Chapter 6

Gene Tagging with Engineered *Ds* Elements in Maize

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Abstract

We describe here protocols for isolating genes in maize using *Dissociation (Ds*) transposons marked with a green fluorescent protein (*GFP*) transgene. The introduced marker enables the phenotypic scoring of the nonautonomous element and the anchoring of unique primers on the element to facilitate the isolation of the adjacent DNA by PCR. Transposons such as *Ds* transpose preferentially to sites closely linked to the *Ds*-launching platform. Based on this transposition behavior, a genetic resource is being created to mobilize a modified *Ds* element from different starting sites in the genome. Enough transgenic lines are being generated to cover most of the maize genome, allowing the targeted tagging of most genes from a *Ds-launching* platform located nearby.

Keywords Transposon tagging, Maize, *Ac–Ds*, GFP-marked *Ds* element, Mapped transposon-launching platforms

1 Introduction

1.1 Tagging Concept

Transposon tagging refers to the use of transposons as forward genetic tools to isolate genes on the basis of their mutant phenotype or as reverse genetics tools to isolate insertion mutations in known genes [1, 2]. In maize, the transposons of choice have been native elements of the *Ac/Ds* [3] and *Mutator* [4] families. In general, two tagging strategies have been used with the *Ac/Ds* transposon system. Non-targeted tagging relies on the ability to select progeny carrying unlinked transposed elements. In contrast, targeted tagging is based on the well-known property of *Ac* and *Ds* to transpose preferentially to closely linked sites [5–9]. Here, we discuss tagging with an engineered *Ds** element carrying an introduced marker that enables the phenotypic tracing of the nonautonomous element and the anchoring of unique primers on the element to facilitate the isolation of the tagged gene by PCR. Since enough transgenic lines are being generated to cover most of the maize genome, we emphasize the tagging of target genes from a *Ds**-launching platform located nearby.

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1.2 Advantages of an Engineered (Marked) Ds Element Over Native Elements

A *Ds** element marked with a transgene, such as the jellyfish gene encoding GFP [10], has the same genetic advantages as *Ac* [8], plus it can be stabilized by segregating away the *Ac* element used originally to mobilize it. These advantages are the following: (a) *Ds** transpositions can be selected phenotypically, making it possible to use convenient seed phenotypes to readily assemble a large collection of independent transposed *Ds** (*trDs**) elements. (b) The marked *Ds** element has a dominant phenotype, so *trDs**s can be identified in heterozygous condition and then homozygosed to screen for mutant phenotypes. (c) The dominant phenotype permits a quick co-segregation analysis of the *trDs** and a new mutation. (d) The visible phenotype allows the mapping of a *trDs** insertion in the genome independently of the molecular mapping of its adjacent sequence. (e) Like *Ac, Ds* tends to transpose to sites that are closely linked to the donor site, making it possible to "saturate" defined regions of the genome with *trDs* insertions. We refer to the collection of *trDs** elements from any one launching pad as a *Ds** insertion library. Placing *Ds** in launching pads at multiple regularly spaced locations will allow selective targeting of the entire genome. (f) The unique transgene sequence (e.g., *GFP*) enables the design of specific primers for the PCR isolation of the DNA adjacent to the *trDs** (*tds* or *transposed Ds* site).

A comprehensive set of lines that will serve as starting points for the production of future *Ds** insertion libraries is presently being generated (Li et al., unpublished). Because elements of the *Ac/Ds* family transpose preferentially to linked sites, about one-third of all transpositions are within 7 cM on either side of the donor site [6–8]. Therefore, based on the 1,727-cM genetic map of [11], the minimum set would consist of 124 lines carrying a uniquely marked element at equally spaced locations in the genome (*see* **Note 1**). In this set, most genes will be within 7 cM of a launching platform and will be, therefore, realistic targets in localized transposon mutagenesis attempts. The active *Ds**-launching platforms are being mapped by matching the sequence of their adjacent DNA, isolated by inverse polymerase chain reaction (iPCR), to the maize genome. The lines have been produced by *Agrobacterium*-mediated transformation of the Hi II hybrid [12] and have been confirmed to carry a single copy of the T-DNA platform. The *Ds** transposon delivery system in the T-DNA is based on the *c1-m2* allele [13], which carries a 2.5-kb *Ds* element in the third exon of the *c1* gene [14] and produces a spotted seed (c-m) phenotype in the presence of *Ac*. The selection scheme for *trDs** elements (*see* Subheading 1.3) takes advantage of the fact that both parents of Hi II are *c1* and produce colorless seed, so it is easy to identify *Ds* transpositions as fully colored seed. A side benefit of this transposant selection system is that most maize lines are *c1* [15, 16], so researchers can cross the *c1-m2(Ds*) Ac* stocks directly to their lines in order to generate *Ds**-tagged mutations of their gene of interest (*goi*).

