An Ac/Ds-mediated Insertional Mutagenesis System for Functional Genomics in Sorghum

Amrish H. Antre, Ramesh S. Bhat, Pushpalatha N.

Department of Biotechnology, University of Agricultural Sciences, Dharwad, 580005, India

Corresponding author email: amrish.antre@gmail.com

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Abstract Transgenic sorghum (cv M 35–1) plants, carrying insertional inactivation tagging Ds, were crossed with iAc plants as the source of transposase to produce mutagenic F₁ plants carrying both Ds and iAc. Screening of F₂ plants for the presence of Ds (Ds⁺), transposed (TDs⁺) and absence of iAc (iA⁺c) could identify eight Ds tagged insertional inactivation tagged mutants. Ds transpositions unlinked to the original site of launch pad (T-DNA+Ds) integration were more frequent (57%) than linked transpositions. Analysis of flanking sequence tags of the Ds among the four mutants showed gene tagging in all. Preliminary phenotyping of the Ds tagged mutants (without ascertaining their zygosity) under green house conditions showed no major difference for morphological traits when compared to M 35–1. With the demonstration of both transiently expressed transposase (TET) system and hybridization of iA⁻c and Ds plants to generate Ds tagged mutants, now it should be possible to develop more mutants for sorghum functional genomics.

Keywords Sorghum; Transposon tagging; Insertional inactivation tagging; Linked and unlinked transposition; Tagged genes

Introduction Sorghum [Sorghum bicolor (L.) Moench] is an important staple food and fodder crop grown worldwide. It is known for high water use efficiency, tolerance to low soil fertility and drought. As a model C₄ grass, sorghum was considered for genome sequencing (Paterson et al., 2009). Approximately 98% of the total predicted genes (34,496) have been placed in their chromosomal context (Paterson et al., 2009). Comparative genomics (Jaiswal et al., 2006), TILLING (Xin et al., 2008), mapping (Billot et al., 2013) and transcriptomics (Dugas et al., 2011) have been widely attempted to identify and study the genes.

Gene disruption with DNA tags provides a direct way of studying gene function (Springer, 2000). T-DNA and transposable element (TE) are the DNA tags used for gene tagging with the latter offering the advantage of developing multiple independent tagged mutants from a few individual starter lines. The ability of TEs to re-transpose from the tagged sites allowing the recovery of revertants offers a unique method of gene function validation. Two-element system of transposon tagging entails a modified autonomous element that expresses a functional transposase but does not undergo transposition. This immobile autonomous element is used along with a non-autonomous element, which can be transposed by the transposase. Two element system with immobile Ac (iAc) and Ds of maize is most widely used for transposon mutagenesis.

Insertional inactivation tagging is the major method of developing mutants through Ds mutagenesis (Springer, 2000; Waki et al., 2013). Insertion of the DNA tag results in gene disruption (inactivation) leading to loss-of-function in insertional inactivation tagging. Previous study with transiently expressed transposase (TET) system in sorghum has demonstrated that insertional inactivated Ds-tagged mutants could be generated as early as in the first tissue culture generation (T₀) (Verma et al., 2011). In addition, mutagenic double transformants were also produced, which could generate mutants in the subsequent generation. But a more general method of developing Ds tagged mutants involves generating independent
transgenics with Ds and iAc, and effecting crosses between them to obtain mutagenic F₁ plants. The F₂ plants showing excision and re-insertion of Ds without containing iAc are recognized as Ds tagged mutants. Alternatively, mutagenic T₀ plants are obtained by double transformation through co-transformation or super-transformation, and Ds-tagged mutants are obtained in subsequent generations. This study describes the development of insertional inactivation tagged mutants in sorghum and checking for Ds transpositions linked and unlinked to original site of Ds integration.

Results

Confirmation of Ds and iAc starter plants

For insertional inactivation, six events of pUR224NA and five events of pNU435 were checked for the T-DNA+Ds copy number. Of the six events of pUR224NA, four events showed the presence of single copy T-DNA+Ds. Among the five events of pNU435, four events carried single copy of T-DNA+Ds as tested by χ² analysis.

Developing mutagenic F₁ plants

For insertional inactivation tagging, seven plants from four different events carrying single copy Ds of pUR224NA were used as female parents. Similarly, eight plants from four different events carrying single copy Ds of pNU435 were used as female parents. Fifteen plants from four different events of pKU352NA were used as male parents (source of transposase). Crossing was done by using simple hand emasculation and pollination.

Confirmation of F₁ plants

From the seven crosses involving four events of pUR224NA parent, a total of 18 true F₁s were obtained. Out of 135 F₁ plants obtained from 8 crosses involving four events of pNU435, 18 were found to be true F₁s. F₁ plants were bagged to ensure selfing. Panicles were harvested at physiological maturity and the F₂ seeds were collected.

Identifying Ds tagged mutants

Screening of 80 F₂ plants involving four events of pNU435 and pUR224NA showed the absence of iAc (iAc⁻), presence of transposed Ds (TDs²) in eight plants, indicating that they were insertional inactivated mutants.

Checking linked and unlinked transpositions of Ds

Of the four Ds tagged mutants obtained from pUR224NA, IIM1 and IIM4 gave an amplification of 450 bp with bar PCR, and three Ds tagged mutants obtained from pNU435, IIM6 gave an amplification of 270 bp with barnase PCR.

