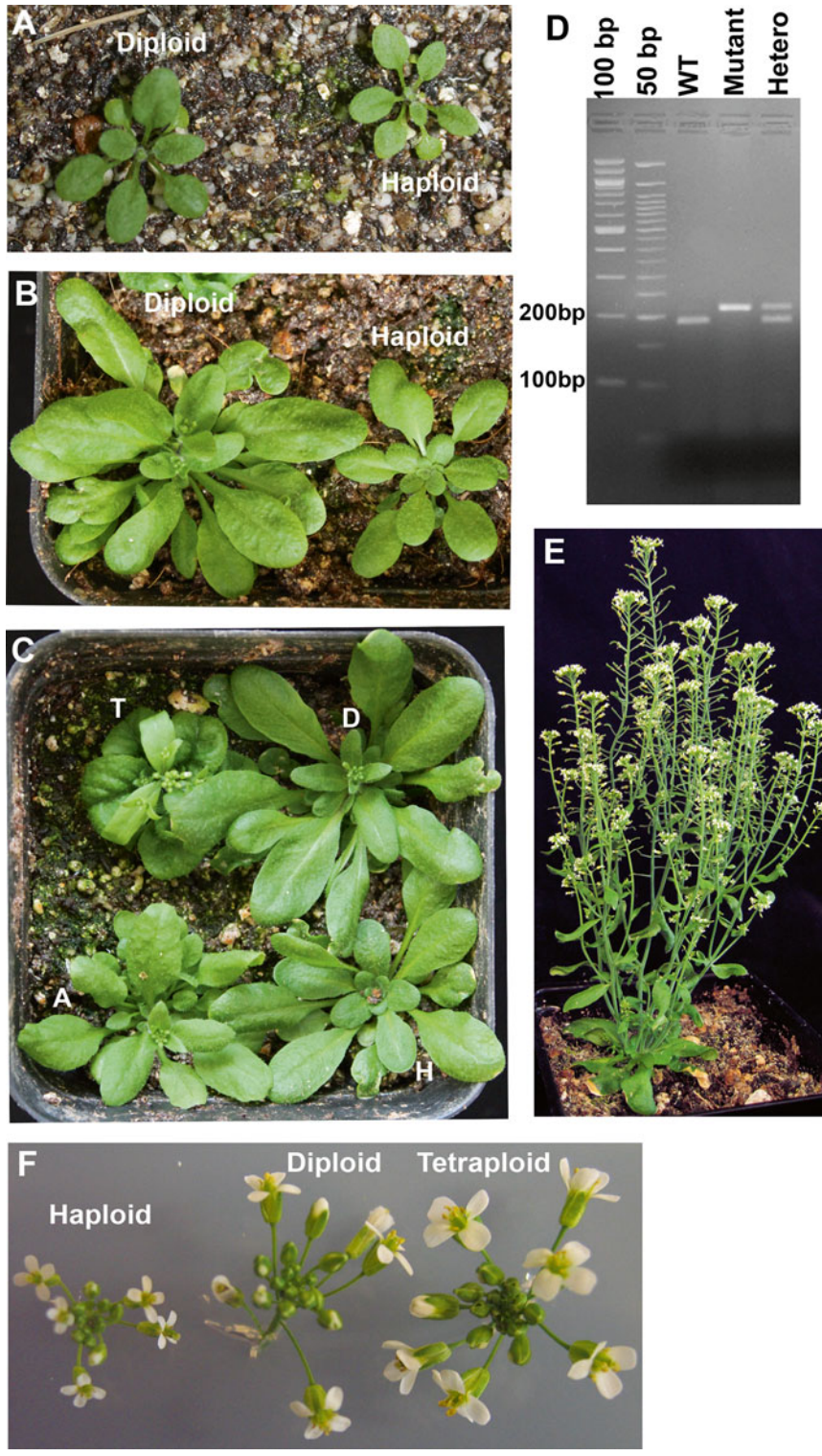

Fig. 2 Phenotypic and genotypic identification of haploid progeny. (**a**) Fifteen-day-old haploid and diploid progeny from GFP-tailswap × WT Col-0 cross. Note that the haploid progeny is approximately half the size of its diploid sibling. The overall morphology remains the same between the two. (**b**) A month old haploid and diploid sibling from GFP-tailswap × WT (HKT 2.4 accession). Note the size difference between them even at later stages of vegetative growth. (**c**) A snap shot of a month old F1 progeny from GFP-tailswap × WT (HKT 2.4 accession) cross without preselection of haploids. *H* Haploid resulting from uniparental genome elimination, *T*

3. In addition, phenotypic analysis of haploids can be complemented by PCR genotyping and/or ploidy analysis by flow cytometry to unambiguously identify haploid plants.

3.6.1 DNA Isolation for Genotyping of Haploids

There are several rapid DNA extraction protocols for dCAPS PCR and genotyping. Adopt the protocol that has been standardized in your lab.

We follow the one described below. Using this method, we find that DNA remains stable for at least a month or more for later use.

1. Gently excise a single rosette leaf using fine scissors and transfer it to a microfuge tube.
2. Crush the tissue to a fine pulp using a bullet blender or paint shaker. Alternatively, if liquid nitrogen is available, snap freeze the tissue (can be stored in −80 °C for later use at this stage) and grind the tissue to a fine powder using a micro pestle.
3. Add 500 μl of CTAB extraction buffer to the ground tissue and vortex it. Add the buffer immediately after grinding/blending (*see* **Note 28**).
4. Add 400 μl of 24:1 chloroform: isoamyl alcohol mixture and gently mix the ingredients.
5. Centrifuge the resultant mixture at 10,621 × *g* for 5 min.
6. Pipette out the aqueous phase to a fresh tube. Avoid pipetting out the tissue debris and the organic phase.
7. To the aqueous phase, add equal volume of isopropyl alcohol and gently invert the tube twice to mix the contents.
8. Centrifuge the contents for 10 min at RT or 4 °C at 17,950 × *g*.
9. Wash the resultant DNA pellet with 70% ethanol.
10. Air-dry the pellet (~15–20 min) by gently inverting the microfuge tube with cap open on a filter paper.
11. Dissolve the DNA pellet in 30 μl of TE buffer or sterile water (*see* **Note 29**).
12. Use 1 μl of the DNA for the PCR reaction.

3.6.2 PCR Genotyping of GFP-tailswap Plants

dCAPS PCR genotyping [22] is done using the oligos specific for identifying *cenh3-1* mutation (*see* **Note 30**). Use the following *Eco*RV dCAPS primers to distinguish the WT allele from the mutant allele

Forward: 5′GGTGCGATTTCTCCAGCAGTAAAAATC3′.

Reverse: 5′CTGAGAAGATGAAGCACCGGCGATAT3′.

1. Setup a standard PCR reaction to a final reaction volume of 15 μl using the above primer combinations.
2. Amplify for 35 cycles (94 °C for 10 s; 58 °C for 10 s; 74 °C for 15 s) with initial denaturation of 94 °C for 2 min and final extension at 74 °C for 2 min. An amplicon of length 215 bp should be visualized upon agarose gel electrophoresis.

3. Prepare the *Eco*RV enzyme (NEB) mastermix (for one reaction: 2 μl NEB 10× cutsmart buffer, *Eco*RV restriction enzyme: 0.2 μl and water: 2.8 μl). The master mix can be scaled up according to the number of samples.
4. Add 5 μl of the mastermix directly to PCR tube containing the amplified products (15 μl). PCR purification is not required.
5. Gently mix the sample, followed by a brief spin and incubate the mixture at 37 °C for at least 6 h to overnight to achieve complete restriction digestion. If using high fidelity *Eco*RV enzyme, the incubation time can be reduced appropriately (*see* **Note 31**).
6. After the necessary incubation period, load the samples (15–20 μl) onto a 2.5 or 3% agarose gel and electrophoresis it for an hour to achieve complete separation of the fragments. Use a 50 or 100 bp ladder as a marker.
7. Visualize/document the gel and score the polymorphisms to identify the genotype of the *CenH3* locus (Fig. 2d) (*see* **Note 32**).
8. In addition to dCAPs genotyping, haploid plants can be distinguished by scoring for the presence or absence of *GFP-tailswap* transgene in the seedling (*see* **Note 33**). Diploid and aneuploid seedlings would show positive amplification for the presence of *GFP-tailswap* transgene whereas haploids won't give any amplification (*see* **Note 34**). Use the following primers to genotype for *GFP-tailswap* insertion.

   Primer 1 for WT and T-DNA: 5′CACATACTCGCTACTGGT CAGAGAATC3′.

   Primer 2 for WT only: 5′CTGAAGCTGAACCTTCGTCTCG3′.

   Primer 3 for the T-DNA: 5′AATCCAGATCCCCCGAATTA3′.

   WT (Primer 1 + Primer 2): 518 bp.

   T-DNA (Primer 1 + Primer 3) = ~450 bp.

   PCR conditions: 35 cycles (94 °C for 15 s; 58 °C for 30 s; 74 °C for 30 s) with initial denaturation of 94 °C for 2 min and final extension at 74 °C for 5 min.
9. Upon flowering, size compare the putative haploid flowers with diploid control. Haploid flowers should approximately be half the size of diploid flowers (Fig. 2f). Also the haploid inflorescence should be sterile with nonelongated siliques (*see* **Note 35**).
10. For further confirmation of haploidy go for cytological (preferably meiosis) examination of fixed flower buds [23]. Haploids should show five chromosomes (*see* **Note 36**).

3.7 Converting Sterile Haploids to Fertile Double Haploids (DH)

Sterile haploid plants have to be converted to fertile diploids (doubled haploids—DH) in order to propagate them. This can be achieved by two ways. The most straightforward and preferred method is to simply harvest the spontaneous DH seeds produced by the haploid plants as a result of meiotic or mitotic doubling events.

1. Let the haploid plants to grow till it stops flowering. Generally haploids tend to flower longer than its diploid counterpart (Fig. 2e) (*see* **Note 37**).
2. Dry the haploid plants for a week or two. The leaves should be crisp and crumble if crushed and the stalks should break if pressed. At this stage, examine the inflorescence in bright light to identify siliques that harbor one or two seeds at the maximum (*see* **Note 38**).
3. Place the inflorescence free of soil on top of a white paper and gently thrash the dried pistils/silique by hand to ensure all pistils split open their valves to release seeds, if any. Make sure no pistils are left unthrashed.
4. Sieve the seeds to separate it from debris. These seeds are spontaneous doubled haploid seeds that can be grown to multiply and maintain the homozygous genotype.

3.8 Colchicine Treatment of Haploid Seedlings to Convert Them to Doubled Haploids

Sterile haploids can also be converted to fertile double haploids by treating them with spindle poison, Colchicine. This method is required only if haploids don't produce any spontaneous doubled haploid seeds.

1. Select 20–25 day old haploid seedlings ideally before flowering.
2. Prepare 0.25% colchicine solution with Silwet (0.2%) as surfactant.
3. Drop 10–20 µl of the solution in the center of the shoot apical meristem (SAM) of the haploid seedlings. Ensure that the applied solution completely soaks the SAM.
4. Let the plant grow and after few days, the plants shall become sick. The leaves and meristems shall show necrotic symptoms. If required, boost the growth with a half strength MS solution.
5. Keep the colchicine treated plants healthy by providing ideal environment for its establishment and growth.
6. One or 2 weeks later, the sick looking plants gradually recover and show healthy growth. Observe for new flush of meristem, which later give rise to inflorescence side shoots.
7. Observe the developing inflorescence. If colchicine treatment has worked, the flowers from the inflorescence will be fully fertile, producing well-elongated siliques indicating successful conversion of a haploid shoot to a diploid. If the treatment is unsuccessful, the flowers will be sterile and pistil won't elongate.
8. Collect the seeds at harvest maturity from the fertile shoots. The resulting seeds yields doubled haploid progeny.

3.9 Crossing GFP-tailswap as a Pollen Parent to Produce Maternal Haploids

GFP-tailswap can also be used as a pollen parent to produce maternal haploids. We have found that some late-formed flowers in the GFP-tailswap inflorescence have reasonable amounts of viable pollen that can be used to pollinate any WT plant to produce maternal haploids (*see* **Note 10** for difference between paternal and maternal haploids).

1. Collect all open flowers from GFP-tailswap parent.
2. Turn on the stereo dissection microscope and examine the anthers from the collected flowers for viable pollen. Zoom the magnification to a point where you can clearly visualize pollen. Viable pollen will be yellow, plump, and oblong in shape and well-hydrated whereas dead pollen will be irregular in shape, pigmented dark, and look shriveled and dehydrated.
3. Gently excise those anthers containing viable pollen and pool them.
4. Dust the pollen from several anthers onto to a single pistil of the emasculated flower bud of any desired parent. Depending on the amount of viable pollen you may require atleast 10–25 anthers/pistil for achieving good seed set.
5. After this step follow the same procedures as described earlier to select and grow haploid progeny.

4 Notes

1. MS basal salt mixture is commercially available from many manufacturers and it can be directly dissolved in water to prepare 1× MS solution as per their recommendations. Some of the salt ingredients that constitute the MS solution may be available in both forms: hydrated and anhydrous. Both forms can be used for preparing the stocks. Hence check the formula weight and weigh appropriate amount for making stock solutions.
2. Sucrose can also be added after autoclaving the MS solution. If so, measure and add appropriate quantity from a 40% sucrose stock solution (filter sterilized); some prefer this to avoid caramelization of sucrose during autoclaving.
3. After autoclaving, the media can be poured onto petriplates or can be stored at room temperature (RT) for later use. To prevent microbial contamination antibiotics such as Ampicillin (100 μg/ml) and/or fungistat such as Nystatin can also be added to the MS media before making the plates. Use laminar flow hood to pour plates to avoid microbial contamination. The solidified MS agar plates can be sealed using parafilm and stored at 4 °C for later use.
4. Soilrite, vermiculite, and perlite are obtained from Keltech Bioenergies Pvt. Ltd. Bangalore, India. Alternatively, any

horticultural growth medium/commercial soil mix or any other formulation that basically supports the growth of *A. thaliana* plants can be used. We autoclave the soil mix to get rid of any microbes and pest. This helps to prevent mold growth in the soil medium. Cocopeat helps to retain moisture for longer time, thus inclusion of it in soil medium minimizes the frequency of fertigation/watering. In the absence of cocopeat the soil medium becomes dry very quickly, thus necessitating frequent watering to keep the soil wet for good growth of plants. Cocopeat can also be replaced with peat or any equivalent soil less substitute.

5. In our experience, we find that fertigation with nitrogenous fertilizers (15 mM KNO_3 and 20 mM NH_4NO_3 solution) once or twice at uniform intervals during active vegetative growth promote lush growth of plants and robust healthy inflorescence with many flowers. This is especially important while growing haploid plants because it maximizes the frequency of spontaneous doubled haploid seed formation by producing more number of flowers which increases the probability of random seed set.
6. GFP-tailswap plants are partially sterile showing more male sterility and good female fertility, an ideal trait for crossing without emasculation. For reasons unknown, male sterility is more pronounced in acropetal flowers (early formed older flowers in the inflorescence) in contrast to basipetal flowers of the inflorescence. The late restoration of male fertility in basipetally formed flowers results in partial seed set as a consequence of self-pollination. Such seeds can be used to raise a homogeneous population of GFP-tailswap plants bypassing the need to maintain the mutation in heterozygous condition. Some proportion of dark brown dead seeds will also be obtained. However, a majority of selfed seeds are viable and give rise to diploid GFP-tailswap progeny (95%) and aneuploid progeny (5%). The aneuploids can be distinguished by the phenotype [6].
7. Seeds from ABRC consists of heterogenous mixture of all genotypes (*CenH3/CenH3*; *GFP-tailswap/GFP-tailswap*, *CenH3/cenh3-1*; *GFP-tailswap/GFP-tailswap*, *cenh3-1/cenh3-1*; *GFP-tailswap/GFP-tailswap*) as they are bulked from a F2 or later generation progeny. Hence one has to identify *cenh3-1/cenh3-1*; *GFP-tailswap/GFP-tailswap* plants for use as HI parent for crossing. This can be achieved easily by the diagnostic lettuce-like compact vegetative growth phenotype of homozygous *GFP-tailswap* plants. The other siblings will have WT phenotype.
8. The phenotype of GFP-tailswap homozygous plants are so prominent that genotyping is not required if you use CS66982 seed stock for haploid induction. Other transgenic and non-transgenic inducers as mentioned in the introduction may not show the diagnostic phenotype and thus you may need to genotype them before crossing.

9. If you prefer to maintain the GFP-tailswap plants in heterozygous condition (*CenH3/cenh3-1*; *GFP-tailswap/GFP-tailswap*) for seed multiplication you can allow those plants to grow.
10. As GFP-tailswap plants are more female fertile than male, it is easy to use GFP-tailswap as a female parent while crossing. Furthermore, we have found parent of origin effect for haploid production. High frequency of haploids (>40%) are produced only when GFP-tailswap is used as female in contrast to using it as male parent (<4% haploids). Hence we recommend to use GFP-tailswap as a female parent to produce haploids. It should be noted that use of GFP-tailswap as female results in the production of paternal haploids where in the haploid progeny contain nuclear genome from the paternal (pollen) parent and cytoplasmic genome from the maternal (Col-0) parent. Maternal haploids (containing both the nuclear and cytoplasmic genome from the WT parent used in a haploid generating cross), can be produced by the reciprocal cross (WT (female) X GFP-tailswap (male)) but the frequency of haploids will be low. This demands huge growing space to screen for haploids. If presence of GFP-tailswap (Col-0) cytoplasmic genome in the resulting haploid is a concern, then we advise developing haploid inducer in the desired accession's cytoplasmic background. To achieve this, cross GFP-tailswap as male (pollen parent) to any desired accession as a female to produce F1 diploid hybrid now harboring the desired accession's cytoplasmic genome. F2 progeny from such hybrid will segregate for GFP-tailswap progeny, which now can be used as a female parent to induce haploids from that accession. The disadvantage of this approach is that additional two generations is required to generate such haploids.
11. As early formed flowers are mostly male sterile, emasculation of flower buds is not required at this stage of crossing. Manual pollination can be done directly on the open flowers of GFP-tailswap plants. This makes the crossing job easier and many flowers can be crossed at a time. Pollination is generally done by gently rubbing the open flowers of pollen parent onto the pistil of GFP-tailswap plant. Alternatively, excise 2–3 anthers with good pollen load using fine forceps and dust the pollen on the stigma of the pistil. However, this is a time consuming process. If large numbers of pollen parent are available, pollen can be harvested using the vacuum collection method [24] to massively pollinate as many GFP-tailswap pistils as possible. This method is ideal, if massive crossing is required to generate a large-scale population of haploids. Pollination can be done with aid of an optivisor or under a stereo dissection microscope to ensure proper deposition of pollen in the stigma. Pollination can be done during anytime of the day, but preferably in the morning as pistils are more receptive in morning than in the evening. Further the anthers release copious load of pollen in the morning than later in the day. Also,

flowers tend to close in the evening masking the anthers and pistils. In case of accidental self-pollination due to occasional male fertility, the resultant selfed progeny can be easily distinguished from the crossed hybrid progeny by its compact, lettuce-like vegetative phenotype.

12. If the cross is unsuccessful, the pistil continues to remain receptive and won't elongate. This serves as a marker to identify successfully pollinated pistil from unpollinated ones.
13. In our experience, we have found that stigma from an unpollinated pistil/flower remains receptive up to a maximum of 4 days post-flower opening.
14. On an average, viable seeds obtained from a single crossed silique may give 2 haploid progeny. Hence if you require 50 haploid progeny, then you need at least 25 successful crosses. Follow this rule of thumb to decide on the number of crosses to be made. Crosses can be continued either on subsequent fresh flowers arising from the same inflorescence or on new inflorescence either from same or a different plant. Ideally, use one GFP-tailswap plant for crossing to one particular genotype. This avoids admixture of seeds when crossing with multiple genotypes.
15. Flowers from later stages of inflorescence (2–3 weeks after flowering of GFP-tailswap) when crossed without emasculation mostly yield high frequency of self-pollinated progeny. This suggests that those flowers produce more viable pollen at later stages of inflorescence development. If you plan to use these flowers for crossing, then those flower buds should be emasculated the previous day before crossing to avoid self-pollination. For emasculation, choose unopened flower buds that are ready to flower the next day. Emasculation is done by gently removing all the anthers from the flower bud using fine tweezers (Dumont No. 5 Swiss tweezers). However, we find it is easy to remove all whorls of flower along with anthers leaving only the pistil for crossing.
16. This promotes proper development of crossed siliques as nutrient resources can now be diverted to developing ovules rather than to produce more flowers. Removal of inflorescence shoots that was not used for crossing helps to prevent tangling of crossed shoots with uncrossed ones and also helps to easily monitor the crossed siliques and also prevents seed contamination from self-fertilized siliques.
17. An easy way to gauge harvest maturity is the time when silique valves change its color from green to tan. At this stage, the valves tend to split open naturally and release seeds with a gentle touch without much force.
18. A typical silique resulting from GFP-tailswap cross (as female), on an average, will produce 30–40 seeds per silique of which

only 10–25% of seeds (depending on the accession) will be viable which are plump with mature embryo. Most of the F1 seeds will be either aborted or inviable as a result of genome elimination. The dead seeds are mostly misshapen, empty, and dark brown. Among the viable progeny varying proportion of haploid, hybrid diploid, and aneuploid seeds will be present.

19. It is best to store only the mature harvested seeds and not the entire siliques. If the siliques are not dried properly, moisture accumulating from the siliques when stored in microfuge tubes, invites mold growth. This may affect seed germination, and if unattended, may kill the harvested seeds.
20. SeedGFP-HI strain is an improved version of GFP-tailswap strain that can be used for preselection of haploid seeds based on GFP fluorescence in the endosperm and cotyledon. This strain is made by transforming GFP-tailswap strain with a second GFP construct expressed under the control of the seed storage protein 2S3 promoter (*At2S3:GFP*) [25]. Because of this construct the whole seed will show GFP fluorescence when observed under stereo dissection fluorescence microscope (*see* Fig. 1d, g). This will not be the case with GFP-tailswap seeds. Both GFP-tailswap and SeedGFP-HI plants on the other hand will show GFP fluorescence at the centromeres of all cells, which can be clearly observed as punctate green foci at 20×–100× magnification using an upright fluorescence microscope.
21. Preselection of haploid seeds can be achieved only if crosses are done using SeedGFP-HI strain. Preselection cannot be made if GFP-tailswap strain (ABRC accession CS66982) is used for crossing. SeedGFP-HI strain will soon be deposited at ABRC and is available to the community on request.
22. In our experience, we have found that seeds that show uniform GFP fluorescence are either selfed GFP-tailswap/hybrid diploid/aneuploid and thus can be removed from the rest of the seed population. Fine needles such as insulin needles can also be used for sorting out the seeds but sometimes the seeds may pop out while sorting and thus there is a risk of losing those seeds when using rigid materials such as fine needles.
23. It is better if the seeds are germinated in MS agar plates as we have found that some proportion of the haploid seeds especially the misshapen seeds are slow germinating and thus better care and attention can be given if they are in the plates. This increases the frequency of recovering a large number of haploid progeny from a cross. Further this also allows for the root-based prescreening for haploid seedling prior to transplantation in soil medium.
24. To uniformly spread dry seeds in MS agar plates, place the seeds on a sterile filter paper and slowly tap the paper from bottom to release one seed at a time so as to ensure uniform placement of seeds. Sterilized seeds can also be presoaked in 100 μM

Gibberellic acid (filter sterilized) O/N before plating. This treatment generally increases the frequency of seed germination, there by maximizing the recovery of slow germinating haploid seeds. Alternatively, Gibberellic acid can also be added in the MS agar plates at a final concentration of 25 μM.

25. The roots from selfed seedlings, hybrid diploid, and aneuploid seedlings will show GFP fluorescence at the centromeres as distinct punctate green foci. In contrast, roots from haploid seedling resulting from loss of all chromosomes from GFP-tailswap parent will not show GFP fluorescence at the centromeres.
26. If you are doing a large-scale crossing experiment, it may be cumbersome to go for root-based screening of all seedlings. In this case you can sow/transplant all the seeds/germinated seedlings respectively without any preselection. Upon growth you observe an array of ploidy siblings (depending on the genotype of accession): 40–50% haploids as a result of uniparental genome elimination, 50–60% aneuploids, 10–15% diploid hybrids. Haploids most often can be phenotypically distinguished from other siblings and rest of the aneuploid plants can be removed. This is highly recommended if the resources for growth of Arabidopsis plants especially the space is not limiting. The haploids can be selected at later stages and other unwanted siblings can be removed during the vegetative growth.
27. Haploid plants are phenotypically identical to diploid plants in overall morphology except for proportional reduction in size. If preselection is not done, it will be easy to compare haploid siblings from diploid hybrid and aneuploid siblings as they might get randomly placed in the same pot while planting facilitating easy comparison among them.
28. Do not allow the powder/pulp to thaw longer, DNA might get degraded due to activity of host nucleases.
29. If desired, include RNAse in the extraction buffer to get rid of contaminating RNA from the resultant DNA preparation. However, it is not an absolute requirement, as it won't interfere with PCR or with genotyping.
30. As the haploid-inducing parent is homozygous for *cenh3-1* point mutation, the F1 hybrid diploid and aneuploid seedlings will be heterozygous for *cenh3-1* allele. In contrast, the haploid seedlings that originates from WT parent will be homozygous for WT *CenH3* allele. *cenh3-1* is a splice acceptor (AG to AA) point mutation in the *CenH3* gene (also known as *HTR12*). The mutation is G161A transition change induced by EMS mutagenesis relative to ATG = +1.
31. When doing dCAPs restriction digestion always include a positive control (homozygous *cenh3-1* mutant, heterozygous *cenh3-1/CenH3* mutant) and a negative control (homozygous WT *CenH3* plant) to ensure complete digestion of the PCR

products. As the mutant allele remains intact without being cut by *Eco*RV enzyme, inclusion of proper controls shall indicate instances of incomplete/partial digestion.

32. PCR amplified product from plants homozygous for mutant *cenh3-1* allele will remain uncut by *Eco*RV restriction enzyme and an intact 215 bp product (slightly above the 200 bp marker) can be seen after electrophoresis, whereas those homozygous for WT *CenH3* will be completely cut to yield 191 bp (slightly below the 200 bp marker) and 24 bp product. However, 24 bp product won't be so prominent and may comigrate with primer fragments. On the other hand, the heterozygous plants will show 215 bp, 191 bp and 24 bp (not prominent) respectively (*see* Fig. 2d).
33. *GFP-tailswap* is a Hygromycin-marked transgene (pCAMBIA 1300) that has the native *CenH3* promoter and terminator, a N-terminal GFP tag, the N-terminal tail domain of histone H3.3, the C-terminal histone fold domain of CenH3 that complements the embryo-lethal phenotype of *cenh3-1* mutant. TAIL-PCR (Thermal asymmetric interlaced PCR) initially indicated that the transgene has landed in chromosome 1. Later, it was discovered that T-DNA integration has induced a reciprocal translocation between chromosome 1 and 4 harboring multiple T-DNA insertions [21]. Nevertheless, we can genotype for the presence or absence using the primers described in the methods. PCR genotyping for the absence of transgene in haploid may be misleading in the event of a failed PCR due to trivial reasons such as poor quality DNA. Hence include proper controls to confirm the negative result.
34. WT diploid seedling contamination cannot be ruled out at this stage but can be distinguished later at reproductive stage by its sterile phenotype (*see* **Note 35**).
35. The sterile nature of haploid plants in combination with smaller size inflorescence (in relative comparison with WT and aneuploid plants) can be used as a diagnostic tool to identify the haploid plants after flowering. If there are any WT diploid contaminants in the population, it can be distinguished at this stage. The WT plants are fully fertile with fully elongated siliques in contrast to haploid plants that are sterile with non-elongated pistils. If required, additional cytological confirmation can be done using fixed immature flower buds, to count for haploid number of chromosomes. This is the gold standard method for confirming the haploid nature of plants. We prefer using fixed immature flower buds for chromosome analysis as both meiotic and mitotic chromosome count can be done simultaneously. Ploidy analysis can also be done by flow cytometry, but it is not fool proof as disomic haploids and haploids with mini chromosomes cannot be distinguished clearly.

36. In addition to diploids ($2n=10$ chromosomes) and higher aneuploids ($>2n=10$ chromosomes) sometimes we do obtain disomic haploids ($n+1$) and haploids with minichromosomes that may resemble true haploids by phenotype [21]. However, they are very low in frequency and can be ignored. The best way to identify is by karyotyping by cytological examination of mitotic and meiotic cells. Meiotic cells are more preferred than mitotic cells as chromosomes are clearly visible and can be unambiguously counted. For example at pachytene, haploids have five univalents whereas diploids will show five bivalents. However, mitotic cells can also be used but most of them will be at interphase. These cells generally show 5–6 chromocenters/nuclei when stained with DAPI.
37. Being sterile, haploid plants tend to flower profusely producing more inflorescence in contrast to diploids. Hence haploids continue to flower for a longer periods of time even after control diploids cease flowering. Seed set in haploid plants depends on chance events that lead to segregation of all five univalent chromosomes to a single pole (5-0 segregation) during meiosis I segregation resulting in the production of viable haploid gametes. Higher the number of flowers, higher the chance of random events producing viable gametes and more the probability of recovering spontaneous doubled haploid seeds.
38. Due to sterile nature of haploids, a majority of siliques will be empty, implying absence of seeds in haploid plants. However, careful examination of dried inflorescence shall reveal instances of spontaneous seed set as a result of meiotic doubling in few siliques with a maximum of upto 2–4 seeds/silique. These are doubled haploid seeds and can be harvested once siliques are dried. These seeds can be released only by manual force to split open the valves. In our experience, we collect around 50–5000 DH seeds/haploid plant depending on the accession. A majority (>95%) of them will be viable and give rise to fertile homozygous diploids. However, for practical purposes, a single viable doubled haploid seed is sufficient to multiply the genotype.

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References

1. Murovec J, Bohanec B (2012) Haploids and doubled haploids in plant breeding. In: Abdurakhmonov IY (ed) Plant breeding. InTech, Rijeka, pp 88–106
2. Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8:377–424
3. Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375
4. Dirks R, van Dun K, de Snoo CB et al (2009) Reverse breeding: a novel breeding approach based on engineered meiosis. Plant Biotechnol J 7:837–845
5. Wijnker E, van Dun K, de Snoo CB et al (2012) Reverse breeding in *Arabidopsis thaliana* generates homozygous parental lines from a heterozygous plant. Nat Genet 44:467–470
6. Ravi M, Marimuthu MPA, Tan EH et al (2014) A haploid genetics toolbox for *Arabidopsis thaliana*. Nat Commun 5:5334
7. Dunwell JM (2009) Patents and haploid plants. In: Forster BP, Jain SM, Touraev A (eds) Advances in haploid production in higher plants. Springer, Dordrecht, pp 97–113
8. Guha S, Maheshwari S (1964) In vitro production of embryos from anthers of Datura. Nature 204:497
9. Ferrie AMR, Caswell KL (2010) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Cult 104:301–309
10. Scholl RL, Feldmann KA (1990) Arabidopsis thaliana (L.): in vitro production of haploids. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 12: Haploids in crop improvement I. Springer, Berlin, pp 309–321
11. Scholl RL, Amos JA (1980) Isolation of doubled-haploid plants through anther culture in *Arabidopsis thaliana*. Z Pflanzenphysiol 96:407–414
12. Gresshoff PM, Doy CH (1972) Haploid *Arabidopsis thaliana* callus and plants from anther culture. Aust J Biol Sci 25:259–264
13. Ravi M, Chan SWL (2010) Haploid plants produced by centromere-mediated genome elimination. Nature 464:615–618
14. Seymour DK, Filiault DL, Henry IM et al (2012) Rapid creation of Arabidopsis doubled haploid lines for quantitative trait locus mapping. Proc Natl Acad Sci U S A 109:4227–4232
15. Maheshwari S, Tan EH, West A et al (2015) Naturally occurring differences in CENH3 affect chromosome segregation in zygotic mitosis of hybrids. PLoS Genet 11:e1004970
16. Ravi M, Kwong PN, Menorca RMG et al (2010) The rapidly evolving centromere-specific histone has stringent functional requirements in *Arabidopsis thaliana*. Genetics 186:461–471
17. Kuppu S, Tan EH, Nguyen H et al (2015) Point mutations in centromeric histone induce post-zygotic incompatibility and uniparental inheritance. PLoS Genet 11:e1005494
18. Wijnker E, Deurhof L, van de Belt J et al (2014) Hybrid recreation by reverse breeding in *Arabidopsis thaliana*. Nat Protoc 9:761–772
19. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
20. Salinas J, Sanchez-serrano JJ (2006) Arabidopsis protocols. Methods in molecular biology, vol. 323, 2nd edn. Humana, Totowa
21. Tan EH, Henry IM, Ravi M et al (2015) Catastrophic chromosomal restructuring during genome elimination in plants. eLife 4:e06516
22. Neff MM, Neff JD, Chory J, Pepper AE (1998) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J 14:387–392
23. Higgins JD, Wright KM, Bomblies K, Franklin FCH (2014) Cytological techniques to analyze meiosis in *Arabidopsis arenosa* for investigating adaptation to polyploidy. Front Plant Sci 4:546
24. Johnson-Brousseau SA, McCormick S (2004) A compendium of methods useful for characterizing Arabidopsis pollen mutants and gametophytically-expressed genes. Plant J 39:761–775
25. Kroj T, Savino G, Valon C et al (2003) Regulation of storage protein gene expression in Arabidopsis. Development 130:6065–6073

Chapter 7

Gametocidal System for Dissecting Wheat Chromosomes

Hisashi Tsujimoto

Abstract

Gametocidal (Gc) system is a novel method of inducing chromosome mutations in wheat by using *Gc* genes of wheat-related species. Many deletion lines were efficiently produced by this system and used for physical mapping of genes and DNA markers. A large stocks with chromosome deletions in homozygous state as well as the lines with *Gc* genes are available in the gene bank of Japan (KOMUGI). In this chapter, I describe the method of inducing breakage in a target chromosome using lines with the *Gc* gene on chromosome 2C of *Aegilops cylindrica* (a wheat-related wild species) and nullisomic–tetrasomics.

Key words Gametocidal chromosome, Addition line, *Aegilops cylindrica*, KOMUGI, Deletion stock, Chromosome dissection, Nullisomic–tetrasomics

1 Introduction

Allohexaploid bread wheat (*Triticum aestivum* L., $2n=42$, AABBDD) has a huge and complex genome comprising A, B, and D homoeologous genome groups. The presence of three sets of homoeologous genes confers more chromosome mutation tolerance to this hexaploid species relative to the diploid species such as *Arabidopsis* and rice. Several diploid wheat progenitor species of the genus *Aegilops* possess the so-called *Gc* genes which are located in different chromosomal regions of their respective genomes (*see* 1 for details). Genetic evidence based on observations in gametogenesis in monosomic alien chromosome addition lines implicated the *Gc* genes as selfish genetic factors [1]. Most often (and especially under intense action of the *Gc* genes), the genetic effect of the *Gc* genes results in a preferential transmission of only *Gc* gene gametes (with the alien chromosome) to the next generation as the non-*Gc* gene gametes become abortive [1].

The Gc system has been exploited to induce chromosome breakage in the gametes and a large number of wheat genetic stocks with chromosome aberration has been maintained in many gene banks such as KOMUGI in Japan (KOMUGI: http://www.

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shigen.nig.ac.jp/wheat/komugi/) and WGRC in the USA (WGRC: https://www.k-state.edu/wgrc/). These genetic stocks are useful to map genes of interest or in creating other novel materials in this species. Also wheat genetic stocks with a pair of alien chromosome named disomic addition lines ($2n=44$, AABBDD + xx, where x is an alien chromosome) are available in the gene bank.

The cross between the disomic addition ($2n=44$, AABBDD + xx) and a normal plant ($2n=42$, AABBDD) produces monosomic addition ($2n=43$, AABBDD + x). The alien chromosome (x) forms univalent in meiosis and is segregated into the female and male gametes (Fig. 1a). Thus, in the next generation,

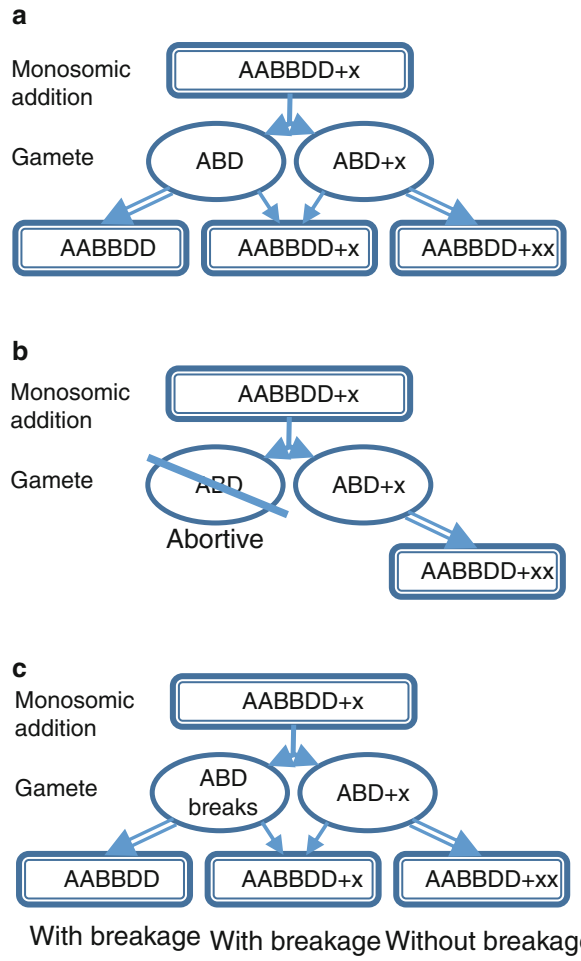

Fig. 1 The behavior of alien chromosome added to wheat. (**a**) Monosomic addition line produces gametes with or without the alien chromosome in a certain ratio. Thus, normal, monosomic addition and disomic addition segregate in the next generation. (**b**) If the alien chromosome carries a *Gc* gene(s) with intense action, the gametes without the alien chromosome becomes abortive because its chromosomes are severely broken by the *Gc* gene(s). (**c**) If the alien chromosome possesses a mild *Gc* gene(s), the slightly broken chromosome(s) is transmitted to the next generation through fertilization

the monosomic addition yields three types of lines: normal (AABBDD), monosomic addition (AABBDD + x), and disomic additions (AABBDD + xx), in a certain ratio (Fig. 1a). However, some alien chromosomes are preferentially transmitted to the next generation because a gene(s) on the alien chromosome induces chromosome breakage in the gametes without the alien chromosome (Fig. 1b). If the effect of the Gc chromosome on chromosome breakage is strong, the resulting gametes with breakage would be abortive (Fig. 1b). As a consequence, only the gametes with the alien chromosome participate in fertilization, and thus the chromosome is transmitted preferentially from both the male and female gametes, generating the disomic addition. On the other hand, if the effect is mild, the gametes with a single or few breakages would remain functional and participate in fertilization, resulting in the appearance of plants with chromosome breakage in the next generation (Fig. 1c).

Because the event of chromosome breakage occurs only in the gametes, the size of breakage in all the cells in a plant is uniform (not chimeric) except the case of presence of dicentric or ring chromosome that generates further breakages in mitoses. Tsujimoto et al. [2] crossed the monosomic addition line of chromosome 2C of *Aegilops cylindrica* (AABBDD + 2C) with nullisomic 1B–tetrasomic 1D that lacks a pair of chromosome 1B and obtained 1327 F_1 plants (Fig. 2). They observed the chromosome 1B in the F_1 plants by C-banding and found that 128 plants (9.6%) of this cross carried one or more aberrations in chromosome 1B (Fig. 3, Table 1). Because these chromosomes are present in the hemizygous state in the F_1 plants, these were used for physical mapping with RFLP markers [2].