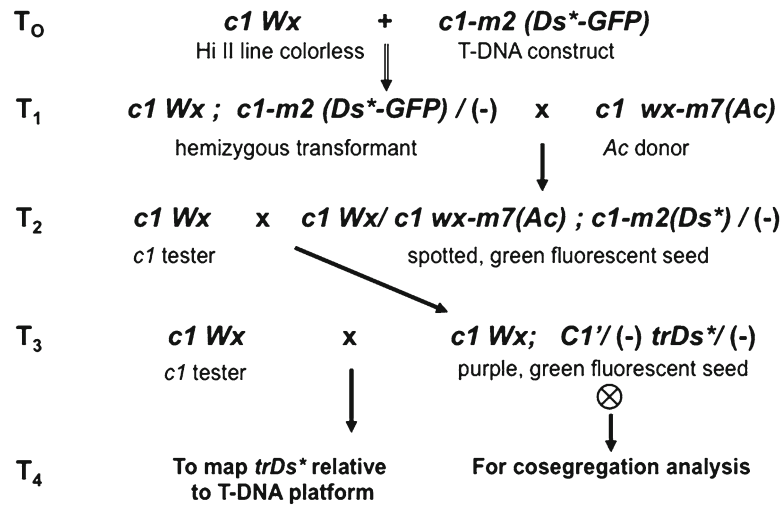

Fig. 1 Genetic scheme to generate: (1) Active *c1-m2(Ds*)* platforms (T_0–T_2) and (2) C′ purple revertants carrying *trDs*-GFP* elements (T_3–T_4)

1.3 Genetic Scheme for Identifying trDs*

Figure 1 outlines the genetic scheme used to generate (a) the T-DNA platforms carrying an active *Ds** element marked with a *GFP* gene expressed behind a seed-specific *α-zein* promoter (generations T_0–T_1) and (b) a *Ds** insertion library from a specific T-DNA platform (generations T_2–T_4). In short, the researcher obtains a specific line from the Maize Stock Center and uses it to pollinate *c1* tester plants (T_2). Transpositions of *Ds** from the modified *c1-m2* gene are selected as purple seeds that retain green fluorescence (T_3). These selections can be self-pollinated to produce *Ds** insertion homozygotes and backcrossed to the *c1* tester to map the location of the *trDs** (T_4).

2 Materials

2.1 Constructs

TAG21 is a pTF102-derived binary vector [12] in which the *gus* gene has been replaced by a modified *c1-m2* allele (Fig. 2a: Li et al., unpublished). In this engineered mutable allele, the *Ds** element carries P(*α-zein*)/*GFP*, a construct in which the *GFP* gene is driven by the *α-zein* promoter [17], conditioning green fluorescence in the endosperm. Germinal transpositions of *Ds** (*α-zein*/*GFP*) are easily identified in single-copy transgenotes as fully purple kernels that retain green fluorescence (Fig. 2b). In most transformants, the c-m spotted phenotype resembles that of the native *c1-m2* allele and segregates as a single Mendelian trait, indicating that the transgene has integrated at a single locus (Fig. 2c).

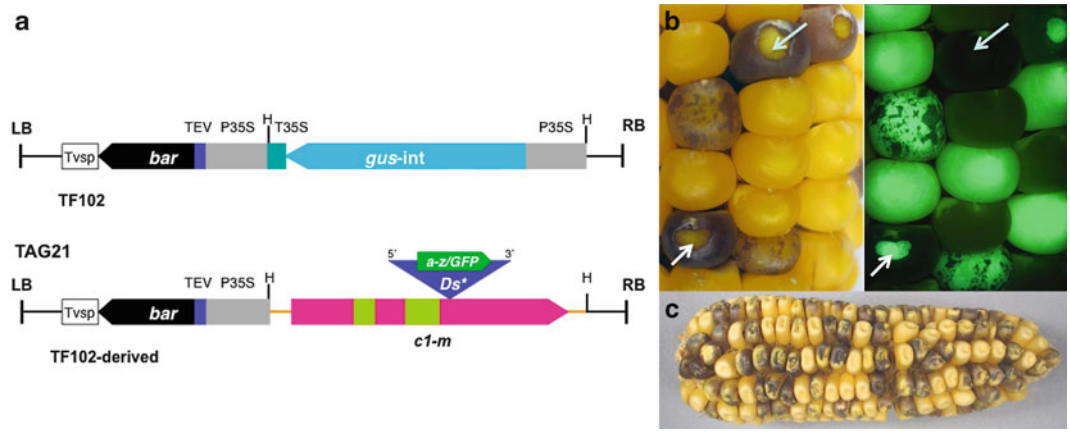

Fig. 2 (a) T-DNA construct used in *Agrobacterium* transformation of Hi II (*c1*) embryos. The *gus* HindIII (H) fragment of pTF102 was replaced with the *c1-m Ds** excision reporter shown in TAG 21 (*LB* left border, *RB* right border). (**b**) Purple kernel selections that retain (*white arrow*) or lose (*blue arrow*) *Ds**(α-*zein*)/*GFP*. (**c**) A testcross ear of an *Agrobacterium*-generated transformant, segregating 1 spotted (c-m):1 colorless (c)

