Chapter 17

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

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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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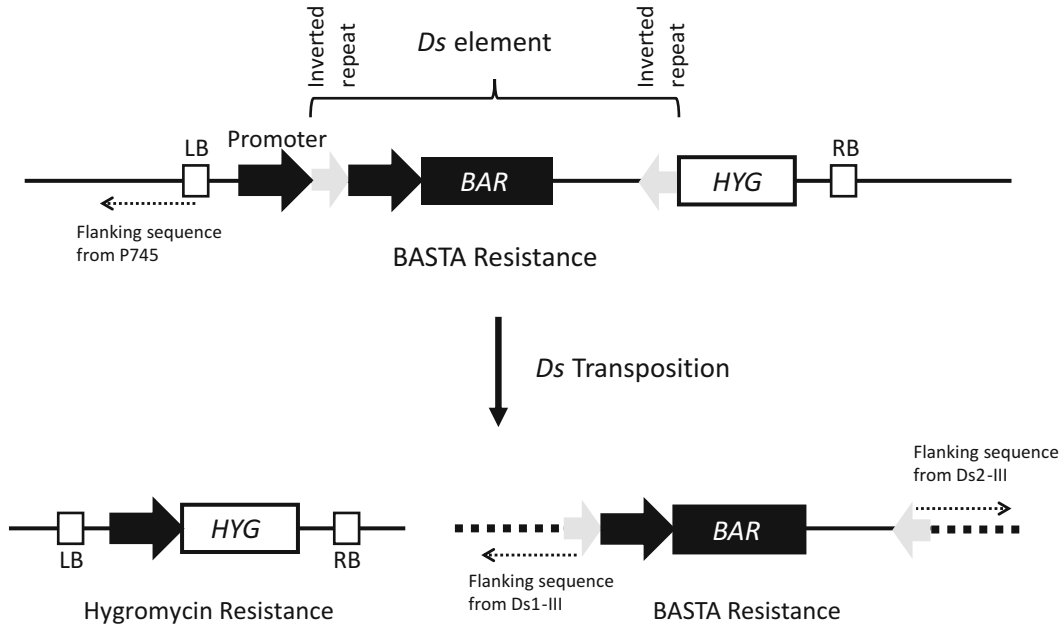

Fig. 1 Schematic representation of *Ds* transposable element system (pDs-Lox, [12]). T-DNA insertions or *Ds* transpositions are selected by resistance to BASTA or hygromycin, respectively. *Ds* transposon is moved by a cut-and-paste process; thus, the *Ds* does not remain in its original location after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

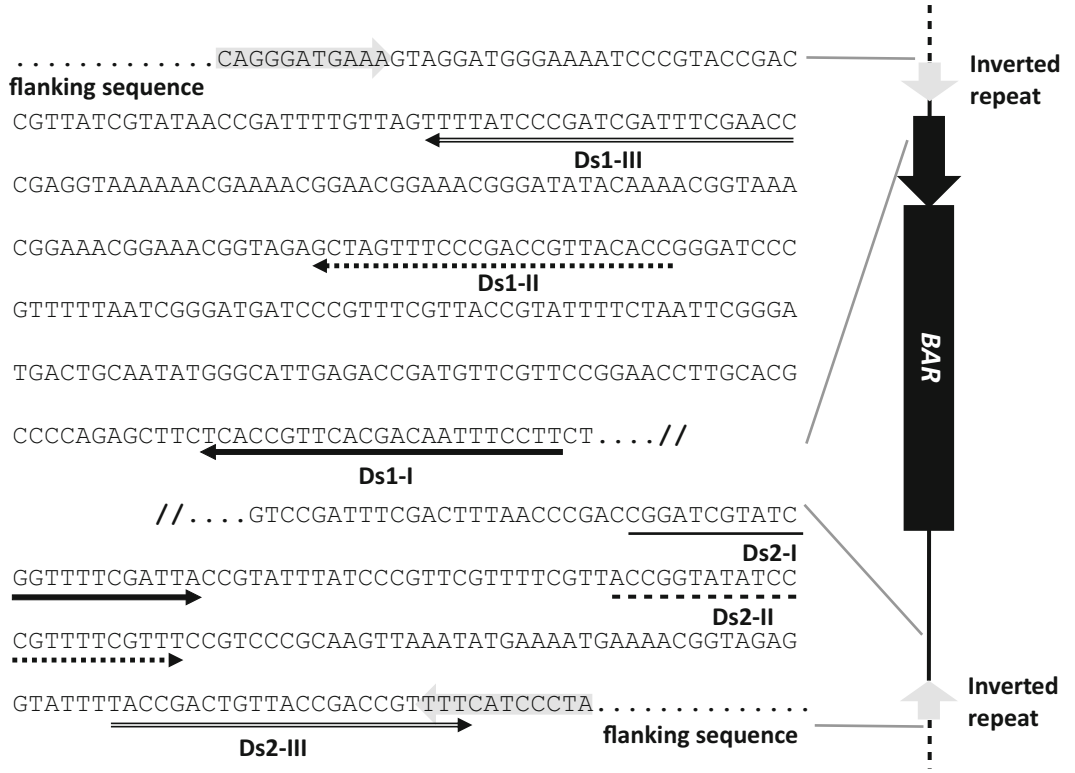

Fig. 2 Sequences of 5′ and 3′ ends of *Ds* element. *Grey arrows* indicate inverted repeats. *Ds* element contains short inverted repeats at end, but internal sequence is identical