Endo and Gill [3] identified plants with a deleted chromosome in the offspring of the AABBDD + 2C or line with chromosome of *Ae. triuncialis* L., or a chromosome segment of *Ae. speltoides*

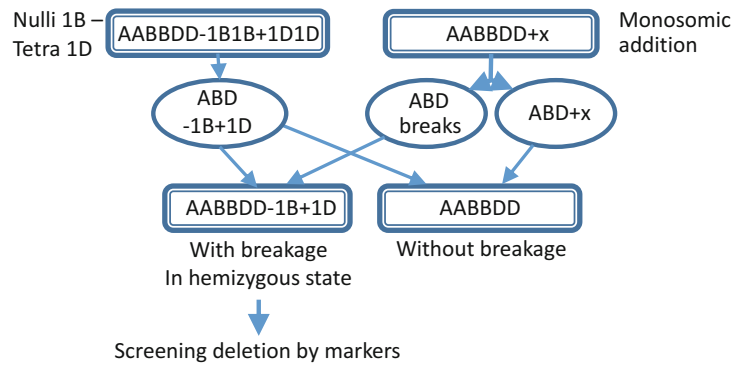

Fig. 2 Breeding procedure to produce deletion lines in the target chromosome. This figure shows the procedure to generate chromosome 1B deletion

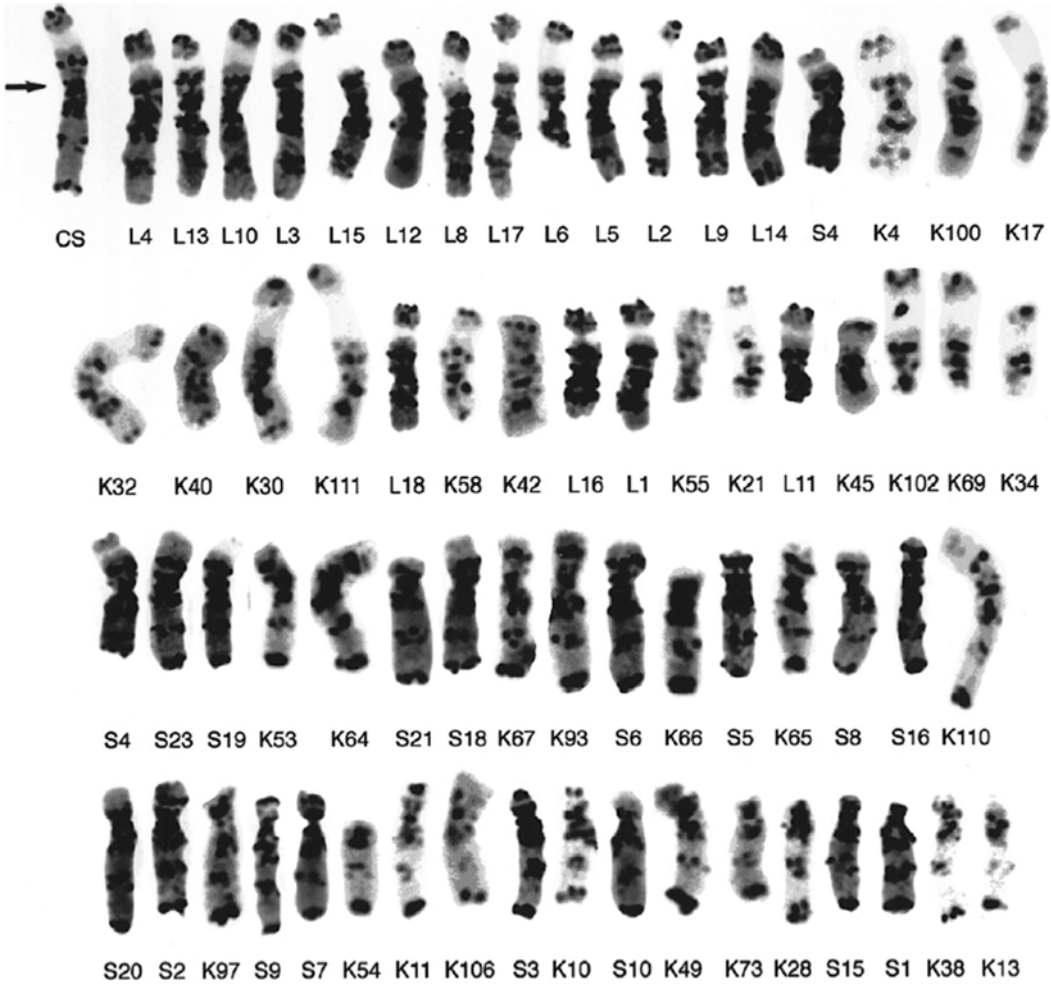

Fig. 3 Deletion and translocation on chromosomes 1B produced by Tsujimoto et al. ([2], chromosomes initiated by K) and Endo and Gill ([3], chromosome initiated by L or S). Cited by Tsujimoto et al. [2]

Table 1
Frequency of aberration on chromosome 1B among 1327 F_1 plants between AABBDD + 2C and nullisomic 1B–tetrasomic 1D [2]

	Short arm	Long arm	Both arms	Total	%
Terminal deletion	38	70	2	110	85.8
Translocation	5	1	1a	7	5.5
Dicentric	4	4	0	8	6.3
Insertion	0	1	0	1	0.8
Highly chimeric	0	0	2	2	1.6
Total	47	76	5	128	
%	36.7	59.4	3.9		100.0

Tausch. They further selected plants possessing the deleted chromosome in the homozygous state. Three hundred and fifty-four deletion stocks are available in the genetic stock center, KOMUGI (Wheat Genetic Resources Database—National BioResouce Project (NBRP) Japan, http://www.shigen.nig.ac.jp/wheat/komugi/). Loss of function, protein, or DNA sequences in the homozygous deletion lines revealed the physical location of the gene. The genetic background of these lines is the "Chinese Spring" (CS). The International Wheat Sequence Consortium did a draft genome reading in this background [4]. Also, many useful genetic stocks as nullisomic–tetrasomics, ditelosomics, and alien chromosome addition lines are available in this background. Consequently, these deletion stocks are especially useful for the genetic and genomic studies of bread wheat.

The Gc chromosome also induces aberration in the alien chromosomes experimentally introduced into the wheat genetic background. Endo [5] and Li et al. [6] selected many alien chromosomes with a deletion or a translocation (Fig. 4) [7] from the offspring of the lines carrying a pair of the alien chromosome and one 2C chromosome (AABBDD + xx + 2C). These wheat lines possessing deleted chromosomes of barley (*Hordeum vulgare* L.) or rye (*Secale cereale* L.) are useful for the physical mapping of these species [7].

Using genetic stocks maintained in KOMUGI, I here explain the method to produce deletions in a target wheat chromosome for mapping. The first step involves the generation of AABBDD + 2C line by crossing between AABBDD + 2C2C and AABBDD because AABBDD + 2C is not available in KOMUGI (Fig. 2).

2 Materials

Firstly, access the homepage of KOMUGI and request the seeds of the normal line CS (LPGKU2269), AABBDD + 2C2C (LPGKU2153), and nullisomic–tetrasomic lines lacking the target chromosome for analysis (LPGKU0043–LPGKU0084). To germinate seeds, prepare filter paper (φ90 mm) and Petri dishes (φ90 mm). For wheat cultivation, prepare plastic pot (φ180 mm) and gardening soil with fertilizer. As the tools for crossing, prepare a pair of forceps with sharp tips, ophthalmologic scissors, vertically long paper envelopes (6 cm width × 14 cm length), and pins. For wheat seed storage, prepare zip-lock plastic bags and silica gels.

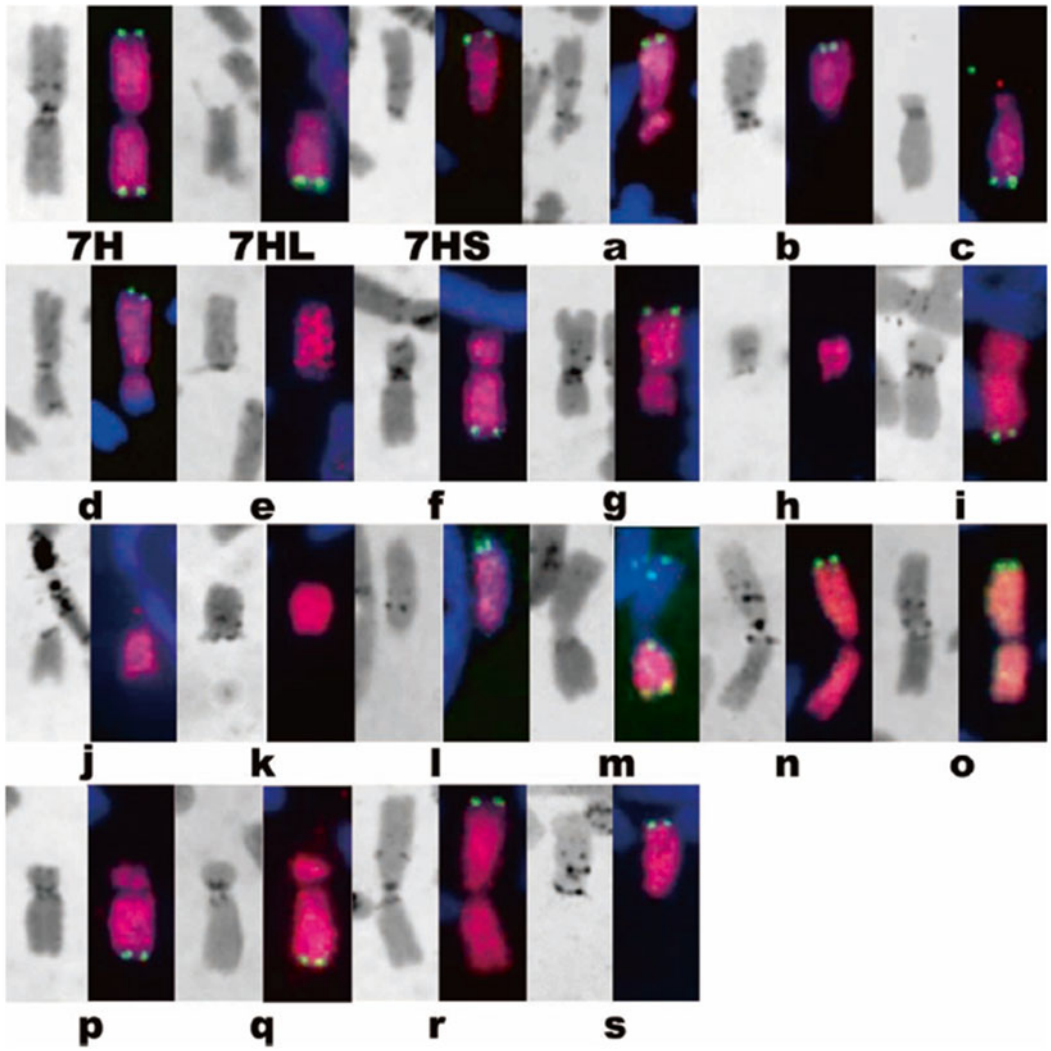

Fig. 4 C-band (*left*) and GISH/FISH (*right*) images of normal 7H, 7H short arm, 7H long arm, and structural aberrations of chromosome 7H (**a**–**s**) of barley. The probes of FISH for barley chromosome detection were barley total genomic DNA (*red*) and barley subtelomeric repeat sequence *Hv*T01 (*green*). Chromosomes were counterstained with DAPI (*blue*). Bar = 10 μm. Cited from Nasuda et al. [7]

3 Methods

3.1 Plant Cultivation

1. Place two sheets of filter paper in a petri dish and moistened with tap water. Remove excess water from the petri dishes, place the seeds of AABBDD + 2C2C and CS in the Petri dishes and cover the lid (*see* **Note 1**).
2. Keep the Petri dishes in the refrigerator at 4 °C for 1 week (*see* **Note 2**).

3. Sow two seeds per pot containing 1 kg of garden soil (10 cm apart and 1 cm in depth). Sprinkle enough water until excess water comes out from the bottom hole. Discard the excess water.
4. Keep the pots in growth chamber (20/10 °C day/night temperature, 40/50 % day/night relative humidity, a 14/10 (day/night) photoperiod, and 80,000 lx light intensity). The coleoptiles should emerge 2 days after sowing. Supply water when the surface of the soil start to dry out (see **Note 3**).
5. After a period of 1 month, change the temperature to 25/20 °C. Provide a small amount of chemical fertilizer at the tillering and heading stages. Spikes should appear after about 2.5 months from seed sowing.

3.2 Production of AABBDD + 2C by Crossing

1. At the emergence of the spike peduncle, emasculate the spikes of AABBDD + 2C2C. Cut off the undeveloped spikelets at the upper part of the spike by scissors and leave 10–12 spikelets in the spike (*see* **Note 4**).
2. In each spikelet, remove the third and upper florets by forceps. Use only the first and second florets for crossing.
3. Gently insert the closed forceps tips to a slit between lemma and palea and open the floret slightly to find anthers. Hold all the anthers by the forceps, remove and emasculate all the florets in the spike (*see* **Note 5**).
4. Cover the spike with a paper envelope to avoid out-pollination. Fasten the paper envelope with a pin; and write the date of emasculation.
5. One day after emasculation, remove the envelope to check the florets. If there is a remaining anther(s), remove it carefully with forceps. Cover the spike again with a paper envelope.
6. On the next day, remove the paper envelope and cut out each floret (glume, lemma, and palea) at the half position to check maturation of the pistil (*see* **Note 6**).
7. For pollination, find flowering spikes of CS, gently take the matured and non-dehiscent anthers and put them on the palm (*see* **Note 7**). Within seconds, the anthers must start to dehisce by the heat of the palm. Carefully pick up an anther and touch onto the stigma hair of the emasculated AABBDD + 2C2C. Cover the spikes again with a paper envelope to avoid outcrossing and write the pollination date on it.
8. At 25–30 days after pollination, the successfully pollinated ovary must have developed into seeds. When the seeds become brownish in color, cut off the spike from the plants and gradually dry at room temperature (*see* **Note 8**). Take out the seeds from the spike and keep in a paper envelope. The resulting seeds are monosomic for chromosome 2C (AABBDD + 2C).

9. Dry the seeds completely by leaving at room temperature for more than 1 week. Keep the seeds in a small zip-lock bag with a few silica gels.

3.3 Production of a Population Including Chromosome Breakage in Hemizygous State

1. After more than 1 month from harvest, sow the seeds of AABBDD + 2C and the targeted nullisomic–tetrasomics in separate pots and cultivate the plants as in Subheading 3.1 above (*see* **Note 9**).
2. Emasculate the AABBDD + 2C spikes and cross as female parent to the nullisomic–tetrasomics. Ensure that enough seeds are obtained (*see* **Note 10**).
3. Select plants with desired chromosome aberration by chromosome observation using the C-banding or PCR markers located on the distal portions of the long and short arms (*see* **Note 11**, Fig. 4).

4 Notes

1. Wheat seeds do not germinate under water. Remove excess water from the petri dish. The seed number should not be more than ten grains in one petri dish. KOMUGI usually provides 5–10 seeds for each line only. The accession number should be written on the filter paper with a pencil.
2. Fresh seed may not germinate because of dormancy. Cold water absorption breaks the dormancy and makes for uniform germination. Because CS requires a short vernalization period, the cold treatment for the seeds promotes smooth heading.
3. The key to wheat cultivation is the light strength and day length. Excess water in the soil is harmful to root growth and causes root rot.
4. When plant growth is bad, the spike may mature before emerging from the leaf sheath. In this case, take out the spike carefully from the sheath and use it for emasculation. When the lowest spikelet does not develop well, it should be removed by forceps.
5. There are three anthers in one floret without exception. The anther may be hidden in the fold of the palea. The anthers suitable for emasculation are green or pale green. If they are yellow or white, the timing for emasculation is too late.
6. If there is dehiscent anther(s) coming out from the spike, the emasculation is unsuccessful. Then, the pollen may have fertilized the pistil of its floret and/or surrounding florets. Such spikes should be cut off and discarded. Mature pistil has white and bright stigma hairs. If the stigma seems to be hard or if the hairs wither away, the timing of pollination is too early or too late, respectively. Fertilized stigma shows withered hair.

7. Suitable spikes to take anthers for pollination are those exhibiting anthers at a few florets. The florets near the flowering florets should hold mature and not-yet non-dehiscent anthers. The color of these anthers is bright yellow. One anther can be used for pollination of two florets.
8. The seeds should not be put in a desiccator. Compulsory desiccation causes deep dormancy.
9. Fresh seeds may have dormancy. In CS, the dormancy is broken 1 month after harvest.
10. If your target chromosome is the chromosome 1A, for example, use nullisomic 1A–tetrasomic 1B (LPKU0043) or nullisomic 1A–tetrasomic 1D (LPKU0044) as the male parent. Then, the F_1's will produce monosomic for 1A. So, the broken chromosomes come into the hemizygous condition. Considering the rate of occurrence of chromosome breakage (about 10%) in a certain chromosome, enough seeds should be prepared. If 100 deletions in the chromosome are required, at least 1000 F_1 seeds are necessary.
11. Chromosome markers or molecular markers at the termini of each chromosome end help to find deletion in the chromosome [7]. For example, Nasuda et al. [7] used a subtelomeric repetitive sequence of barley chromosomes (HvT01) as the probe of FISH (Fig. 4). Lack of one of the FISH signals localizing on each chromosome end is an indication of the occurrence of chromosome deletion on the chromosome. Likewise, PCR markers localizing at the very terminal ends help to detect the deletion.

Acknowledgment

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References

1. Tsujimoto H (2005) Gametocidal genes in wheat as the inducer of chromosome breakage. Wheat Inf Serv 100:33–48
2. Tsujimoto H, Yamada T, Hasegawa K, Usami T, Endo TR, Ogihara Y, Sasakuma T (2001) Large-scale selection of lines with deletion in chromosome 1B in wheat and application for fine deletion mapping. Genome 44:501–508
3. Endo TR, Gill BS (1996) The deletion stocks of common wheat. J Hered 87:295–307
4. International Wheat Genome Sequence Consortium (2014) A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. Science 345:1251788
5. Endo TR (2009) Cytological dissection of barley genome by gametocidal system. Breed Sci 59:481–486
6. Li JJ, Nasuda S, Endo TR (2013) Dissection of rye chromosomes by gametocidal system. Genes Genet Syst 88:321–327
7. Nasuda S, Kikkawa Y, Ashida T, Rafiqul Islam AKM, Sato K, Endo TR (2005) Chromosomal assignment and deletion mapping of barley EST markers. Genes Genet Syst 80:357–366

Chapter 8

CRISPR/Cas-Mediated Site-Specific Mutagenesis in *Arabidopsis thaliana* Using Cas9 Nucleases and Paired Nickases

Simon Schiml, Friedrich Fauser, and Holger Puchta

Abstract

The CRISPR/Cas system has recently become the most important tool for genome engineering due to its simple architecture that allows for rapidly changing the target sequence and its applicability to organisms throughout all kingdoms of life. The need for an easy-to-use and reliable nuclease is especially high in plant research, as precise genome modifications are almost impossible to achieve by *Agrobacterium*-mediated transformation and the regeneration of plants from protoplast cultures is very labor intensive. Here, we describe the application of the Cas9 nuclease to *Arabidopsis thaliana* for the induction of heritable targeted mutations, which may also be used for other plant species. To cover the concern for off-target activity, we also describe the generation of stable mutants using paired Cas9 nickases.

Key words Gene technology, Double-strand break repair, Engineered nucleases, Genome engineering, Targeted mutagenesis

1 Introduction

The molecular basis of a type II CRISPR (for clustered regularly interspaced short palindromic repeats) system from *Streptococcus pyogenes* was unraveled in 2012 [1]. The CRISPR-associated (Cas) protein Cas9 was shown to be a nuclease that interacts with two short RNAs, the CRISPR-RNA (crRNA) and the trans-activating crRNA (tracrRNA). The crRNA binds to the target site on the DNA by Watson–Crick pairing and Cas9 cleaves the DNA strands with two nuclease domains (termed RuvC-like domain and HNH motif), each cleaving one of the two DNA strands. It was also shown, that the two RNAs could be fused to form a chimeric single-guide RNA (sgRNA) without losing cleavage activity. Furthermore, it was demonstrated that, by mutating a single amino acid in the active site of one of the nuclease domains, (substitutions D10A or H840A) Cas9 could be converted to a nickase.

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The first demonstration of Cas9 as a programmable, engineered nuclease for the targeted induction of mutations in human cells [2, 3] marks one of the most important contributions to the field of genome engineering over the last years. Since then, the CRISPR/Cas system has successfully been applied to a wide range of organisms [4–8]. For plants, there has always been a tremendous need for tools to manipulate genomes in a site-specific manner, since transgenes transferred via *Agrobacterium tumefaciens* integrate completely haphazardly. Therefore, the CRISPR/Cas system was rapidly demonstrated to work in protoplast and callus cultures of *Arabidopsis thaliana*, tobacco, rice, and wheat [9–11]. Furthermore, it was reported by several groups that CRISPR-induced mutations in plants can be inherited, which is the most important step for reliable biotechnological applications [12–14].

However, despite the possibilities that CRISPR/Cas offers there are still enduring doubts about off-target activity. Although this has been intensively studied with different methods [15–19], it is still not completely clear whether the 23 nts target sequence guarantee sufficient specificity. To overcome this problem, a Cas9 nickase can be applied together with two sgRNAs, which guide the enzyme to cleave each of the two DNA strands. Thus, a double-strand break is formed and mutations are induced by nonhomologous end-joining [20, 21]. We have demonstrated that this approach can also be applied to plants, producing heritable mutations at a comparable frequency as for the Cas9 nuclease [22].

Here, we describe the design and cloning procedure of our CRISPR/Cas vector system as well as the generation of homozygously mutated lines for *A. thaliana* [13]. The target specificity of the sgRNA is determined by cloning a pair of annealed oligonucleotides into a respective plasmid harboring the sgRNA backbone. Subsequently, the complete sgRNA construct is transferred to a binary vector that contains the Cas9 expression cassette. The final vector can then be transformed into plants via *A. tumefaciens*. We also show the procedure to apply the paired Cas9 nickases to *A. thaliana* [22]. The modified sgRNA vector allows for parallel cloning of both sgRNA constructs. Two consecutive cloning steps combine both sgRNA sequences with the Cas9 expression system, again resulting in a binary vector for *Agrobacterium*-mediated transformation. This vector system may not only be used for the Cas9 nickase but also for the production of paired nucleases that create site- and sequence-specific deletions in the genome.

2 Materials

2.1 Plant Material

Transformable plant material according to your standard transformation protocol (e.g., *A. thaliana* plants as previously described [23]).

2.2 Vectors

All vectors described can be obtained upon request to the authors or from the Arabidopsis Biological Resource Center (ABRC, donations CD3-1927 to CD3-1932). Sequence information on all plasmids is obtainable at www.botanik.kit.edu/crispr.

1. pChimera

   Cloning vector that was synthesized by GeneArt (Life Technologies Inc., Carlsbad, CA, USA). Contains the sgRNA expression system flanked by *Avr*II restriction sites and can be used for classical cloning into the binary Cas9 expression vector pCAS9-TPC. Confers resistance to ampicillin.

2. pEn-Chimera

   Based on the pGEM®-T-Easy backbone (Promega Corp., Fitchburg, WI, USA); contains the sgRNA expression system flanked by attR sites for Gateway cloning into the binary Cas9 expression vector pDe-CAS9. Confers resistance to ampicillin.

3. pEn-C1.1

   Identical to pEn-Chimera except for additional *Bsu*36I and *Mlu*I restriction sites on each side between the sgRNA and the attR site. Confers ampicillin resistance.

4. pCAS9-TPC

   Based on the backbone of pPZP201 [24], a binary plasmid for *Agrobacterium*-mediated transformation. Harbors the Cas9 expression cassette, the sgRNA from pChimera can be added by classical cloning with *Avr*II. Confers spectinomycin resistance in bacteria, the T-DNA contains a resistance gene against phosphinothricin (PPT) (*see* **Note 1**).

5. pDe-CAS9

   Resembles pCAS9-TPC, a Gateway destination sequence was added that contains a *ccdB* gene, to allow addition of the sgRNA from pEn-Chimera or pEn-C1.1 by Gateway cloning. Confers spectinomycin resistance in bacteria, the T-DNA contains a resistance gene against PPT has to be propagated in *ccdB*-resistant *E. coli* strain.

6. pDe-CAS9-D10A

   Identical to pDe-CAS9, the D10A exchange was accomplished by PCR-based site-directed mutagenesis. The plant resistance was changed to kanamycin.

2.3 Reagents

1. DNA-oligonucleotides

   All oligonucleotides were ordered in desalted purity and were used at a concentration of 50 pmol/µl. Table 1 lists all primers that are described in the Subheading 3.

2. Restriction enzymes *Bbs*I and *Avr*II with buffers as supplied (*see* **Note 2**).
3. Taq DNA Polymerase for colony-PCRs (*see* **Note 3**).
4. T4 DNA ligase with buffer as supplied.
5. Alkaline phosphatase with buffer as supplied.
6. LR Clonase II Enzyme mix (includes Proteinase K).
7. TE-buffer pH 8: 10 mM Tris–HCl, 1 mM EDTA.
8. LB-agar plates: 10 g/L pepton, 5 g/L yeast extract, 10 g/L NaCl, 7.5 g/L agar.
9. GM-agar plates: 4.9 g/L Murashige & Skoog, 10 g/L sucrose, adjust to pH 5.7, 8 g/L agar.
10. Antibiotics/herbicides: ampicillin, spectinomycin, kanamycin, and PPT.
11. Bacterial strains:

For propagation of *ccdB* encoding vectors (pDe-CAS9, pDe-CAS9-D10A), use a *ccdB*-resistant *E. coli* strain, e.g., DB3.1 (Life Technologies Inc.). All cloning steps are performed with NEB5α (New England Biolabs), a derivate of DH5α. For plant transformation, use *A. tumefaciens* strain GV3101 (*see* **Note 2**).

Table 1
Sequences of all primers

Oligo name	Sequence 5′–3′	Orientation
M13 rev	CACAGGAAACAGCTATGAC	rv
SS42	TCCCAGGATTAGAATGATTAGG	fw
SS43	CGACTAAGGGTTTCTTATATGC	rv
SS102	CACCATGTTATCACATCAATCC	rv
SS61	GAGCTCCAGGCCTCCCAGCTTTCG	fw
SS72	CTTCTATCGCCTTCTTGACG	rv
SS143	CAAGAAAGCTGGGTCCTCAG	rv
SS144	GTCCGGACGTCTTAATTAACC	rv

3 Methods

The basic principle of our cloning system is made up by two cloning steps. The first step involves the specification of the sgRNA for the target sequence. This is achieved by annealing and subsequent cloning of two complementary oligonucleotides into a linearized plasmid. After confirmation of the cloning products, the sgRNA scaffold is then transferred into a binary vector that contains the Cas9 expression cassette. This second cloning step can either be performed by conventional, restriction-based cloning or via Gateway cloning. For the paired nickases, the sgRNAs have to be transferred in two subsequent cloning steps.

3.1 Cloning of sgRNAs

1. Digest the sgRNA vector (pChimera, pEn-Chimera, pEn-C1.1, *see* **Note 4**) with *Bbs*I. Mix 1 μg of plasmid miniprep with 2 μl restriction enzyme buffer and 1 μl *Bbs*I, add ddH_2O to a total volume of 20 μl. Incubate at 37 °C for at least 2 h or overnight.
2. Purify the digested vector with a PCR purification kit, there is no need to purify it from a gel. Elute in 30 μl ddH_2O and adjust the final concentration to 5 ng/μl (*see* **Note 5**).
3. Pick your 20 nt CRISPR target sequence upstream of an NGG PAM (protospacer adjacent motif, *see* **Notes 6–8**). Order the following oligonucleotides (Fig. 1):

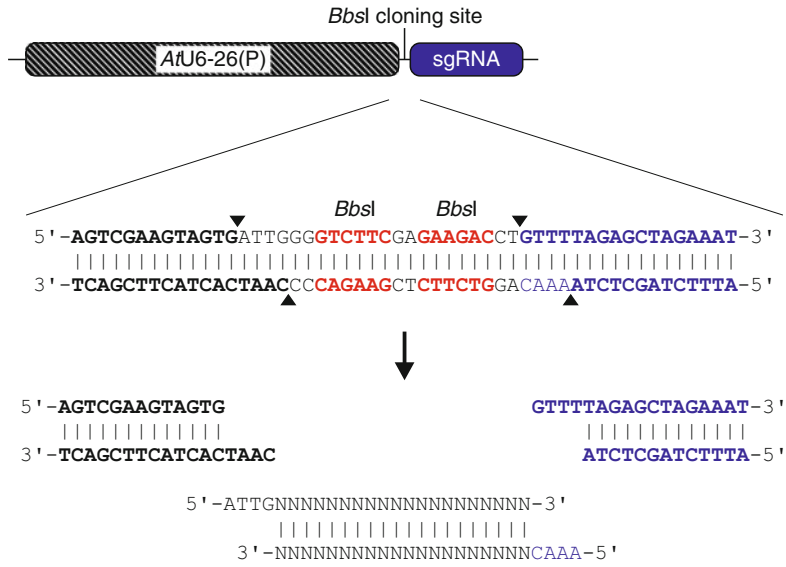

Fig. 1 Cloning principle for the specification of sgRNAs. The *Bbs*I recognition sites are cleaved out of the vector, generating defined sticky ends. Two complementary oligonucleotides with appropriate overhangs are cloned into the cutting site

Forward: 5′-ATTG-20 nts protospacer-3′

Reverse: 5′-AAAC-20 nts reverse complement of your protospacer-3′

4. Mix 2 µl of each oligo (50 pmol/µl) with 46 µl of ddH_2O. Incubate for 5 min at 95 °C, cool at room temperature for 10 min.
5. Mix 2 µl of digested sgRNA vector with 3 µl of the annealed oligos. Add 1 µl T4 ligase buffer, 3 µl ddH_2O, and 1 µl T4 ligase. Incubate for at least 2 h at room temperature. Transform 5 µl into *E. coli* NEB5α and plate on LB with ampicillin (100 mg/l).
6. Check colonies by colony-PCR (*see* **Note 3**). Use your forward protospacer oligo together with M13 rev, anneal at 56 °C. The expected band is at approx. 370 bp; you will find at least 70% of the clones to be correct, so checking 4–6 colonies is sufficient.
7. Purify plasmid DNA of one or two clones and sequence the plasmids with primer SS42 to verify correct integration of your spacer.

3.2 Transfer of sgRNA to Binary Vector by Conventional Cloning

1. Assemble your sgRNA in pChimera as described in Subheading 3.1.
2. Digest your sgRNA vector and pCAS9-TPC with *Avr*II by mixing 1–2 µg plasmid DNA with 2 µl restriction enzyme buffer and 1 µl *Avr*II (*see* **Note 9**), add ddH_2O to a total volume of 20 µl. Incubate for at least 2 h at 37 °C.
3. Separate sgRNA from vector backbone in a 1 or 2% agarose gel. The desired band is at approx. 530 bps. Purify with a gel purification kit, elute in ddH_2O.
4. Purify digested pCAS9-TPC backbone directly from the reaction, elute in 30 µl ddH_2O. Dephosphorylate the backbone by adding 3.5 µl phosphatase buffer and 1.5 µl alkaline phosphatase. Incubate for 20 min at 37 °C and heat inactivate the enzyme as recommended.
5. Prepare ligation by mixing 5 µl of sgRNA insert with 5 µl dephosphorylated vector, 2 µl T4 ligase buffer, 7 µl ddH_2O, and 1 µl T4 ligase (*see* **Note 10**). Incubate for at least 3 h (or overnight) at RT. Transform 10 µl of the reaction into NEB5α and plate on LB with spectinomycin (100 mg/l).
6. Check colonies by colony-PCR. Use primers SS42/SS43, anneal at 60 °C. The expected band is at approx. 1 kb. Check 5–10 clones or more if necessary.
7. Purify plasmid DNA of one or two clones. Check the plasmids by control digestion with *Afl*II and *Nhe*I, expected bands are at approx. 5.9, 5, and 3.8 kb.
8. Correct vectors are ready for transformation into *A. tumefaciens* and subsequent plant transformation.

3.3 Transfer of sgRNA by Gateway Cloning

1. Assemble your sgRNA in pEn-Chimera as described in Subheading 3.1.
2. Adjust your sgRNA vector to 50 ng/μl and the destination vector to 100 ng/μl.
3. Prepare Gateway-reaction: Mix 2 μl of your specified pEn-Chimera plasmid with 3 μl of pDe-CAS9. Add 4 μl TE-buffer and 1 μl of LR Clonase II. Vortex and centrifuge the reaction and incubate for at least 2 h at room temperature.
4. Add 1 μl Proteinase K and incubate for 10 min at 37 °C (*see* **Note 11**).
5. Transform completely into NEB 5α and plate on LB with spectinomycin (100 mg/l).
6. Check by colony-PCR with primers SS42/SS43. Anneal at 60 °C, the expected band is at about 1 kb.
7. Purify plasmid DNA of one or two clones. Control by restriction digestion with *Afl*II and *Nhe*I, expected bands are at approx. 5.9, 5, and 3.8 kb.
8. Correct vectors are ready for transformation into *A. tumefaciens* for plant transformation.

3.4 Cloning of Constructs with Paired Nickases

1. Assemble both sgRNAs in pEn-C1.1 as described in Subheading 3.1.
2. Digest your first sgRNA vector and pDe-CAS9-D10A with *Bsu*36I and *Mlu*I (*see* **Notes 12** and **13**). Use 1–2 μg of plasmid along with 2 μl restriction enzyme buffer and 1 μl of each enzyme, add ddH_2O to a total volume of 20 μl. Incubate for at least 1 h at 37 °C.
3. Purify your sgRNA fragment from a gel, to separate it from its vector backbone. The fragment size is about 530 bp. The digested pDe-CAS9 can be purified directly from the reaction.
4. Prepare ligation using 5 μl of digested pDe-CAS9-D10A and 5 μl of digested pEn-C1.1, 2 μl T4 buffer, 6 μl ddH_2O, and 1 μl T4 ligase (*see* **Note 10**). Incubate for at least 3 h or overnight at room temperature.
5. Transform 10 μl of the reaction into a *ccdB*-resistant *E. coli* strain (e.g., DB3.1), plate on LB containing 100 mg/l spectinomycin.
6. Check clones by colony-PCR. Use primers SS42/SS102 (*see* **Note 13**), anneal at 60 °C, expected band is at approx. 1 kb.
7. Purify plasmid DNA of one or two clones. Control by restriction digestion with *Afl*II and *Nhe*I. Expected bands are at 7.5, 5.7, and 3.8 kb.

8. Adjust to 50 ng/µl. Adjust your second sgRNA-plasmid to 100 ng/µl.
9. Prepare Gateway-reaction: mix 2 µl sgRNA-plasmid with 3 µl destination vector (already containing the first sgRNA construct). Add 4 µl TE-buffer and 1 µl LR Clonase II. Vortex and centrifuge; incubate for at least 2 h at room temperature.
10. Add 1 µl Proteinase K and incubate for 10 min at 37 °C.
11. Transform completely into NEB 5α and plate on LB with 100 mg/l spectinomycin.
12. Check clones by colony-PCR using primers SS61/SS143. Anneal at 60 °C, the expected band is at approx. 1 kb.
13. Purify plasmid DNA of one or two clones. Control by digesting with *Afl*II and *Nhe*I. Expected bands are at 5.8, 5.7, 3.8, and 0.6 kb. Confirm by sequencing with primer SS144 (*see* **Note 14**).
14. Correct vectors are ready for *A. tumefaciens* mediated transformation.

3.5 Mutant Screening in A. thaliana

1. Transform your vector into *A. tumefaciens.*
2. Perform stable Agrobacterium-mediated transformation into *A. thaliana* (e.g., floral dip transformation as described in [23]).
3. Select for primary transformants on GM plates by using 7 mg/l PPT (nuclease) or 30 mg/l Kanamycin (nickase).
4. Cultivate 20 or more primary transformants to obtain progeny seeds (T2 generation, *see* **Notes 15** and **16** and Fig. 2).
5. Check T2 lines for correct Mendelian segregation on the respective selection medium to identify single-locus lines (25% should not be able to germinate).
6. For ten correctly segregating lines sow at least ten seeds each on medium or soil without selection marker (*see* **Note 17**).
7. After 10–14 days, extract DNA from your plants with a rapid extraction protocol [25].
8. Test samples for mutagenesis events. This can be done in numerous ways, the most common methods are T7 endonuclease assay, restriction digestion assay, or high-resolution melting analysis.
9. Confirm positively tested samples by Sanger sequencing (*see* **Note 18**).
10. Cultivate mutated plants to obtain T3 seeds.
11. For each T3 line, sow 20 seeds on respective selection medium to validate absence of the T-DNA (*see* **Note 19**).
12. For transgene-free T3 lines, test up to ten plants for the presence of the mutation by Sanger sequencing.

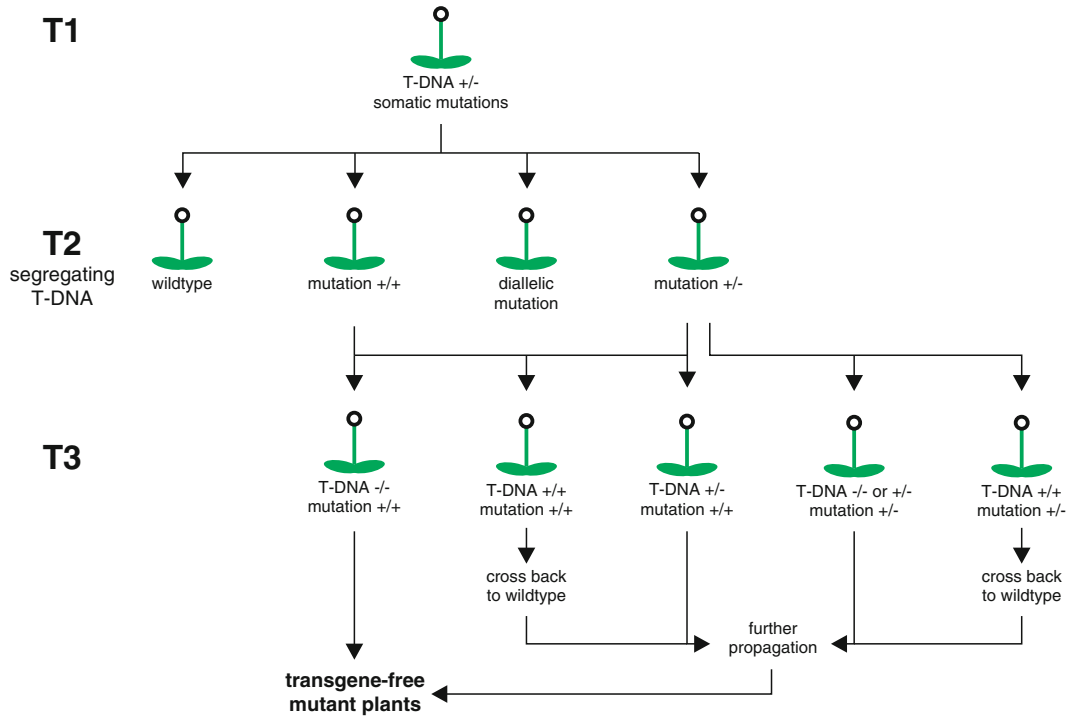

Fig. 2 Simplified overview of possible mutagenesis outcomes. Somatic mutagenesis events can occur in transgenic T1 plants. In T2, the T-DNA segregates in a Mendelian fashion for single-locus lines. Heritable mutations can be heterozygous, homozygous, or diallelic (transheterozygous). A transgene-free, homozygously mutated plant can occur in T2 as well as in later generations. T2 plants tested positively for a mutagenesis event should therefore be cultivated to T3 and the offspring should be tested for the presence of the T-DNA and for the mutation genotype. Note that in any heterozygous mutant or wild-type plant with at least one T-DNA allele, ongoing mutagenesis can create chimeric plants

4 Notes

1. Figure 3 summarizes the final constructs. Note that all elements can be exchanged to fit your specific needs (e.g., different promoter or plant resistance).
2. Enzymes and strains listed are according to what we use in the lab. Since the procedures are designed to be standard cloning steps, just use the normal material that your lab is used to.
3. Colony-PCR is used to identify correct colonies on your transformation plate. This is generally an optional but recommended step, as it reduces the number of plasmid isolations you have to perform. However, a robust *Taq*-polymerase is needed, that can cope with inhibiting substances in the reaction. We routinely use DreamTaq polymerase (ThermoFisher Scientific). Also, it is recommended to make 50 µl reactions, so that inhibitors are more diluted.