2.2 Stocks

Most transgenic lines produce purple revertants (C′) in sufficiently high numbers (3–4 %) to serve as potential sources of transposed *Ds** elements. About half of C′ revertants carry new *trDs** based on retention of green fluorescence and GFP-hybridizing bands in Southern blots, a result consistent with the one-half fraction seen in other screens based on *Ac/Ds* excision [7]. The germinally active single-copy T-DNA platforms are mapped by isolating adjacent DNA via iPCR, sequencing it, and comparing it with the maize genome sequence. The genomic location of 82 platforms has been mapped so far (Fig. 3) and that of 150 other lines is presently being mapped. Stocks for all these *Ds**-launching platforms will be available from the Maize Genetics Stock Center.

2.3 Molecular Biology Solutions

All of the buffers, reagents, and other solutions used in the enzyme reactions (*see* Subheading 3) are found in kits provided by the manufacturers of the respective enzymes. Other reagents are prepared as follows.

1. DNA extraction buffer:
   (a) 0.1 M NaCl.
   (b) 50 mM EDTA (pH 8.0).
   (c) 50 mM Tris–HCl (pH 8.0).
   (d) 0.1 M DIECA (diethyldithiocarbamic acid sodium salt).
   (e) 1 % N-Laurylsarcosine Na-salt.
   (f) 8.5 mM β-mercaptoethanol.
2. CTAB solution (2 %):
   (a) 2 % CTAB (N-hexadecyl-N,N,N trimethylammonium bromide).
   (b) 50 mM Tris–HCl (pH 7.5), 10 mM EDTA (pH 8.0).

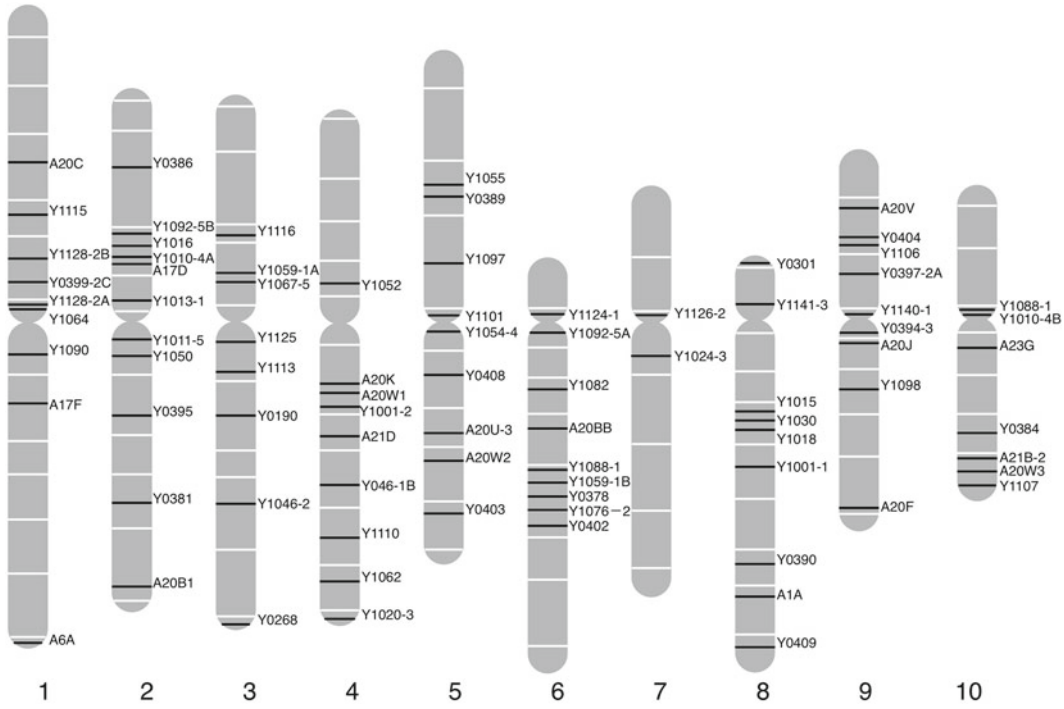

Fig. 3 Location of 82 T-DNA platforms currently mapped in the genome. The black marks identify the location of the mapped T-DNA platforms; the white marks, the location of the bin core markers. The space between the two core markers corresponds to roughly 20 cM in the genetic map

3. SOC medium:
   (a) 950 ml double-distilled water.
   (b) 20 g Bacto-tryptone.
   (c) 5 g Bacto-yeast extract.
   (d) 0.5 g NaCl.
   (e) 2.5 ml of 1 M KCl.

   Adjust the pH to 7.0 with 10 N NaOH and then make up to a final volume of 1 L using double-distilled water. Autoclave to sterilize, and then store the medium at room temperature (21–25 °C). Add 20 ml sterile 1 M glucose immediately before use.
4. LB + ampicillin plates:
   (a) 10 g Bacto-tryptone.
   (b) 5 g Bacto-yeast extract.
   (c) 10 g NaCl.
   (d) 15 g Bacto-agar to double-distilled water (such that the final volume is 1 L).