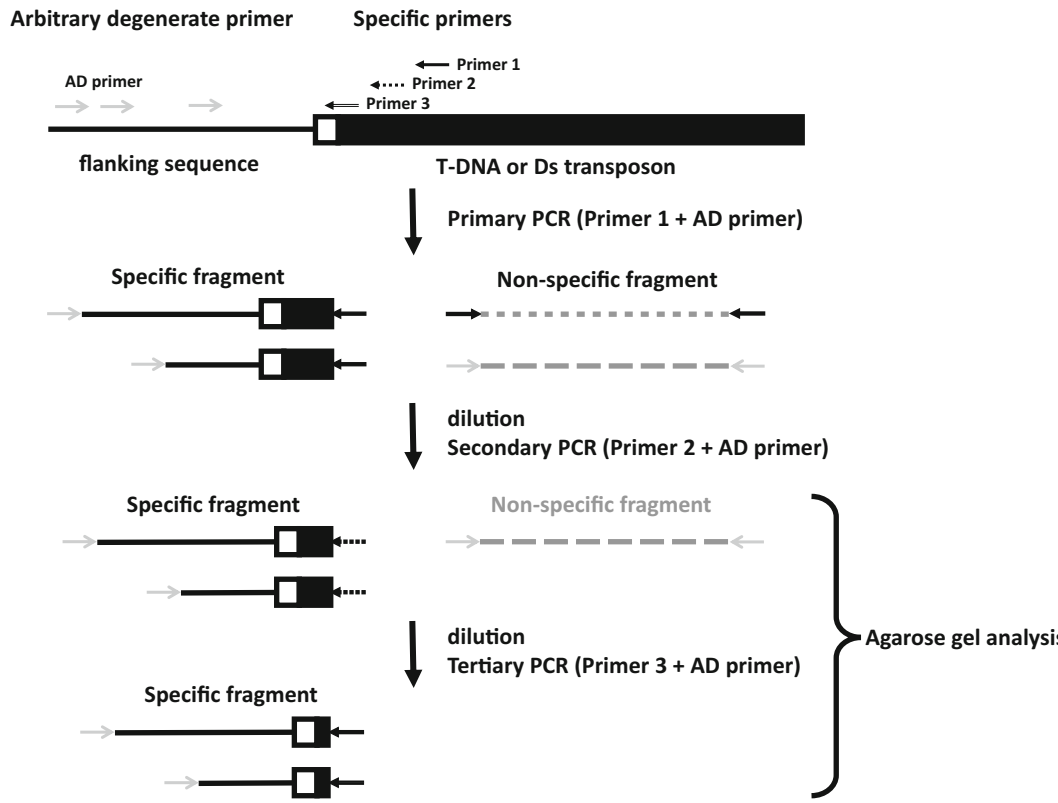

Fig. 3 Schematic representation of TAIL-PCR. One side of T-DNA or *Ds* element is shown

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).

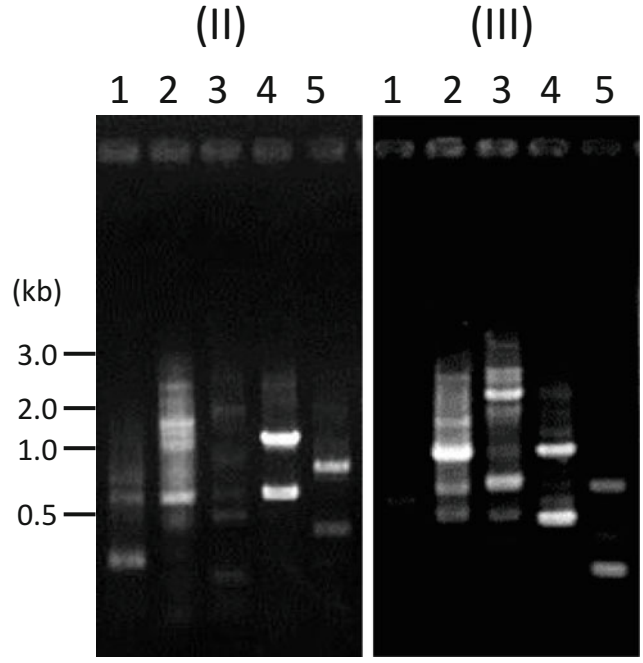

Fig. 4 TAIL-PCR products. Agarose gel images of TAIL-PCR products from secondary (II) and tertiary (III) reactions. *Lanes 1–5* show products from individual lines

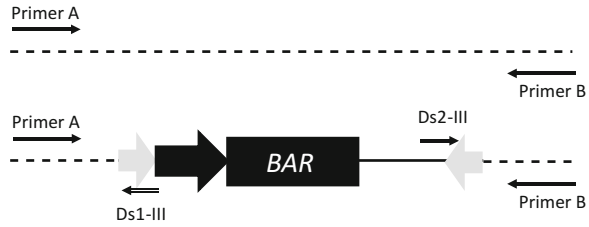

Fig. 5 Confirmation of *Ds* insertion. DNA amplification will be observed when Primer A–Ds1-III, Primer B–Ds2-III, and Primer A–Primer B are used

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.

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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