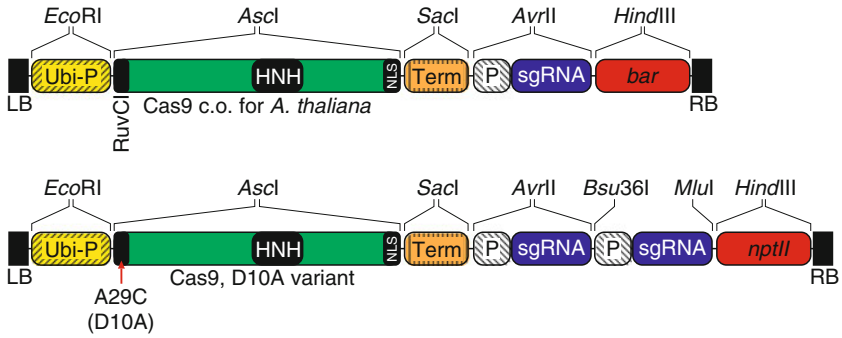

Fig. 3 Final T-DNA constructs for nuclease (*top*) and paired nickases (*bottom*). The shown restriction sites may be used to exchange any of the contained elements to fit your specific situation

4. Generally, pChimera is for conventional cloning, pEn-Chimera is for Gateway cloning, and pEn-C1.1 is for paired sgRNAs.
5. The digested vector can be stored at −20 °C, so there is no need to do a new *Bbs*I restriction reaction for every sgRNA.
6. The design of spacers can be done manually. There are only few limitations: avoid presence of an *Avr*II restriction site, if you want to do conventional cloning. For paired nickases, one of your spacers should be devoid of *Bsu*36I and/or *Mlu*I. Furthermore, avoid having more than five subsequent T's in your spacer sequence, as this can terminate expression of the sgRNA. The spacer sequence does not have to start with a G.
7. If you prefer using design tools, we recommend CCTop [26] or CRISPR-P [27]. The latter is especially useful, if you want to identify mutants via the disruption of a restriction site.
8. It is also possible to pick CCN as PAM if this fits better for your specific experiment. In this case, your forward-oligo (still starting with ATTG) should contain the reverse-complementary sequence of the 20 nts downstream of the PAM, while the reverse oligo directly resembles your target sequence.
9. Use undiluted plasmid miniprep for the digestion reaction, normally ranging in concentrations from 100 to 500 ng/μl.
10. Simply taking 5 μl of each ligation fragment without caring for concentrations proved to be working well. However, if you experience problems with your cloning, changing to a threefold molar excess of the insert fragment should improve ligation success.
11. Proteinase K treatment is crucial. There will be no correct clones, if this step is skipped.
12. Depending on your spacer design, it is also possible to use only one of the two enzymes. However, in this case, it is necessary to dephosphorylate the vector prior to ligation.

13. The described procedure can also be used for paired nucleases, to induce specific deletions. Just use pDe-CAS9 instead of pDe-CAS9-D10A. The reverse primer for the first colony-PCR changes to SS72 and the control digestion bands differ slightly.
14. Make sure to identify the 0.6 kb band, as it indicates the presence of both sgRNA cassettes. The sequencing covers both sgRNA cassettes in one run, so it confirms your sgRNAs are both present and intact.
15. The exact number may vary depending on the bacterial strain and the transformation method. Make sure to have at least ten individual single-locus lines.
16. It is possible, yet not necessary to check for nuclease activity in primary transformants. This can be done by T7 endonuclease or restriction digestion assay.
17. Again, the exact number can vary and is hard to predict. Testing 100 plants should be enough to identify a mutant; however, this number can be scaled up easily.
18. Plants are likely to be heterozygous in this stage. A double peaked chromatogram can be analyzed with Poly Peak Parser [28].
19. Transgene-free T3 offspring represents the optimal situation, indicating that the respective T2 mother plant was already transgene free. If the mutation and/or the T-DNA are still heterozygous in T3, the number of plants and lines can be scaled up or the procedure can be repeated in T4. If the T-DNA is homozygous (i.e., all plants grow on selection medium), it is possible to cross back to wild-type plants.

Acknowledgments

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References

1. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821
2. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823
3. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA-guided human genome engineering via Cas9. Science 339:823–826
4. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31:233–239
5. Friedland AE, Tzur YB, Esvelt KM, Colaiácovo MP, Church GM, Calarco JA (2013) Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat Methods 10:741–743
6. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh J-RJ, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31:227–229

7. Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J, O'Connor-Giles KM (2013) Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics 194:1029–1035
8. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153:910–918
9. Li J-F, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol 31:688–691
10. Nekrasov V, Staskawicz B, Weigel D, Jones JDG, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. Nat Biotechnol 31:691–693
11. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu J-L et al (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31:686–688
12. Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang D-L, Wang Z, Zhang Z, Zheng R, Yang L et al (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc Natl Acad Sci U S A 111:4632–4637
13. Fauser F, Schiml S, Puchta H (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. Plant J 79:348–359
14. Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N et al (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12:797–807
15. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31:822–826
16. Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim J-S (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res 24:132–141
17. Kuscu C, Arslan S, Singh R, Thorpe J, Adli M (2014) Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. Nat Biotechnol 32:677–683
18. Smith C, Gore A, Yan W, Abalde-Atristain L, Li Z, He C, Wang Y, Brodsky RA, Zhang K, Cheng L et al (2014) Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. Cell Stem Cell 15:12–13
19. Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP et al (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol 33:187–197
20. Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 31:833–838
21. Ran FA, Hsu PD, Lin C-Y, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y et al (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154:1380–1389
22. Schiml S, Fauser F, Puchta H (2014) The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. Plant J 80:1139–1150
23. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735–743
24. Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25:989–994
25. Zhang J, Stewart JM (2000) Economical and rapid method for extracting cotton genomic DNA. J Cotton Sci 4:193–201
26. Stemmer M, Thumberger T, Keyer DS, Wittbrodt J, Mateo JL (2015) CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. PLoS One 10:e0124633
27. Lei Y, Lu L, Liu H-Y, Li S, Xing F, Chen L-L (2014) CRISPR-P: a web tool for synthetic single-guide RNA design of CRISPR-system in plants. Mol Plant 7:1494–1496
28. Hill JT, Demarest BL, Bisgrove BW, Su Y-C, Smith M, Yost HJ (2014) Poly peak parser: Method and software for identification of unknown indels using sanger sequencing of polymerase chain reaction products. Dev Dyn 243:1632–1636

Chapter 9

Targeted Mutagenesis in Rice Using TALENs and the CRISPR/Cas9 System

Masaki Endo, Ayako Nishizawa-Yokoi, and Seiichi Toki

Abstract

Sequence-specific nucleases (SSNs), such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) system, are powerful tools for understanding gene function and for developing novel traits in plants. In plant species for which transformation and regeneration systems using protoplasts are not yet established, direct delivery to nuclei of SSNs either in the form of RNA or protein is difficult. Thus, *Agrobacterium*-mediated transformation of SSN expression constructs in cultured cells is a practical means of delivering targeted mutagenesis in some plant species including rice. Because targeted mutagenesis occurs stochastically in transgenic cells and SSN-mediated targeted mutagenesis often leads to no selectable phenotype, identification of highly mutated cell lines is a critical step in obtaining regenerated plants with desired mutations.

Key word Sequence-specific nuclease, TALENs, CRISPR/Cas9, Targeted mutagenesis, DNA double-strand breaks

1 Introduction

The TALENs and CRISPR/Cas9 systems both rely on endonucleases that initiate DNA double-strand breaks (DSBs) at virtually any genomic target sequence and both are used for targeted mutagenesis [1]. While both technologies are popular, it is better to select one or the other as appropriate depending on the purpose. The TALENs system has the advantage of little limitation in target sequence because TALEs contain multiple 33- to 35-amino acid repeat domains that each recognize a single base pair (Fig. 1a) [2, 3]. On the other hand, when *Streptococcus pyogenes* Cas9 (*Sp*Cas9) and guide RNAs (gRNAs) are used, CRISPR/Cas9 targets must immediately precede an NGG site protospacer adjacent motif (PAM) sequence recognized by Cas9 (Fig. 1b) [4–7]; it is usually not difficult to locate NGG sites for knockouts, but this constraint sometimes causes problems for other applications. Nevertheless,

Minoru Murata (ed.), *Chromosome and Genomic Engineering in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1469, DOI 10.1007/978-1-4939-4931-1_9,

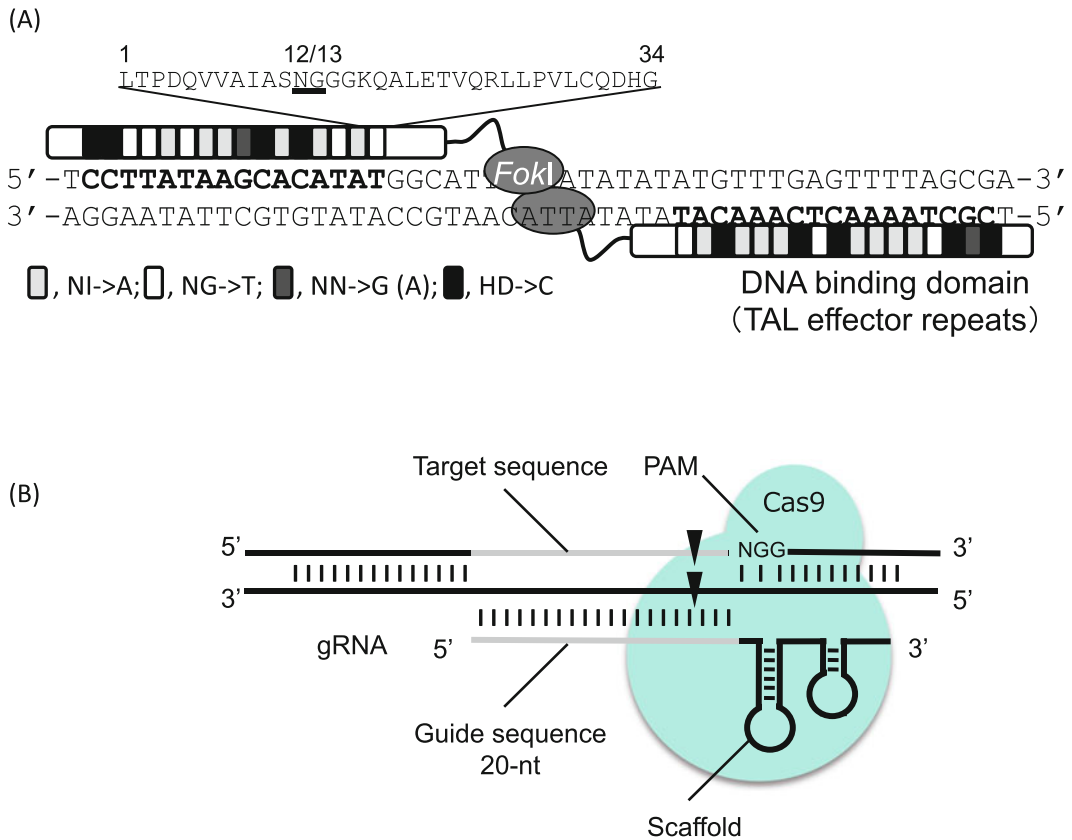

Fig. 1 Schematic representation of TALENs- and CRISPR/Cas9-mediated DNA cleavage. (**a**) Schematic diagram of TALENs binding to target DNA. The TALEN consists of N- and C-terminal regions containing nuclear localization signals, a central DNA-binding domain (TAL effector repeats) with typically 34 amino acid repeats, and a DNA cleavage domain with the restriction enzyme *Fok* I. The amino acid sequences of each TAL effector repeat are highly conserved, but the 12th and 13th amino acids are variable and recognize a single base pair (NI = A, NG = T, NN = G or A and HD = C). (**b**) Schematic diagram of Cas9 and gRNA complex binding to target DNA. A limitation of the CRISPR/Cas9 system is the 5′-NGG PAM sequence on the genomic DNA. The 20-bp target sequence preceding a PAM sequence must exist on the gRNA. Note that the PAM sequence required for target recognition by Cas9 is never present as part of the gRNA itself

CRISPR/Cas9 has the advantage of being able to knock out multiple genes at the same time because the target specificity of CRISPR/Cas9 relies not on protein/DNA recognition but rather on ribonucleotide complex formation; thus, mutations can be introduced in multiple genes when multiple guide RNAs (gRNAs) determining different target sequences are expressed together with Cas9. In contrast to TALENs, which rely on protein domains to confer DNA-binding specificity, Cas9 forms a complex with a small guide RNA (gRNA) that directs the enzyme to its DNA target via Watson-Crick base pairing. Consequently, the system is simple and fast to design for each application and requires only the production of a short oligo RNA to direct DNA binding to the target locus.

2 Materials

2.1 Vector Construction

1. Several types of assembly kit for TALENs (https://www.addgene.org/talen/) and various CRISPR/Cas9 plasmids for use in plants are available from Addgene (https://www.addgene.org/crispr/plant/). When pU6gRNA and pZH_gYSA_MMCas9 [8], (*see* **Note 1**) are used for construction of Cas9, gRNA, selection marker all-in-one vector, materials 2–5 are needed.
2. Restriction enzymes: *Bbs* I, *Asc* I, *Pac* I.
3. Gel Extraction Kit (e.g., QIAquick, Qiagen Germany).
4. Ligation Kit.
5. *E. coli* DH5α competent cells.

2.2 Agrobacterium-Mediated Transformation

2.2.1 Plant and Agrobacterium Materials

1. Mature rice seeds (*Oryza sativa L.* cv. Nipponbare).
2. *Agrobacterium tumefaciens* strain EHA105 [9].

2.2.2 Media for Agrobacterium-Mediated Transformation

1. AB, AAM, 2N6AS, N6D, ReIII, HF medium: *See* ref. 10 and Nishizawa-Yokoi et al., Chapter 10.

Autoclaved media are poured as 50 mL in each dish (9 cm in diameter, 2 cm in depth). All media are stored at 4 °C. Media are incubated at room temperature and the lids are opened to dry water drops on the surface of the medium in a clean bench just before use.

2. 2,4-Dichlorophenoxy acetic acid (2,4-D), naphtalene acetic acid (NAA), kinetin, acetosyringone, meropenem, hygromycin B, G418: *See* ref. 10 and Nishizawa-Yokoi et al., Chapter 10.

2.3 Selection and Regeneration of TALENs or Cas9- and gRNA-Transformed Calli

1. Selection medium (1L): After autoclaving N6D medium, add 1 mL of 25 mg/mL meropenem and 50 mg/L hygromycin B.
2. Regeneration (ReIII) medium (1L): After autoclaving, add 1 mL of 12.5 mg/mL meropenem and 50 mg/L hygromycin B.
3. Hormone-free (HF) medium (1L): After autoclaving, add 1 mL of 12.5 mg/mL meropenem.

2.3.1 Media for Selection of Transgenic Calli

2.4 Detection of Mutations

1. Agencourt chloropure (Beckman Coulter, USA).

2.4.1 DNA Extraction

2.4.2 Polymerase Chain Reaction

1. For PCR amplification, 2 μL of 5× PCR reaction buffer, 0.8 μL of 2.5 mM dNPTs, 0.25 μL of each 10 μM primer, 1 μL of 1/100 diluted genomic DNA, and 0.25 units of Taq DNA polymerase (e.g., PrimeSTAR GXL DNA polymerase, Takara Bio Inc.) are mixed with distilled water to give a final volume of 10 μL.

2.4.3 Cleaved Amplified Polymeric Sequences (CAPS) Analysis

1. PCR product (500 bp to 1 kb).
2. Restriction enzymes.
3. 1 or 2% agarose gel.
4. Digital gel imaging system (e.g., Chemi Doc imaging system, BioRad, USA).

2.4.4 Analysis of Mutation Patterns in Calli by Sequencing

1. Gel Extraction Kit (e.g., QIAquick, Qiagen).
2. PCR Cloning Kit (e.g., Zero Blunt TOPO, Thermo Fisher Scientific, USA).
3. E. coli DH5α Competent Cells.
4. BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).
5. Sequencer (e.g., ABI PRISM 2100 Genetic Analyzer, Applied Biosystems, USA).

2.4.5 Guide-It Resolvase Assay

1. PCR product (500 bp to 1 kb).
2. Guide-it Mutation Detection Kit (Takara Bio Inc.).

2.4.6 Heteroduplex Mobility Shift Assay (HMA)

1. PCR product (100–200 bp).
2. Polyacrylamide gel: 20% (w/v) polyacrylamide (a mixture of 99% acrylamide and 1% methylenebisacrylamide) in 1× TBE,

0.5% (w/v) ammonium persulfate (APS), 0.01% (v/v) *N*,*N*,*N*′,*N*′ Tetramethylethylene diamine (TEMED).

3. 30% Acrylamide: for 100 ml, 29.7 g of acrylamide, 0.3 g of methylenebisacrylamide.
4. 10× TBE: for 1 L, combine 108 g of Tris base, 55 g of boric acid, 40 mL of 0.5% EDTA (pH 8.0). Autoclave for 20 min.

3 Methods

3.1 Selecting Target Sequence

3.1.1 TALENs

In nature, the target sites of TAL effectors derived from *Xanthomonas* start mostly from T at the 5′-end. Thus, a T at the 5′-end is required to select the target site of the TAL effector DNA-binding domain in many cases. The TAL effector repeat arrays of the DNA-binding domain can be designed easily using software such as TAL effector Nucleotide Targeter (TALE-NT 2.0, https://tale-nt.cac.cornell.edu/, 11) and Scoring Algorithm for Predicting TALE(N) Activity (SAPTA, http://bao.rice.edu/Research/BioinformaticTools/TAL_targeter.html, 12).

3.1.2 CRISPR/Cas9

When *Streptococcus pyogenes* Cas9 (*Sp*Cas9) is used, a requirement for the guide sequence is the 20-bp target sequence preceding a 5′-NGG protospacer adjacent motif (PAM) sequence recognized by Cas9 (Fig. 1b). *Sp*Cas9 cuts 3-nt upstream of the PAM site. If the expected cleavage site is on the restriction enzyme recognition site, mutation can be detected by CAPS analysis. If the Guide-it resolvase assay or HMA is used, restriction enzyme sites do not need to be considered.

3.2 Vector Construction

3.2.1 TALENs

Several platforms, including Golden Gate assembly technology [13], REAL [14] and FLASH [15], have been developed for the construction of TAL effector repeat arrays. Among them, Golden Gate assembly technology has been used widely and validated in multiple organisms. Using this system, TAL effector repeat arrays are constructed by digestion with a type IIS restriction enzyme and ligation of each TAL repeat and are subsequently cloned into a vector carrying the TAL backbone and catalytic domain of the *Fok* I endonuclease. For induction of TALEN-mediated target mutagenesis in rice, expression vectors carrying expression cassettes of TALEN pairs driven by mono- or polycistronic expression constructs (for details, *see* ref. 16).

3.2.2 CRISPR/Cas9

Details of the construction method differ depending on the vectors used for expressing Cas9 and gRNA. Examples of vector construction strategies used in [8] are as follows: only the 20-nt target sequence of the gRNA needs to be replaced for targeting different genomic sites while the scaffold sequence of the gRNA remains the same. Therefore, the gRNA cloning vector allows the target sequence to be swapped

easily. For example, pU6gRNA [8] contains two *Bbs* I sites in place of the gRNA target sequence such that digestion leaves overhangs complementary to the annealed oligo overhang (Fig. 2a). Because the cleavage site of *Bbs* I, 5′-GAAGAC(N)2/-3′ is outside of its recognition sequence, we can avoid the addition of unnecessary sequence in this step. Once a target 20 bp is determined, forward and reverse oligos consisting of the 20-nt target site with 5′ and 3′ overhangs complementary to the digested gRNA cloning vector are annealed and cloned into the gRNA cloning vector. Because the OsU6 RNA polymerase III promoter prefers to start transcripts with a guanine (G) nucleotide, a G is included immediately 5′ of the target for inserts cloned in the pU6gRNA vector (Fig. 2a).

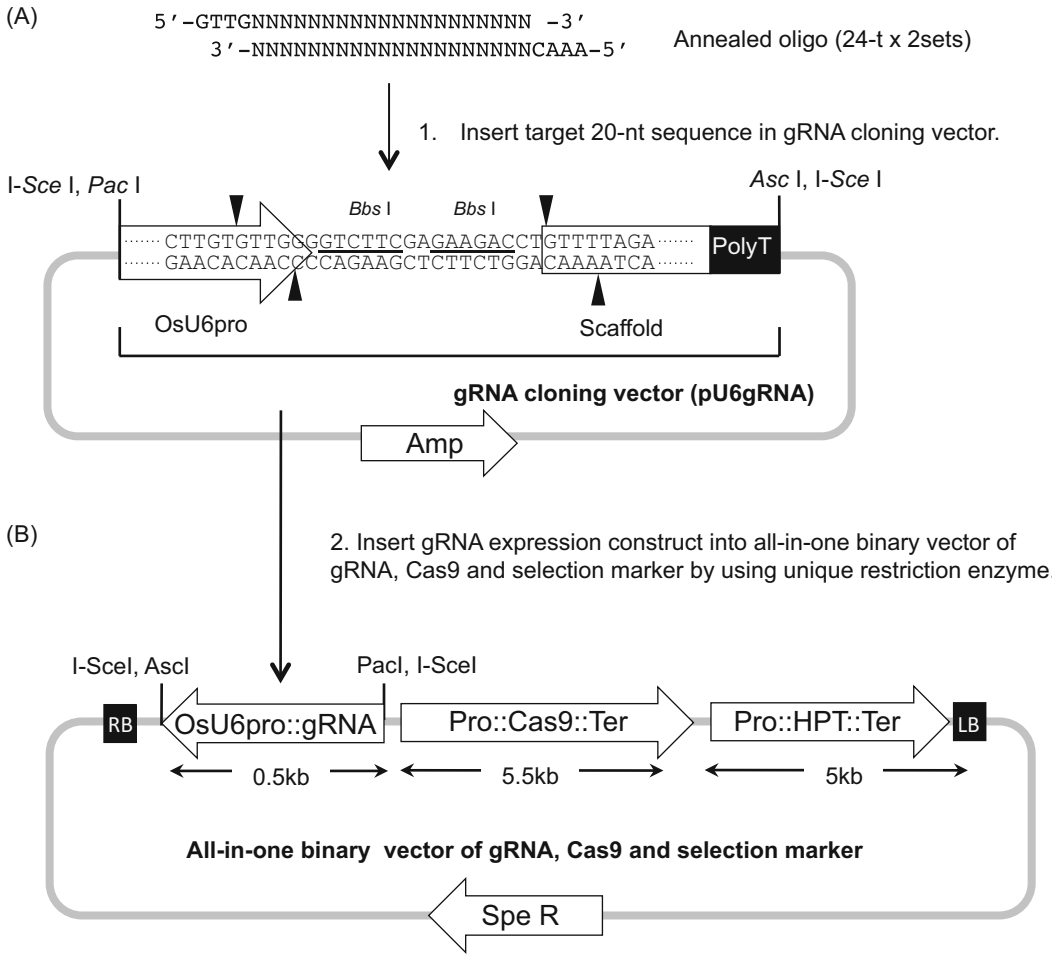

Fig. 2 Strategy for CRISPR/Cas9 vector construction. (**a**) Schematic representation of cloning the target sequence into a plasmid containing the OsU6 promoter and the gRNA scaffold. The annealed target sequence oligos contain overhangs to allow ligation into the pair of *Bbs* I sites in the sgRNA vector, pU6gRNA. (**b**) Schematic representation of cloning the gRNA expression construct into a binary vector containing Cas9 and HPT expression constructs. I-*Sce* I or combination of *Asc* I and *Pac* I can be used for this cloning

For stable rice transformation, the gRNA expression construct OsU6pro::gRNA::polyT completed on pU6gRNA must be placed in a binary vector containing the Cas9 expression construct and selection marker. A restriction enzyme reaction (I-*Sce* I only or the combination of *Asc* I and *Pac* I) can be used to replace the gRNA expression construct when pZH_gRNA_MMCas9 (Fig. 2b, [8]) is used as an all-in-one binary vector expressing Cas9, gRNA and the selection marker.

Details:

1. Combine 1 μL of each oligo DNA (100 μM) and 48 μL of ddH_2O. Boil 5 min and leave at room temperature for 20 min to anneal the oligos.
2. Digest the gRNA cloning plasmid, pU6gRNA as follows: combine 2 μg of pU6gRNA plasmid DNA, 5 μL of 10× NEB cut smart buffer, 1 μL of *Bbs* I enzyme (5 unit/μL) and ddH_2O to 50 μL. Incubate at 37 °C for 2–16 h. Run the digested products on 1% (w/v) agarose gel in TAE buffer and purify the linearized vector using a QIAquick PCR purification kit.
3. Ligate the annealed gRNA oligos into the digestion vector using a Rapid DNA ligation kit as follows: combine 4 μL of digested pU6gRNA (10 ng/μL), 4 μL of annealed oligo, 2 μL of 5× DNA dilution buffer, 10 μL of 2× T4 DNA ligation buffer, and 1 μL of T4 DNA ligase. Incubate at room temperature for 5 min and transform the ligation reaction into competent *E. coli* cells according to the manufacturer's instructions.
4. Isolate plasmid DNA and sequence the plasmid using primer OsU6-2F(5′-TGCTGGAATTGCCCTTGGATCATGAACCAA-3′) to verify that the clones harbor the corrected gRNA.
5. Digest the all-in-one Cas9, gRNA, and selection marker vector (pZH_gYSA_MMCas9) and the completed gRNA cloning vector (from **step 4)** with *Asc* I and *Pac* I and purify the resulting 17,257 bp (pZH_gYSA_MMCas9) and 486 bp (pU6gRNA) fragments.
6. Ligate *Asc* I-, *Pac* I-digested all-in-one vector and gRNA expression construct (OsU6pro::gRNA) (from **step 6**) to complete the Cas9, gRNA, selection marker all-in-one binary vector.

3.3 Agrobacterium-Mediated Transformation Using Primary Callus of Rice

1. *Agrobacterium* strain EHA105 harboring an all-in-one binary vector (from Subheading 3.2.2) containing Cas9, gRNA and selection marker expression cassettes is used for transformation. Transformation and selection steps basically follow the method published by [10].
2. Transgenic calli grown on selection medium are transferred onto regeneration medium (ReIII) containing 25 mg/L meropenem and the appropriate concentration of selection agents, and grown at 28 °C under constant light for 10–14 days (*see* **Note 2**).

3. Shoots arising from callus on ReIII medium are transferred to HF medium containing 25 mg/L meropenem and grown at 28 °C under constant light for 2 weeks.

3.4 Detection of Mutation in Rice Calli (*See* Note 3)

Extract DNA from transgenic calli using Agencourt chloropure according to the manufacturer's instructions.

3.4.1 DNA Isolation

3.4.2 Amplification of the Targeted Sequence

Design PCR primers that should amplify a 0.5–2 kb fragment including the gRNA-targeted sequence and set up the following PCR. When CAPS assay or surveyor nuclease is to be used for detecting mutations, the targeted sequence is better to be located near the center of the PCR products. This facilitates separation of mutated and non-mutated PCR products on agarose gels (*see* **Note 4**).

Genomic DNA	1 μL
5× PCR reaction buffer	2 μL
2.5 mM dNTPs	0.8 μL
10 μM forward primer	0.25 μL
10 μM reverse primer	0.25 μL
ddH_2O	5.45 μL
Taq DNA polymerase (e.g., PrimeStar GXL)	0.25 μL
Total	10 μL

PCR conditions

Cycle 1, 95 °C for 2 min; Cycles 2, 95 °C for 15 s and 68 °C for 1 min, repeat 35 times; Cycle 3, 68 °C for 7 min.

3.4.3 CAPS Assay

When an appropriate restriction enzyme recognition sequence is located on the expected cleavage site, PCR and subsequent restriction enzyme reactions can be used to detect the mutation of interest (Fig. 3). Use the PCR product from Subheading 3.4.2 to set up the following restriction enzyme reaction.

PCR product	5 μL
10× buffer	2 μL
ddH_2O	12.5 μL
Restriction enzyme	0.5 μL
Total	20 μL

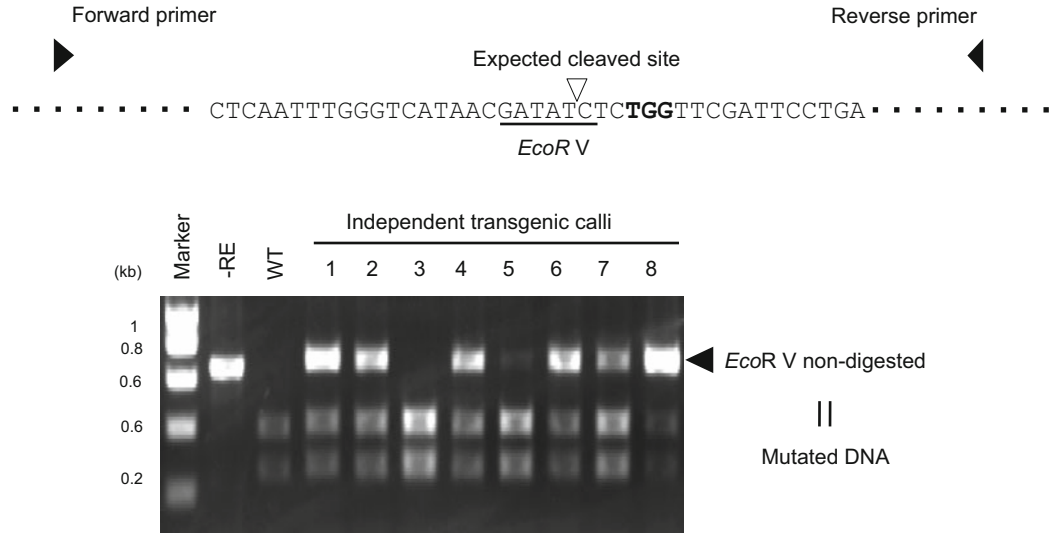

Fig. 3 Example of CAPS analysis using transgenic calli of Cas9 and gRNA. First, genomic DNAs from Cas9, gRNA-transformed calli, or regenerated plants are amplified by PCR. PCR products are then digested with restriction enzyme, which recognizes the wild-type target sequence. Mutated DNAs are resistant to the restriction enzyme reaction because of loss of the restriction enzyme recognition sequence and are revealed as uncleaved bands (indicated by *black arrowhead*) in agarose gels. Mutation frequency can be measured from the relative intensity of cleaved and uncleaved bands

Mix the reaction well and spin down. Incubate the reaction at the appropriate temperature for 2 h. Run the digested DNA on an agarose gel. Uncleaved bands detected in Cas9-, gRNA-transformed cells are proof of mutation.

3.4.4 Estimation of the Precise Mutation Frequency

An approximate mutation frequency can be estimated by measuring the intensity of the PCR amplicon and cleaved bands with gel quantification software.

Mutation $(\%) = A/(A+B+C) \times 100$ (A, intensity of the non-digested PCR product; B and C, intensity of the digested PCR product).

3.4.5 Analysis of Mutation Patterns in Calli by Sequencing

Purify the undigested bands using a gel purification kit according to the manufacturer's instructions and clone these fragments into pENTR-D TOPO using a Zero Blunt TOPO PCR cloning kit. Identify the mutations by sequencing of plasmid DNA isolated from overnight cultures of single colonies.

3.5 Guide-It Resolvase Assay

This mutation detection method is based on a mismatch-specific DNA endonuclease (Fig. 4). All steps are performed according to the manufacturer's instructions provided in the Guide-it Mutation Detection Kit.

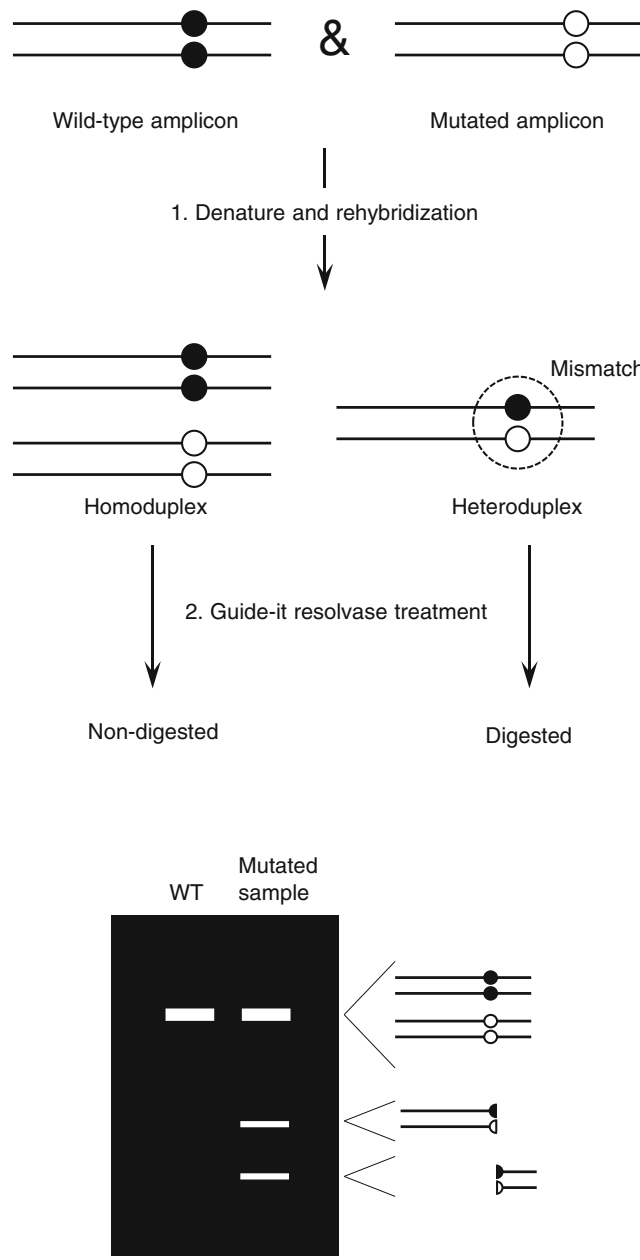

Fig. 4 Schematic representation of Guide-it resolvase assay. First, genomic DNAs from Cas9-, gRNA-transformed calli, or regenerated plants are amplified by PCR. PCR products are then denatured and re-annealed using a thermal cycler. Guide-it resolvase digests heteroduplex DNA at mismatches and an extrahelical loop is formed by single or multiple nucleotides

3.6 Heteroduplex Mobility Shift Assay (HMA)

HMA is based on the denaturing and annealing of PCR-amplified nucleotide strands that are not fully complementary and therefore generate homo- and hetero-duplexes. Heteroduplexes can be separated from homoduplexes by polyacrylamide gel electrophoresis

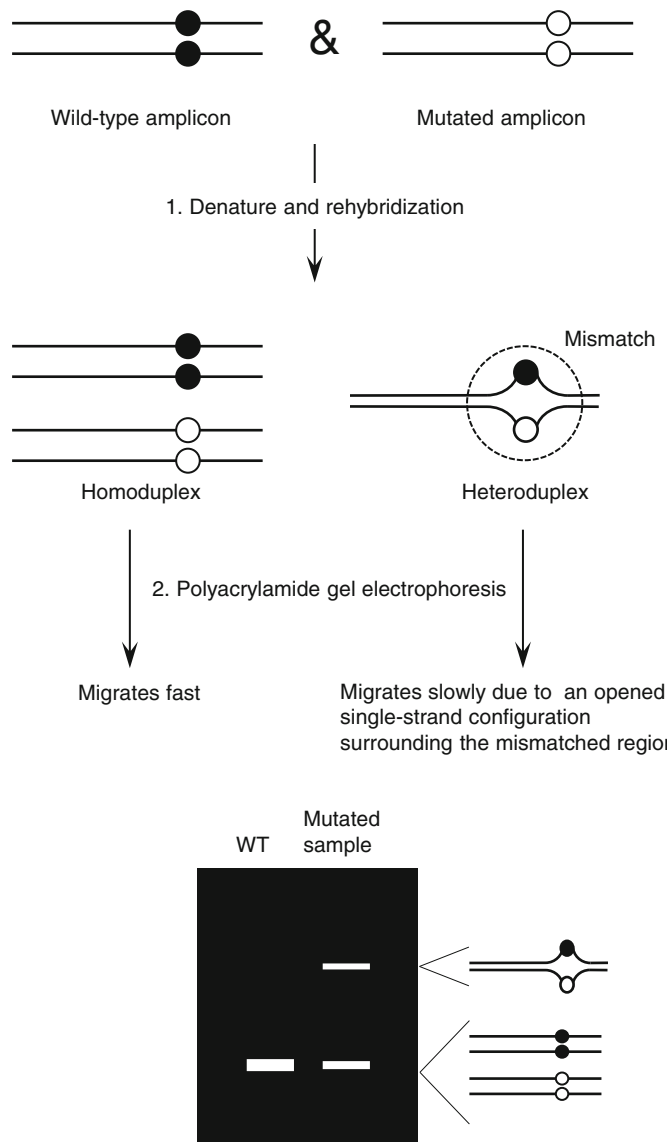

Fig. 5 Schematic representation of HMA. First, genomic DNAs from Cas9-, gRNA-transformed calli or regenerated plants are amplified by PCR. PCR products are then denatured and re-annealed using a thermal cycler. Heteroduplex and homoduplex forms are separated by polyacrylamide gel electrophoresis

because heteroduplex migrates more slowly due to an opened single-strand configuration surrounding the mismatched region (Fig. 5) (for details, *see* ref. 17). PCR amplifications (100–200 bp) are mixed with loading dye and electrophoresed on 20% acrylamide gels in 1× TBE running buffer at 200 V for 1 h. After electrophoresis, gels are stained with ethidium bromide and images captured using a gel imaging system.

4 Notes

1. These plasmids are not deposited to Addgene yet but available to academic investigators for non-commercial research purposes. Please contact to Seiichi Toki (stoki@affrc.go.jp) or Masaki Endo (mendo@affrc.go.jp) for the request.
2. G418 often inhibits regeneration. Thus, it is better to eliminate G418 from ReIII medium when transgenic calli are fully isolated on selection medium.
3. Because mutation efficiency sometimes differs between independent transgenic callus clones due to different expression levels of Cas9 and gRNA, we recommend to check mutation frequency in the clone before going to the regeneration step.
4. Use wild-type genomic DNA as a negative control.

Acknowledgments

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References

1. Gaj T, Gersbach CA, Barbas CF III (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31:397–405
2. Mak AN, Bradley P, Cernadas RA, Bogdanove AJ, Stoddard BL (2012) The crystal structure of TAL effector PthXo1 bound to its DNA target. Science 335:716–719
3. Deng D, Yan C, Pan X, Mahfouz M, Wang J, Zhu JK, Shi Y, Yan N (2012) Structural basis for sequence-specific recognition of DNA by TAL effectors. Science 335:720–723
4. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821
5. Garneau JE, Dupuis MÈ, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468:67–71
6. Sapranauskas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V (2011) The Streptococcus thermophilus CRISPR/Cas system provides immunity in *Escherichia coli*. Nucleic Acids Res 39:9275–9282
7. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature 507:62–67
8. Mikami M, Toki S, Endo M (2015) Comparison of CRISPR/Cas9 expression constructs for efficient targeted mutagenesis in rice. Plant Mol Biol 88:561–572
9. Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Res 2:208–218
10. Saika H, Onodera H, Toki S (2012) Visual selection in rice: a strategy for the efficient identification of transgenic calli accumulating transgene products. Methods Mol Biol 847:67–74
11. Doyle EL, Booher NJ, Standage DS, Voytas DF, Brendel VP, VanDyk JK, Bogdanove AJ

(2012) TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res 40(Web Server issue):W117–W122

12. Lin Y, Fine EJ, Zheng Z, Antico CJ, Voit RA, Porteus MH, Cradick TJ, Bao G (2014) SAPTA: a new design tool for improving TALE nuclease activity. Nucleic Acids Res 42:e47
13. Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39:e82
14. Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, Yeh JR (2011) Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat Biotechnol 29:697–698
15. Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK (2012) FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol 30:460–465
16. Cermak T, Starker CG, Voytas DF (2015) Efficient design and assembly of custom TALENs using the Golden Gate platform. Methods Mol Biol 1239:133–159
17. Ota S, Hisano Y, Muraki M, Hoshijima K, Dahlem TJ, Grunwald DJ, Okada Y, Kawahara A (2013) Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. Genes Cells 18:450–458

Chapter 10

Seamless Genome Editing in Rice via Gene Targeting and Precise Marker Elimination

Ayako Nishizawa-Yokoi, Hiroaki Saika, and Seiichi Toki

Abstract

Positive–negative selection using hygromycin phosphotransferase (*hpt*) and diphtheria toxin A-fragment (*DT-A*) as positive and negative selection markers, respectively, allows enrichment of cells harboring target genes modified via gene targeting (GT). We have developed a successful GT system employing positive–negative selection and subsequent precise marker excision via the *piggyBac* transposon derived from the cabbage looper moth to introduce desired modifications into target genes in the rice genome. This approach could be applied to the precision genome editing of almost all endogenous genes throughout the genome, at least in rice.