Autoclave to sterilize, cool to 55 °C, add 1 ml 100 mg/ml ampicillin (amp), and then pour into sterile 10 cm petri dishes. Store at 4 °C and dispose of unused plates after 3 weeks.

5. LB + ampicillin medium:
   (a) 10 g Bacto-tryptone.
   (b) 5 g Bacto-yeast extract.
   (c) 10 g NaCl to double-distilled water (such that the final volume is 1 L).
   Autoclave to sterilize and then store at room temperature. To 1 L add 1 ml 100 mg/ml ampicillin. After the addition of ampicillin store the medium for a maximum period of 2 weeks at 4 °C.

2.4 Molecular Biology Reagents

1. 1 kb DNA ladder.
2. Agarose (electrophoresis grade).
3. Ampicillin.
4. Bacto-agar.
5. β-mercaptoethanol.
6. Bacto-tryptone.
7. Bacto-yeast extract.
8. BigDye® Terminator v3.1 Cycle Sequencing Kit.
9. Buffer P1.
10. Buffer P2.
11. Buffer P3.
12. CTAB (N-hexadecyl-N,N,N trimethylammonium bromide).
13. DIECA (diethyldithiocarbamic acid sodium salt).
14. EDTA.
15. Efficiency™ DH5α™ Competent Cells.
16. Epicentre End-It™ DNA End-Repair kit.
17. Ethanol (99.7 % (vol/vol), AnalaR)).
18. Ethidium bromide solution (10 mg/ml).
19. Expand High Fidelity PCR System, dNTPack.
20. Glucose.
21. HPLC-purified oligonucleotides (*see* Table 2).
22. NEB buffer 2.
23. N-Laurylsarcosine Na-salt.
24. pGEM®-T Easy Vector.
25. Potassium chloride, KCl.
26. Qiagen Multiplex PCR kit.
27. QIAquick Gel Extraction Kit.
28. QIAquick PCR Purification Kit.

29. RNase A.
30. Sodium chloride, NaCl.
31. Sodium hydroxide, NaOH.
32. T4 DNA ligase (400 U/μl; supplied with 10× buffer).

2.5 Supplies and Equipment

The following supplies and equipment are used in Subheading 3.

1. Nebulizer Kit.
2. 1.5-ml microtubes.
3. PCR tubes and lids (strips of 8).
4. Thin-walled 96-well PCR plates.
5. Deep-well 96-well plates.
6. Millipore filter plates.
7. Bio-Rad S1000™ thermal cycler.
8. 37 °C incubator.
9. Heating block.
10. Gel-running equipment including gel tank and power supply.
11. Micro-centrifuge.
12. Sorvall RC6+ centrifuge.
13. MicroAmp® Optical 96-Well Reaction Plate.
14. 3730 DNA Analyzer.

3 Methods

3.1 Isolation of C' Revertants with trDs-GFP

The first step in setting up a targeted gene tagging experiment is to identify one or two transposon-launching platforms located as near as possible to the *goi* and to request the corresponding stocks from the Maize Genetics Stock Center in Champagne-Urbana, IL (http://maizecoop.cropsci.uiuc.edu/). As in any gene tagging experiment, a large number of independent transpositions is desirable. Using the parent with the *Ds**-launching platform as male is much more efficient for the following reasons: (a) The average germinal transposition frequency is about two times higher in the male than in the female. (b) Postmeiotic or gametophytic transpositions, which lead to non-corresponding kernels carrying a C′ endosperm and a c-m embryo (false selections), occur much more frequently in the female gametophyte than in the male gametophyte. and (c) Premeiotic transpositions, which lead to clusters of individuals with the same transposition event (jackpots), are rare in the male, allowing the same tassel to be used in multiple pollinations over a number of days without undue concern about recovering the same event multiple times. The average T_2 testcross ear (Fig. 1) will have 1–2 *trDs** elements. Thus, a collection of 1,000 independent

transposition events can be obtained from about 700 ears. That number of ears would be produced in 700 pollinations of *c1* tester plants by *c1-m2(Ds-GFP)* plants (T2 in Fig. 1). Since each male plant can be used multiple times, no more than 50 male plants will be needed if the *c1* females are hand pollinated (*see* **Note 2**).

1. Plant 500–1,000 seeds of *c1* tester, either from the investigator's own stocks or obtained from the Maize Stock Center, to serve as female parents, and 50–100 seeds of the heterozygous *c1-m2(Ds*)/(-); wx-m7/Wx* stock, obtained from the Maize Stock Center, to serve as male parents (*see* **Note 3**): If the latter seed stock is limited, selfing the hemizygotes first to increase the seed would also result in an increase in the number of *c1-m2(Ds*)* alleles from 1 to 1.3 per individual among the spotted seed. In this case, the fine spotted seed from the selfed ear (*Ac* homozygotes) should be avoided because the frequency of transposition is reduced at higher doses of *Ac* [18, 19].
2. As the plants mature, protect all ears of the *c1* tester line with ear shoot bags (e.g., Lawson 217) prior to silk emergence.
3. Pollinate all *c1* plants with pollen of the *Ds** stock (collected in tassel bags, such as Lawson 404) and cover pollinated ears with the same bags. The number of the *Ds** parent plant can be recorded on the bag if the investigator wants to keep track of the parental origin of all C′ selections, although premeiotic events are rare (*see* **Note 4**).
4. After harvesting and drying the ears to ≤13 % moisture, screen all ears for fully purple kernels (i.e., C′ revertants). Then, inspect the C′ selections for green fluorescence under a blue light source (400–500 nm wavelength). Gently sand the top surface of individual C′ kernels to expose the underlying endosperm, as is done when staining for wx starch (Fig. 2b), and score the presence of reinserted *trDs** elements on the basis of green fluorescence of the exposed endosperm. Use Dark Reader Hand Lamp (HL32T, blue filter to remove green/red light) and Dark Reader viewing glasses, equipped with an amber filter to remove blue light, both of which are purchased from Clare Chemical Research, Inc (*see* **Note 5**).
5. Grow out the purple, green fluorescent seed selections (C′ GFP), preferably in the greenhouse or a similar protected environment, number them, and collect one leaf from each 4-week-old plant for future DNA analysis. Freeze the leaf in liquid nitrogen (or dry ice) and store the frozen leaf in a regular (noncycling) freezer until ready to extract DNA.
6. Self-pollinate and/or cross the C′ GFP selections to a *c1* tester. The option here will depend on several factors, such as what the researcher wants to accomplish at this stage, the time frame of the experiment, and the amount of greenhouse growing space.

3.2 Mutant Screen

1. To isolate a *Ds**-tagged allele of a gene defined genetically (i.e., with a known mutant phenotype), self-pollinate the T_3 C′ GFP selections in order to homozygose the *trDs** element and cross them as males to plants carrying the known reference mutant allele of the gene in order to identify new, putative *Ds**-tagged, alleles in the T_4 generation (Fig. 1). Alternatively, if a line with the reference mutant allele is available in a *c1* mutant version (very likely, as most colorless seed stocks are *c1* mutants), use that line as female in crosses to the *c1-m2(Ds*)/*(-) parent (Fig. 1, T_2). In that case, the screen for new mutations putatively tagged by *Ds** could be conducted in the T_3, rather than the T_4 (*see* **Note 6**).
2. To isolate a *Ds**-tagged allele of a gene defined only molecularly (*goi*) (i.e., with an unknown mutant phenotype), self-pollinate all selections and proceed to analyze them molecularly (*see* Subheading 3.6) to identify a *Ds** insertion in *goi*.

3.3 Mutant Analysis

1. To obtain preliminary information that a mutant phenotype is, indeed, caused by the insertion of a *trDs** element, one can perform a co-segregation analysis of the self-progeny of the pertinent *Ds** transposant, preferably in the absence of *Ac* (*see* **Note 7**). If the *trDs** has caused a new dominant mutation, all the mutants should be green fluorescent and vice versa. If the *trDs** has caused a new recessive mutation, all the mutants should be green fluorescent and all the green fluorescent nonmutant should segregate the mutation upon selfing. Secondary transpositions of *Ds** would result in exceptions to these outcomes. This is a correlation type of analysis and one can only conclude that the mutation is likely to be transposon tagged, not that it is tagged, regardless of the size of the experiment.
2. To confirm that a mutant phenotype is caused by the insertion of a *trDs** element, one conducts a reversion analysis. Mutant individuals carrying *Ac*, preferably in heterozygous condition, are self-pollinated and their self-progenies are screened for wild-type revertants. If the mutation was *Ds** tagged, the revertants should lose *Ds** from the mutant gene. This is best ascertained by a molecular analysis of the revertants, which usually involves PCR and sequencing, although Southern blots were traditionally used for this purpose. Since germinal transpositions of *Ac* and *Ds* are in the 1–5 % range, a population of several hundred individuals should be screened for reversion.

3.4 Isolation of the tds Site from a Mutant Allele by iPCR

The insertion site of a *trDs** element (*tds* site) in a *Ds**-mutagenized gene is readily isolated by iPCR amplification from total genomic DNA using unique primers from the *GFP* transgene and nonspecific primers from the *Ds* 5′ or 3′ ends. *Ds* primers are inevitably nonspecific because the different classes of *Ds* elements in the maize genome are highly redundant.

1. Digest 5 μg of maize genomic DNA (*see* Subheading 3.7) with a restriction enzyme that does not cut the known sequence between the ends of the iPCR primers to be used. Use several enzymes to be sure of obtaining a fragment of appropriate length. The reaction should contain:
   (a) Maize genomic DNA 5 μg.
   (b) 10× Restriction buffer 5 μl.
   (c) 10× BSA (if needed) 5 μl.
   (d) Restriction enzyme 20 U.
   (e) Distilled water to a final volume of 50 μl.