Key word Gene targeting, Positive–negative selection, Marker excision, *piggyBac* transposon, Homologous recombination

1 Introduction

Homologous recombination (HR)-mediated gene targeting (GT) is currently the only universal technology that can introduce desired modifications precisely into a target gene. However, in most multicellular organisms, the repair of DNA double strand breaks (DSBs) by the HR pathway occurs at very low frequency, resulting in low GT efficiency. Thus, positive–negative selection has been applied in several organisms, including rice, to eliminate cells carrying the transgene integrated randomly into other gene loci and to enrich true GT cells harboring the HR-modified target locus. Terada et al. [1] succeeded in applying this system to establish an efficient GT system in rice by using the hygromycin phosphotransferase (*hpt*) gene as a positive selection marker and the diphtheria toxin A-fragment (*DT-A*) gene as a negative selection marker. Using positive–negative selection with *hpt* and *DT-A*, the designed modification of a target gene via GT has become a reproducible and general approach, at least in rice [2]. In addition, improved cytosine deaminase (codA D314A), which has increased

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deaminase activity for 5-fluorocytosin, or antisense RNA of an antibiotic resistance gene can also be used as negative selection markers for the enrichment of GT cells [3, 4].

To obtain transgenic plants harboring only the desired mutation in the target locus, the positive selection marker inserted into the targeted locus must be removed completely from the GT locus after the selection of GT cells. The *piggyBac* transposon—a DNA transposon derived from the moth (*Trichoplusia ni*)—inserts into the host genome at TTAA elements and excises without leaving a footprint at the excised site [5]. The *piggyBac* transposon has been used for transgenesis, insertional mutagenesis, and marker excision not only in insects but also in mammalian cells [6, 7]. We found that *piggyBac* can transpose accurately and efficiently also in rice cells [8]. More recently, *piggyBac* has been applied successfully to the excision of the positive selection marker from a GT locus [9]. Thus, seamless genome editing via GT and subsequent precise *piggyBac*-mediated marker elimination could be applied universally in rice (*see* **Note 1**).

Here, we provide protocols for the targeted modification of endogenous genes via GT with positive–negative selection using *hpt* and *DT-A* and subsequent marker excision via *piggyBac* transposon in rice (Figs. 1 and 2).

2 Materials

2.1 Agrobacterium-Mediated Transformation

1. Mature rice. seeds (*Oryza sativa L.* cv. Nipponbare).
2. *Agrobacterium tumefaciens* strain EHA105 [10].

2.1.1 Plant and Agrobacterium Materials

2.1.2 GT Vector

The GT vector (Fig. 1) carries the *DT-A* gene (under the control of the maize poly-ubiquitin 1 gene promoter or rice elongation factor promoter) at both ends of the T-DNA flanking the left and right border sequences, respectively, as well as a region of ca. 6.0-kb homologous to the target gene locus harboring the desired mutation. The *piggyBac* transposon carrying a rice actin terminator and *hpt* expression cassette is inserted into a central TTAA site in the homology arm of the target gene locus. If there are no TTAA sites adjacent to the target region of a desired mutation, the *piggyBac* transposon can be inserted into an artificially created TTAA site in the intron of the target gene. This artificially synthesized TTAA site remains after *piggyBac* excision in the target gene.

2.1.3 piggyBac Transposase Expression Vector

piggyBac transposes accurately and efficiently in rice upon expression of a mammalian codon-optimized hyper-active *piggyBac* transposase (hyPBase) carrying seven amino acid substitutions [11]. The hyPBase expression vector carries an expression cassette consisting of

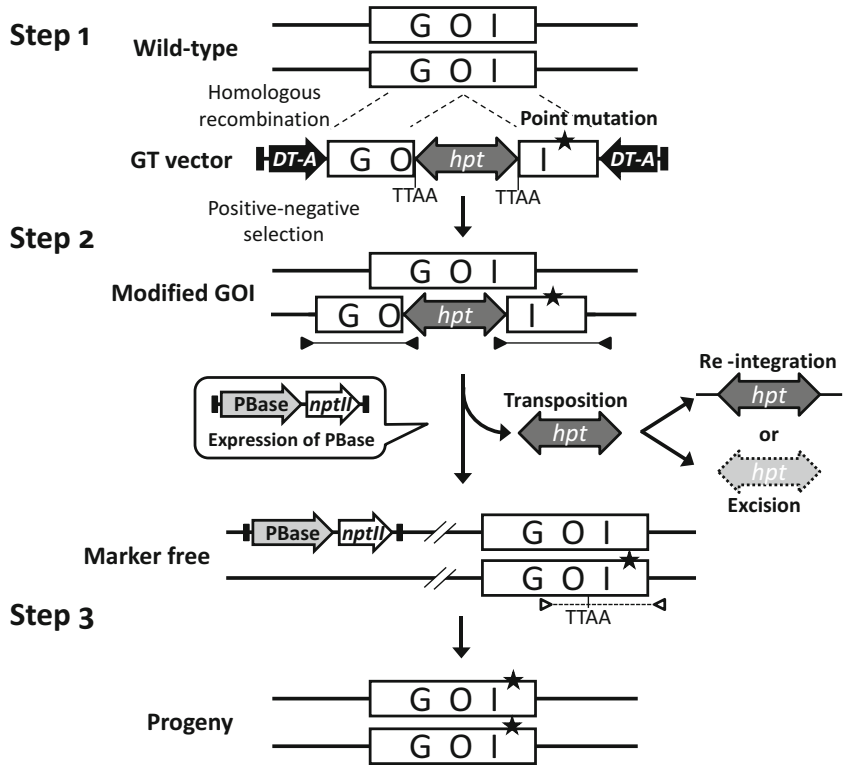

Fig. 1 Strategy to introduce a desired mutation into a target gene via GT and subsequent marker excision from the GT locus using the *piggyBac* transposon. (Step 1) Introduction of point mutations into the target gene via GT. *Wild-type* and *GT vector* schematics show the structure of the wild-type gene of interest (GOI) locus and the GT vector, respectively. The GT vector comprises target gene sequences with a point mutation (*star*), the *piggyBac* transposon carrying the *hpt* gene as a positive selectable marker, and expression cassettes of diphtheria toxin fragment A (*DT-A*) as a negative selectable marker. GT cells isolated using positive/negative selection are subjected to PCR analysis with the primer set indicated by *black arrowheads*. (Step 2) Marker excision from the GT locus using *piggyBac* transposon. *Modified GOI* and *Marker free* schematics reveal the structure of the target locus modified via GT and the modified target locus containing only the desired mutations following hyPBase-mediated marker excision, respectively. *White arrowheads* indicate the primer sets used for PCR to verify the presence of the TTAA element at the *piggyBac*-excised site and the desired mutation in the target gene. Since the hyPBase expression cassette might have integrated into the host genome, marker-free and homozygous plants with the modified GOI can be obtained via self-pollinating or out-crossing (Step 3)

hyPBase driven by the maize polyubiquitin1 gene promoter and a neomycin phosphotransferase II (*nptII*) expression cassette.

2.1.4 Media for Agrobacterium-Mediated Transformation

1. AB medium: 5 g/L glucose, 3 g/L K_2HPO_4, 1.3 g/L $NaH_2PO_4{\cdot}2H_2O$, 1 g/L NH_4Cl, 150 mg/L KCl, 10 mg/L $CaCl_2{\cdot}2H_2O$, 2.5 mg/L $FeSO_4{\cdot}7H_2O$ and 15 g/L Bacto-agar, pH 7.2. After autoclaving, add 1.2 mL of 1 M $MgSO_4{\cdot}7H_2O$ and the appropriate selective agent depending on the vector and *Agrobacterium* strain.

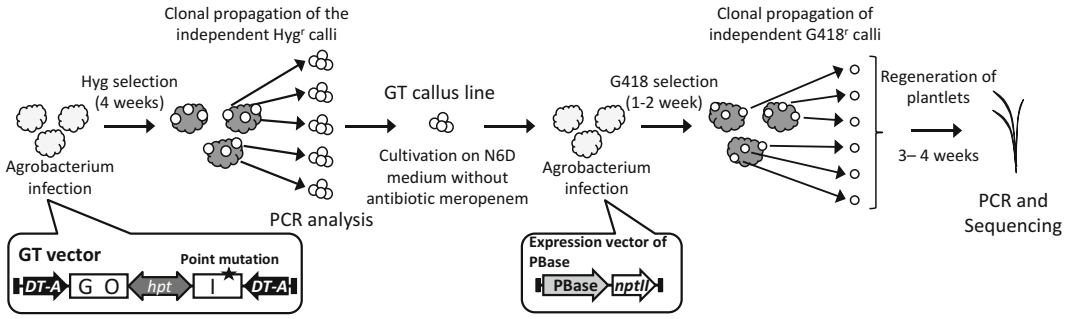

Fig. 2 Experimental strategy for precise genome modification using the *piggyBac* transposon. Four-week-old rice calli were inoculated with *Agrobacterium* harboring the GT vector and were selected on N6D medium containing 50 mg/L hygromycin sulfate and 25 mg/L meropenem for 4 weeks. Genomic DNA extracted from hygromycin-resistant calli was subjected to PCR analysis with primer sets shown in Fig. 1 to identify transgenic calli in which a GT event had occurred at the target locus. GT callus lines were transferred to N6D medium without meropenem and cultured for 4 weeks. GT calli were infected with *Agrobacterium* harboring a PBase expression vector. PBase transgenic calli were selected and regenerated on medium with 35 mg/L G418 and 25 mg/L meropenem. Regenerated plants were subjected to marker excision analysis by PCR and sequencing

2. AAM medium (liquid medium): 68.5 g/L sucrose, 36 g/L glucose, 250 mg/L $MgSO_4 \cdot 7H_2O$, 150 mg/L $CaCl_2 \cdot 2H_2O$, 150 mg/L $NaH_2PO_4 \cdot 2H_2O$, 3 g/L KCl, 40 mg/L Fe-EDTA, 10 mg/L $MnSO_4 \cdot 4\text{-}6H_2O$, 2 mg/L $ZnSO_4 \cdot 7H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 0.75 mg/L KI, 3 mg/L H_3BO_3, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 100 mg/L myo-inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 0.5 g/L casamino acids, 7.5 mg/L L-glycine, 176.7 mg/L L-arginine, 0.9 g/L L-glutamine and 0.3 g/L L-aspartic acid, pH 5.2. Autoclave.
3. 2N6AS medium: 30 g/L sucrose, 10 g/L glucose, 0.3 g/L casamino acids, 2 mg/L L-glycine, N6-vitamin, N6 salts, 2 mg/L 2,4-D and 4 g/L gelrite, pH 5.2. After autoclaving, add 0.2 mL of 100 mg/mL acetosyringone.
4. N6D medium: 30 g/L sucrose, 0.3 g/L casamino acids, 2.878 g/L L-proline, 2 mg/L L-glycine, N6-vitamin (100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 1 mg/L Thiamine HCl), N6 salts [Chu(N6)Medium Salt Mixture, Wako Pure Chemical Industries, Osaka, Japan], 2 mg/L 2,4-D and 4 g/L gelrite (for hygromycin selection) or 8 g/L Bacto Agar (for G418 selection), pH 5.8. Autoclave. After autoclaving, add 2 mL of 12.5 mg/mL meropenem and 1 ml of 50 mg/L Hygromycin B or 35 mg/L Geneticin (G418).
5. ReIII medium: 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, MS Medium salt Mixture (Wako Pure Chemical Industries, Japan), 2 mg/L glycine, N6-vitamins, 20 μg/L 1-naphthalene acetic acid, 2 mg/L kinetin and 4 g/L gelrite

(for hygromycin selection) or 8 g/L Bacto Agar (for G418 selection), pH 5.8. After autoclaving, add 2 mL of 12.5 mg/mL meropenem and 1 ml of 50 mg/L Hygromycin B or 35 mg/L Geneticin (G418).

6. HF medium: 30 g/L sucrose, MS Medium salt Mixture, 2 mg/L glycine, N6-vitamins and 4 g/L gelrite (for hygromycin selection) or 8 g/L Bacto Agar (for G418 selection), pH 5.8.

2.1.5 Stock Solutions

1. 2,4-Dichlorophenoxy acetic acid (2,4-D): 5 mg/mL. Dissolve the powder in dimethyl sulfoxide (DMSO). After filter-sterilization, store at 4 °C.
2. Kinetin: 0.2 mg/mL. Dissolve in a small amount of 1 N KOH or 1 N NaOH, make up volume with water, and store at 4 °C.
3. Naphtalene acetic acid (NAA): 0.2 mg/mL. Dissolve in a small amount of 1 N KOH or 1 N NaOH, make up volume with water, and store at 4 °C.
4. Acetosyringone (3′,5′-dimethoxy-4′hydroxy-acetophenone): 100 mg/mL. Dissolve the powder in DMSO. After filter-sterilization, store at 4 °C.
5. Meropenem: 12.5 mg/mL. Dissolve the powder in hot ddH_2O. After filter-sterilization, store at 4 °C.
6. Hygromycin B: 50 mg/mL. Dissolve the powder in ddH_2O. After filter-sterilization, store at 4 °C.
7. G418: 35 mg/mL. Dissolve the powder in ddH_2O. After filter-sterilization, store at 4 °C.

2.2 Identification of GT Calli

2.2.1 DNA Extraction

Agencourt chloropure (Beckman Coulter, USA).

2.2.2 Polymerase Chain Reaction

KOD FX neo (TOYOBO, Japan).

2.2.3 Purification of PCR Products for Sequencing Analysis

Agencourt AMPure XP (Beckman Coulter, USA).

2.3 Selection of Marker-Free Plants

2.3.1 Southern Blot Analysis

Nucleon PhytoPure (GE Healthcare, USA).

2.3.2 Polymerase Chain Reaction

PrimeSTAR GXL DNA polymerase (Takara, Japan).

3 Methods

3.1 Agrobacterium-Mediated Transformation of GT Vector into Rice Calli

1. Dehulled mature seeds of rice are sterilized and inoculated on callus induction N6D medium [12]. Seeds are incubated at 33 °C for 2–3 weeks.
2. Actively proliferating rice calli (approximately 5 mm in diameter) are subcultured on fresh N6D medium for 4 days before *Agrobacterium* infection.
3. *Agrobacterium* (EHA105) harboring the GT vector is inoculated onto AB medium containing appropriate antibiotics in Petri dishes and incubated at 28 °C in the dark for 3 days before *Agrobacterium* infection.
4. Approximately 5 g of calli (in a volume of approximately 5 mL) are collected in a 50 mL tube (BD falcon, USA). *Agrobacterium* harboring the GT vector is suspended in 40 mL of AAM medium ($OD_{600} = 0.03–0.1$) supplemented with 20 mg/L acetosyringone in a fresh 50 mL tube, and is then added to the 50 mL tube containing calli. The bacterial suspension and calli are mixed gently by inverting for 1.5 min. We usually use a strainer to discard the bacterial suspension solution. After discarding the bacterial suspension, calli are placed on three or four pieces of sterilized filter paper (70 mm) in Petri dishes to remove excess bacterial suspension and are transferred onto a sterilized filter paper that is then placed on 2N6-AS medium and moistened with 0.5 mL of AAM medium supplemented with 20 mg/L acetosyringone. Calli on 2N6-AS medium are co-cultivated at 25 °C in the dark for 3 days.
5. After 3 days of co-cultivation, calli are collected in a 50 mL tube and washed ten times with sterilized water with 25 mg/L meropenem solution. Washed calli are placed onto three or four pieces of sterilized filter paper in Petri dishes to remove excess solution. Approximately 100 pieces of calli are placed onto N6D medium containing 50 mg/L hygromycin and 25 mg/L meropenem and grown at 33 °C.
6. After a 2-week selection period, all calli are transferred onto fresh N6D medium containing 50 mg/L hygromycin and 25 mg/L meropenem and grown at 33 °C for another 2 weeks. Hygromycin-resistant calli do not appear at this time.
7. Hygromycin-resistant calli are transferred onto fresh N6D medium containing 50 mg/L hygromycin and 25 mg/L meropenem and are clonally propagated at 33 °C for 2 weeks.

3.2 Identification of GT Calli Using PCR Analysis

1. Genomic DNA is extracted from small pieces of hygromycin-resistant calli using Agencourt chloropure (Beckman Coulter, USA) according to the manufacturer's protocol.

2. PCR amplifications targeting both upstream and downstream of the insertion site of the positive selection marker are performed with KOD FX neo (TOYOBO, Japan) using primer sets that anneal to the positive selection marker and the endogenous target locus, which is not present on the GT vector (Fig. 1). Transgenic calli yielding positive PCR signals derived from the target locus carrying *piggyBac* with the positive selection marker are regarded as true GT lines. The proportion of PCR-positive calli per total hygromycin-resistant calli is approximately 1 % [1].
3. To confirm the introduction of a desired mutation into the target gene and TTAA elements located in the insertion site of the *piggyBac* transposon, PCR fragments targeting both upstream and downstream of the insertion site of the positive selection marker are purified by Agencourt AMPure XP (Beckman Coulter, USA) according to the manufacturer's protocol, and sequenced using a primer annealing approximately 300-bp distant from the mutation position or TTAA elements (*see* **Notes 2** and **3**).
4. True GT calli are transferred onto fresh N6D medium containing 50 mg/L hygromycin without meropenem and grown at 33 °C for 2–4 weeks to remove the meropenem (*see* **Note 4**).

3.3 Marker Excision via piggyBac Transposition

1. *Agrobacterium* (EHA105) harboring the hyPBase expression vector is inoculated onto AB medium containing appropriate antibiotics in Petri dishes and incubated at 28 °C in the dark for 3 days before *Agrobacterium* infection.
2. Cultured true GT calli on N6D medium without meropenem are transformed with *Agrobacterium* harboring the hyPBase vector and are co-cultivated according to Subheading 3.1 **step 4**. Co-cultivated calli with *Agrobacterium* are washed according to Subheading 3.1 **step 4**, then transferred onto N6D medium containing 35 mg/L G418 and 25 mg/L meropenem and grown at 33 °C for 2 weeks.
3. G418-resistant calli are transferred onto fresh N6D medium containing 35 mg/L G418 and 25 mg/L meropenem and are propagated clonally at 33 °C for 2 weeks.
4. To analyze the copy number of the hyPBase expression vector using Southern blot analysis, genomic DNA is extracted from approximately 20 lines of hyPBase transgenic calli using Nucleon PhytoPure (GE Healthcare, USA) in accordance with the manufacturer's protocol. Southern blot analysis is performed with specific DNA probes for the hyPBase gene according to a standard protocol (*see* **Note 5**).
5. Transgenic calli carrying a single copy of the hyPBase expression cassette are selected and transferred onto regeneration

medium (ReIII) containing 35 mg/L G418 and 25 mg/L meropenem and grown at 28 °C under constant light for 10–14 days (*see* **Note 6**).

6. Some pieces of green callus are transferred to fresh ReIII medium and grown at 28 °C under constant light for 2 weeks.
7. Shoots arising from callus on ReIII medium are transferred to HF medium containing 35 mg/L G418 and 25 mg/L meropenem and grown at 28 °C under constant light for 2 weeks.

3.4 Selection of Marker-Free Plants

1. Shoots derived from hyPBase transgenic calli are divided into individual plants and each is assigned a number.
2. Genomic DNA is extracted from leaves of regenerated plants using Agencourt chloropure (Beckman Coulter) according to the manufacturer's protocol.
3. To confirm whether the selection marker has been eliminated from GT-modified target locus via transposition of *piggyBac*, PCR amplifications are performed with KOD FX neo (TOYOBO) using the primer set shown in Subheading 3.2 **step 2** (Fig. 1). In addition, to detect the existence of re-integrated *piggyBac* transposon, positive selection marker-specific fragments are amplified with KOD FX neo. If the selection marker is excised from the target locus without concomitant re-integration of *piggyBac* transposon, the DNA fragment cannot be amplified with the PCR conditions used here.
4. DNA fragments carrying the *piggyBac*-excised site and the desired mutation inserted into the target gene via GT are amplified with PrimeSTAR GXL DNA polymerase (Takara) using a primer set specific for the endogenous target gene, and are purified using Agencourt AMPure XP. Subsequently, these fragments are cloned into the vector pCR-Blunt II-TOPO using TOPO cloning methods (Life Technologies, USA) and sequenced to check the TTAA element at the *piggyBac*-excised site and the presence of the desired mutation in the target gene.
5. To analyze segregation of the target gene modified via GT and the hyPBase expression vector, T_1 progeny plants are obtained from self-pollinating regenerating plants.

4 Notes

1. We find that a combination of introduction of DSBs by sequence-specific nucleases and their repair via single-strand annealing (SSA) also allows for marker excision without leaving unnecessary sequences from the GT locus (Unpublished data).
2. The desired mutations are not always inserted into the target locus via GT.

3. If mutations introduced into the target gene via GT generate recognition sites for a restriction enzyme, the presence of the mutation in the target gene can be detected by cleaved amplified polymorphic sequences (CAPS) analysis combined with PCR amplification of the target gene and digestion with that restriction enzyme.
4. The expected band size derived from modified target gene locus via GT in PCR-positive calli should also be checked by Southern blot analysis.
5. To obtain progenies in which a hyPBase expression cassette segregates out, transgenic calli lines carrying a single copy of the hyPBase expression cassette should be selected by Southern blot analysis.
6. If G418-resistant regenerated plants are not obtained, use ReIII and MSFH medium solidified with 4 g/L gelrite containing 25 mg/L meropenem without G418.

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References

1. Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20:1030–1034
2. Shimatani Z, Nishizawa-Yokoi A, Endo M, Toki S, Terada R (2015) Positive-negative-selection-mediated gene targeting in rice. Front Plant Sci 5:748
3. Osakabe K, Nishizawa-Yokoi A, Ohtsuki N, Osakabe Y, Toki S (2014) A mutated cytosine deaminase gene, codA (D314A), as an efficient negative selection marker for gene targeting in rice. Plant Cell Physiol 55:658–665
4. Nishizawa-Yokoi A, Nonaka S, Osakabe K, Saika H, Toki S (2015) A universal positive-negative selection system for gene targeting in plants combining an antibiotic resistance gene and its antisense RNA. Plant Physiol 169:362–370
5. Cary LC, Goebel M, Corsaro BG, Wang HG, Rosen E, Fraser MJ (1989) Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology 172:156–169
6. Ryder E, Russell S (2003) Transposable elements as tools for genomics and genetics in Drosophila. Brief Funct Genomic Proteomic 2:57–71
7. Woodard LE, Wilson MH (2015) piggyBacing models and new therapeutic strategies. Trends Biotechnol 33:525–533
8. Nishizawa-Yokoi A, Endo M, Osakabe K, Saika H, Toki S (2014) Precise marker excision system using an animal-derived piggyBac transposon in plants. Plant J 77:454–463
9. Nishizawa-Yokoi A, Endo M, Ohtsuki N, Saika H, Toki S (2015) Precision genome editing in

plants via gene targeting and piggyBac-mediated marker excision. Plant J 81:160–168

10. Hood E, Gelvin S, Melchers L, Hoekema A (1993) New Agrobacterium helper plasmids for gene-transfer to plants. Transgenic Res 2:208–218
11. Yusa K, Zhou L, Li M, Bradley A, Craig N (2011) A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci U S A 108:1531–1536
12. Saika H, Onodera H, Seiichi T (2012) Visual selection in rice: a strategy for the efficient identification of transgenic calli accumulating transgene products. In: Dunwell JM, Wetten AC (eds). Transgenic plants. Humana, Totawa. Methods Mol Biol 847:67–74

Chapter 11

Development of Genome Engineering Tools from Plant-Specific PPR Proteins Using Animal Cultured Cells

Takehito Kobayashi, Yusuke Yagi, and Takahiro Nakamura

Abstract

The pentatricopeptide repeat (PPR) motif is a sequence-specific RNA/DNA-binding module. Elucidation of the RNA/DNA recognition mechanism has enabled engineering of PPR motifs as new RNA/DNA manipulation tools in living cells, including for genome editing. However, the biochemical characteristics of PPR proteins remain unknown, mostly due to the instability and/or unfolding propensities of PPR proteins in heterologous expression systems such as bacteria and yeast. To overcome this issue, we constructed reporter systems using animal cultured cells. The cell-based system has highly attractive features for PPR engineering: robust eukaryotic gene expression; availability of various vectors, reagents, and antibodies; highly efficient DNA delivery ratio (>80 %); and rapid, high-throughput data production. In this chapter, we introduce an example of such reporter systems: a PPR-based sequence-specific translational activation system. The cell-based reporter system can be applied to characterize plant genes of interested and to PPR engineering.

Key words Animal cultured cell, DNA-binding protein, Genome editing, HEK293T cell, Translational activation, Pentatricopeptide repeat, PPR, RNA-binding protein

1 Introduction

Recent advances in genome editing tools, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), Pentatricopeptide repeat (PPR), and clustered regularly interspaced short palindromic repeat (CRISPR) systems, enable rational modifications of specific genes in living cells [1–3]. The pentatricopeptide repeat (PPR) motif is a sequence-specific RNA/DNA-binding module prevalent in flowering plants [4, 5]. Elucidation of the RNA/DNA binding mechanism enabled application of PPR as an alternative option for genome editing and novel genome-scale RNA manipulation tools [6, 7].

The RNA/DNA binding capacities and sequence-specific binding activities of several PPR proteins have been characterized by in vitro biochemical assays, including electrophoretic mobility

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shift assay, using recombinant PPR proteins produced in *E. coli*. However, the acquisition of active recombinant PPR proteins has been a major obstacle to studying PPRs due to the insoluble nature of PPR proteins, thereby delaying understanding of PPR-RNA/DNA binding characteristics and subsequent engineering. We have investigated various expression and reporter systems for PPR analyses and found that animal cell-based reporter systems greatly facilitate PPR engineering. Animal cultured cells have highly attractive features: robust eukaryotic gene expression; high-efficiency DNA delivery (>80%); availability of various vectors, regents, and antibodies; and quantitative, high-throughput data production (96-well format). Here, we introduce an example of such reporter systems: a PPR-based sequence-specific translational activation system using animal cultured cells.

Eukaryotic translation has been well characterized, and several factors are required for translational initiation [8]. Eukaryotic translation initiation factor 4E (eIF4E) for recognition of the 5′ cap structure, eIF4A as an RNA helicase, and eIF4G as a scaffold protein act cooperatively in translational initiation. Various artificial translational activation systems have been developed using a fusion of the eIF proteins and sequence-specific RNA-binding proteins such as λN, MS2, PUFs, and IRP-1 [9–11]. Thus, we designed a system by (1) fusing a PPR protein with eIF4G (in effector plasmid) and (2) inserting a PPR binding site at the 5′-UTR of a reporter gene (in reporter plasmid) to enable PPR-specific recruitment of eIF4G for translational activation (Fig. 1).

2 Materials

2.1 Instruments

1. Facility for molecular biology experiments (for vector construction, etc.).
2. Inverted microscope (e.g., DM IL S40, Leica Microsystems, Wetzlar, Germany).
3. CO_2 incubator (e.g., KM-CC17RH2, Panasonic Healthcare, Tokyo, Japan).
4. Bio clean bench (e.g., MHE-S1300A2, Panasonic Healthcare, Tokyo, Japan).
5. Aspirator (e.g., SP-30, Air Liquide Medical Systems, Bovezzo BS, Italy).
6. Centrifuge (with a swing rotor) (e.g., LC-200, Tomy Seiko, Tokyo, Japan).
7. Ultra-low temperature freezer (−80 °C) (e.g., MDF-C8V, Panasonic Healthcare, Tokyo, Japan).
8. Microplate reader (e.g., EnSight Kaleido, PerkinElmer, Waltham, MA, USA).

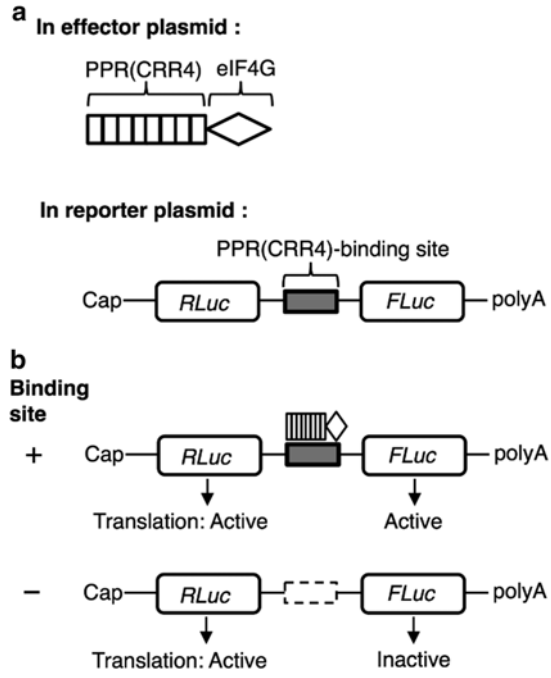

Fig. 1 Schematic representation of the PPR-based reporter system for translational activation. (**a**) The system contains effector and reporter plasmids. The effector plasmid expresses a fusion protein of PPR motifs and eIF4G for translational activation. The well-characterized PPR protein CRR4 was used in this study [12, 13]. The reporter plasmid transcribes di-cistronic mRNA carrying a *Renilla* luciferase (*RLuc*) gene and a firefly luciferase (*FLuc*) gene. The PPR (CRR4) binding site was inserted 5′ of *FLuc*. (**b**) *RLuc* should be similarly translated both in the presence and absence of the binding site. Thus, RLuc activity is used as a transfection control. However, translation of *FLuc* should be activated only in the presence of the binding site by artificial recruitment of eIF4G via PPR (CRR4) binding. Translation of *FLuc* should be inactive (occur less frequently) without the PPR binding site

2.2 Cell Culture

1. HEK293T cell line (*see* **Note 1**).
2. Dulbecco's modified Eagle's medium, DMEM, high glucose (*see* **Note 2**).
3. 100× penicillin-streptomycin solution.
4. Fetal bovine serum (FBS) (*see* **Note 3**).
5. EDTA-NaCl solution: 10 mM EDTA and 0.85 % (w/v) NaCl. Adjust pH to 7.2–7.4. Sterilized by autoclaving and stored at room temperature.
6. 100 × 20 mm cell culture dish (e.g., Greiner bio one, Frickenhausen, Germany).
7. 10 mL disposable pipet.
8. 15 and 50 mL conical centrifuge tubes.
9. 1.8 mL cryo storage vial (e.g., Nunc; Thermo Fisher Scientific, Waltham, MA, USA).

10. Cryogenic freezing container (e.g., Nalgene; Thermo Fisher Scientific, Waltham, MA, USA).
11. Bambanker (e.g., Lymphotec, Tokyo, Japan).

2.3 Transient Transfection

1. Effector plasmid (100 ng/μL; containing a fusion gene of PPR and eIF4G) based on pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA, USA) (*see* Subheading 3.1 and **Note 4**).
2. Reporter plasmid (100 ng/μL; containing the luciferase gene with a PPR-binding sequence at the 5′-UTR) based on pcDNA3.1.
3. Poly-L-lysine-coated 96-well plate (e.g., AGC Techno glass, Shizuoka, Japan).
4. 1× phosphate-buffered saline, PBS (−): 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl. Adjust pH to 7.4. Sterilized by autoclaving and stored at room temperature.
5. Hemacytometer (for cell counting) (e.g., Improved Neubauer Type Cell counter plate, Watson, Hyogo, Japan).
6. Transfection reagent (e.g., HilyMax, Dojindo Molecular Technologies, Kumamoto, Japan).

2.4 Luciferase Assay

1. Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA.).
2. 96-well luminometer plate (e.g., PerkinElmer, Waltham, MA, USA).

3 Methods

3.1 Vector Construction

The reporter assay requires an effector plasmid and a reporter plasmid, both based on pcDNA3.1. The effector plasmid contains a fusion gene of PPR protein and a partial domain of human eIF4G (amino acids 567–1559) (Fig. 1a). The PPR motifs (amino acids 53–613) of CRR4 were used in this study [12, 13]. The reporter plasmid contains two open reading frames (ORFs) for reporter genes *Renilla* luciferase (*RLuc*) and firefly luciferase (*FLuc*), transcribed bicistronically (Fig. 1a). *RLuc* is located 5′ of *FLuc* and used as an expression control. The PPR binding site was inserted at the 5′-UTR of the *FLuc* ORF and consists of three repeats of the CRR4 recognition sequence (13 nts each: 5′-UAUCUUGUCUUUA-3′) interrupted by 4 nts of random sequences (ATCG and GATC). Cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation signal were used for both the effector and reporter genes. For the control experiments, the effector plasmid without eIF4G was constructed by fusing the PPR to a FLAG epitope tag. A control reporter plasmid with no PPR binding site was also constructed.

3.2 Cell Culture

The procedures should be aseptically performed. All equipment should be sterilized with 70% ethanol prior to the procedure. A schematic overview of the reporter assay is depicted in Fig. 2.

3.2.1 Starting Cell Culture from the Frozen Cell Stock

1. Place 9 mL DMEM in a sterile 15-mL conical tube.
2. Swiftly thaw 1 mL HEK293T cells stocked in a cryo-tube by incubating in a water-bath at 37 °C.
3. Gently transfer the cells into the 15-mL conical tube containing 9 mL DMEM.
4. Centrifuge at 1100 × *g* for 2 min at room temperature. Remove the supernatant.
5. Resuspend the cells in 10 mL DMEM supplemented with 10% FBS.
6. Transfer the suspended cells to the 100-mm dish. Place the dish in a humidified incubator at 37 °C with 5% CO_2. Cultured cells should be subcultured after 24 h incubation when beginning from a frozen stock.

3.2.2 Subculturing for Cell Maintenance

To keep cells healthy (*see* **Note 5**), maintain cultures between 10 and 80% confluency, that is a percentage of the surface coverage by cells. Subculturing is typically performed every 3 days (twice per week) or as required depending on the cell growth rate. Further, the cell line should be replaced by a frozen cell stock once per month to keep the passage number low. Low passage number and cell health are important factors for efficient DNA transfection.

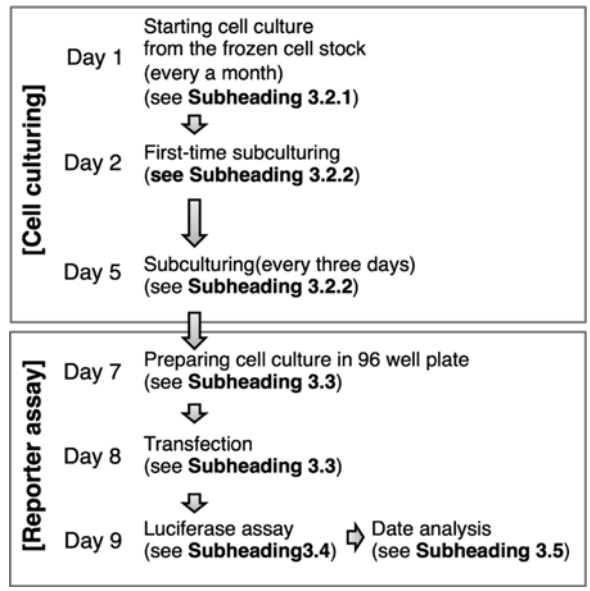

Fig. 2 Experimental procedure for the reporter assay using HEK293T cells. The overall procedure of the reporter assay is shown step by step. Detailed procedures are described in the text

1. Prepare the required number of new 100 mm dishes. Add 8 mL DMEM and 1 mL FBS to each dish in advance.
2. Carefully aspirate the medium from the dish containing cultured cells (*see* **Note 6**).
3. Gently add 2 mL EDTA-NaCl solution to the adherent cells on the internal surface of the dish to avoid detaching the cells. Gently swirl the dish to spread the solution evenly. Carefully aspirate the solution from the dish. Detach the cells by tapping the dish.
4. Add 10 mL DMEM to the cells in the dish and resuspend with gentle pipetting.
5. Dispense 1 mL cell suspension (10% of cultured cells) in the pre-prepared dish containing 9 mL medium. Disperse the cells by slowly swirling the dish.

3.2.3 Freezing Cell Stocks for Future Use

Frozen cell stocks can be prepared using Bambanker reagent and cultured cells at ~50% confluency during log-phase growth. Use of Bambanker easily enables long-term storage with a high recovery rate.

1. Detach 2-day cultured cells from the dish following the subculturing procedure (*see* Subheading 3.2.2). Add 5–10 mL DMEM to the dish and gather the cells in a 50-mL conical tube.
2. Centrifuge at 1100 × *g* for 2 min at room temperature and remove the supernatant.
3. Resuspend the cells in 1 mL Bambanker per dish.
4. Rapidly transfer the cell suspension into a cryo-tube and close the lid firmly.
5. Place the vials in the cryogenic freezing container at –80 °C for 12 h (*see* **Note 7**).
6. Transfer the vial to a normal sample box and store at –80 °C or in liquid nitrogen.

3.3 Transient Transfection

1. Before starting, prepare the required number of dishes containing 2-day cultured cells and ensure that the cells are healthy (*see* **Note 8**). As a rough estimate, a single dish can be used for 96 assays.
2. Detach the 2-day cultured cells from the dish following the subculturing procedure (*see* Subheading 3.2.2) and transfer the cell suspension to a 50-mL conical tube.
3. Centrifuge at 1100 × *g* for 2 min at room temperature and remove the supernatant.
4. Completely disperse the cell clumps in 10 mL DMEM supplemented with 10% FBS by pipetting.
5. Count the cells using a hemacytometer and an inverted microscope. Adjust the cell concentration to 1–2 × 10^5 cells/mL by suspending in the appropriate volume of DMEM with 10% FBS.

6. Add 200 μL aliquots ($2–4 \times 10^4$ cells) of the cell suspension to each well of a 96-well culture plate and incubate at 37 °C with 5 % CO_2 overnight. A single well is used for a single assay.
7. The following day, carefully replace the medium in each well with 100 μL fresh DMEM with 10 % FBS.
8. Prepare a mixture of 400 ng effector plasmid (4 μL of 100 ng/μL) and 100 ng reporter plasmid (1 μL of 100 ng/μL) in a single well of a new 96-well PCR plate (or a new 0.2 mL tube). The mixture (5 μL) is used for a single assay.
9. Dilute 1 μL HilyMAX in 10 μL serum-free DMEM per sample.
10. Add 11 μL of the dilution to all the well containing the plasmids. Mix by pipetting.
11. Incubate at room temperature for 15 min. Transfer the total amount of the mixture to the well containing the cultured cells. Incubate at 37 °C with 5 % CO_2 for 24 h.

3.4 Luciferase Assay

The dual-luciferase assay is performed using a Dual-Glo Luciferase Assay System according to the manufacturer's instructions with minor modifications.

1. At 24 h post-transfection, replace medium with 40 μL 1× PBS per well.
2. Add 40 μL Dual-Glo luciferase reagent to each well and mix by pipetting.
3. Incubate at room temperature for 10 min and transfer the mixture to a new 96-well white luminometer plate.
4. Measure the firefly luminescence for *FLuc* gene expression in a microplate reader.
5. Dilute Dual-Glo Stop & Glo substrate 100-fold with the Stop & Glo buffer. Add 40 μL diluted solution to each well.
6. Incubate at room temperature for at least 10 min and then measure *Renilla* luminescence for *RLuc* gene expression.