   Incubate at enzyme's optimum temperature for 5 h to overnight.
2. Stop the reaction by heating at 65 °C (or recommended temperature) for 15 min. Purify restriction products through a Qiagen PCR purification column or by standard ethanol precipitation.
3. Resuspend DNA in 100 μl water for intramolecular ligation by T4-DNA ligase:
   (a) Purified DNA digestion products 100 μl.
   (b) 10× T4 DNA ligase reaction buffer 20 μl.
   (c) T4 DNA ligase (from NEB: 400 U/μl) 1 μl.
   (d) Distilled water 79 μl.

   Incubate 200-μl ligation reaction at 16 °C overnight.
4. Stop the reaction by heating at 65 °C for 15 min. Purify ligation products through a Qiagen PCR purification column or by standard ethanol precipitation.
5. Resuspend DNA in 200 μl water, and use 1 μl as template for the 25-μl iPCR reaction.
6. The sequences and combinations of PCR primers are shown in Table 1.
7. Load 3 μl of the reaction on a 1× TAE 0.8 % agarose gel to visualize the amplified iPCR products (*see* **Note 8**). Purify iPCR products of a desired size and analyze on a 0.8 % agarose gel to confirm the recovery and to measure the approximate concentration for direct sequencing with an ABI BigDye Terminator V3.1 Cycle Sequencing Kit.
8. To determine the physical and genetic locations of *tds* sites, compare the flanking sequences with the B73 RefGen_v2 database provided in the MaizeGDB Genome Browser (http://gbrowse.maizegdb.org/cgi-bin/gbrowse/maize_v2/). BlastN results include their physical locations, such as chromosome number, contig number, and start and end positions in the pseudo

Table 1
Combinations of PCR primers to isolate *trDs flanking sequences**

*trDs** End	1st-round PCR	2nd-round PCR
5′-end	GFP-1/Ds-10	GFP-3/Ds-9
3′-end	GFP-2/Ds-13	GFP-4/Ds-14

Primer sequence:
GFP-1: 5′-GTCGCCACCATGGTGAGCAA-3′
GFP-2: 5′-GCGGCCGCTTTACTTGTACA-3′
GFP-3: 5′-CGTAAACGGCCACAAGTTCA-3′
GFP-4: 5′-TCGTCCATGCCGAGAGTGAT-3′
Ds-9: 5′-CGGTTATACGATAACGGTCG-3′
Ds-10: 5′-ACCTCGGGTTCGAAATCGAT-3′
Ds-13: 5′-CCGGTATATCCCGTTTTCGT-3′
Ds-14: 5′-TTTCGTTTCCGTCCCGCAA-3′

molecule, as well as their genetic locations, such as bin number and recombination coordinate number. The MaizeGDB Genome Browser provides additional helpful information, such as coding capacity and EST support for the query sequence.

3.5 Isolation of the tds Site from a Mutant Allele by Adapter-Ligated PCR

Splinkerette PCR is an alternative to iPCR for the isolation of the host sequence adjacent to a *trDs** [20]. This method involves the ligation of adapters with a hairpin at one end that are designed to eliminate the amplification of sequences adjacent to the many native *Ds* elements.

1. Preparation of the splinkerette adaptor with HPLC-purified "long-strand adaptor" and "short-strand adaptor" oligonucleotides (*see* Table 2):
   (a) Dissolve the oligonucleotides in 5× NEB buffer 2 to a concentration of 50 μM.
   (b) Add 50 μl of each adaptor stock to a PCR tube and vortex to mix.
   (c) The adaptor mix is denatured and annealed by heating it to 95 °C for 5 min and then cooling to room temperature at the rate of 1 °C every 15 s in a thermal cycler. Store the adaptor mix at −20 °C.
2. Shear 2 μg genomic DNA to 2–4 kb DNA fragments with a nebulizer.
3. Blunt-end the DNA using Epicentre End-It™ DNA End-Repair kit.
4. Combine and mix the following components in a microfuge tube:
   (a) 1–34 μl genomic DNA to end-repair (300 ng).
   (b) 5 μl 10× End-repair buffer.
   (c) 5 μl dNTP mix.

Table 2
Oligonucleotides required for splinkerette PCR protocol

Name	Sequence (5′–3′)
Adaptors	
Long-strand adaptor	CGAAGAGTAACCGTTGCTAGGAGAGACC GTGGCTGAATGAGACTGGTGTCGACA CTAGTGG
Short-strand adaptor	CCACTAGTGTCGACACCAGTCTCTAATTT TTTTTTTCAAAAAAA
1st-round primers	
Splink1	CGAAGAGTAACCGTTGCTAGGAGAGACC
GFP-12 (5′-end)	CAGCTCCTCGCCCTTGCTCACCA
GFP-10 (3′-end)	TCCGCCCTGAGCAAAGACC
2nd-round primers	
Splink2	GTGGCTGAATGAGACTGGTGTCGAC
Ac5′-178 (5′-end)	GTGAAACGGTCGGGAAACTAGCTCTAC
Ds-13 (3′-end)	CCGGTATATCCCGTTTTCGT

(d) 5 μl ATP.
(e) *x*μl sterile water to a reaction volume of 49 μl.
(f) 1 μl End-repair enzyme mix.

Total reaction volume: 50 μl.

Incubate at room temperature for 45 min. Stop the reaction by heating at 70 °C for 10 min. Place the tube on ice.

5. Set up the following splinkerette adaptor ligation reaction:
   (a) 50 μl blunt-end DNA.
   (b) 1 μl Adaptor mix (25 μM).
   (c) 10 μl 10× T4 DNA ligase buffer.
   (d) 1 μl T4 DNA ligase.
   (e) 38 μl sterile water.

   Total reaction volume: 100 μl.

   Incubate the ligation reaction at 16 °C overnight. Heat-inactivate the T4 DNA ligase at 65 °C for 15 min. Use the QIAquick Gel Extraction Kit to clean up the ligation reaction. Elute the DNA in 40 μl of 10 mM Tris–HCL (pH 8.5) and store it at –20 °C.
6. Use 5 μl of purified splinkerette adaptor-ligated genomic DNA as template for first-round PCR amplification.

The PCR program is as follows:

(a) 2 min at 94 °C.

(b) 35 cycles of 20″ at 94 °C.

(c) 30″ at 56 °C.

(d) 3′ at 72 °C.

(e) 7′ at 72 °C.

Use 1 μl of 1/1,000 diluted primary PCR product as template for second-round PCR amplification following the above PCR program. (*See* Table 2 for primers used in first- and second-round PCR).

7. Visualize the secondary PCR products on a 1× TAE 1 % agarose gel, size-select the 0.5–1 kb products, and recover them using QIAquick Gel Extraction Kit.
8. Use Taq DNA polymerase to perform A-overhang reaction of recovered PCR products at 72 °C for 30 min.
9. Ligate the PCR products with pGEM®-T Easy Vector and transform Subcloning Efficiency™ DH5α™ Competent Cells.
10. Culture the transformed colonies in deep-well 96-well plates. For each individual, pick 12 colonies. Purify plasmids from the colonies and sequence them with BigDye Terminator on an ABI 3730 DNA Analyzer.
11. Determine the physical and genetic locations of the *tds* site as described in **step 8** of the previous section.

3.6 Isolation of the tds Site from a Gene Defined Only Molecularly

The *tds* site of a gene defined only molecularly can be efficiently identified in DNA pools of C′ GFP revertants (*see* **Note 9**). The following is a simplified account of the analysis of DNA from 960 such individuals.

1. Germinate individual C′ GFP revertants and array in ten plates of 12 × 8 format.
2. For each plate, collect 2-week-old seedling leaves from all 96 individuals and pool roughly equal amounts of leaf tissue for DNA extraction.
3. Test the DNA pools by a multiplex PCR with two *GFP* primers from the 5′ and 3′ ends of *Ds** (*see* Table 1) and one primer from *goi*. Several *goi* primers may be needed to cover the full length of the desired gene. A PCR band corresponding to a new junction between *Ds** and *goi* will only be amplified in pools containing DNA from an individual with a new *trDs** insertion in *goi*.
4. Extract individual DNAs from all of the C′ revertants in the positive pool and array them in a 96-well plate.

5. Pool DNAs into 8 row pools and 12 column pools.
6. Test the 20 DNA pools by PCR. The intersection of the positive row and column pools identifies the well containing DNA from the desired individual.

3.7 Isolation of Genomic DNA from Maize Leaf Tissues

1. Grind the leaf tissue (~5 g) into fine powder (in liquid N_2), transfer into a 50 ml centrifuge tube, add 20 ml 1× DNA extraction buffer, mix well by shaking, and put on ice for 30 min.
2. Bring up the volume to 45 ml by adding chloroform, and mix well by inverting the tubes four to six times.
3. Spin at 3,300 × *g* for 15 min.
4. Transfer the top phase into a fresh 50 ml centrifuge tube. Add the same volume of phenol, chloroform, isoamyl alcohol (25:24:1) and mix well by flipping the tubes four to six times.
5. Spin at 3,300 × *g* for 15 min for separation of phases.
6. Transfer the aqueous phase (above the white interface layer) into a fresh 50 ml centrifuge tube. Add the same volume of chloroform and mix well by flipping the tubes four to six times.
7. Spin at 3,300 × *g* for 15 min for separation of phases.
8. Transfer the aqueous phase (above the white interface layer) into a fresh 50 ml centrifuge tube. Add the same volume of 100 % ethanol and mix well by flipping the tubes four to six times.
9. Spin at 3,300 × *g* for 15 min.
10. Pour away the supernatant and air-dry the pellet.
11. Resuspend the pellet in 5 ml 1× TE (pH 8.0) with RNaseA (final concentration of 25 μg/ml), and incubate at 37 °C for 30 min or until the pellet is totally dissolved.
12. Add 5.7 ml 2 % CTAB mixture (*see* Subheading 2.3) and 0.7 ml 5 M NaCl in the RNaseA-treated DNA samples.
13. Vortex gently to allow the DNA to precipitate out of solution (*see* **Note 10**).
14. Drip off the supernatant, keeping the precipitated DNA in the centrifuge tube.
15. Add 10 ml 70 % ethanol (0.15 M NaCl) and wash the precipitated DNA for 15 min in order to remove salts.
16. Pour off the wash solution and wash again with 10 ml 70 % ethanol for 15 min. Use a 1-ml pipette tip to hold the DNA against the tube wall and pour off the ethanol wash.
17. Pipette the DNA which has adhered to the tip into a 2 ml Eppendorf tube, allow it to air-dry, and dissolve in 1× TE (pH 8.0).