3.5 Data Analysis

1. Calculate FLuc/RLuc values for each assay to normalize the transfection efficiencies and experimental errors among the assays.
2. Determine the fold activation of reporter gene expression by dividing the value (FLuc/RLuc) for the experimental plasmid (with translational activation domain; eIF4G) by that of the control plasmid (with FLAG-tag) in the presence and absence of PPR binding site, respectively. Example data are shown in Fig. 3.

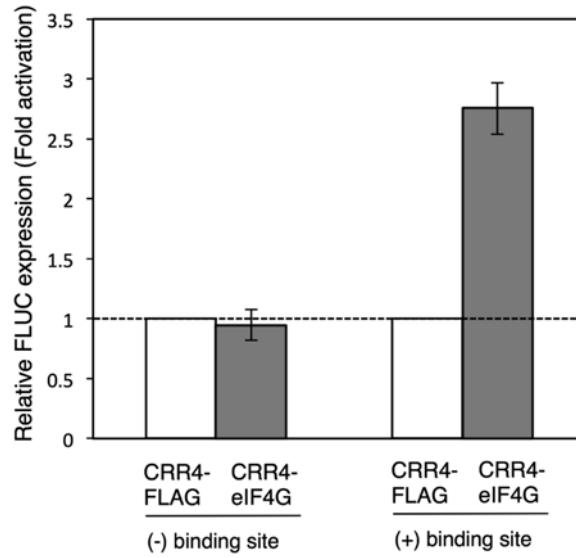

Fig. 3 Selective translational activation is dependent on CRR4-eIF4G fusion and the PPR binding site. The experiments are performed using effector plasmids containing the CRR4-FLAG fusion protein (without translational activator) or the CRR4-eIF4G fusion protein (with translational activator), respectively shown by *white* and *gray bars*, and reporter plasmids with and without the PPR binding site. The result demonstrates 2.75-fold translational activation specifically in the presence of both the PPR-eIF4G fusion protein and the binding site. Values indicate means and standard deviations ($n = 3$)

4 Notes

1. HEK293T is a human embryonic kidney cell line that expresses the large T antigen from Simian virus 40. The cell line allows easy culturing and high-efficiency transfection using a variety of methods. HEK293T cells are available from RIKEN BRC (ja.brc.riken.jp) or ATCC (www.atcc.org).
2. DMEM is supplemented with 1× penicillin-streptomycin solution to prevent microbial contamination.
3. FBS should be heat-inactivated at 56 °C for 30 min and stored at 4 °C before use.
4. Plasmid purity is crucial for transfection efficiency. Plasmids must be isolated using transfection-grade quality purification kits.
5. Daily growth rate is an index of cell health. To avoid inhibiting growth, cells should always be grown with sufficient space and nutrients.
6. HEK293T cells easily detach from the culture dish surface and must be handled gently while replacing the culture medium.

7. A cryogenic freezing container is an insulated box that can control freezing speed (approximately −1 °C/min at −80 °C), allowing cryopreservation in a normal, non-programmable −80 °C freezer.
8. We use cells at 50–80% confluency for transfection. However, the suitable degree of confluency depends on the transfection reagent. In addition, the ratio of transfection reagent (μL) to plasmid DNA (μg) should be optimized according to the manufacturer's instructions. The described procedure is optimized for HEK293T cells using HilyMAX as the transfection reagent in a 96-well culture plate.

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References

1. Gaj T, Gersbach CA, Barbas CF 3rd (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31:397–405
2. Kim H, Kim JS (2014) A guide to genome engineering with programmable nucleases. Nat Rev Genet 15:321–334
3. Hilton IB, Gersbach CA (2015) Enabling functional genomics with genome engineering. Genome Res 10:1442–1455
4. Andrés C, Lurin C, Small ID (2007) The multifarious roles of PPR proteins in plant mitochondrial gene expression. Physiol Plant 129:14–22
5. Nakamura T, Yagi Y, Kobayashi K (2012) Mechanistic insight into pentatricopeptide repeat proteins as sequence-specific RNA-binding proteins for organellar RNAs in plants. Plant Cell Physiol 53:1171–1179
6. Barkan A, Rojas M, Fujii S, Yap A, Chong YS, Bond CS, Small I (2012) A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. PLoS Genet 8:e1002910
7. Yagi Y, Nakamura T, Small I (2014) The potential for manipulating RNA with pentatricopeptide repeat proteins. Plant J 78:772–782
8. Van Der Kelen K, Beyaert R, Inzé D, De Veylder L (2009) Translational control of eukaryotic gene expression. Crit Rev Biochem Mol Biol 44:143–168
9. Cao J, Arha M, Sudrik C, Bugaj LJ, Schaffer DV, Kane RS (2013) Light-inducible activation of target mRNA translation in mammalian cells. Chem Commun (Camb) 49:8338–8340
10. Cao J, Arha M, Sudrik C, Schaffer DV, Kane RS (2014) Bidirectional regulation of mRNA translation in mammalian cells by using PUF domains. Angew Chem Int Ed Engl 53:4900–4904
11. De Gregorio E, Preiss T, Hentze MW (1999) Translation driven by an eIF4G core domain in vivo. EMBO J 18:4865–4874
12. Kotera E, Tasaka M, Shikanai T (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 433:326–330
13. Okuda K, Nakamura T, Sugita M, Shimizu T, Shikanai T (2006) A pentatricopeptide repeat protein is a site recognition factor in chloroplast RNA editing. J Biol Chem 281:37661–37667

Chapter 12

Chromosomal Allocation of DNA Sequences in Wheat Using Flow-Sorted Chromosomes

Petr Cápal, Jan Vrána, Marie Kubaláková, Takashi R. Endo, and Jaroslav Doležel

Abstract

Flow cytometry enables chromosomes to be sorted into different groups based on their characteristics, such as relative DNA content and the presence of repetitive DNA sequences. Despite the recent progress in the analysis of plant genome organization and chromosome structure, there is a need for easy methods to assign DNA sequences to individual chromosomes. Here, we describe an easy way to allocate genes or DNA sequences to chromosomes in wheat using flow-sorted chromosomes combined with fluorescence in situ hybridization and PCR analyses.

Key words Chromosomal allocation, Transgene, Flow cytometry, Chromosome sorting, Single chromosome amplification, Chromosome genomics, FISH, Wheat

1 Introduction

Chromosomal assignment of a DNA sequence of interest is often the first step toward further analyses of the sequence; moreover, it has a practical benefit in breeding for agronomically useful traits brought by transgenes. In wheat, aneuploid analysis has been employed to identify critical chromosomes carrying specific genes and DNA sequences. A series of aneuploid stocks are available in durum and common wheat, thanks to their tetraploid and hexaploid nature, respectively. The aneuploids of common wheat, such as monosomics, nullitetrasomics, and ditelosomics, lack a single homologous chromosome $(2n-1)$, a single pair of homologous chromosomes $(2n-2)$, and a pair of chromosome arms $(2n-2t)$, respectively [1, 2]. They have been used to allocate genes to specific chromosomes or chromosome arms [3]. In practice, the chromosomal location of a gene can be determined by examining the progeny of hybrids between a target wheat cultivar or line carrying the critical gene and appropriate

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aneuploid lines. However, this type of analysis is laborious and time consuming, and requires cytological skills. A monosomic analysis to determine the chromosomal location of a gene, for example, requires crosses between the 21 monosomic lines of common wheat and the target line carrying the target gene, growing the 21 F_1 hybrids to produce F_2 progeny by self-pollination, and examination of segregation ratios (presence vs. absence) of the gene in all 21 F_2 progeny lines. Considering the fact that the monosomic chromosome is transmitted preferentially to the progeny through pollen, the segregation of the F_2 progeny derived from the critical monosomic line does not fit to the ratio of three present to one absent. Thus, we can identify the critical chromosome carrying the gene. However, we need to grow the 21 monosomic lines for two generations and count the chromosome numbers of the individual plants of the monosomic, F_1 and F_2 lines, because the progeny of those lines from self-pollination is expected to be euploid ($2n=42$), monosomic ($2n=41$), or nullisomic ($2n=40$) for specific chromosomes.

A flow cytometer equipped with a sorter can sort thousands of chromosomes represented in different groups of chromosomes that are discriminated on the basis of a flow karyotype; even single chromosomes can be sorted, provided they can be discriminated from other chromosomes based on their optical parameters [4]. A few hundred flow-sorted chromosomes are enough for direct PCR analysis. Even single flow-sorted chromosomes can be amplified separately and the amplified DNA can be analyzed by PCR [5]. Chromosomes flow-sorted onto a microscope slide can be identified by fluorescence in situ hybridization (FISH). In common wheat, all 21 chromosomes are identifiable by FISH using two kinds of repetitive sequences, GAA microsatellites and Afa family repeats [6]. Hence, we can locate specific DNA sequences on specific chromosomes in a much shorter time and less laboriously by combining PCR and FISH analyses with chromosome flow sorting. In this chapter, we provide a detailed protocol for the chromosomal allocation of genes and other DNA sequences in wheat by flow cytometric chromosome sorting combined with FISH and PCR analyses of flow-sorted chromosomes. The procedure for chromosome sorting using flow cytometry is practically the same as described by Vrána et al. [7].

2 Materials

2.1 Plant Material

Seeds of *Triticum aestivum* L. ($2n=6x=42$, bread or common wheat), *T. durum* L. ($2n=4x=28$, macaroni or durum wheat), and genetic stocks of common wheat including transgenic lines.

2.2 Laboratory Reagents and Solutions

2.2.1 Laboratory Reagents and Solutions for Chromosome Sample Preparation and Flow Cytometry

1. Solution A: 45 mM H_3BO_3 (280 mg), 20 mM $MnSO_4 \cdot H_2O$ (340 mg), 0.4 mM $CuSO_4 \cdot 5H_2O$ (10 mg), 0.8 mM $ZnSO_4 \cdot 7H_2O$ (22 mg) and 0.08 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (10 mg) in deionized water (100 mL). Store at 4 °C.
2. Solution B: 0.05 mM concentrated H_2SO_4 (0.5 mL) in deionized water (100 mL). Store at 4 °C.
3. Solution C: 18 mM Na_2EDTA (3.36 g) and 20 mM 2.79 g $FeSO_4$ (20 mM) in deionized water. Heat the solution to 70 °C while stirring until the color turns yellow-brown. Cool down, adjust the volume with deionized water (500 mL) and store at 4 °C.
4. Hoagland's stock solution (10×): 4.7 g $Ca(NO_3)_2 \cdot 4H_2O$ (40 mM), 2.6 g $MgSO_4 \cdot 7H_2O$ (20 mM), 3.3 g KNO_3 (65 mM), 0.6 g $NH_4H_2PO_4$ (10 mM), 5 mL solution A, and 0.5 mL solution B, in deionized water. Adjust volume to 500 mL. Prepare just before use.
5. Hoagland's nutrient solution (1×): 100 mL Hoagland's stock solution (10×) and 5 mL solution C in deionized water. Adjust volume to 1000 mL. Prepare just before use.
6. Hoagland's nutrient solution (0.1×): 10 mL Hoagland's stock solution (10×) and 0.5 mL solution C in deionized water. Adjust volume to 1000 mL. Prepare just before use.
7. 1 mM Hydroxyurea (HU) solution: dissolve 60.8 mg hydroxyurea in 800 mL 0.1× Hoagland's nutrient solution. Prepare just before use.
8. 1.25 mM HU solution: dissolve 76 mg hydroxyurea in 800 mL 0.1× Hoagland's nutrient solution.
9. 2 mM HU solution: dissolve 121.6 mg hydroxyurea in 800 mL 0.1× Hoagland's nutrient solution. Prepare just before use.
10. 2.5 mM HU solution: dissolve 152 mg hydroxyurea in 800 mL 0.1× Hoagland's nutrient solution. Prepare just before use.
11. Amiprophos methyl (APM) stock solution (20 mM): dissolve 60.86 mg APM in 10 mL ice-cold acetone and store at –20 °C, in 1 mL aliquots.
12. APM working solution (2.5 μM): 101.3 μL APM stock solution in 800 mL deionized water. Prepare just before use.
13. APM working solution (10 μM): 405.2 μL APM stock solution in 800 mL deionized water. Prepare just before use.
14. Oryzalin stock solution (10 mM): dissolve 86.59 mg oryzalin in 25 mL ice-cold acetone. Store at –20 °C, in 1 mL aliquots.
15. Oryzalin working solution (2.5 μM): 200 μL oryzalin stock solution in 800 mL deionized water. Prepare just before use.
16. Oryzalin working solution (5 μM): 400 μL oryzalin stock solution in 800 mL deionized water. Prepare just before use.

17. Oryzalin working solution (10 μM): 800 μL oryzalin stock solution in 800 mL deionized water. Prepare just before use.
18. Tris-base buffer: 10 mM Tris (606 mg), 10 mM Na_2EDTA (1.861 g), 100 mM NaCl (2.922 g) in deionized water (500 mL). Adjust pH to 7.5 using 1 N NaOH.
19. Formaldehyde 2% fixative: 13.5 mL formaldehyde in Tris buffer. Adjust volume to 250 mL. Prepare just before use.

    Formaldehyde 4% fixative: 27 mL formaldehyde in Tris buffer. Adjust volume to 250 mL. Prepare just before use.
20. LB01 buffer: 15 mM Tris (0.363 g), 2 mM Na_2EDTA (0.149 g), 0.5 mM spermine·4HCl (0.0348 g), 80 mM KCl (1.193 g), 20 mM NaCl (0.234 g), 0.1% (v/v) Triton X-100 (200 μL) in deionized water (200 mL). Adjust pH to 9. Filter through a 0.22 μm filter to remove small particles. Add 220 μL β-mercaptoethanol and mix well. Store at −20 °C, in 8 mL aliquots.
21. 4′,6-Diamidino-2-phenylindole (DAPI) stock solution (0.1 mg/mL): dissolve DAPI in deionized water by stirring. Filter through a 0.22 μm filter to remove small particles. Store at −20 °C, in 0.5 mL aliquots.

2.2.2 Laboratory Reagents and Solutions for FISH

1. P5 buffer: 10 mM Tris (30.28 mg), 50 mM KCl (93.2 mg), 2 mM $MgCl_2·6H_2O$ (10.17 mg) and 5% sucrose (1.25 g) in deionized H_2O (25 mL). Adjust pH to 8 using 1 N HCl. Store at −20 °C, in 1 mL aliquots.
2. 20× SSC stock solution: 3 M NaCl (175.3 g) and 300 mM $Na_3C_6H_5O_7·2H_2O$ (88.2 g) in deionized H_2O (1000 mL). Adjust pH to 7. Sterilize by autoclaving. Store at room temperature.
3. 4× SSC washing buffer: 20× SSC (200 mL) and 0.2% Tween 20 in deionized H_2O.
4. 2× SSC washing buffer: 20× SSC (100 mL) in deionized H_2O. Prepare just before use.
5. 0.1× SSC stringent washing buffer: 20× SSC (5 mL), 0.1% Tween 20 and 2 mM $MgCl_2·6H_2O$ in deionized H_2O. Prepare just before use.
6. Hybridization mix: 40% formamide (10 μL), 20× SSC (1.25 μL), 0.625 μL calf thymus (250 ng/μL), labeled DNA probe(s) (1 ng/μL). Add 50% dextran sulfate (final volume 25 μL). Prepare just before use. Labeled DNA probes (either directly labeled with fluorescent probes, or labeled by digoxigenin or biotin) can be prepared by PCR using primers for GAA microsatellite repeats, $(GAA)_7$ and $(TTC)_{10}$, and primers for Afa family repeats, 5′-GAT GAT GTG GCT TTG AATGG-3′ and 5′-GCA TTT CAA ATG AAC TCT GA-3′ [8].
7. Detection of digoxigenin-labeled probes: FITC-labeled anti-digoxigenin antibody raised in sheep.

8. Detection of biotin-labeled probes: Cy3-labeled streptavidin antibody.
9. Blocking solution: dissolve 0.5 g blocking reagent in 50 mL 4× SSC. Autoclave. Store at –20 °C, in 1 mL aliquots.
10. Vectashield antifade solution containing DAPI.
11. 1 M NaOH stock solution: Dissolve 4 g of NaOH in 100 mL deionized or distilled water. Store the stock solution at room temperature.
12. 0.015 M NaOH/ethanol solution: 1 M NaOH (1.5 mL) in 98.5 mL 70% ethanol. Prepare before use (*see* **Note 1**).

2.2.3 Reagents and Solutions for FISHIS

1. 10 M NaOH: dissolve solid NaOH in deionized water. Store at room temperature.
2. 1 M Tris-HCl: dissolve Tris in deionized water by stirring, adjust the pH to 7.5 using 1 N HCl. Store at 4 °C.
3. $(GAA)_7$ microsatellite probe labeled with FITC moiety on both 3′ and 5′ ends: Dissolve the probe to 100 μM concentration with 2× SSC according to manufacturer's instructions. Prepare working solution by adding 2× SSC to final concentration 80 ng/μL. Store in the dark at –20 °C, in 20 μL aliquots.

2.2.4 Laboratory Reagents and Solutions for PCR

1. 10× PCR buffer with 1.5 mM $MgCl_2$.
2. 10 mM dNTPs solution.
3. Primers: Dissolve the primers in water as stated by the manufacturer to obtain a 100 μM concentration. Mix 10 μL of the corresponding forward and reverse primers with 180 μL water and store at –20 °C.
4. Taq polymerase, 2 U/μL.

2.2.5 Laboratory Reagents and Solutions for Single Chromosome Amplification

1. GE Healthcare GenomiPhi V2 kit (GE Healthcare Life Sciences).
2. 1 M Tris–HCl (pH 8): Dissolve 12.11 g Tris-base in 80 mL dd H_2O and adjust the pH to 8 with concentrated HCl. Adjust the volume to 100 mL with dd H_2O. Store at 4 °C.
3. 0.5 M EDTA: Dissolve 18.61 g EDTA-Na_2 in 80 mL dd H_2O and adjust the pH to 8 with 5 M NaOH. Adjust the volume to 100 mL with dd H_2O. Autoclave and store at 4 °C.
4. Denaturation buffer: Dissolve 280 mg KOH (final conc. 600 mM) and 15.4 mg DTT (final conc. 100 mM) in 800 μL dd H_2O. Add 20 μL 0.5 M EDTA (final conc. 10 mM) and adjust volume to 1 mL. Pass through a Millex-GS 0.22 μm filter unit with a sterile syringe into an autoclaved 1.5 mL Eppendorf tube. UV irradiate in a UV crosslinker device (Stratalinker 2400 set for 1200 mJ). Store at -20 °C in 50 μL aliquots (*see* **Note 2**).

5. Neutralization buffer: Mix 200 μL 3 M HCl (final conc. 600 mM) with 800 μL 1 M Tris–HCl, pH 8. Pass through a Millex-GS 0.22 μm filter unit with a sterile syringe into an autoclaved 1.5 mL Eppendorf tube. Store at −20 °C in 50 μL aliquots.
6. Reaction buffer for single chromosome sorting: Mix 100 μL Sample buffer from the GenomiPhi V2 kit with 10 μL proteinase K (10 mg/mL, Sigma-Aldrich, cat. No. P4850). Prepare just before use.

2.3 Consumables, Laboratory Equipment, and Instruments

1. Glass petri dishes.
2. Filter papers.
3. Incubator with temperature control.
4. Plastic box (roughly 20 cm × 10 cm × 12 cm) covered with a lid with drilled holes (3 mm in diameter).
5. Aquarium aeration system.
6. Temperature-controlled water bath.
7. Razor blade or mechanical tissue homogenizer.
8. Sample tubes for flow cytometer.
9. 4 × 4 cm nylon mesh filters with pore size 20 and 50 μm.
10. pH meter.
11. Flow cytometer and sorter equipped with 355 and 488 nm lasers.
12. 0.2 mL PCR tubes or 96-well microtiter plates.
13. Microscope slides and coverslips.
14. Blower (camera equipment).
15. Parafilm.
16. Rubber cement.
17. Humidity chamber with temperature control.
18. Fluorescence microscope equipped with filters for DAPI, FITC, and Texas Red fluorescence.
19. Laboratory pipettes.
20. Sterile pipette tips with filters.
21. PCR thermocycler.
22. Biosafety cabinet.
23. Millex-GS 0.22 μm filter unit (Merck Millipore).
24. Plastic syringe, 5 mL.
25. Hot plate.
26. Vortex.
27. Mini-centrifuge.
28. UV crosslinker (for example Stratalinker 2400).
29. Homogenizer (for example Polytron PT 1300D or similar).

3 Methods

3.1 Sample Preparation

1. Soak seeds in a beaker with deionized water at room temperature for 15 min.
2. Prepare a petri dish by placing a filter paper on the bottom, cover with a paper towel and put another filter paper over the top. Presoak with water and place the wet seeds equally all over the area of the dish.
3. Let the seeds germinate in the dark at 25 °C until the length of the roots reaches 2–3 cm (usually it takes 3 days).
4. Stretch the seedling roots through drilled holes of a plastic lid and place the lid with the seedlings onto a plastic box filled with water. Ensure that the roots are fully immersed in water and proceed to the next step.
5. Transfer the plastic lid with the seedlings onto a plastic box containing a 2 mM HU solution, and incubate in the dark at 25 °C for 18 h using an aeration system. Ensure that the roots are fully immersed in the solution.
6. Transfer the plastic lid with the seedlings onto a plastic box with 0.1× Hoagland solution and incubate them for 5.5 h in the same conditions as described in **step 5**. Ensure that the roots are fully immersed in the solution.
7. Transfer the plastic lid with the seedlings onto a box filled with 2.5 μM APM solution and incubate for 2 h in the same conditions as described in **step 5**. Ensure that the roots are fully immersed in the solution.
8. Rinse the seedlings thoroughly by submerging the lid in deionized water.
9. Transfer the lid with the seedlings onto a box with a mixture of cold water and ice cubes and place it in a refrigerator for 18 h. Ensure that the roots are fully immersed in water.
10. Cut off the terminal 1 cm of approximately 60 roots using a scalpel and place the root tips into a 50 mL beaker filled with deionized water.
11. Remove the deionized water completely using a 5 mL pipette and fill the beaker with 2% formaldehyde fixative solution. Chill the beaker in a water bath at 5 °C for 20 min.
12. Remove the fixative completely and wash the root tips three times with Tris buffer at 5 °C, 5 min each time. Fill the beaker with Tris buffer and keep it on ice.
13. On a clean glass petri dish, excise the 1–2 mm apex of every root with a scalpel, and transfer the apices into a sample tube with 1 mL LB01 buffer.
14. Grind the apices in the LB01 buffer using a mechanical homogenizer (20,000 rpm) for 13 s.

15. Filter the suspension through a 50 μm nylon mesh into a new sample tube and keep the tube on ice until use.

3.2 Chromosome Labeling Using FISH in Suspension (FISHIS)

1. Filter 300 μL of chromosome suspension through a 20 μm nylon mesh into 1.5 mL tube and place the tube on ice.
2. Add 10 M NaOH to reach pH 13.
3. Incubate the suspension for 20 min on ice.
4. Adjust the pH in the range of 8–9 using Tris–HCl.
5. Immediately add 1 μL of $(GAA)_7$ probe working solution and let the suspension incubate at room temperature for 1 h in the dark.
6. Keep the suspension on ice until the flow cytometry.

3.3 Chromosome Flow Sorting

1. Filter the chromosome suspension through a 20 μm nylon mesh into the tube suitable for flow sorter and stain the suspension with DAPI at final concentration of 2 μg/mL (20 μL of stock solution per 1 mL sample).
2. Start and set-up the flow sorter.
3. Load the corresponding experiment in the flow sorter's software and open the histograms and dot plots. Firstly, gate the area representing chromosomes on FSC vs. DAPI dot plot. Work with this gating on subsequent dot plots—FITC vs. DAPI when analysing FISHIS-labeled chromosomes or DAPI-W vs. DAPI-A when only DAPI stained chromosomes are analysed.
4. Draw a new gate around chromosome(s) of interest on corresponding dot plot.
5. Run the sample, analyse at least 20,000 chromosomes and save the data.
6. Sort the chromosomes of interest in sufficient quantity into appropriate vessels (sample tubes or microscope slides).

3.4 FISH Analysis with Sorted Chromosomes

1. Sort approximately 2000 chromosomes onto a 5 μL drop of P5 buffer on a microscope slide. Allow the drop to air dry and store in the dark at room temperature until use.
2. Add 25 μL of hybridization mix to slide, place a coverslip and seal with rubber cement.
3. Place the slide on a hot plate at 80 °C for 45 s.
4. Move the slide into a humidity chamber and incubate overnight at 37 °C.
5. Transfer the slide into container filled with 2× SSC and carefully remove the coverslip using tweezers. Wash at 42 °C for 10 min.
6. Wash in 0.1× SSC at 42 °C for 5 min.
7. Incubate in 2× SSC at 42 °C for 10 min.
8. Remove the container from incubator. Replace the solution with heated (42 °C) 2× SSC solution, and incubate the slide at room temperature for 10 min.

9. Wash in 4× SSC at room temperature for 10 min.
10. Remove the slide from the container and apply 60 μL of 1% blocking solution over the area with the chromosomes. Cover the slide with a piece of parafilm and incubate at room temperature for 10 min. Remove the parafilm, and repeat the incubation in blocking solution two more times.
11. Add the solution of fluorescently labeled antibody (follow manufacturer's instructions regarding concentration) in 60 μL of 1% blocking solution, and incubate at 37 °C for 1 h. This step is omitted in the case of directly labeled fluorescent probes.
12. Wash the slide three times in 4× SSC solution at 40 °C for 10 min.
13. Apply 10 μL of antifade solution containing DAPI onto the slide and place a cover slip on the solution for microscopic observation.
14. By fluorescence microscopy, identify the chromosomes based on the standard FISH karyotype of common wheat (Vrána et al. [6]) (Fig. 1).

Steps 2–12 can be replaced with **steps 15–24**. This alternative procedure also works.

15. Dip the slide into a 0.015 M NaOH solution at room temperature for 5 min.
16. Transfer the wet slide into a 2× SSC solution at room temperature, and leave it there for 3 min.
17. Rinse the slide briefly with distilled water and air dry it quickly using a blower.
18. Apply 10 μL of hybridization mixture onto the slide and place a cover slip on the mixture.
19. Place the slide in a leak-proof plastic box moistened inside with a damp paper towel, securely put the lid on it, and keep the box at 37 °C for 6 h to overnight, taking care that the box does not dry inside.
20. Remove the coverslip using tweezers and immerse the slide in 2× SSC at room temperature for 3 min.
21. Rinse the slide briefly with distilled water and air dry it quickly using a blower.
22. Apply 7–10 μL of antibody solution onto the slide and place a cover slip on the mixture.
23. Place the slide in a leak-proof plastic box moistened inside with a damp paper towel, put the lid on it, and keep the box at 37 °C for 1 h.
24. Remove the coverslip using tweezers, and rinse the slide briefly with distilled water. Air-dry it quickly using a blower.

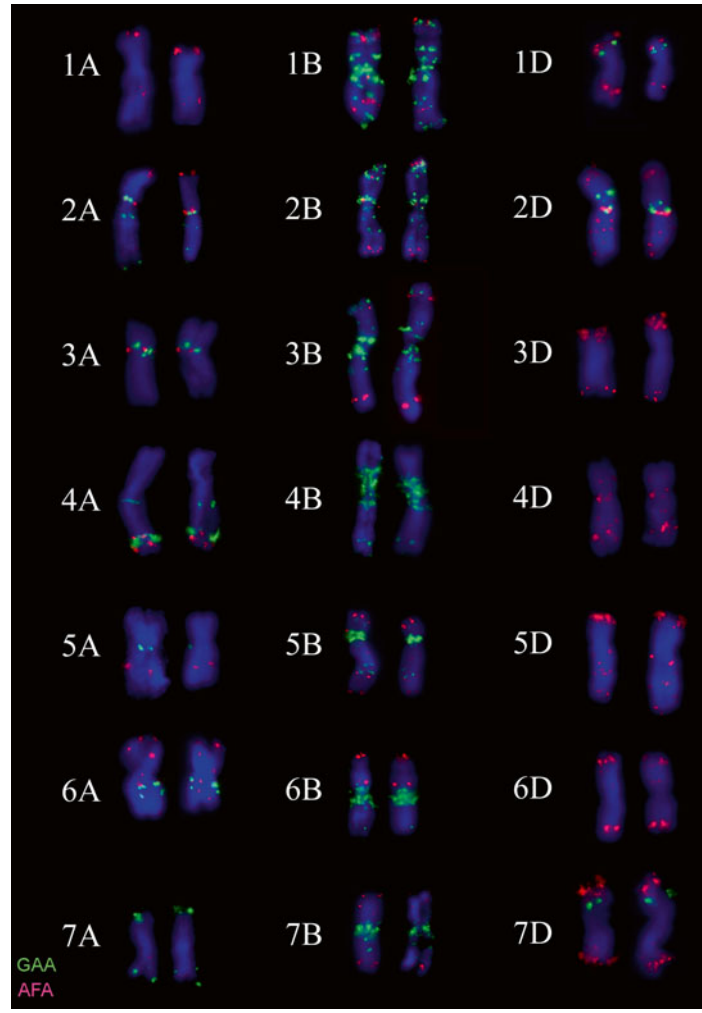

Fig. 1 Two-color FISH karyotype constructed from chromosomes that were flow-sorted from common wheat cv. Chinese Spring. FISH probes are GAA microsatellites (*green* signals) and Afa family repeats (*red* signals)

3.5 PCR Analysis with Sorted Chromosomes

1. Sort 500 chromosomes into a 0.2 mL tube or a well of a 96-well microtiter plate containing 4.5 μL ddH_2O (*see* **Note 3**).
2. Put the sorted chromosomes in tubes/plates into a freezer (−20 °C) for at least 2 h and thaw them subsequently (*see* **Note 4**).
3. Prepare PCR master mix from the reagents listed in Table 1. Make a sufficient volume for the number of reactions, i.e., 10% more than the calculated required volume.
4. Add 10 μL of the master mix to each tube or well of a microplate, seal the plate with foil suitable for thermocycling and spin down using a microfuge or mini-centrifuge.
5. Perform PCR in a standard thermocycler with the following conditions: Initial denaturation of 95 °C for 7 min (*see* **Note 5**),

Table 1
PCR premix

Component	Concentration	Volume (μL)	Final concentration
dd H_2O		5.1	
PCR buffer	10×	1.5	1×
dNTPs	2 μM	1.5	200 nM
F + R primers	5 μM	1.5	500 nM
Taq polymerase	2 U/μL	0.4	0.05 U/μL

35 cycles of 95 °C denaturation for 30 s, 52–65 °C annealing for 30 s, and 72 °C extension for 30 s, followed by a final extension at 72 °C for 5 min.

6. Mix 5 μL of each respective PCR reaction with 1 μL of loading dye, load the mixture onto a 1.5 % agarose gel, and run the gel to resolve the bands.

3.6 Single Chromosome Amplification and PCR

Perform all steps in as sterile conditions as possible. Use filter pipette tips exclusively and sterilize equipment and working surfaces by UV irradiation to minimize contamination.

1. Pipette 3 μL of reaction buffer for single chromosome sorting into a sterile 0.2 mL tube.
2. Set the flow cytometer into single-cell sorting mode and lower the sample rate to avoid doublet formation and to obtain the best possible chromosome discrimination.
3. Sort one chromosome into the 3 μL freshly prepared reaction mix and immediately spin down using a mini-centrifuge (*see* **Note 6**).
4. Incubate at 50 °C for 18 h.
5. Inactivate proteinase K in a thermocycler at 85 °C for 15 min. Digested chromosomes can be stored at −20 °C at this point until further processing.
6. Add 1.5 μL of denaturation buffer to the tube containing a single, digested chromosome, spin down, and incubate at 30 °C for 10 min.
7. Stop the denaturation reaction by adding 1.5 μL of neutralization buffer and spin down. Keep the tube on ice until use.
8. Prepare a master mix by combining 4 μL of sample buffer, 9 μL of reaction buffer and 1 μL of enzyme per reaction (all reagents are from GE healthcare GenomiPhi V2 whole genome amplification kit).

9. Add 14 μL of the master mix to 6 μL of the sample in each respective tube, spin down, mix gently by flicking the tubes, and spin down again.
10. Incubate in a thermocycler at 30 °C for 4 h, then heat at 65 °C for 10 min to inactivate the enzyme, and store at −20 °C.
11. DNA generated this way can be handled as genomic DNA, although a purification step is recommended.

3.7 Chromosomal Allocation of DNA Sequences

Identification of a chromosome carrying a target DNA sequence can be conducted as shown in Fig. 2.

1. Sort chromosomes simultaneously both onto a microscope slide (about 2000 chromosomes) and into a PCR tube (about 500 chromosomes) from the same region on the flow karyotype (*see* Fig. 2a). Do this for each of the regions.
2. Check the presence of the target sequence by PCR (*see* Fig. 2b) (*see* **Note 7**).
3. Conduct FISH to identify the chromosomes from the region in which the presence of the target sequence has been confirmed (*see* Fig. 2c) (*see* **Note 8**).
4. Conduct single chromosome sorting and amplification of the most likely chromosome or chromosomes that carry the target sequence.
5. Conduct PCR analysis using the primer sets for the target sequence and for the chromosome-specific markers (*see* Fig. 2d) (*see* **Note 9**).

4 Notes

1. Note that the concentration of NaOH is one-tenth of the concentration for chromosome preparations made by the squash method [9].
2. Pipette the aliquots with sterile filter tips into presterilized 0.2 mL tubes. Do not store the frozen lysis buffer longer than 1 month. Vortex lysis buffer vigorously after thawing to dissolve DTT properly.
3. Each chromosome is sorted in a 1.1 nL drop; therefore the final volume in a tube is 0.5 μL (=4.5 μL + (500 × 0.0011 μL)).
4. A freeze/thaw cycle assures the accessibility of chromatin. Multiple cycles can be repeated. This step can be substituted with proteinase K digestion of chromosomes, but the freeze-thaw method is cheaper and easier.
5. Although the standard time of initial denaturation is 3 min, for sorted chromosomes it is advisable to prolong the time up to 7 min in order to denature compact chromosomes properly.

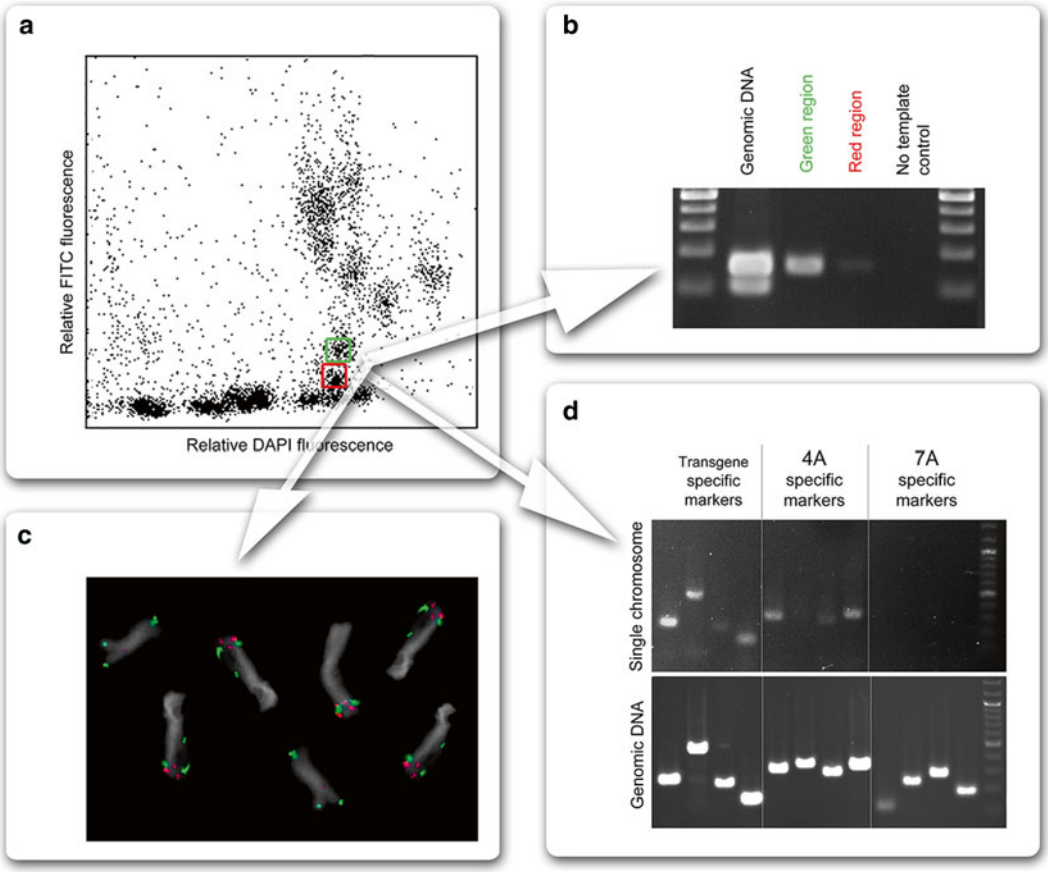

Fig. 2 Hypothetical procedure for chromosomal allocation of a transgene in common wheat using flow-sorted chromosomes. (**a**) Bi-variate flow karyotype of a common wheat line carrying the transgene. Individual populations on the dot plot represent chromosomes with specific intensity of DAPI fluorescence (*x* axis) and GAA-FITC probe fluorescence (*y* axis). In this experiment, chromosomes in separate regions (e.g. those surrounded by *green* and *red rectangles*) were sorted separately for PCR and FISH analyses. (**b**) PCR analysis for the transgene with 500 chromosomes sorted from each of the regions designated by *red* and *green rectangles*. PCR amplification using one of the transgene markers occurred only in the sorted chromosomes from the *green rectangle*. This indicates that the transgene is carried by a chromosome among the chromosomes represented in the *green rectangle*. (**c**) Two-color FISH image of the chromosomes sorted from the same *green rectangle*. The majority of the sorted chromosomes were chromosome 4A; however, about 10 % of them were chromosome 7A. Incidentally, the majority of chromosomes sorted from the red rectangle were chromosome 7A. This fact suggests that chromosome 4A most probably carries the transgene. (**d**) PCR analysis of a single chromosome sorted from the region surrounded by a green rectangle. Clear PCR amplification occurred with all five markers for the transgene and with three out of four markers for chromosome 4A. No 7A-specific marker was amplified from the same single chromosome. This concurrent PCR amplification of the transgene-specific markers and chromosome 4A-specific markers confirms that chromosome 4A carries the transgene

6. Conduct every centrifugation step with a mini-centrifuge at room temperature. Pipette every reagent, apply it to the wall of a tube, and spin down without allowing the pipette tip to touch the surface of the liquid in the tube.

7. It is efficient to pool chromosomes from multiple regions for PCR analysis in narrowing down the critical region.
8. The most frequent chromosome is to be selected for the next step, i.e. single chromosome sorting and amplification.
9. Single chromosome amplification is not always successful, but concurrent PCR amplification of both the target and chromosome-specific markers in a few samples ensures the identification of the chromosome carrying the target sequence.

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References

1. Sears ER (1954) The aneuploids of common wheat. Missouri Agr Exp Sta Res Bull 572:1–58
2. Sears ER, Sears LMS (1978) The telocentric chromosomes of common wheat. Proc. 5th Int. Wheat Genet. Symp. New Delhi, India, pp 389–407
3. Law CN, Snape JW, Worland AJ (1987) Aneuploidy in wheat and its uses in genetic analysis. In: Lupton RGH (ed) Wheat breeding: its scientific basis. Springer, Netherlands, pp 71–108
4. Doležel J, Vrána J, Cápal P, Kubaláková M, Burešová V, Šimková H (2014) Advances in plant chromosome genomics. Biotechnol Adv 32:122–136
5. Cápal P, Blavet N, Vrána J, Kubaláková M, Doležel J (2015) Multiple displacement amplification of the DNA from single flow-sorted plant chromosome. Plant J. doi:10.1111/tpj.13035
6. Vrána J, Kubaláková M, Šimková H, Číhalíková J, Lysák MA, Doležel J (2000) Flow-sorting of mitotic chromosomes in common wheat (*Triticum aestivum* L.). Genetics 156:2033–2041
7. Vrána J, Cápal P, Číhalíková J, Kubaláková M, Doležel J (2016) Flow sorting plant chromosomes. In: Kianian SF, Kia PMA (eds) Plant cytogenetics. Humana, Totowa
8. Vrána J, Šimková H, Kubaláková M, Číhalíková J, Doležel J (2012) Flow cytometric chromosome sorting in plants: the next generation. Methods 57:331–337
9. Sakai K, Nasuda S, Sato K, Endo TR (2009) Dissection of barley chromosome 3H in common wheat and comparison of 3H physical and genetic maps. Genes Genet Syst 84:25–34

Chapter 13

Image Analysis of DNA Fiber and Nucleus in Plants

Nobuko Ohmido, Toshiyuki Wako, Seiji Kato, and Kiichi Fukui

Abstract

Advances in cytology have led to the application of a wide range of visualization methods in plant genome studies. Image analysis methods are indispensable tools where morphology, density, and color play important roles in the biological systems. Visualization and image analysis methods are useful techniques in the analyses of the detailed structure and function of extended DNA fibers (EDFs) and interphase nuclei. The EDF is the highest in the spatial resolving power to reveal genome structure and it can be used for physical mapping, especially for closely located genes and tandemly repeated sequences. One the other hand, analyzing nuclear DNA and proteins would reveal nuclear structure and functions. In this chapter, we describe the image analysis protocol for quantitatively analyzing different types of plant genome, EDFs and interphase nuclei.

Key words Image analysis, Extended DNA fibers (EDFs), Isolated nuclei, CHIAS-straight, CHIAS-interphase

1 Introduction

Applications of a wide range of visualization methods in plant genome studies are now essential for obtaining quantitative data of plant genomes. Fluorescence in situ hybridization (FISH) on plant genome is effective for the physical mapping of cloned DNA markers [1, 2]; however, the special resolution is limited to 1–2 Mb on mitotic metaphase chromosomes and 100–500 kb on meiotic pachytene chromosomes [3]. The special resolution of gene mapping on interphase nuclei is about tenfold higher than that on chromosomes, because chromatin is much less condensed in the nucleus. Stretched chromatin fibers have been used as an effective alternative procedure for mapping adjacent DNA probes with a higher spatial resolution [3]. FISH on extended DNA fibers (EDFs), known as fiber-FISH, shows the highest spatial resolution to obtain genome structure, because all the proteins interacted with DNA compaction are removed and the genome DNAs are extended. It was used to detect the molecular organization of genome structures such as telomeres in japonica and indica rice

Minoru Murata (ed.), *Chromosome and Genomic Engineering in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1469, DOI 10.1007/978-1-4939-4931-1_13,

[4]. Fiber-FISH facilitates accurate analysis of repetitive sequences in the eukaryotic genomes and is a valuable in large genome sequencing projects.

Images of interphase nuclei have been analyzed by various methods. Both the fluorescent and conventional nuclear staining methods can show uneven DNA density in a nucleus. In some plants, they show heterochromatic chromocenters (CCs) as heavily stained granular regions. Interphase nuclei of Orchidaeceae show many CCs with different sizes by using conventional staining method [7]. The numerical parameters of CCs within a nucleus are used to determine the type of resting chromosome, which is a useful cytological characteristic that can serve as an indicator of cross compatibility among orchid plant species. The CCs in *Arabidopsis thaliana* are discrete nuclear domains of mainly pericentric heterochromatic regions. FISH with specific sequences would reveal the relationship between the specific sequence and nuclear structure. *Arabidopsis* CCs harbor major tandem and dispersed repetitive sequences [5]. Immunostaining can visualize specific DNA molecule such as methylated cytosine and specific nuclear protein. The *Arabidopsis* CCs carry epigenetic markers of silent chromatin, such as dimethylated histone H3K9 and methylated DNA [6]. Fluorescent protein is also used for visualizing specific nuclear protein [7].

The visualized information of DNA or protein in EDFs and nuclei could be analyzed quantitatively by image analysis methods. We demonstrate that CHIAS-straight and CHIAS-interphase enable the quantitative analysis of fibrous signals on EDFs and granular signals in interphase nuclei, respectively.

2 Materials

2.1 Software and Equipment

1. Personal computer with ImageJ (version 1.48 or later) software [8]. ImageJ requires an appropriate version of Java software.
2. Image analysis software Chromosome Image Analyzing System (CHIAS). It can be downloaded from the CHIAS website (http://www2.kobe-u.ac.jp/~ohmido/index03.htm).
3. Samples on poly-L-lysine-coated slide glass (e.g., Matsunami Glass, Osaka, Japan).

   Preparation method for EDF-FISH samples are described in Chapter 14.
4. Cytospin 4 (Thermo Scientific, Waltham, MA, USA).
5. Hydrophobic pen (e.g., DAKO or PAP pen).
6. Fluorescence microscope (e.g., BX50, Olympus).
7. Cooled charge coupled device (CCD) camera (e.g., SPOT-RT3, Diagnostic Instruments. Inc.).

2.2 Reagents

1. Nuclei isolation buffer (NIB): 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES, pH 5.5), 0.2 mM sucrose, 2.5 mM ethylene-diamine-tetraacetic acid (EDTA), 0.1 mM spermine, 2.5 mM dithiothreitol (DTT), 10 mM NaCl, 10 mM KCl, 0.5 mM spermidine, and 0.5% Triton X 100.
2. Stretching/Lysis buffer: 0.5% w/v sodium dodecyl sulfate (SDS), 100 mM Tris–HCl, and 5 mM EDTA (pH 7.0).
3. Pre-fixation solution: 4% paraformaldehyde in phosphate-buffered saline (PBS) (*see* **Note 1**).
4. Fluorescent staining solution: 1 μg/mL 4, 6 diamidino-2-phenylindole (DAPI) in sterilized water.
5. Vectashield (e.g., Vector Laboratories, Burlingame, CA, USA).

3 Methods

3.1 Preparation of Nuclei

1. Chop ten fresh and soft plant seedlings in 500 μL NIB using two single-edge razor blades in a glass petri dish or plastic partitioned box placed on ice.
2. Filter the solution on ice using a 50-μm nylon mesh filter to remove cell debris.
3. Collect 50–100 μL of filtered solution and centrifuge in Cytospin 4 at 500 rpm for 5 min. The preparation protocol of EDFs are demonstrated in Chapter 14.
4. Draw a circle around nuclei preparation regions with a hydrophobic pen, place a few drops of 4% paraformaldehyde on the circled area, and leave for 15 min. Wash in PBS and H_2O briefly.
5. Dehydrate with 70 and 100% ethanol each for 5 min and air-dry.
6. Incubate in 1 μg/mL DAPI for 10 min. Wash in H_2O briefly.
7. Mount in Vectashield to check preparation quality.

3.2 CHIAS: Straight

CHIAS, which runs as a plug-in of the public-domain image-processing software ImageJ (http://imagej.nih.gov/ij/) developed by the National Institute of Health (NIH; Bethesda, MD, USA) using the Java programming language [9]. ImageJ is the host application for CHIAS IV [10]; for both operating systems, the software requires version 1.48 or later of ImageJ (http://rsb.info.nih.gov/ij/download.html). The CHIAS IV plug-in file must then be installed. The "CHIAS4_.jar" file was obtained from the CHIAS website (*see* **Note 4**).

CHIAS-Straight is an appropriate application to measure the image of fiber-FISH and immunostained chromatin fiber [11]. Routinely 25–100 signal tracks are screened to measure the length of fluorescent signal tracks on EDFs.

1. Download the plug-in files CHIAS-Straight from the CHIAS website. The plug-in is distributed free of charge, but a registration is required for download based on the user instruction.
2. Install the plug-in file 'CHIAS_st.jar' and copy it into the 'plug-ins' folder of the 'ImageJ' folder.
3. Click 'CHIAS-Straight' on the menu bar to display the command menu (Fig. 1A).
4. Select command:
   (a) Open: Select image of fiber-FISH (Fig. 1B).
   (b) Set scale: Enter the distance in pixels based on the magnification of the CCD camera and microscope.
   (c) Select segmented line tool: Decide the width of the region of interest in pixels and draw each segment line along the fiber image click by click;
   (d) Straighten: Turn the selected region to the straightened image (Fig. 1C).
   (e) Select line tool: Measure the intensity of fluorescent signals and then draw a line to measure the selected region click by click (Fig. 1D).
   (f) Measure: Measure the length and density of selected line in the straightened image;
   (g) Plot profile: Plot the intensity of selected line (Fig. 1E).
   (h) Save as: Save image data and/or tables as an Excel file.

3.3 CHIAS-Interphase

1. Download the plug-in files CHIAS-interphase from the CHIAS website (*see* **Note 4**). The plug-in is distributed free of charge, but a registration is required for download based on the user instruction.
2. Install the plug-in file 'CHIASi_.jar' and copy it into the 'plug-ins' folder of the 'ImageJ' folder.
3. Click 'CHIAS-interphase' on the menu bar to display the command menu (Fig. 2A).
4. Select command:
   (a) Open image: Open the image of interphase nucleus with red, green, and blue (RGB) colors (Fig. 2B).
   (b) Set Density Slice: Set threshold and click the red region on the image (see Note 2) to turn it into a shape surrounded by a yellow line (Fig. 2C).
   (c) Erase Back Ground: Erase background noise (Fig. 2D).
   (d) Signal control: Set the signal region of each R and G for FISH or immunostaining images and click 'setDensity' ('Red' for red signal and 'Green' for green signal) (Fig. 3A).
   (e) Scan and EraseBG: Scan data of the selected regions and erase background noise (Fig. 3B).

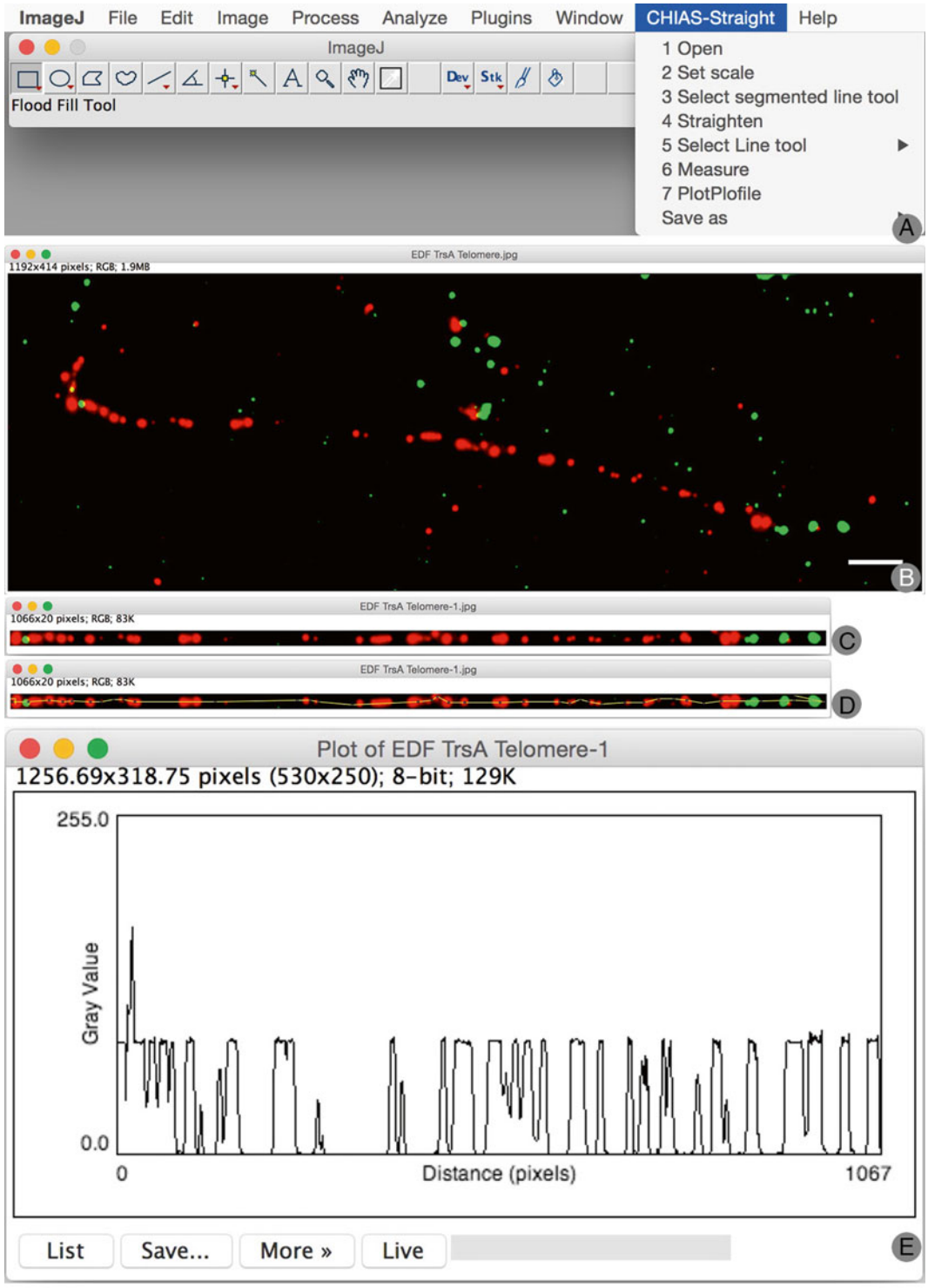

Fig. 1 Procedure of 'CHIAS-straight', (**A**) 'CHIAS-Straight' menu, (**B**) Original EDF image, (**C**, **D**) Straighten EFFs images, (**E**) Plot of the signal intensity of EDFs

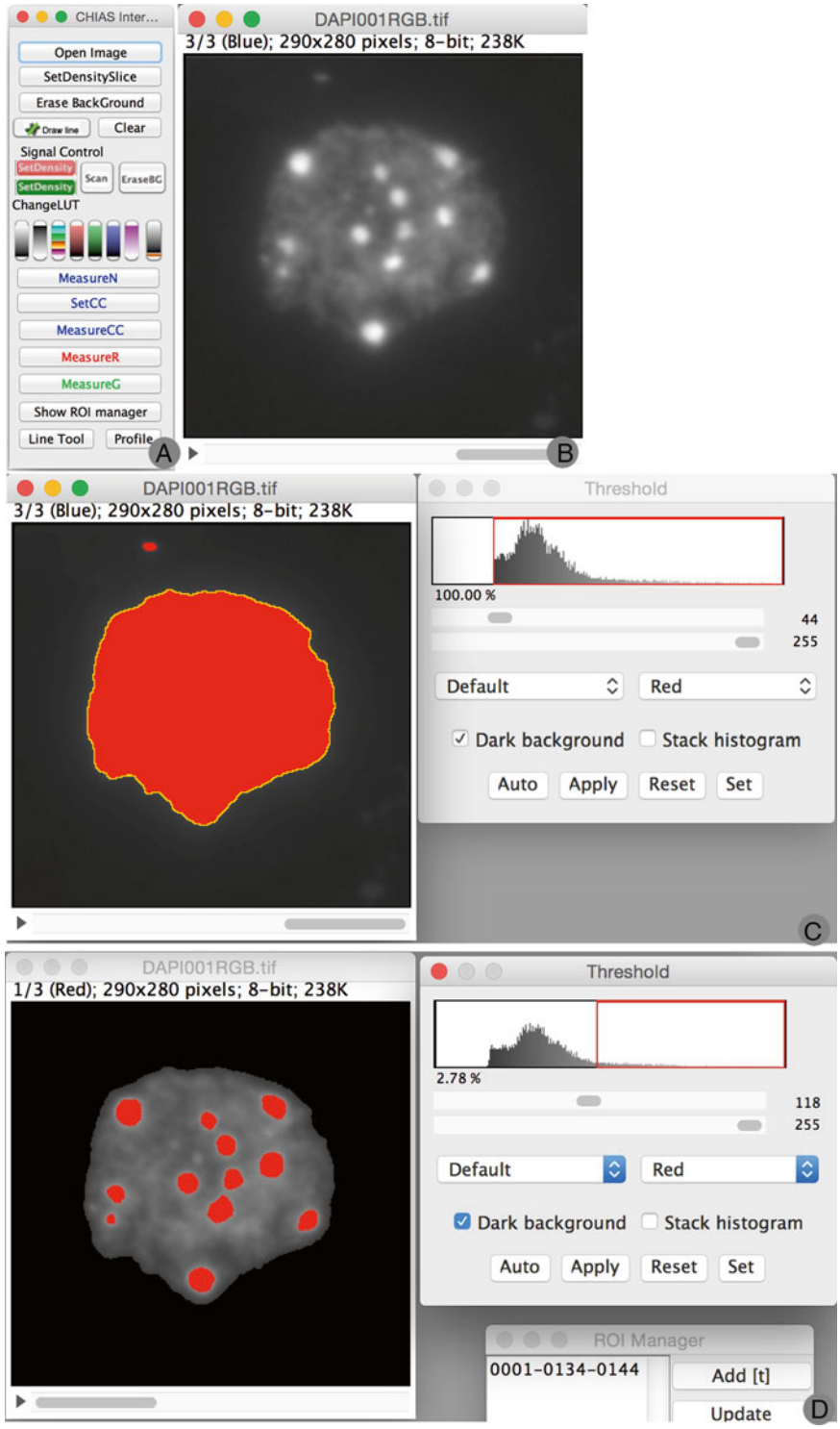

Fig. 2 Procedure of 'CHIAS-Interphase'-1, (**A**) 'CHIAS- interphase' menu, (**B**) Original nuclei image consisting by RGB, (**C**) Set Density slice image and Erase Back Ground, (**D**) Set signals regions

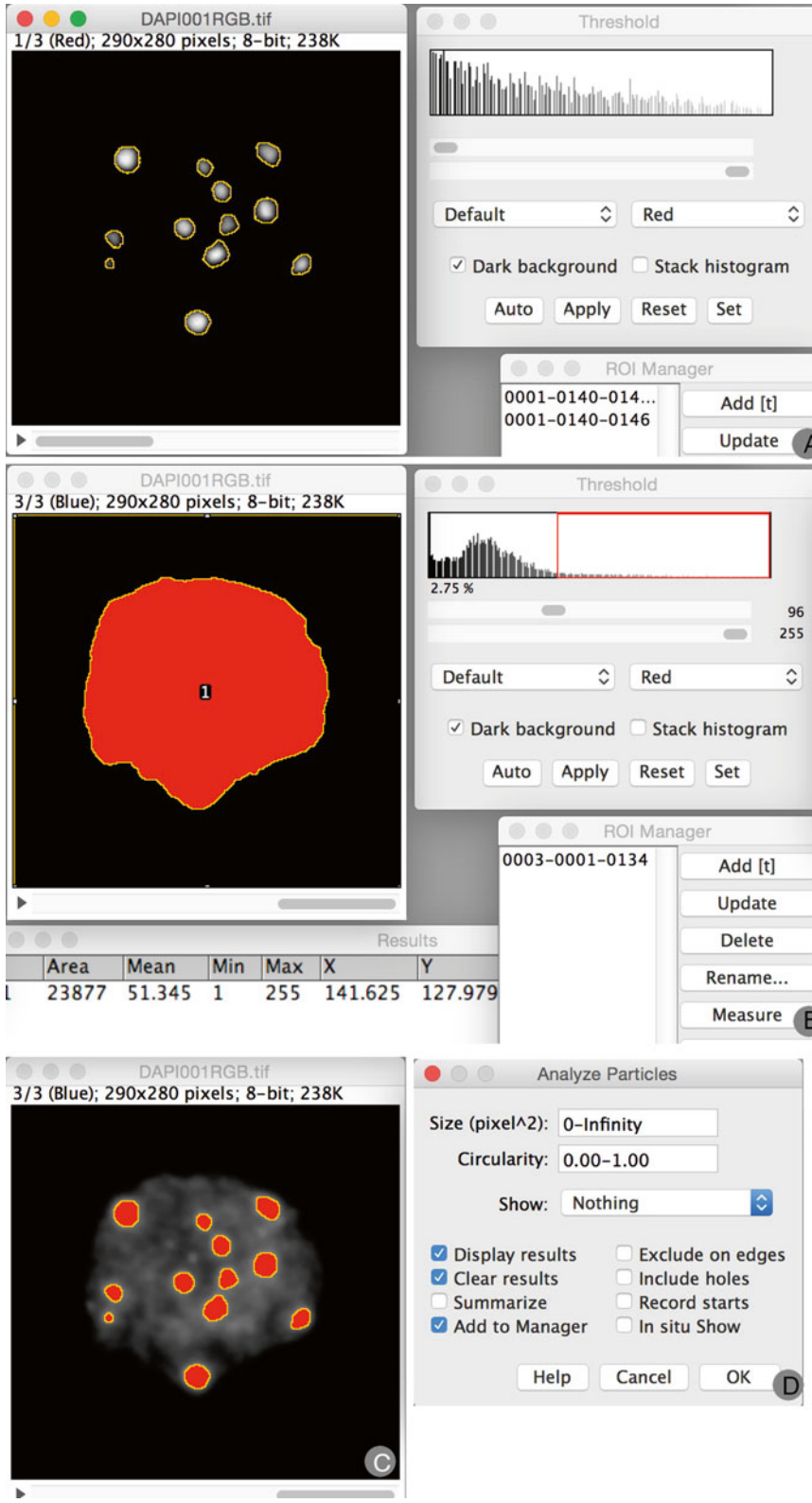

Fig. 3 Procedure of 'CHIAS-Interphase'-2, (**A**) Scan signal and Erase background, (**B**) Measure nucleus, (**C**) Set CC, (**D**) Measure CCs

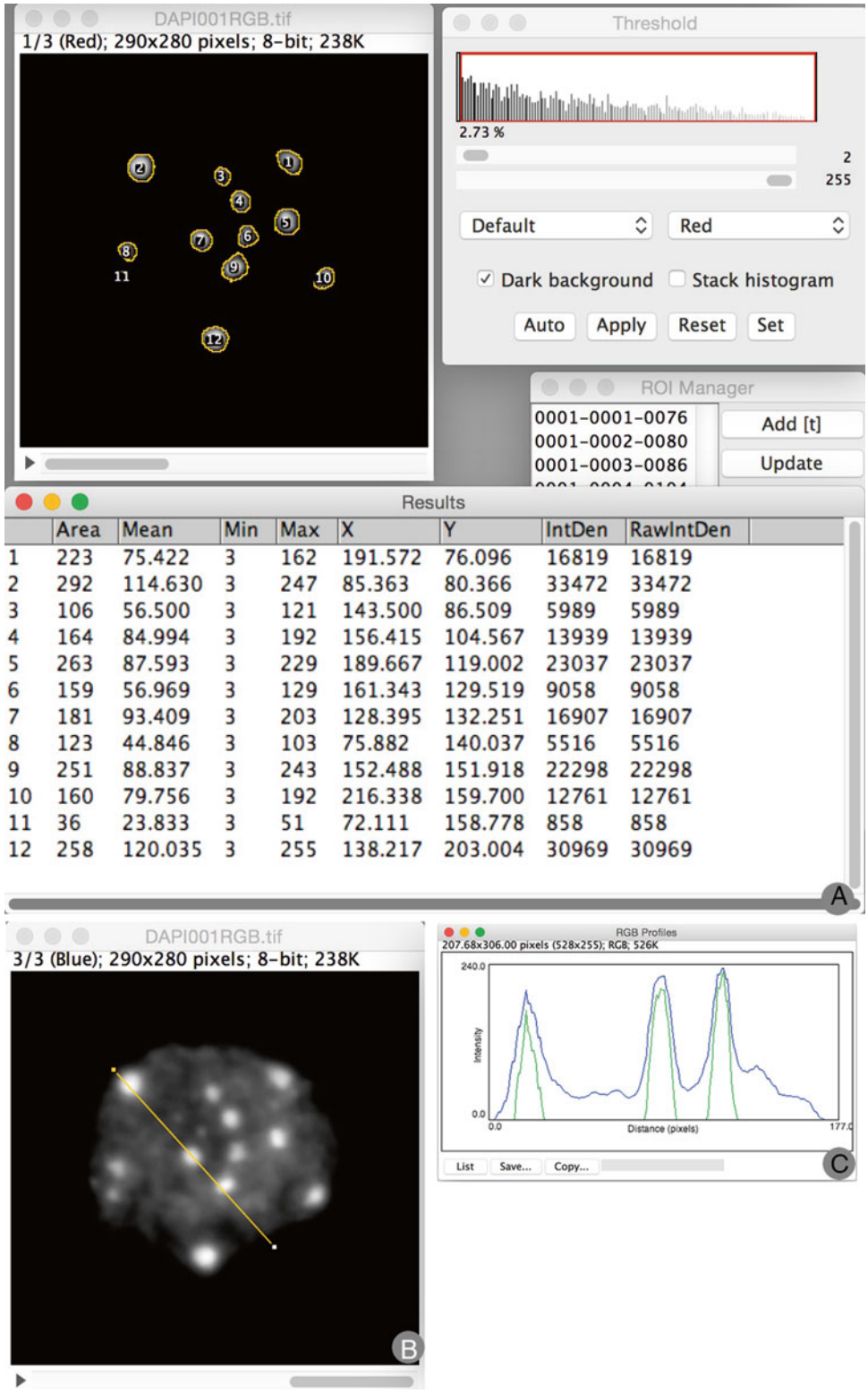

Fig. 4 Procedure of 'CHIAS-Interphase'-3, (**A**) Measure R and G, (**B**) Set the regions to measurement, (**C**) Profile curves

(f) Measure N: Measure nuclear area and individual signal intensities and save the results as 'N' (Fig. 3B).

(g) Set CC: Click the red regions one by one, with pressing the 'shift' key, to encircle the chromocenter or granular regions in yellow lines (Fig. 3C).

(h) Measure CCs: Measure intensity, number, and area sizes of the selected regions, and click 'OK' to save the results (*see* **Note 3**) (Fig. 3D).

(i) Measure R and Measure G: Measure the red and green signal areas and click 'OK' to save the results (Fig. 4A).

(j) Line Tool and Profile: Click the regions of your interest to encircle them in yellow lines and show the profile curves of three images (Fig. 4B, C).

4 Notes

1. Paraformaldehyde in PBS must be freshly prepared. Heat 25 mL H_2O at 60 °C and add 25 μL 1 M NaOH, 2 g paraformaldehyde (e.g., Sigma-Aldrich, St. Louis, MO, USA) in a fume cupboard. Dissolve with stirring and at 60 °C heating. Cool and add 25 mL 2× PBS. Adjust pH to approximately 7.0 using 5 μL drops of 30% H_2SO_4.
2. Do not click any other command (Auto, Apply, Reset, or Set).
3. Click 'No' to 'All three slices processed?'
4. Contact to http://www2.kobe-u.ac.jp/~ohmido/index03.htm or N.O. to get CHIAS-Interphase and Straight

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References

1. Ohmido N, Fukui K (2004) Recent advances in FISH analysis of plant chromosomes. Recent Res Devel Biochem 5:267–279
2. Fukui K, Ohmido N, Khush GS (1994) Variability in rDNA loci in genus Oryza detected through fluorescence in situ hybridization. Theor Appl Genet 87:893–899
3. de Jong JH, Fransz P, Zabel P (1999) High resolution FISH in plants - techniques and applications. Trends Plant Sci 4:258–263
4. Ohmido N, Kijima K, Akiyama Y, de Jong JH, Fukui K (2000) Quantification of total genomic DNA and selected repetitive sequences reveals concurrent changes in different DNA families in indica and japonica rice. Mol Gen Genet 263:388–394
5. Fransz PF, de Jong JH, Lysak M, Castiglione MR, Schubert I (2002) Interphase chromosomes in *Arabidopsis* are organized as well defined chromocenters from which euchromatin loops emanate. Proc Natl Acad Sci U S A 99:14584–14589
6. Soppe WJ, Jasencakova Z, Houben A, Kakutani T, Meister A, Huang MS, Jacobsen SE,

Schubert I, Fransz PF (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in Arabidopsis. EMBO J 21:6549–6559

7. Matsunaga S, Ohmido N, Fukui K (2006) Chromosome dynamics in tobacco BY-2 cultured cells. Biotechnology in agriculture and forestry, tabacco NY-2 cells. In: Nagata T, Matsuoka K, Inze D (eds) Cellular dynamics to omics. Springer, Heidelberg, pp 51–63
8. Wako T, Fukui K (2003) Quantitative analysis of nuclear chromocenter in *Spiranthes sinensis* (Pers.) Ames. Bioimages 11:97–103
9. Abramhoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics Int 11:36–42
10. Kato S, Ohmido N, Fukui K (2009) Image analysis of small plant chromosomes by using an improved system, CHIAS IV. Chromosome Sci 12:43–50
11. Ohmido N, Kijima K, Ashikawa I, de Jong JH, Fukui K (2001) Visualization of the terminal structure of rice chromosomes 6 and 12 using multicolor FISH to chromosomes and extended DNA fibers. Plant Mol Biol 47:413–421

Chapter 14

Detection of Transgenes on DNA Fibers

Fukashi Shibata

Abstract

Fluorescence in situ hybridization (FISH) was developed for detecting specific DNA sequences directly on mitotic or meiotic chromosomes. However, the resolution of FISH on chromosomes is limited by condensed structure of chromatin, and it is difficult to differentiate two target sites close to each other. To overcome this issue, the objects was changed to stretched DNA fibers, and this fiber FISH technique has now been used for revealing genome structure at molecular level. Hybridization and detection procedures of fiber FISH are common with FISH on chromosomes. Therefore, application of fiber FISH is not difficult for the researchers of some experience in ordinary FISH. DNA fibers can be released from nuclei fixed on glass slides using a detergent. The DNA fibers were shred in FISH procedure, and the resultant fragments became small bead-like shape. This makes FISH signals on DNA fibers a series of dots. The size of DNA in the dot is estimated to be approximately 1 kb, it corresponding to the resolution of fiber FISH. This makes it possible to analyze structures of transgenes on DNA fibers in detail.

Key words DNA fiber, FISH, Transgene, T-DNA

1 Introduction

Fluorescence in situ hybridization (FISH) is a powerful tool for mapping DNA or genes directly onto the nuclei and/or chromosomes. However, it had a distinct limitation on resolution, because of high-order packaging of DNA molecules into chromosomes. Such limitation has been overcome by using extended chromatin and DNA molecules [1]. Since then this sort of fiber FISH techniques has been improved with modifications and widely used to analyze the mammalian genome structures [2]. The first application of this technique to plants was made by Fransz et al. [3], who succeeded in extending DNA fibers from isolated nuclei of *Arabidopsis thaliana* and tomato leaves. To date, in plant sciences, the fiber FISH has been used to analyze genome structures of chloroplasts as well as nuclei [4].

There are a number of protocols for preparation of DNA fibers from plant nuclei. The process consists of two steps: fixation of nuclei on glass slide, and release of DNA fibers from nuclei with a

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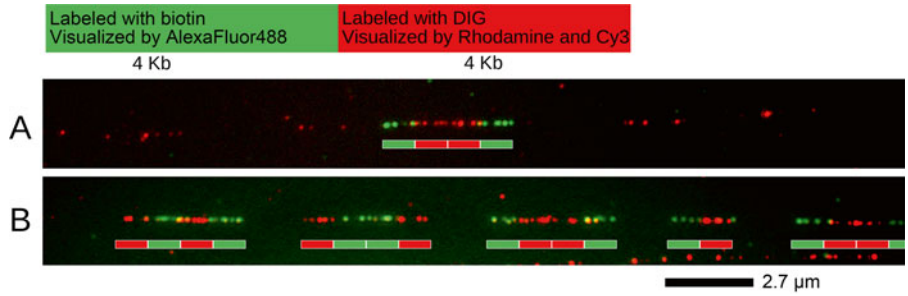

Fig. 1 T-DNAs visualized on a single DNA fiber prepared from a transgenic *A. thaliana*. The length of T-DNA detected in this image is estimated to be approximately 8 kb, corresponding theoretically to 2.7 μm. The first half of T-DNA is labeled with biotin and visualized by Alexa Fluor 488 (*Green*), the second half is labeled with DIG and visualized by rhodamine and Cy3 (*Red*). (**A**) Two copies of T-DNA are shown to be inserted in the tail-to-tail fashion. (**B**) Nine copies of T-DNA are inserted in the head-to-head, tail-to-tail, head-to-tail, tail-to-tail fashions. Bar = 2.7 μm. Compared with actual length of the signals, the length of DNA fibers are about 70 % of theoretical length

detergent(s). The protocol described below is based on the technique developed for analysis of T-DNA inserted in *Arabidopsis thaliana* genome [5] (Fig. 1). In this protocol, the Cytospin™ 4 Cytocentrifuge (Thermo Fisher Sci.) was introduced for preparing DNA fibers. The Cytospin has been widely used for preparing nuclei or chromosome spread from animal cells, because under a certain condition nuclei are preferentially fixed onto the surface of glass slides, whereas other smaller organelles such as mitochondria are flowed away during centrifugation. Hence, it is possible to make clean and long DNA fibers when appropriate number of nuclei is loaded on glass slides. The organelles attached on the glass slides not only disturb smooth extension of DNA fibers but also interferer FISH signals due to their autofluorescence.

Hybridization and detection are basically same with FISH on chromosomes [6]. Probes are labeled with biotin or DIG and detected using Streptavidin or anti-DIG antibody labeled with fluorochrome. However signals on DNA fibers are very weak, for detecting weak signals, signals must be amplified using antibody. Antibodies used for amplifying signals are anti-streptavidin antibody conjugated with biotin and anti-Sheep antibody. The combination of the fluorochrome can be arbitrarily changed.

2 Materials

2.1 Stock Buffers

1. 20× SSC: 3 M NaCl, 0.3 M trisodium citrate. Sterilize and store at room temperature (RT).
2. 10× PBS: 1.3 M NaCl, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4. Sterilize and store at RT.

3. Maleic acid buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5. Dissolve maleic acid and NaCl in 800 mL distilled water (DW) and adjust pH to 7.5 with solid NaOH and 8 M NaOH solution. Adjust volume to 1 L with DW. Sterilize and store at RT.
4. 10% blocking reagent stock solution: Dissolve 10% blocking reagent (Roche) in maleic acid buffer, and stir at 60 °C until dissolved. Sterilize and store at −20 °C.

2.2 Reagent and Equipment for Preparation of DNA Fibers

1. 0.6 M sorbitol: Sterilize and store at RT.
2. Enzyme solution: 2% Cellulase Onozuka RS (Kinki Yakult, Japan), 0.5% Pectolyase Y-23 (Seishin, Japan) in 0.6 M sorbitol (*see* **Note 1**). Store at −20 °C.
3. 75 mM KCl: Sterilize and store at RT.
4. STE buffer [7]: 0.5% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA). Store at RT.
5. DAPI solution: 0.1 μg 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Prepare at the time of application.
6. Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific).
7. Single Cytofunnel (Thermo Fisher Sciientific).
8. Poly-L-lysine (PLL) coated glass slide.

2.3 Reagent for Probe Labeling

1. Hybridization buffer: 10% (w/v) Dextran sulfate (e.g., GE Healthcare, No. 17-0340-01) in 50% (v/v) deionized formamide in 2× SSC. Store at −20 °C.
2. DNA labeling kit with biotin (Biotin Nick Translation Mix, Roche).
3. DNA labeling kit with digoxigenin (DIG) (DIG Nick Translation Mix, Roche).

2.4 Reagent and Equipment for Hybridization

1. 4% (w/v) paraformaldehyde in PBS (*see* **Note 2**). Pour into a staining jar and store at 4 °C.
2. 2× SSC: Dilute 20× SSC tenfold with DW. Pour into a staining jar.
3. 70% (v/v) ethanol: Pour into two staining jars. Put one staining jar into a freezer at least 30 min before use.
4. 100% ethanol: Pour into a staining jar.
5. Denaturation buffer: 50% formamide in 2× SSC. Mix 25 mL formamide, 5 mL 20× SSC, 20 mL DW in a 50 mL tall-form beaker and store at 4 °C (*see* **Note 3**). Preheat to 70 °C in a water bath.
6. Siliconized coverslips (24 × 32 mm).

2.5 Reagent for Detection

1. 2× SSC: Dilute 20× SSC tenfold with DW. Prepare in two staining jars and preheat to 37 °C in a water bath.
2. TN buffer: 0.1 M Tris-HCl, 0.15 M NaCl pH 7.5. Sterilize and store at RT.

3. Washing buffer: 0.05% Tween 20 in TN buffer. Prepare six staining jar of washing buffer at the time of use.
4. Blocking buffer: 1% blocking reagent (Roche) in TN buffer. Dilute 10% blocking reagent stock solution with TN buffer. Store at −20 °C.
5. Detection reagent: Add 1/200 volume Streptavidin, Alexa Fluor 488 conjugate (2 mg/mL, Molecular Probes) and 1/200 volume Anti-Digoxigenin-Rhodamine, Fab fragments (200 μg/mL, Roche) to blocking buffer. Prepare detection reagent 100 μL per a slide.
6. Amplification reagent: Add 1/100 volume Cy3 AffiniPure Donkey Anti-Sheep IgG (H+L) (1.5 mg/mL, Jackson Immuno. Res.) and 1/100 volume Biotinylated Anti-Streptavidin Antibody (0.5 mg/mL, Vector Lab.) to blocking buffer. Prepare amplification reagent 100 μL per slide.
7. Mounting reagent: ProLong Gold Antifade Reagent (Molecular Probes).

3 Methods

3.1 Preparation of DNA Fibers from A. thaliana Leaves (See Note 4)

3.1.1 Preparation of Protoplasts

1. Collect 5 cm^2 in total of leaf pieces and cut to the width of 1 mm with a razor blade.
2. Put the sliced leaves in a small petri dish (30 mm diameter) with 10 mL enzyme solution at RT with gently agitation for 1–2 h until protoplasts are released.
3. Filter protoplast suspension into a 15 mL centrifuge tube through a 100 μm nylon mesh.
4. Centrifuge the filtrates at 200 rpm for 5 min.
5. Remove the supernatant.
6. Add 5 mL of 0.6 M sorbitol solution and suspend gently with a Pasteur pipette.
7. Centrifuge the cell suspension at 200 rpm for 5 min.
8. Remove the supernatant, and repeat once from **step 6**.
9. Suspend the pellet in 2 mL of 0.6 M sorbitol solution.

3.1.2 Preparation of DNA Fibers

1. Dilute the protoplast suspension into 1/100–200 with 75 mM KCl (*see* **Note 5**).
2. Stir vigorously with a vortex mixer (*see* **Note 6**).
3. Transfer 400 μL of the protoplast solution to a Single Cytofunnel (Thermo Fisher Sci.). The glass slides used here must be coated with PLL (*see* **Note 7**).
4. Centrifuge at 2000 rpm for 2 min by Cytospin (*see* **Note 8**).

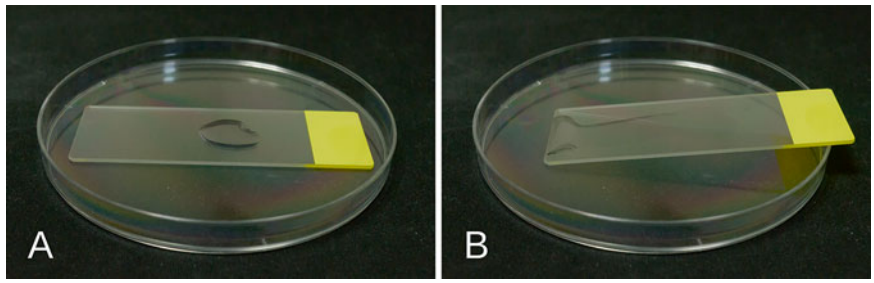

Fig. 2 (**A**) Application of STE buffer on nuclei, (**B**) Slide tilting to extend DNA fibers

5. Pick up a glass slide and place it in a petri dish (90 mm diameter). Apply 50 μL STE buffer. Tilt the slide immediately at the edge of a petri dish (*see* **Note 9**) (Fig. 2).
6. When STE buffer flow off, remove the extra buffer accumulated in the downstream, and air-dry.
7. Fix the slides in the fixative (3 vol. ethanol: 1 vol acetic acid) for 2 min.
8. Incubate the slides at 60 °C for 3 h. The slides can be stored at −20 °C until use.