4 Notes

1. More recently, the estimated genetic size of the maize genome has grown to about 2,000 cM [21], raising the number of equidistant *Ds*-launching platforms required to cover it to 140.
2. Of 1,000 *Ds** transpositions, one-third or about 333 are expected to fall within 7 cM on either side of the *c1-m2(Ds*)* donor locus. Assuming a genetic map of 1,727 cM [11] and a gene number of 32,000 [21], there are roughly 259 genes in the average 14-cM interval. So, if transposition was randomly distributed in the 14 cM flanking the donor locus, each gene would be hit 1.3 times among 333 transpositions. That assumption is clearly not valid as the probability of hitting a specific gene is higher close to the donor locus [6–8]. The distributions of linked and unlinked transpositions have been examined for a limited set of transpositions from only a handful of loci, the largest number studied to date being the 1,228 *Ac* transpositions from *wx-m7(Ac)* mapped by Cowperthwaite et al. [8]. In that study, 387 transpositions fell within 7 cM of the *wx* donor locus, of which 249 fell within 4.6 cM of *wx*. Therefore, doubling or tripling the number of desirable transpositions would be advisable for genes known to be >5 cM away from the *c1-m2(Ds*)* platform being used. Even 3,000 *Ds** transpositions could be generated from no more than 2,000 pollinations, a still reasonably small transposon tagging experiment.
3. Several plantings of the *c1* tester line should be made by the investigator to ensure that enough *c1* female plants "nick" (i.e., flower concurrently) with the *c1-m2(Ds*) Ac* male plants. The latter are in a Hi II x W22 hybrid background, so they flower earlier than most inbreds. In the average NJ summer, they shed pollen between 62 and 68 days after planting. Most of our experience is based on *c1-m2(Ds*) Ac* that have flowered in late July after a mid to late May planting.
4. It is advisable to spread the pollen from individual *c1-m2(Ds*) Ac* plants over several days to dilute out potential premeiotic transpositions, which lead to clusters of C′ revertants carrying the same *trDs** element.
5. An alternative, but more expensive, piece of equipment that serves the same purpose as the Dark Reader Hand Lamp is a fluorescent dissecting microscope, such as the Leica MZ16 that was used for Fig. 2b. Kernel green fluorescence can also be visualized under the blue light provided by a conventional LCD projector.
6. Screening only the T_3 C′ GFP selections from the cross to the reference mutation is an extremely efficient way of identifying mutations with an adult plant phenotype and can be done in

the greenhouse. A mutation with a seed phenotype can be directly screened in the testcross ears, making sure that any mutant seeds are green fluorescent, so as to eliminate unwanted self-contaminants. A mutation with a seedling phenotype can be screened on sand benches in the greenhouse by germinating only the T_3 C′ GFP selections, if space is limited, or the entire T_3 GFP population, if space is plentiful. The latter is not an efficient method, but has the advantage that all *Ds** transpositions would be included in the screen: those co-segregating with the *C1′* excision product (purple kernels), those co-segregating with the *c1-m2(Ds*)* parental allele (spotted), and those co-segregating with *c1-m2(Ds*)*, but without *Ac* (colorless). The last two can arise from replicative transpositions of *Ds** [22].

7. It is preferable to conduct the co-segregation analysis in the absence of *Ac* because secondary transpositions of *Ds** will affect the outcome. The *wx-m7(Ac)* allele, which is the source of *Ac*, will most likely be segregating in the T_3 selfed ear and most of the *Wx/Wx* progeny in that ear will have lost *Ac* by segregation.
8. It is always very informative to run an agarose gel to check the first-round PCR products, and the pattern of the amplicons on the gel can serve as guidance for the performance of the second-round PCR.
9. If the sequence, but not the mutant phenotype, is known, the PCR identification of a *Ds** insertion into the desired sequence is best carried out in pools of individuals carrying a *trDs**. An example of a pooling strategy employed to identify a transposon-tagged sequence can be found in Das and Martienssen [23].
10. Do not spin down DNA by centrifugation because the pelleted DNA is hard to dissolve.

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