3.2 Probe Labeling

1. Prepare following labeling reaction mixture in a microcentrifuge tube.

   Probe DNA (1 μg) X μL

   DW (16-X) μL

   Biotin (or DIG)-Nick translation Mix 4 μL
2. Incubate the tube at 15 °C for overnight (*see* **Note 10**).
3. Add 0.5 μL 0.5 M EDTA and incubate at 60 °C for 10 min to stop reaction.
4. Add 200 μL hybridization buffer. Store at −20 °C. When using probes labeled with biotin and DIG, mix them in equal volume. Before use, denature the probe solution at 80 °C for 10 min, and chill immediately on ice.

3.3 Hybridization

1. Fix DNA fibers on slide glasses in 4% (w/v) paraformaldehyde in PBS at RT for 5 min.
2. Wash the slides in 2× SSC at RT for 5 min.
3. Soak the slides in 70% (v/v) ethanol for 5 min.
4. Soak the slides in 100% ethanol for 5 min.
5. Air-dry the slides at RT.
6. Denature the DNA fibers on glass slides in denaturation buffer at 70 °C for 1 min.

7. Soak the slides immediately in chilled 70% (v/v) ethanol at least for 5 min.
8. Soak the slides in 100% ethanol at RT for 5 min.
9. Dry the slides.
10. Apply 20 μL denatured probe solution and covered with a siliconized coverslip (24 × 32 mm) (*see* **Note 11**). Seal the coverslip with rubber gum.
11. Place the slides in a humid chamber and incubate at 37 °C for overnight.

3.4 Detection

1. Remove the coverslips and soak the slides immediately in 2× SSC in a staining jar.
2. Incubate at 37 °C for 15 min.
3. Transfer the slides into another staining jar with 2× SSC and incubate at 37 °C for 15 min.
4. Soak in TN buffer at RT for at least 5 min.
5. Remove the extra buffer on slides, apply 200 μL blocking buffer and covered with a piece of Parafilm (Bemis Flexible Packaging).
6. Place the slides with a Parafilm in a humid chamber and incubate at RT for at least 5 min.
7. Remove the Parafilm and drain off blocking buffer.
8. Apply 100 μL detection reagent, and cover with a Parafilm, and place in humid chamber (*see* **Note 12**).
9. Incubate at 37 °C for 1 h.
10. Remove Parafilm and soak in washing buffer. Incubate at RT for 10 min with gently shaking.
11. Transfer to new washing buffer and incubate at RT for 10 min with gently shaking.
12. Remove extra buffer on slide apply 100 μL amplification reagent covered with a piece of Parafilm, place in humid chamber.
13. Incubate at 37 °C for 1 h.
14. Remove Parafilm and soak in washing buffer. Incubate at RT for 10 min with gently shaking.
15. Transfer to new washing buffer and incubate at RT for 10 min with gently shaking.
16. Remove extra buffer on slide apply 100 μL detection reagent covered with a piece of Parafilm, place in humid chamber.
17. Incubate at 37 °C for 1 h.
18. Remove Parafilm and soak in washing buffer. Incubate at RT for 10 min with gently shaking.
19. Transfer to new washing buffer and incubate at RT for 10 min with gently shaking.

20. Wash in DW.
21. Drying slides at 37 °C for 30 min.
22. Mount with 20 μL ProLong Gold Antifade Reagent (Molecular Probes).
23. Drying slides for overnight at RT (*see* **Note 13**).

Observe signals under fluorescence microscopy using appropriate filters (*see* **Note 14**).

4 Notes

1. Because the cellulase is hard to be dissolved in 0.6 M sorbitol, stir the solution overnight at 4 °C.
2. Dissolve paraformaldehyde in PBS with stirring at 60 °C until dissolved completely. Store at 4 °C. This solution can be used for several months.
3. Deionization of formamide is not required. Denaturation buffer can be stored for more than 6 months and can be used several times.
4. This method can apply to *Nicotiana tabacum* [8]. If you want to use other species, refer to other protocols for making protoplast, and change process of DNA fiber extension by trial and error.
5. Dilution rates of protoplasts should be decided by the number of nuclei counted at **step 4**.
6. This procedure breaks protoplasts and nuclei are released from protoplasts.
7. The PLL-coating on glass slides disappears in a few months.
8. After the first trial, check the number of nuclei on slides by applying 50 μL DAPI solution with a coverslip. Count the number of nuclei with a ×40 objective lens. In the best condition, approximately five nuclei appear in the field of view. When there are too many nuclei, reduce the number of protoplasts added at **step 1**. Similarly, when too many organelles are retained on the slides, reduce the number of protoplasts added at **step 1**. If no nuclei are observed, check the efficacy of PLL coating.
9. After the first trial, check the quality of DNA fibers. Stain DNA fibers with DAPI solution and observe using a ×40 objective lens. Some protocols recommend to use YOYO-1 for observation of DNA fibers. However, DNA fibers can be usually visualized with DAPI itself. If no DNA fibers appear on glass slides, doubt the PLL-coating efficacy first.
10. The reaction should be performed overnight. The shorter reactions cause high backgrounds.

11. Siliconized coverslip is recommended for use, because they are easily removed at **step 1** of detection.
12. After this step, keep the slides in the dark during washing and incubation.
13. Anti-fading effects of the ProLong Gold antifade reagent does not work before cure of reagent.
14. Weak and small signals of T-DNA are hard to find. First, adjust focus to the background or dust on surface of glass slides, using a set of excitation and emission filters appropriate to the fluorochrome used for detection of FISH signals, and find out signals on a glass slide.

References

1. Heng HH, Squire J, Tsui LC (1992) High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. Proc Natl Acad Sci U S A 89:9509–9513
2. Weler H-UG, Wang L, Mullikin JC, Zhu Y, Cheng J-F, Greulich KM, Bensimon A, Gray JW (1995) Quantitative DNA fiber mapping. Hum Mol Genet 4:1903–1910
3. Fransz PF, Alonso-Blanco C, Liharska TB, Peeters AJ, Zabel P, de Jong JH (1996) High-resolution physical mapping in *Arabidopsis thaliana* and tomato by fluorescence *in situ* hybridization to extended DNA fibres. Plant J 9:421–430
4. Lilly JW, Havey MJ, Jackson SA, Jiang J (2001) Cytogenomic analyses reveal the structural plasticity of the chloroplast genome in higher plants. Plant Cell 13:245–254
5. Murata M, Shibata F, Hironaka A, Kashihara K, Fujimoto S, Yokota E, Nagaki K (2013) Generation of an artificial ring chromosome in *Arabidopsis* by Cre/*LoxP*-mediated recombination. Plant J 74:363–371
6. Shibata F, Hizume M (2015) Multi-color fluorescence *in situ* hybridization. Cytologia 80:385–392
7. Pich U, Schubert I (1998) Terminal heterochromatin and alternative telomeric sequences in *Allium cepa*. Chromosome Res 6:315–321
8. Nagaki K, Shibata F, Suzuki G, Kanatani A, Ozaki S, Hironaka A, Kashihara K, Murata M (2011) Coexistence of NtCENH3 and two retrotransposons in tobacco centromeres. Chromosome Res 19:591–605

Chapter 15

Three-Dimensional, Live-Cell Imaging of Chromatin Dynamics in Plant Nuclei Using Chromatin Tagging Systems

Takeshi Hirakawa and Sachihiro Matsunaga

Abstract

In plants, chromatin dynamics spatiotemporally change in response to various environmental stimuli. However, little is known about chromatin dynamics in the nuclei of plants. Here, we introduce a three-dimensional, live-cell imaging method that can monitor chromatin dynamics in nuclei via a chromatin tagging system that can visualize specific genomic loci in living plant cells. The chromatin tagging system is based on a bacterial operator/repressor system in which the repressor is fused to fluorescent proteins. A recent refinement of promoters for the system solved the problem of gene silencing and abnormal pairing frequencies between operators. Using this system, we can detect the spatiotemporal dynamics of two homologous loci as two fluorescent signals within a nucleus and monitor the distance between homologous loci. These live-cell imaging methods will provide new insights into genome organization, development processes, and subnuclear responses to environmental stimuli in plants.

Key words Chromatin dynamics, Chromatin tagging system, *lacO*/LacI-EGFP, Live-cell imaging, Time-lapse imaging

1 Introduction

The spatiotemporal dynamics and arrangement of chromatin directly contribute to the regulation of biological processes in the nuclei of eukaryotes, including plants [1, 2]. In plants, respective chromatin domains are randomly organized in nuclei, whereas they are organized non-randomly in animal nuclei [3, 4]. Recently, a genome-wide chromosome confirmation capture (Hi-C) method revealed the three-dimensional genome structure in interphase nuclei of *Arabidopsis thaliana* [5, 6]. Hi-C revealed that any local interaction domains, called topological associated domains, were not found in the nuclei of *A. thaliana*, unlike in humans and flies. Therefore, the three-dimensional chromatin structure in plants might be more complicated than in animals. Fluorescence in situ hybridization (FISH) has contributed to the investigation of the

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subnuclear distribution of DNA sequences, such as centromeric or telomeric repetitive sequences and ribosomal genes. However, FISH requires the fixation of cells and high temperatures for DNA denaturation. Unfortunately, FISH cannot be used to analyze chromatin dynamics in living cells.

The chromatin tagging system was developed as a technique to monitor chromatin dynamics and arrangement in living cells [7]. This technique is based on a bacterial operator/repressor system. The repressor, which is fused to a fluorescent protein, binds a tandem array of operators that are inserted into a specific genomic locus. Visualization of the specific genomic loci by the chromatin tagging system enables investigation into the dynamics of RNA transcription, DNA replication, and DNA repair at different genomic loci in mammalian cells and yeasts [8–10]. In a plant chromatin study, this technique was used to observe the spatial arrangement of chromatin domains in *A. thaliana* plants that contained a *lacO* operator/LacI repressor-enhanced green fluorescent protein (*lacO*/LacI-EGFP) system or a *tetO* operator/TetR repressor-enhanced yellow fluorescent protein (*tetO*/TetR-EYFP) system under the control of the cauliflower mosaic virus (CaMV) 35S promoter [11–13]. However, in these lines, overexpression of the repressor protein-fluorescent protein (RP-FP) often induced silencing, and the pairing frequency of the tandem operator array was higher than that of other genomic loci [14]. A recent refinement of promoters for the chromatin tagging system solves these problems. RP-FP expression under the RPS5A and GC1 promoters, which are specifically activated in meristematic tissues or guard cells, respectively, does not induce silencing or effect the chromatin arrangement [15]. Here, we introduce a method to analyze chromatin dynamics and arrangement in the nuclei of roots or leaves via three-dimensional, live-cell imaging using a *lacO*/LacI-EGFP system. Importantly, we used this system to quantitatively analyze the distance between homologous loci, as well as the movements of genomic loci in living plant cells. Recently, our experiments using this system revealed that chromatin arrangement was changed by DNA damages in *A. thaliana* [16]. Therefore, our method could reveal the function of various chromatin regulators and improve our knowledge of chromatin dynamics in response to plant development, growth, and environmental stimuli.

2 Materials

2.1 The lacO/LacI-EGFP System in A. thaliana

1. The line expressing *lacO*/*pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP [15]. This transgenic line was used to image chromatin dynamics in the nuclei of roots.
2. The line expressing *lacO*/*pro35S*::LacI-EGFP and *proGC1*::LacI-EGFP [15]. This transgenic line was used to image chromatin dynamics in the nuclei of a guard cell in a cotyledon.

2.2 Seeding for Live-Cell Imaging in Roots

1. 70% Ethanol.
2. A sterile solution: 0.1% [v/v] Triton X-100, 5% sodium hypochlorite.
3. 1/2 Murashige and Skoog (MS) gellan gum medium in a 35 mm glass dish (*see* **Note 1**).
4. Surgical tape.
5. A black stand tilted to 45° (Fig. 1).

2.3 Seedling for Live-Cell Imaging in Cotyledons

1. 70% Ethanol.
2. A sterile solution: 0.1% Triton X-100, 5% sodium hypochlorite.
3. A 1/2 MS agar medium: 1/2 MS salts, 1% [w/v] sucrose, 1.0% [w/v] agar.
4. 1/2 MS liquid solution: 1/2 MS Salts, 1% [w/v] sucrose.
5. A 1-well glass chamber slide.
6. Paper wipes.
7. Surgical tape.

2.4 Live-Cell Imaging of Chromatin Dynamics

1. An inverted fluorescence microscope equipped with 40× and 60× objectives, a confocal scanning unit, and a scientific complementary metal oxide semiconductor CMOS (sCMOS) camera.

3 Methods

3.1 Seeding for Imaging of Chromatin Dynamics in the Nuclei of Roots

1. Add 1 mL of 70% ethanol to a tube containing seeds of a plant that expresses *lacO/pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP. Invert the tube a few times to mix.
2. Remove the ethanol from the tube. Add 1 mL of the sterile 0.1% Triton X-100 and 5% sodium hypochlorite solution to the tube and invert it approximately five times to mix.
3. After 5 min, remove the sterile solution from the tube. Wash the seeds three times with sterile distilled water.
4. Prepare the medium in the dish (*see* Subheading 2.2), and hollow out one-third of the medium using a spatula. Place approximately five seeds on the side of medium and place the lid on the dish (Fig. 1). Wind surgical tape around it.
5. Place the dish at 4 °C for 1 day and then move it to a 22 °C incubator. Put the dish on the stand tilled at 45° (Fig. 1).
6. Grow the seeds at 22 °C on a 16-h light/8-h dark cycle for 5 days.

3.2 Time-Lapse Imaging of Chromatin Dynamics in the Nuclei of Roots

1. Put the dish on the stage of an inverted fluorescence microscope with a 40× objective.
2. Move the region of interest (e.g., the meristematic or elongation zone) to the center of the visual field and determine the *z* range.

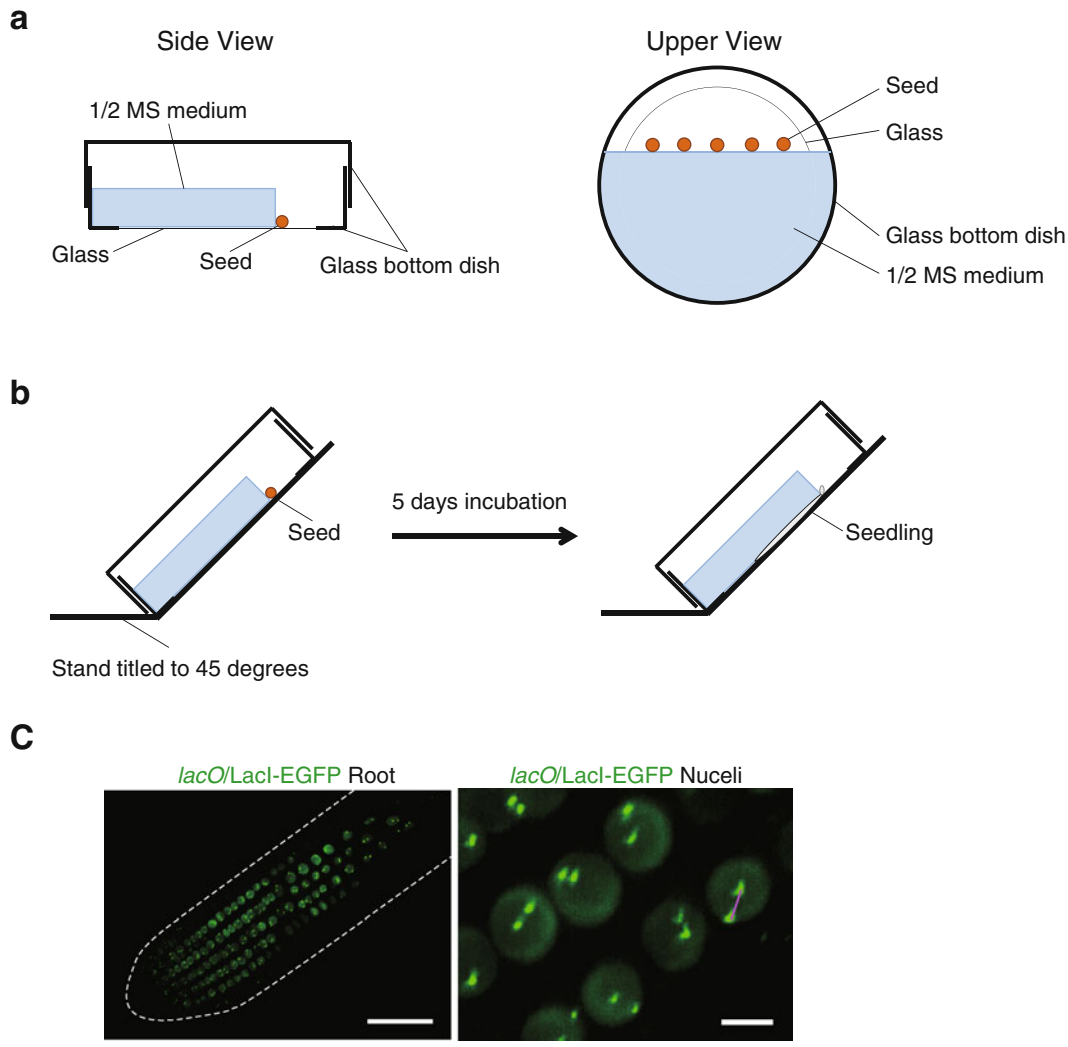

Fig. 1 Preparation for the imaging of chromatin dynamics in root nuclei. (**a**) A schematic figure of *A. thaliana* seeding for the imaging of chromatin dynamics in root nuclei. Sterilized seeds were placed on the edge of the 1/2 MS medium at even intervals in a glass-bottom dish. (**b**) A schematic figure of the incubation of *A. thaliana* seeds for the imaging of chromatin dynamics in root nuclei. The glass-bottom dish is placed onto a stand tilted to 45°. After 5 days, the root extends into the space between the 1/2 MS medium and the glass. (**c**) A root (*left*, scale bar = 50 μm) and nuclei (*right*, scale bar = 5 μm) of *A. thaliana* expressing *lacO*/*pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP. The bright foci of LacI-EGFP can be detected in the green nuclei. A *magenta line* indicates the distance of homologous loci (*right*)

For example, in the case of the root epidermis, the z range is approximately 15 μm (*see* **Note 2**).

3. Set the conditions for the time-lapse imaging, time interval, and duration. For example, to observe cell division in the meristematic zone, the time interval is 10 min and the duration is 3 h (*see* **Note 3**).

4. Perform the time-lapse imaging. As roots continue to grow during observation, the object being measured might need to be moved to the center of the visual field (*see* **Note 4**).

3.3 Seeding for Imaging of Chromatin Dynamics in the Nuclei of Guard Cells of a Cotyledon

1. Add 1 mL of 70% ethanol to the tube containing the seeds of plants expressing *lacO/pro35S*::LacI-EGFP and *proGC1*::LacI-EGFP.
2. Remove the ethanol from the tube. Add 1 mL of the sterile 0.1% Triton X-100 and 5% sodium hypochlorite solution to the tube and invert it approximately five times to mix.
3. After 5 min, remove the sterile solution from the tube. Wash the seeds three times with distilled water.
4. Prepare the 1/2 MS agar medium. Sow the seeds on the medium.
5. Store the plate at 4 °C for 1 day and then move it to a 22 °C incubator. Grow the seeds at 22 °C on a 16-h light/8-h dark cycle for 5 days.

3.4 Preparation of Samples for Time-Lapse Imaging of Chromatin Dynamics in the Nuclei of Guard Cells of a Cotyledon

1. Five days after moving the medium to the incubator, excise the cotyledons from the seedlings using tweezers.
2. Put ~5 cotyledons, adaxial side up, on a 1-well slide chamber and add the 1/2 MS liquid solution.
3. Soak paper wipes in the 1/2 MS liquid solution and fold them in accordance with the size of the slide.
4. Cover the cotyledons with the wet paper wipes and place the lid on the slide chamber (Fig. 2). Wind surgical tape around it (*see* **Note 5**).

3.5 Time-Lapse Imaging of Chromatin Dynamics in the Nuclei of Guard Cells of a Cotyledon

1. Place the chamber on the stage of an inverted fluorescence microscope with a 60× objective.
2. Cotyledons cannot be visualized by light from above because they are covered with paper wipes. Therefore, visualize the cotyledons using their intrinsic fluorescence under an excitation light.
3. Determine the *z* range. For example, in the case of guard cells, the *z* range is about 5–10 μm (*see* **Note 2**).
4. Perform the time-lapse imaging. Because leaves do not move during observation, the position of the object being measured does not need adjustment (*see* **Note 5**).

4 Notes

1. Mix 1/2 MS salts, 1% sucrose, and 0.6% gellan gum in sterile distilled water and adjust the pH to 5.9 with 0.1 N KOH. Autoclave the medium for 20 min at 121 °C. After autoclaving, pour 6 mL of the medium into a 35 mm glass dish. Store the medium in the dish at 4 °C. The transparency of gellan gum in the medium is

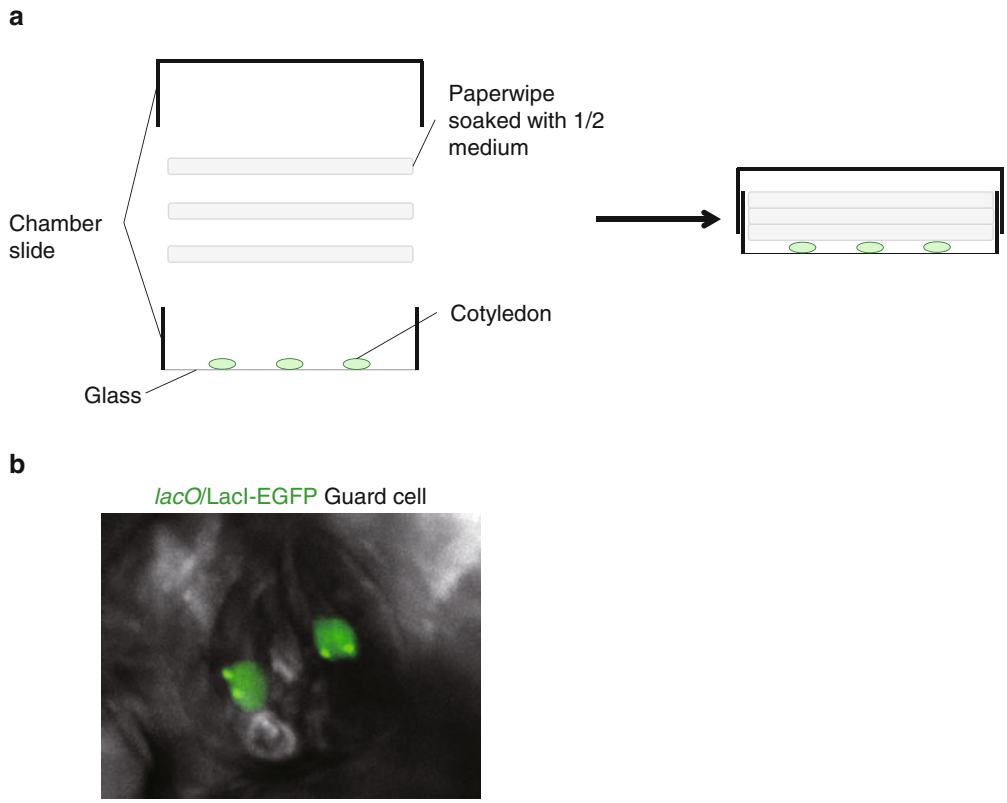

Fig. 2 Preparation for the imaging of chromatin dynamics in the nuclei of guard cells of a cotyledon. (**a**) A schematic figure of the seeding for the imaging of chromatin dynamics in guard cell nuclei. Excised cotyledons of *A. thaliana* are placed onto the *bottom* of the glass chamber. Accumulated Kimwipes soaked with 1/2 MS liquid solution are placed gently on the cotyledons. Finally, the Kimwipes are pressed down by the chamber cover. (**b**) Guard cell nuclei in *A. thaliana* expressing *lacO*/*pro35S*::LacI-EGFP and *proGC1*::LacI-EGFP. The fluorescent image merged with the bright-field image shows guard cells on the underside of a cotyledon. The bright foci of LacI-EGFP can be detected in the green nuclei

higher than that of agar. Therefore, it is desirable to use gellan gum to make the medium in the experiment.

2. The exposure time should be short to prevent *lacO*/LacI-EGFP signals from shifting in the z-stacks.
3. Mitotic nuclei can be observed in meristematic zone of plant lines transfected with *lacO*/*pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP. However, it is difficult to discern their phase in detail. We recommend producing double-labeled cell lines, for example, the line transfected with the *lacO*/LacI-EGFP and the histone H2B-red fluorescent protein (H2B-RFP) systems.
4. For image analysis, we usually use ImageJ software (http://imagej.nih.gov/ij/). LP -StackLine (LPixel, http://lpixel.net/) and manual tracking (http://rsb.info.nih.gov/ij/plugins/track/track.html) can be used to measure chromatin dynamics.

5. The materials and methods used to observe the *lacO*/LacI-EGFP signal in the nuclei of cotyledons are mainly based on those in a previously report [17, 18]. This method is also effective for long-time observations of cellular dynamics, such as stomatal aperture, cell division, and organellar movement in plants.

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References

1. Misteli T (2007) Beyond the sequence: cellular organization of genome function. Cell 128:787–800
2. Matsunaga S, Katagiri Y, Nagashima Y et al (2013) New insights into the dynamics of plant cell nuclei and chromosomes. Int Rev Cell Mol Biol 305:253–301
3. Cremer T, Cremer M (2010) Chromosome territories. Cold Spring Harb Perspect Biol 2:a003889
4. Schubert I, Shaw P (2011) Organization and dynamics of plant interphase chromosomes. Trends Plant Sci 16:273–281
5. Feng S, Cokus SJ, Schubert V et al (2014) Genome-wide Hi-C analyses in wild-type and mutants reveal high-resolution chromatin interactions in *Arabidopsis*. Mol Cell 55:694–707
6. Grob S, Schmid MW, Grossniklaus U (2014) Hi-C analysis in *Arabidopsis* identifies the KNOT, a structure with similarities to the flamenco locus of *Drosophila*. Mol Cell 55:678–693
7. Belmont AS, Straight AF (1998) *In vivo* visualization of chromosomes using *lac* operator-repressor binding. Trends Cell Biol 8:121–124
8. Dion V, Kalck V, Horigome C et al (2012) Increased mobility of double-strand breaks requires Mec1, Rad9 and the homologous recombination machinery. Nat Cell Biol 14:502–509
9. Zhao R, Nakamura T, Fu Y et al (2011) Gene bookmarking accelerates the kinetics of post-mitotic transcriptional re-activation. Nat Cell Biol 13:1295–12304
10. Saner N, Karschau J, Natsume T et al (2013) Stochastic association of neighboring replicons creates replication factories in budding yeast. J Cell Biol 202:1001–1012
11. Kato N, Lam E (2001) Detection of chromosomes tagged with green fluorescent protein in live *Arabidopsis thaliana* plants. Genome Biol 2:research0045
12. Matzke AJM, van der Winden J, Matzke M (2003) Tetracycline operator/repressor system to visualize fluorescence-tagged T-DNAs in interphase nuclei of *Arabidopsis*. Plant Mol Biol Rep 21:9–19
13. Matzke AJM, Huettel B, van der Winden J et al (2005) Use of two-color fluorescence-tagged transgenes to study interphase chromosomes in living plants. Plant Physiol 139:1586–1596
14. Jovtchev G, Watanabe K, Pecinka A et al (2008) Size and number of tandem repeat arrays can determine somatic homologous pairing of transgene loci mediated by epigenetic modifications in *Arabidopsis thaliana* nuclei. Chromosoma 117:267–276
15. Matzke AJM, Watanabe K, van der Winden J et al (2010) High frequency, cell type-specific visualization of fluorescent-tagged genomic sites in interphase and mitotic cells of living *Arabidopsis* plants. Plant Methods 6:2
16. Hirakawa T, Katagiri Y, Ando T et al (2015) DNA double-strand breaks alter the spatial arrangement of homologous loci in plant cells. Sci Rep 5:11058
17. Kato N, Reynolds D, Brown ML et al (2008) Multidimensional fluorescence microscopy of multiple organelles in Arabidopsis seedlings. Plant Methods 4:9
18. Iwata E, Ikeda S, Matsunaga S et al (2011) GIGAS CELL1, a novel negative regulator of the anaphase-promoting complex/cyclosome, is required for proper mitotic progression and cell fate determination in *Arabidopsis*. Plant Cell 23:4382–4393

Chapter 16

Chromatin Immunoprecipitation for Detecting Epigenetic Marks on Plant Nucleosomes

Kiyotaka Nagaki

Abstract

Due to high resolution and reproducibility, chromatin immunoprecipitation (ChIP) has been used as a standard tool to investigate epigenetic marks including modified histones and specific histone variants (e.g., centromere-specific histone H3, CENH3) in this decade. Here, I describe a sensitive and low-background ChIP protocol for a wide range of plant species.

Key words Chromatin immunoprecipitation (ChIP), Epigenetic marks, Histone modification, Histone variants, CENH3

1 Introduction

Chromatin immunoprecipitation (ChIP) consists of several steps including fixation, isolation of nucleosomes, and immunoprecipitation of the nucleosomes using specific antibodies. Then the precipitated nucleosomes have been analyzed by several different ways including blot hybridization, hybridization on DNA chips (ChIP-chip), real-time quantitative polymerase chain reaction (ChIP-qPCR), or sequencing using next-generation sequencer (ChIP-seq). In the primitive ChIP analyses using chromatins from plant species, precipitated DNA had been analyzed by blot hybridization, but the sensitivity was not so high [1–5]. The current detection methods, ChIP-qPCR and ChIP-seq, are sensitive enough to analyze single-copy DNA sequences even in the plant species having large genomes [6–9]. This increased sensitivity has made ChIP more common in plant science, but there are some difficulties including how to isolate good chromatins from different species and tissues, how to select good antibodies, and how to reduce ChIP background. Here, I describe a ChIP protocol including some special knacks to solve the above problems.

Minoru Murata (ed.), *Chromosome and Genomic Engineering in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1469, DOI 10.1007/978-1-4939-4931-1_16,

2 Materials

Prepare all solutions using ultrapure water (e.g., milli-Q water) and analytical grade reagents, and store all reagents at room temperature (unless indicated specially).

2.1 Fixation

1. 10× Phosphate-buffered saline (PBS): Add 800 ml water to a 1 l beaker, and solve 80.0 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4. Adjust pH to 7.4 with 1 M HCl, and make up to 1 l with water.
2. Fixation buffer: Add 100 ml of 1× PBS in a 200 ml glass bottle. Add 1 g paraformaldehyde in the bottle and, incubate at 80 °C to solve paraformaldehyde. Add 200 μl of Triton-X100, and store at 4 °C. Use within a week.
3. A vacuum pump.
4. 1 M Glycine solution: Add 80 ml water to a 100 ml beaker. Solve 7.5 g glycine and make up to 100 ml with water.
5. Paper towels.
6. Liquid nitrogen.

2.2 Isolation of Nucleosome

1. A mortar and pestle.
2. 1 M Tris–HCl pH 7.5: Add 800 ml water to a 1 l beaker, and add 121.1 g Tris base and 60 ml 12 M HCl. Adjust pH to 7.5 with 1 M HCl, and make up to 1 l with water.
3. 1 M $CaCl_2$ solution: Add 80 ml water to a 100 ml beaker. Solve 11.1 g $CaCl_2$. Make up to 100 ml with water.
4. 1 M $MgCl_2$ solution: Add 80 ml water to a 100 ml beaker. Solve 20.3 g $MgCl_2 \cdot 6H_2O$, and make up to 100 ml with water.
5. Protease inhibitor solution: Available as tablets (e.g., cOmplete™ ULTRA tablets, Sigma-Aldrich, St. Louis, MO, USA). Solve one tablet to 1 ml water, and store at −20 °C.
6. Phenylmethanesulfonyl fluoride (PMSF) solution: Solve 17 mg phenylmethanesulfonyl fluoride into 10 ml isopropanol.
7. TBS with protease inhibitors: Add 800 ml water to a 1 l beaker, and add 10 ml 1 M Tris–HCl pH 7.5, 3 ml 1 M $CaCl_2$, and 2 ml 1 M $MgCl_2$, and make up to 1 l with water. Add 1 ml/l protease inhibitor solution and 1 ml/l PMSF solution before use.
8. Miracloth (Merck Millipore, Billerica, MA, USA).
9. 12% (w/v) Sucrose in TBS with protease inhibitors: Add 80 ml TBS with protease inhibitors to a 100 ml beaker. Solve 12.0 g sucrose, and make up to 100 ml with TBS with protease inhibitors.
10. MNase digestion buffer: Add 80 ml water to a 100 ml beaker. Add 5 ml 1 M Tris–HCl pH 7.5, 100 μl 1 M $CaCl_2$, and 400 μl 1 M $MgCl_2$ into the water. Make up to 100 ml with water.

11. 1 U/μl MNase solution: Add 50 μl water in a bottle containing 50 U nuclease, micrococcal (Sigma-Aldrich, #N5386-50UN). Mix gently to solve all powder in the bottle. Transfer the solution into a 1.5 ml tube. Store at –20 ° C.
12. 0.1 U/μl MNase solution: Dilute 1 U/μl MNase solution ten times by water. Store at –20 ° C.
13. 0.5 M EDTA (pH 8.0): Add 800 ml water to a 1 l beaker. Add 186.1 g disodium ethylenediaminetetraacetate $2H_2O$ and 20.0 g NaOH to the water. Adjust pH to 8.0 with NaOH. Make up to 1 l with water.
14. Lysis buffer: Add 100 μl 1 M Tris–HCl to 100 ml water. Add 1 ml/l protease inhibitor solution and 1 ml/l PMSF solution before use.

2.3 Chromatin Immunoprecipitation

1. 1 M NaCl: Add 80 ml water to a 100 ml beaker. Solve 5.8 g NaCl into the water. Make up to 100 ml with water.
2. Incubation buffer: Add 80 ml water to a 100 ml beaker. Add 2 ml 1 M Tris–HCl pH 7.5, 1 ml 0.5 M DETA pH 8.0, and 5 ml 1 M NaCl into the water. Make up to 100 ml with water. Add 1 ml/l protease inhibitor solution and 1 ml/l PMSF solution before use.
3. Normal sera of rabbit or mouse (e.g., MP bio, Santa Ana, CA, USA #55989).
4. Anti-histone H3 antibody (e.g., Abcam, Cambridge, UK #ab1791) or anti-histone H4 antibody (e.g., medical and biological laboratories, Nagoya, JAPAN #MABI0400).
5. Antibodies for your interested modifications.
6. Dynabeads® Protein A or G (Thermo Fisher Scientific, Waltham, MA, USA).
7. Magnetic tube stands.
8. Wash buffer: Add 80 ml water to a 100 ml beaker. Add 5 ml 1 M Tris–HCl pH 7.5, 2 ml 0.5 M DETA pH 8.0, and 5 ml 1 M NaCl, and make up to 100 ml with water.
9. 10% (w/v) SDS solution: Add 80 ml water to a 100 ml beaker. Solve 10.0 g sodium dodecyl sulfate (SDS), and make up to 100 ml with water.
10. Elution buffer: Add 80 ml water to a 100 ml beaker. Add 2 ml 1 M Tris–HCl pH 7.5, 1 ml 0.5 M DETA pH 8.0, 5 ml 1 M NaCl, and 10 ml 10% (w/v) SDS, and make up to 100 ml with water.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Fixation

This fixation steps are not necessary for native-ChIP (*see* **Note 1**).

1. Soak 1–10 g fresh plant materials (*see* **Note 2**) in 10–100 ml the fixation buffer.
2. Vacuum (−50 kPa) for 1 min and release it to infiltrate the fixation buffer.
3. Stir it for 10 min.
4. Add 1 M glycine (one-quarter volume of the fixation buffer) to stop the fixation.
5. Stir it for 5 min.
6. Remove the buffer.
7. Add 1× PBS (two volume of the fixation buffer).
8. Stir it for 5 min at 4 °C.
9. Repeat **steps 6–8** twice (three times in total).
10. Dry briefly the materials by blotting with paper towels.
11. Freeze in liquid nitrogen.
12. Store at −80 °C.

3.2 Isolation of Nucleosome

1. Grind the frozen plant material to a fine powder using a mortar and pestle in liquid nitrogen.
2. Suspend the powder in 100 ml of TBS with protease inhibitors.
3. Filter the suspension through Miracloth into 50 ml tubes.
4. Spin the tube at 600 × *g* for 10 min at 4 °C.
5. Remove supernatant by decantation.
6. Suspend the pellet in 50 ml of 12% (w/v) sucrose in TBS with protease inhibitors.
7. Spin the tube at 600 × *g* for 10 min at 4 °C.
8. Remove the supernatant by decantation.
9. Suspend the pellet in 3 ml of MNase digestion buffer (*see* **Note 3**).
10. Transfer the suspension into three separate 1.5 ml tubes.
11. Add 0.5, 1, and 3 μl of MNase solution to the three tubes, respectively (*see* **Note 4**).
12. Incubate the tubes at 37 °C for 10 min.
13. Add 15 μl of 0.5 M EDTA to stop digestion.
14. Spin the tubes at 6000 × *g* for 1 min.
15. Transfer supernatants into new 1.5 ml tubes.
16. Keep the supernatants on ice.
17. Suspend the pellet at **step 14** in 1 ml of lysis buffer.
18. Mix these suspensions by vortex mixer for 1 h at 4 °C.
19. Spin the tubes at 6000 × *g* for 1 min.

20. Transfer supernatants into new 1.5 ml tubes.
21. Keep the supernatants on ice.
22. Pick up 50 μl each of the supernatants at **steps 16** and **21**.
23. Incubate the picked supernatants at 65 °C for at least 4 h to remove the cross-link (*see* **Note 5**).
24. Purify DNA from the suspension by phenol/chloroform extraction and ethanol precipitation or DNA purification kits (e.g., Qiagen).
25. Check amounts and sizes of the nucleosome by electrophoresis with 2% (w/v) agarose gel using a half of the purified DNA.

3.3 Immunoprecipitation of Nucleosomes

1. Transfer supernatants at **steps 16** and/or **21** of Subheading 3.2 containing optimum size of nucleosomes for your purpose (*see* **Note 6**) into a 15 ml tube.
2. Add the same volume of incubation buffer.
3. Transfer equal volume of the mixture into a set of 1.5 ml tubes (*see* **Note 7**).
4. Add 2–5 μl of normal sera or antibodies per tube (*see* **Note 8**).
5. Mix these at 4 °C overnight.
6. Add 10 μl of equilibrated Dynabeads Protein A or G per tube (*see* **Note 9**).
7. Mix these for 30 min (*see* **Note 10**).
8. Capture the beads by magnet, and remove supernatant.
9. Add 1 ml of wash buffer.
10. Mix these for 10 min.
11. Capture the beads by magnet, and remove supernatant.
12. Repeat **steps 9–11** twice (three times in total).
13. Suspend the beads in 200 μl of wash buffer.
14. Transfer the suspensions into new 1.5 ml tubes (*see* **Note 11**).
15. Capture the beads by magnet, and remove supernatant.
16. Suspend the beads in 100 μl of elution buffer.
17. Incubate at 65 °C for 4–16 h to remove the cross-link (*see* **Note 5**).
18. Mix these by vortex mixer for 1 min.
19. Capture the beads by magnet, and transfer the supernatants into new 1.5 ml tubes.
20. Purify DNA from the supernatants by phenol/chloroform extraction and ethanol precipitation with glycogen or DNA purification kits (e.g., Qiagen).
21. Solve the purified DNA into 20 μl each of TE.
22. Store 4at −20 °C.
23. Use 0.5 μl/tube of the DNA solutions for qPCR.

4 Notes

1. In general, the native ChIP (ChIP without fixation) does not include fixation artifacts (e.g., misfixed unrelated chromatins). Since bindings between histones and DNA in nucleosomes are strong, only intact nucleosomes precipitate in the native ChIP. Though epigenetic marks (including methylations and acetylations of histones and specific histone variants) are detectable in the native ChIP, some modifications are unstable and not easily detected in the native ChIP. If you would encounter such difficulties perform fixation before ChIP. Also, if your target is not involved in nucleosomes (e.g., transcription factors), fixation is necessary.
2. Amounts of plant materials for ChIP are related to many different factors (genome sizes of the plants, abundance of target sequences, efficiencies of antibodies, detection methods, etc.). For example, theoretically, one hundred times more nucleosomes are required for onion (genome size = 16 Gb) than *Arabidopsis thaliana* (genome size = 160 Mb). Use 1–10 g of the plant materials for pilot experiments.
3. For isolating nucleosomes, there are two different ways, MNase digestion and sonication. In general, MNase digestion is milder than sonication, because sonication possibly damages the nucleosome complex. The MNase digestion produces distinct ladder patterns composed of mono- to the integer polymer nucleosomes in electrophoreses, whereas sonication usually produces unclear and smear pattern (Fig. 1). Especially, only MNase digestion can produce monomers without linker DNA. However, MNase may have selectivity for DNA sequences.
4. Since nucleosomes of Poaceae species are more sensitive than other species, use 0.1 U/μl MNase solution for Poaceae species, but 1 U/μl MNase solution for others. Digestion efficiency is affected by purity of the nucleosome suspension, so prepare three different concentrations of the enzyme to get optimum nucleosomes for your ChIP (*see* **Note 6**).
5. For the native ChIP, this step is not necessary.
6. Nucleosomes containing more than monomer are easier to precipitate than monomeric nucleosomes, but these make ChIP results unclear. For example, ChIP using only monomeric nucleosomes clearly determines status of the position about 200 bp resolutions, and the resolutions are low (600 bp for dimer and 1 kb for trimer) in ChIP using longer nucleosomes (Fig. 2). On the other hand, qPCR using DNA from monomeric nucleosomes are more difficult than using DNA from longer nucleosomes (Fig. 3). For example, when you

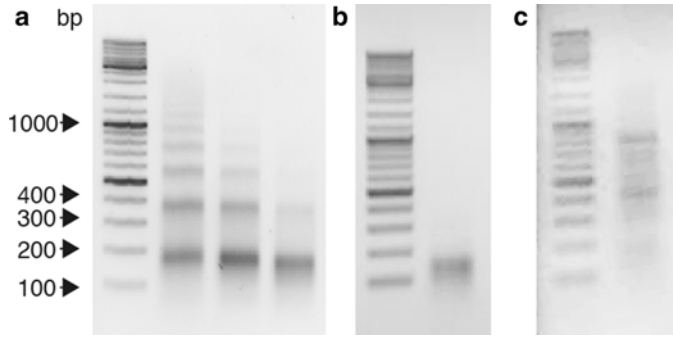

Fig. 1 Quality and quantity check of nucleosomes by electrophoresis. Inverted images of electrophoreses of DNA from isolated nucleosomes using 2% (w/v) agarose gel. (**a**) A serial dilution of MNase digestions. (**b**) DNA from mono-nucleosomes produced by an MNase digestion. (**c**) DNA from nucleosomes produced by a sonication

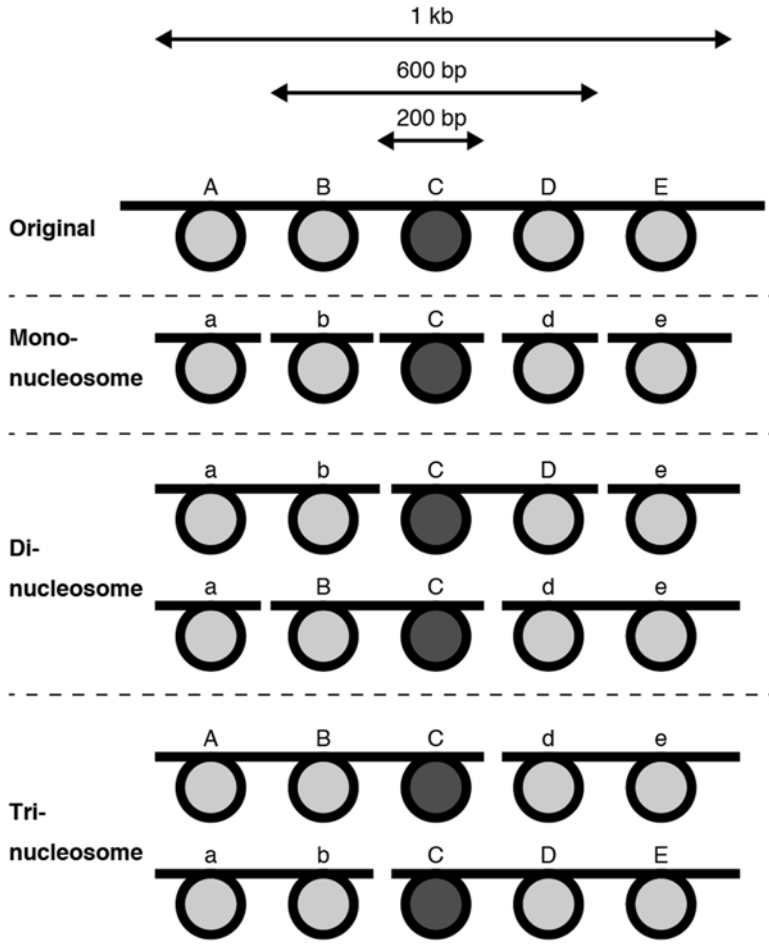

Fig. 2 Resolutions of ChIP-qPCR with mono-, di-, and tri-nucleosomes. PCR sites are indicated on fragments alphabetically. *Capital* and *lowercase letters* indicate PCR positive and negative, respectively. A nucleosome with *dark color* has a target epigenetic mark. In ChIP-qPCR with mono-nucleosomes, only the site C is positive, and there is no false positive. In ChIP-qPCR with di-nucleosomes, the sites B and D are false positive. In ChIP-qPCR with tri-nucleosomes, the sites A, B, D, and E are false positive

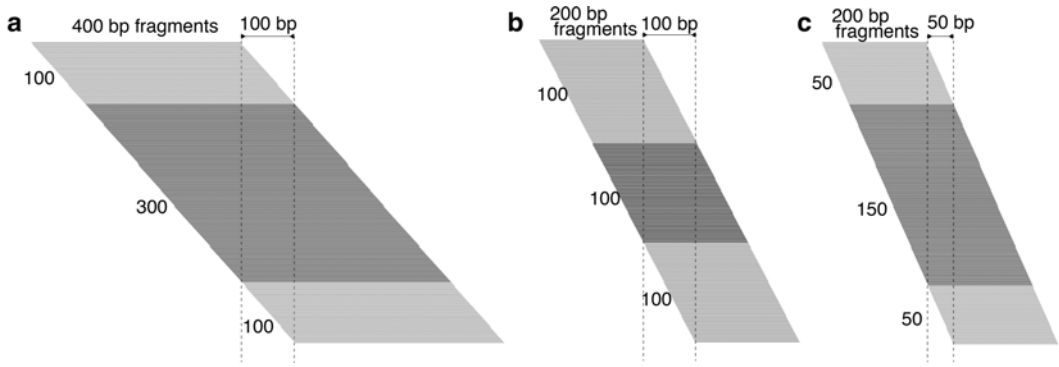

Fig. 3 Usable template contents in DNA pools from mono- and di-nucleosomes. *Arrowheads* indicate primer sites. DNA fragments containing both or one of the primers are indicated as *black lines* or *gray lines*, respectively. (**a**) A 100 bp PCR product and DNA pool from di-nucleosomes. (**b**) A 100 bp PCR product and DNA pool from mono-nucleosomes. (**c**) A 50 bp PCR product and DNA pool from mono-nucleosomes

amplify a 100 bp PCR product using a DNA pool containing 400 bp fragments from di-nucleosome, 60% of the fragments are estimated to act as templates (Fig. 3a). However, when you amplify corresponding to a 100 bp PCR product using a DNA pool containing 200 bp fragments from mono-nucleosome, only 33% of the fragments containing the region act as templates (Fig. 3b). To get similar sensitivity for the case using a DNA pool containing 400 bp fragments and 100 bp PCR products, you should design a primer set to amplify 50 bp fragment for the mono-nucleosome (Fig. 3c).

7. Prepare at least three tubes for negative control, positive control, and antibodies of your interest for statistic tests.
8. Use normal sera of rabbit or mouse as a negative control, and anti-histone H3 or anti-histone H4 antibodies as a positive control for modified histones. The amounts of antibodies depend on quality and concentration of the antibodies and amount of target modifications on nucleosomes. Use 2–5 μl each of antibodies for pilot experiments. I strongly recommend to check the quality of antibodies by immunostaining before use, because the quality of antibodies is dependent on the product and lot. *Arabidopsis* nuclei are a good material for checking the qualities of antibodies against modified histones (Fig. 4).
9. The magnetic beads drastically reduce the ChIP background compared with the agarose or sepharose beads, probably because the magnetic beads have less nonspecific binding to nucleosomes and DNA. Since binding specificities of protein A and G to the antibodies from different animals are different, select beads optimum for the antibodies. Transfer enough

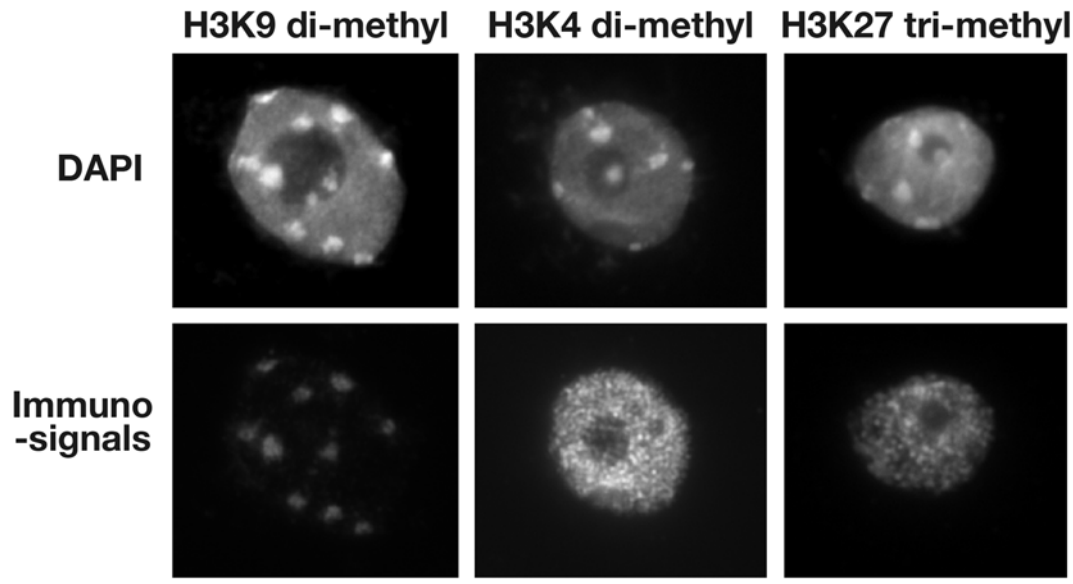

Fig. 4 Typical images of immunostaining for *Arabidopsis* nuclei using anti-modified histone antibodies. Basically, heterochromatic modifications (e.g., H3K9 di- or tri-methyl) are observed on chromocenters in interphase nuclei, but euchromatic modifications (e.g., H3K4 di- or tri-methyl) are observed out of chromocenters in interphase nuclei. However, a heterochromatic modification specific for open reading frames of inactive genes, H3K27 tri-methyl, shows similar pattern to the euchromatic modification on the nuclei

amount (10 μl/tube) of protein A or G Dynabeads into a 1.5 ml tube, and wash the beads three times using 1 ml of incubation buffer. Then, suspend the equilibrated beads into the original volume of Incubation buffer.

10. Do not precipitate the beads through the incubation. It reduces binding efficiency of the bead to antibodies.
11. This is a very important step to avoid contamination of non-specifically bounded nucleosomes on tube wall.

References

1. Nagaki K, Talbert PB, Zhong CX, Dawe RK, Henikoff S, Jiang JM (2003) Chromatin immunoprecipitation reveals that the 180-bp satellite repeat is the key functional DNA element of *Arabidopsis thaliana* centromeres. Genetics 163:1221–1225
2. Zhong CX, Marshall JB, Topp C, Mroczek R, Kato A, Nagaki K, Birchler JA, Jiang J, Dawe RK (2002) Centromeric retroelements and satellites interact with maize kinetochore protein CENH3. Plant Cell 14:2825–2836
3. Houben A, Schroeder-Reiter E, Nagaki K, Nasuda S, Wanner G, Murata M, Endo TR (2007) CENH3 interacts with the centromeric retrotransposon cerebra and GC-rich satellites and locates to centromeric substructures in barley. Chromosoma 116:275–283
4. Nagaki K, Murata M (2005) Characterization of CENH3 and centromere-associated DNA sequences in sugarcane. Chromosome Res 113:195–203
5. Tek AL, Kashihara K, Murata M, Nagaki K (2011) Functional centromeres in *Astragalus sinicus* include a compact centromere-specific histone H3 and a 20-bp tandem repeat. Chromosome Res 19:969–978
6. Neumann P, Navrátilová A, Schroeder-Reiter E, Koblížková A, Steinbauerová V, Chocholová E,

Novák P, Wanner G, Macas J (2012) Stretching the rules: monocentric chromosomes with multiple centromere domains. PLoS Genet 8:e1002777

7. Nagaki K, Yamamoto M, Yamaji N, Mukai Y, Murata M (2012) Chromosome dynamics visualized with an anti-centromeric histone H3 antibody in *Allium*. PLoS One 7:e51315
8. Gong Z, Wu Y, Koblizkova A, Torres GA, Wang K, Iovene M, Neumann P, Zhang W, Novak P, Buell CR, Macas J, Jiang J (2012) Repeatless and repeat-based centromeres in potato: implications for centromere evolution. Plant Cell 24:3559–3574
9. Nagaki K, Tanaka K, Yamaji N, Kobayashi H, Murata M (2015) Sunflower centromeres consist of a centromere-specific LINE and a chromosome-specific tandem repeat. Front Plant Sci 6:912

Chapter 17

Mapping of T-DNA and *Ac/Ds* by TAIL-PCR to Analyze Chromosomal Rearrangements

Satoru Fujimoto, Sachihiro Matsunaga, and Minoru Murata

Abstract

Insertion mutagenesis using known DNA sequences such as T-DNA and transposons is an important tool for studies on gene function in plant sciences. The transposons *Activator* (*Ac*)/*Dissociation* (*Ds*) have been systematically used to manipulate plant chromosomes. For both of these applications, the recovery of genomic DNA sequences flanking the insertions is required to estimate the sizes and/or scales of the reconstituted chromosomes. In this chapter, we describe the protocols for thermal asymmetric interlaced PCR (TAIL-PCR) for isolation of genomic sequences flanking DNA inserts in plant genomes.

Key words TAIL-PCR, Flanking sequence, T-DNA, Transposon, *Ac/Ds*

1 Introduction

Gene disruption is an important technique to investigate gene function. Targeted gene disruption via homologous recombination is applicable for a number of eukaryotes ranging from yeasts to animals. Because the frequency of homologous recombination is quite low in flowering plants, the success of gene targeting has been limited [1]. Recently, genome editing techniques such as TAL effector nuclease and CRISPR/Cas9 systems have been adapted for use in plants, making it easier to produce knockout mutants [2]. However, their application in large-scale chromosomal and genomic reconstructions has not yet been established [3]. In contrast, the maize transposon system using *activator/dissociation* (*Ac/Ds*), which was originally developed for insertional mutagenesis, is widely used to induce relatively large chromosomal deletions in combination with site-specific recombination systems (i.e., Cre/*LoxP*, Flp/*Frt*) that originated from non-plant organisms [4]. The *Ac* is a single-component system that carries the transposase (TPase) gene required for its own transposition [5]. A single-component system does not require genetic crossing for transposition. Because of the mobility of *Ac*, however, it is difficult

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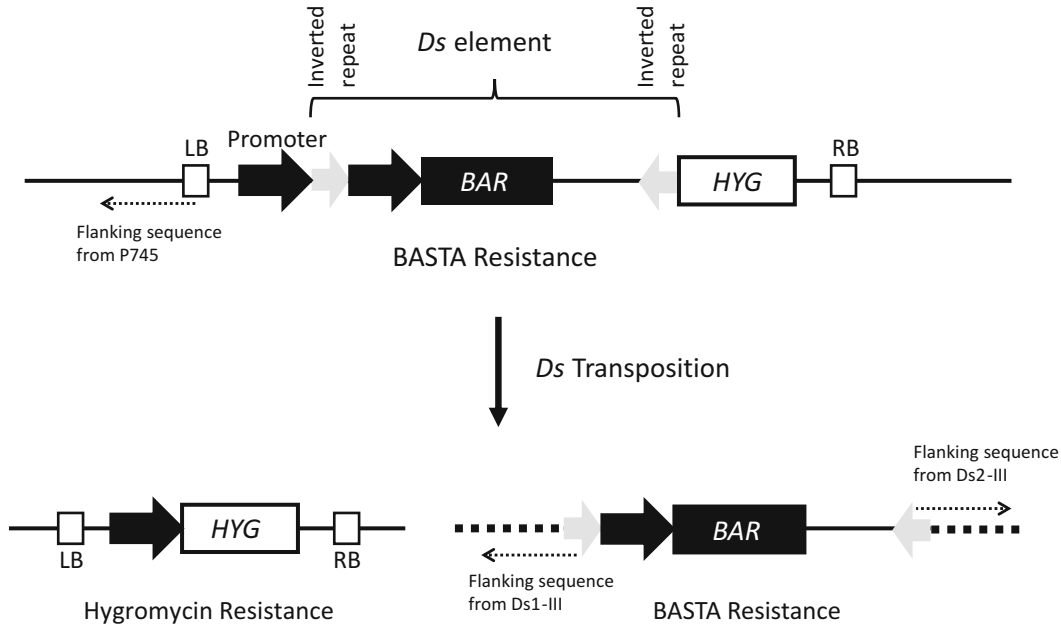

Fig. 1 Schematic representation of *Ds* transposable element system (pDs-Lox, [12]). T-DNA insertions or *Ds* transpositions are selected by resistance to BASTA or hygromycin, respectively. *Ds* transposon is moved by a cut-and-paste process; thus, the *Ds* does not remain in its original location after being inserted into a new location

to establish lines with a stable *Ac* position. To overcome this problem, the *Ac/Ds* two-component system was developed [6–9]. The *Ac/Ds* system comprises an *Ac* TPase gene derived from an autonomous *Ac* transposon, and a non-autonomous element, *Ds,* which is unable to transpose without the *Ac* TPase. To monitor transposition, it is preferable that *Ds* is inserted between a promoter and a resistance marker gene. If *Ds* transposition occurs, the gene will be activated, expressing resistance. The transposition can be fixed by crossing with a wild-type plant to remove the *Ac* TPase gene (Fig. 1). Although the *Ac/Ds* transposons themselves can induce chromosomal breakage and rearrangements [10], the combination with the Cre*/LoxP* system induces more efficient chromosomal rearrangements [11, 12] and can be used to generate artificial ring chromosomes [13]. However, because T-DNA insertion and *Ds* transposition occur mostly at random [14, 15], their inserted and transposed positions on chromosomes should be determined to estimate the scales of chromosomal reconstruction or the size and structure of artificial minichromosomes.

Several methods have been developed to determine the genomic sequences flanking T-DNA or transposons. One of the common methods is inverse PCR [16]. Whereas standard PCR amplifies a DNA fragment between two inward primers, inverse

PCR amplifies DNA sequences that are flanked with one end of a known DNA sequence. The individual restriction fragments are converted into circles by self-ligation, and the DNA can be used directly for PCR amplification with appropriate primer sets designed from the inserted DNA sequences. Some pretreatments are required before inverse PCR, such as restriction-enzyme digestion of genomic DNA followed by self-ligation. Another method to amplify unknown sequences adjacent to known DNA is thermal asymmetric interlaced (TAIL)-PCR [17, 18], which does not require any pretreatments. TAIL-PCR consists of two or three nested insertion-specific primers that anneal at relatively high temperatures during a series of reactions (Fig. 2), in combination with arbitrary degenerate (AD) primers that anneal at relatively low temperatures. AD primers are degenerate primers that anneal throughout the genome. The relative amplification efficiencies of specific products versus nonspecific products can be thermally controlled. From the primary to the tertiary reaction, the primers get closer to the edge of the inserted DNA (Fig. 3) (*see* **Note 1**). TAIL-PCR does not need special DNA manipulations before PCR, and the product specificity can be estimated by agarose gel electrophoresis. The TAIL-PCR reaction can be completed in only 1 day.

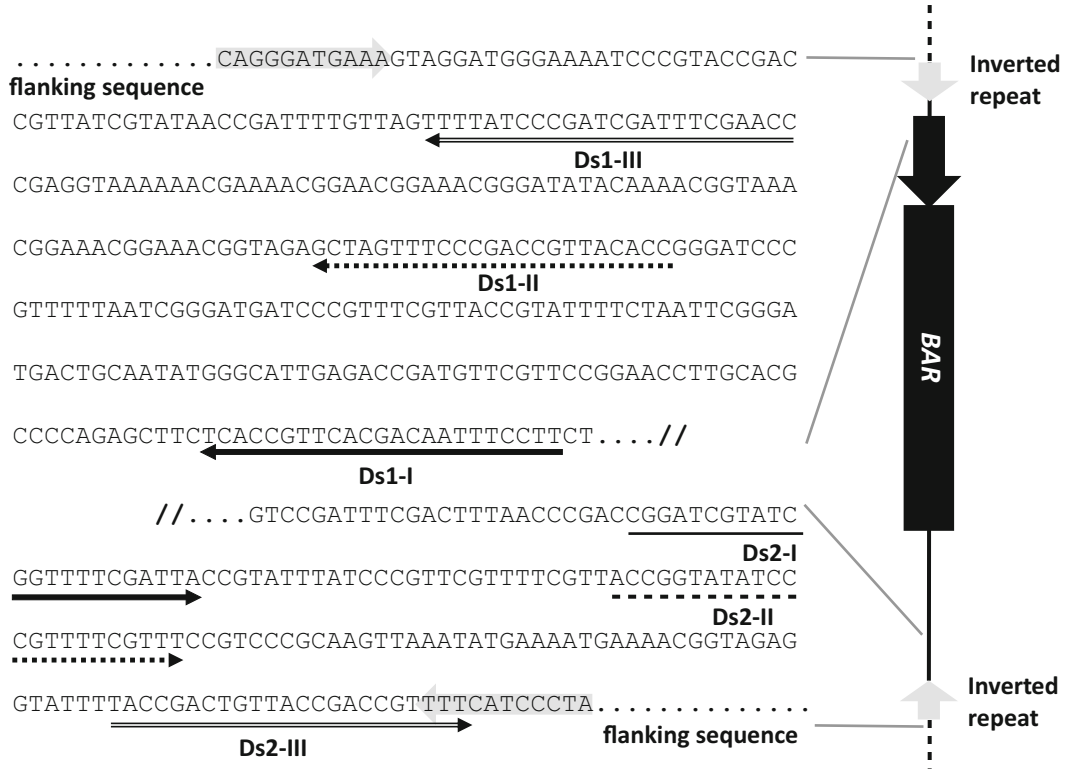

Fig. 2 Sequences of 5′ and 3′ ends of *Ds* element. *Grey arrows* indicate inverted repeats. *Ds* element contains short inverted repeats at end, but internal sequence is identical

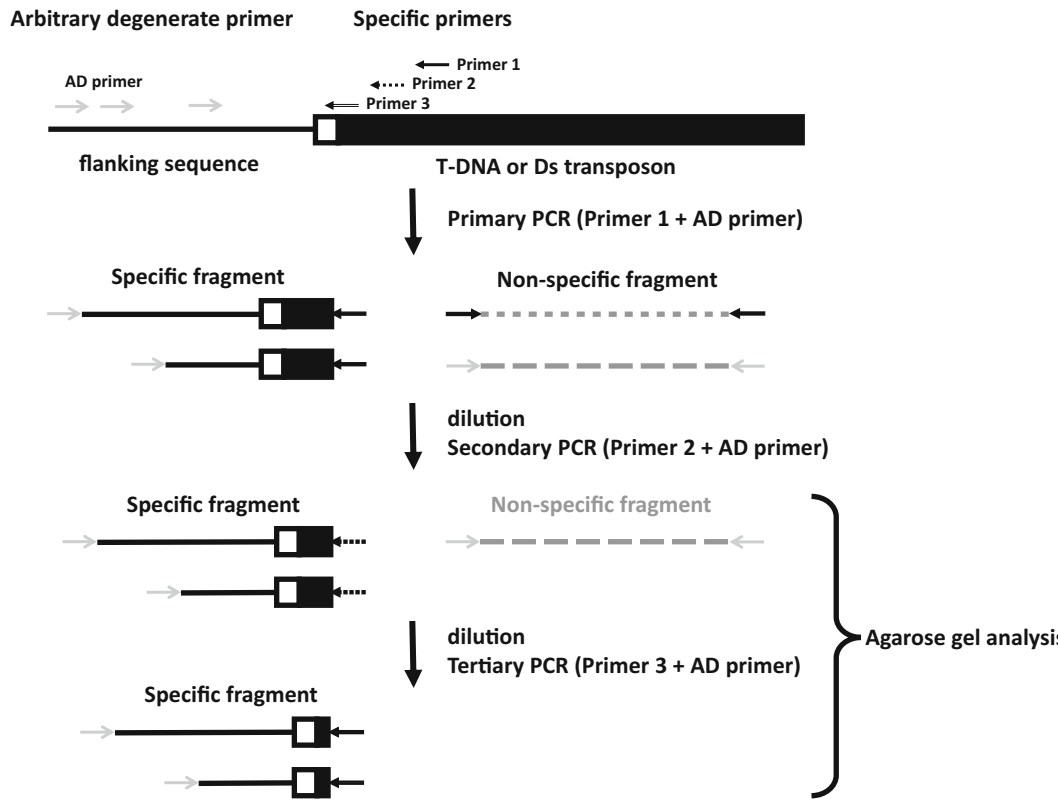

Fig. 3 Schematic representation of TAIL-PCR. One side of T-DNA or *Ds* element is shown

Thus, this method is very effective for obtaining the genomic sequences flanking known DNA inserts such as T-DNA or *Ds*.

Here, we provide a detailed TAIL-PCR method for isolating the flanking sequences of T-DNA or *Ds* transposable elements inserted into plant genomes, to predict chromosomal rearrangements.

2 Materials

2.1 DNA Extraction from Plants

1. Plant with T-DNA and/or a *Ds* transposable element. The site of *Ds* transposition via the *Ac* TPase can be determined after crossing to remove the *Ac* gene.
2. Plant DNA isolation kit (e.g., DNeasy Plant Mini Kit, Qiagen, Hilden, Germany).
3. Extraction buffer: 200 mM Tris–HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS [19].
4. Disposable grinders or tooth picks.
5. Isopropanol.
6. Ethanol: 70% (v/v).
7. TE buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA.

2.2 Thermal Asymmetric Interlaced PCR (TAIL-PCR)

1. Ex Taq polymerase (e.g., Takara Bio Inc., Kusatsu, Japan).
2. Specific primers (Table 1) (*see* **Note 1**).
3. AD primers:
   AD2-1: NGTCGASWGANAWGAA (N = A,G,C or T, S = C or G, W = A or T).
   AD17: TCNGSATWTGSWTGT (N = A,G,C or T, S = C or G, W = A or T).
4. Thermal cycler (e.g., Veriti® Thermal Cycler, Applied Biosystems, Foster City, CA, USA).
5. TAE buffer: 4.84 g Tris base, 1.14 ml acetic acid, 2 ml 0.5 M EDTA (pH 8.0), adjust to the volume to 1.0 l with ddH_2O.
6. Agarose.
7. Agarose gel electrophoresis apparatus (e.g., Mupid System, Advance, Tokyo, Japan).
8. Agarose gel extraction kit (e.g., Wizard SV Gel and PCR Clean-up System, Promega, Madison, WI, USA).

Table 1
Specific primers to amplify DNA adjacent to T-DNA or *Ds* in TAIL-PCR

Target	Specific primer	Primer name	Sequence
T-DNA of pBin19 derivatives (Left border) [20] (*see* **Note 2**)	1	LBal	TGGTTCACGTAGTGGGCCATCG
	2	LBb1.3	ATTTTGCCGATTTCGGAAC
	3	LBb1	GCGTGGACCGCTTGCTGCAACT
T-DNA of pDs-Lox (Left border, Fig. 1) [12]	1	LT6	AATAGCCTTTACTTGATTGGCGTAAAAG
	2	P745	AACGTCCGCAATGTGTTATTAAGTTGTC
Ds of pDs-Lox [12] after transposition (5′ end, Figs. 1 and 2)	1	Ds1-1	AAGGAAATTGTCGTGAACGGTGA
	2	Ds1-II	GGTGTAACGGGAAATAGC
	3	Ds1-III	GGTTCGAAATCGATCGGGATAAA
Ds of pDs-Lox [12] after transposition (3′ end, Figs. 1 and 2)	1	Ds2-I	CGGATCGTATCGGTTTTCGATTA
	2	Ds2-II	ACCGGTATCCCGTTTTCGTTT
	3	Ds2-III	TACCGACTGTTACCGACCGTTTT

3 Methods

3.1 DNA Extraction from Plants

Extract DNA from 50 mg leaf tissue with a plant DNA isolation kit according to the manufacturer's protocol.

Alternatively, because TAIL-PCR is a robust method, rapid and crude techniques to extract plant genomic DNA, such as that described by Edwards et al. [19], can be used to obtain a large number of DNA samples.

1. Crush leaf tissue (3 mm × 3 mm) in 100 μl extraction buffer in a 1.5 ml tube using a disposable grinder or tooth pick.
2. Centrifuge the extract at top speed for 2 min and transfer supernatant to a new 1.5 ml tube.
3. Mix the supernatant with an equal volume of isopropanol. Centrifuge the mixture at top speed for 5 min, and then wash the pellet with 70% (v/v) ethanol. Vacuum-dry the pellet and dissolve in 40 μl TE.

3.2 Thermal Asymmetric Interlaced PCR (TAIL-PCR)

1. Program the thermal cycler for primary TAIL-PCR as follows:

   TAIL-PCR1:

   94 °C for 1 min

   (94 °C for 1 min, 65 °C for 1 min, 68 °C for 3 min) × 5

   94 °C for 1 min, 30 °C for 1.5 min, 68 °C (ramp 10%) for 3 min

   (94 °C for 1 min, 65 °C for 1 min, 68 °C for 3 min, 94 °C for 1 min, 65 °C for 1 min, 68 °C for 3 min, 94 °C for 1 min, 44 °C for 1 min, 68 °C for 3 min) × 13
2. Prepare reaction mixture for primary TAIL-PCR as follows:

   0.5 μl Extracted genomic DNA

   6.9 μl ddH_2O

   1.0 μl 10× Ex Taq buffer

   0.8 μl 2.5 mM dNTPs

   0.2 μl Specific primer 1 (e.g., LT6 for T-DNA of pDs-Lox, Ds1-I for 5′ *Ds* of pDs-Lox) (10 μM) (Table 1)

   0.5 μl AD primer (one of the AD primers) (100 μM)

   0.1 μl Ex Taq polymerase
3. Run program TAIL-PCR1. The program is completed in approximately 4–5 h.
4. Program for secondary TAIL-PCR as follows:

   TAIL-PCR2:

   94 °C, 1 min

(94 °C for 1 min, 65 °C for 1 min, 68 °C for 3 min, 94 °C for 1 min, 65 °C for 1 min, 68 °C for 3 min, 94 °C for 1 min, 44 °C for 1 min, 68 °C for 3 min) × 13

5. Prepare reaction mixture for secondary TAIL-PCR as follows:

   0.5 µl 1/10 dilution of primary PCR product

   7.0 µl ddH_2O

   1.0 µl 10× Ex Taq buffer

   0.8 µl 2.5 mM dNTPs

   0.2 µl Specific primer 2 (e.g., P745 for T-DNA of pDs-Lox, Ds1-II for 5′ *Ds* of pDs-Lox) (10 µM) (Table 1)

   0.4 µl AD primer (100 µM)

   0.1 µl Ex Taq polymerase

6. Run program TAIL-PCR2. The program is completed in approximately 3.5–4 h.

7. Prepare reaction mixture for tertiary TAIL-PCR, if applicable; otherwise skip to **step 9**.

   0.5 µl 1/10 dilution of secondary PCR product

   7.0 µl ddH_2O

   1 µl 10× Ex Taq buffer

   0.8 µl 2.5 mM dNTPs

   0.2 µl Specific primer 3 (e.g., Ds1-III for 5′ *Ds* of pDs-Lox) (10 µM) (Table 1)

   0.4 µl AD primer (100 µM)

   0.1 µl Ex Taq polymerase

8. Run program TAIL-PCR2.

9. Electrophorese 1 µl PCR product on a 1.2% (w/v) agarose gel, stain with ethidium bromide, and visualize under ultraviolet light (Fig. 4).

10. Extract all DNA fragments using an agarose gel extraction kit according to the manufacturer's protocol (*see* **Note 3**).

11. Sequence DNA fragments with the specific primers used at the last step (e.g., P745 for T-DNA of pDs-Lox, Ds1-III for 5′ *Ds* of pDs-Lox) (Table 1).

12. Conduct DNA sequence analyses using the BLASTN (nucl query vs nucl db) program (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi) for the plant genome. If the band is correct, the sequences will begin with the T-DNA or *Ds* border sequence, followed by plant genome sequences. To confirm whether the authentic flanking sequences have been amplified, design primer sets around the boundary region (Primers A and B in Fig. 5).

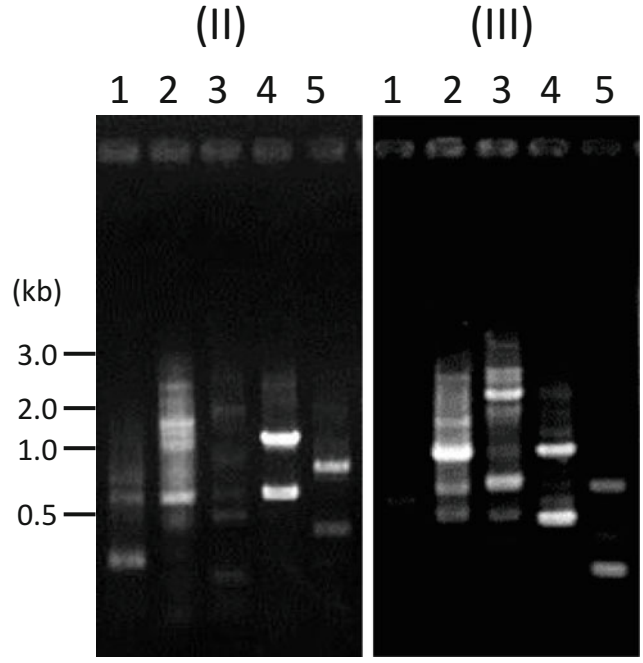

Fig. 4 TAIL-PCR products. Agarose gel images of TAIL-PCR products from secondary (II) and tertiary (III) reactions. *Lanes 1–5* show products from individual lines

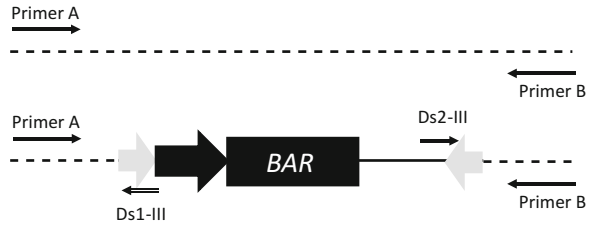

Fig. 5 Confirmation of *Ds* insertion. DNA amplification will be observed when Primer A–Ds1-III, Primer B–Ds2-III, and Primer A–Primer B are used

4 Notes

1. The border primers should be specific to the borders of the T-DNA or to the boundary sequence of the *Ds* transposable element. Because T-DNA integration occurs from the right border, the left-border side of the T-DNA is frequently truncated. Thus, successful TAIL-PCR amplification from the LB increases the probability of full-length T-DNA integration. A number of binary vectors have been developed from differ-

ent *Agrobacterium* strains, with divergent border sequences. If your binary vectors are not commonly used, check the T-DNA sequences and design primer sets with Tm > 65 °C from the border sequences.

2. pBin19 derivatives [20] include pBI101, pBI121, pRok2 (Salk lines [21]), and others.
3. All amplified bands should be used for sequencing. When a single band appears, purify the PCR product using exonuclease and shrimp alkali phosphatase (e.g., ExoSAP-IT (Affymetrix, Inc, Cleveland, OH, USA)). However, in the case of *Agrobacterium*-mediated T-DNA transfer, multiple T-DNA insertions often occur (multiple copies at single locus and/or multiple loci). Some of them contain truncated T-DNA regions or binary vector backbone sequences. A single-locus insertion line can be selected based on segregation of resistance to antibiotics, and the number of insertions can be determined by Southern blot hybridization analysis.

Acknowledgements

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References

1. Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20:1030–1034
2. Puchta H, Fauser F (2013) Gene targeting in plants: 25 years later. Int J Dev Biol 57:629–637
3. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 42:10903–10914
4. Osborne BI, Wirtz U, Baker B (1995) A system for insertional mutagenesis and chromosomal rearrangement using the Ds transposon and Cre-*lox*. Plant J 7:687–701
5. Schmidt R, Willmitzer L (1989) The maize autonomous element *Activator* (*Ac*) shows a minimal germinal excision frequency of 0.2%–0.5% in transgenic *Arabidopsis thaliana* plants. Mol Gen Genet 220:17–24
6. Hehl R, Baker B (1989) Induced transposition of *Ds* by a stable *Ac* in crosses of transgenic tobacco plants. Mol Gen Genet 217:53–59
7. Lassner MW, Palys JM, Yoder JI (1989) Genetic transactivation of *Dissociation* elements in transgenic tomato plants. Mol Gen Genet 218:25–32
8. Masterson RV, Furtek DB, Grevelding C, Schell J (1989) A maize *Ds* transposable element containing a dihydrofolate reductase gene transposes in *Nicotiana tabacum* and *Arabidopsis thaliana*. Mol Gen Genet 219:461–466
9. Bancroft I, Bhatt AM, Sjodin C, Scofield S, Jones JD, Dean C (1992) Development of an efficient two-element transposon tagging system in *Arabidopsis thaliana*. Mol Gen Genet 233:449–461
10. Weil CF, Wessler SR (1993) Molecular evidence that chromosome breakage by *Ds* elements is caused by aberrant transposition. Plant Cell 5:515–522
11. Zhang S, Raina S, Li H, Li J, Dec E, Ma H, Huang H, Fedoroff NV (2003) Resources for targeted insertional and deletional mutagenesis in *Arabidopsis*. Plant Mol Biol 53:133–150
12. Woody ST, Austin-Phillips S, Amasino RM, Krysan PJ (2007) The *WiscDsLox* T-DNA col-

lection: an Arabidopsis community resource generated by using an improved high-throughput T-DNA sequencing pipeline. J Plant Res 120:157–165

13. Murata M, Shibata F, Hironaka A, Kashihara K, Fujimoto S, Yokota E, Nagaki K (2013) Generation of an artificial ring chromosome in Arabidopsis by the Cre/*LoxP*-mediated recombination. Plant J 74:363–371
14. Parinov S, Sevugan M, Ye D, Yang WC, Kumaran M, Sundaresan V (1999) Analysis of flanking sequences from *Dissociation* insertion lines: a database for reverse genetics in Arabidopsis. Plant Cell 11:2263–2270
15. Krysan PJ, Young JC, Jester PJ, Monson S, Copenhaver G, Preuss D, Sussman MR (2002) Characterization of T-DNA insertion sites in *Arabidopsis thaliana* and the implications for saturation mutagenesis. OMICS 6:163–174
16. Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623
17. Liu YG, Whttier RF (1995) Thermal asymmetric interlaced PCR: Automatable amplification and sequencing of insert and fragments from P1 and YAC clones for chromosome walking. Genomics 25:674–681
18. Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463
19. Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19:1349
20. Frisch DA, Harris-Haller LW, Yokubaitis NT, Thomas TL, Hardin SH, Hall TC (1995) Complete sequence of the binary vector Bin 19. Plant Mol Biol 27:405–409
21. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301:653–657

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