Determining tagged gene among mutants

Site of insertion of Ds was checked in four mutants (IIM2, IIM3, IIM6, and IIM7) that showed unlinked transposition. Blast results showed that flanking sequence obtained from IIM2 had homology on chromosome number 6 and Ds insertion was between 767 bp and 768 bp of gene coding rice cyclin family protein (cyclin-T₁–4). Similarly, IIM3 showed insertion of Ds in Sb07g017390 on chromosome number 7.

Ds tagged mutant IIM6 obtained from the launch pad of pNU435 had showing homology with region on chromosome number 8. The Ds insertion was between 1,287 bp and 1,288 bp of Sb08g021000 gene. A FST of 29 bp was recovered from IIM7, and it showed homology to region on chromosome number 1. The insertion inactivation Ds tag was inserted between 3,033 bp and 3,034 bp of Sb01g008710 gene.

Phenotypic evaluation of the mutants

The Ds tagged mutants were observed in for the traits like days to 50 percent flowering, plant height at maturity, number of leaves per plant, number of internodes per plant, panicle length, panicle breadth, number of primaries per panicle, test weight, number of grains per panicle and grain yield per plant as defined by Sorghum Descriptor (IPGRI, 1993).

Discussion

From the large number of Ds launch pad and iAc plants produced in our previous studies in the background of M 35–1, a popular variety of sorghum, those receiving the Ds from pUR224NA and pNU435 were used as the launch pad lines for insertion inactivation tagging. Plants receiving the iAc from pKU352NA were used as the source of transposase for both the types of tagging.
In general, starter lines with low copy launch pads are used (Upadhyaya et al., 2002) for transposon mutagenesis. Such Ds starter lines not only simplify the mutant analysis, but also ensure higher activity of Ds (Kolesnik et al., 2004). In this regard, improvements in the Ds constructs have played a key role in generating starter lines with low copy launch pad as it has been demonstrated with pNU435 (Upadhyaya et al., 2006; Bhat et al., 2011). In this study, Ds launch pad lines carrying single copy T-DNA+Ds as identified by Southern hybridization (Verma et al., 2011) and segregation analysis were selected for initiating transposon tagging. Further, a few plants with 2~3 copies of the launch pad as checked by Southern hybridization were also selected (Figure 1). Ds starter plants showing normal phenotype were used for generating Ds-tagged mutants.

In total, four events each for pUR224NA, pNU435 and pKU352NA were employed for crossing since the use of multiple events of Ds starter lines provides an opportunity for genome saturated mutagenesis (Upadhyaya et al., 2006). Of the twenty two crosses effected, 15 involved the parents with insertional inactivation tagging Ds. (Table 1). F₁s carrying both iAc and Ds (iAc⁺ and Ds⁺) were regarded as mutagenic as they can give rise to Ds tagged mutants. Since the parents used for crossing were not confirmed to be homozygous, the F₁s were tested for the presence of Ds and iAc. From the seven crosses involving pUR224NA Ds parents, 18 F₁ plants showed the presence of both iAc and Ds. Amplification of 497 bp amplicon with iAc (3333:3829) PCR confirmed the presence of iAc. Similarly, the presence of Ds was confirmed with the amplification of a 436 bp product with uidA PCR. Another 18 F₁s originating from eight crosses involving pNU435 parental lines were also mutagenic for insertion inactivation tagging. The F₁ plants showed amplification with uidA PCR and iAc (3333:3829) PCR. Overall, 36 mutagenic F₁s were obtained for insertion inactivation tagging. F₁ plants were bagged to ensure selfing. Seeds were harvested at physiological maturity and the F₂ plants were raised.

F₂ plants that were devoid of iAc but carried the transposed Ds (TDs⁺) as confirmed by Ds excision and Ds re-insertion were selected as Ds tagged stable mutants. Of the 80 F₂ plants involving pUR224NA Ds parent, only 27 plants (against expected 60) carried the Ds as checked by uidA PCR (Figure 2A). Origin of such a low frequency of Ds carrying plants could be due to excision without re-integration of the Ds in the genome as reported earlier (Lazarow et al., 2012).

Among the 27 plants carrying Ds, 11 were devoid of iAc, indicating that the Ds in such plants is stable. Of the 11 plants, four plants showed Ds excision as they could amplify 460 bp amplicon with 35S:bar Ds excision PCR. Remaining seven plants failed to amplify 460 bp product, indicating that the Ds was intact within the T-DNA. As expected, 35S:bar Ds excision PCR with the plasmid DNA of pUR224NA failed to amplify the 460 bp product (Figure 2B). Therefore, those four plants that did not carry iAc (iAc⁻), but carried transposed Ds (TDs⁺) were considered as stable insertional inactivation tagged mutants (IIM1, IIM2, IIM3 and IIM4) (Table 2).

pUR224NA has bar gene as the Ds excision reporter. Upon spraying Basta to 10 day old 20 F₂ seedlings from the cross involving pUR224NA_T₀(50) event of Ds launch pad, only four plants survived, indicating the Ds excision in these plants. Subsequently, the Basta surviving plants were checked with 35S:bar Ds

Figure 1 Southern hybridization for Ds launch pad lines
Note: 1: pUR224NA-T₀(40)–T₁(1)–T₂(5)–T₃(8)–T₄; 2: pUR24NA-T₀(50)–T₁(7)–T₂

In total, four events each for pUR224NA, pNU435 and pKU352NA were employed for crossing since the use of multiple events of Ds starter lines provides an opportunity for genome saturated mutagenesis (Upadhyaya et al., 2006). Of the twenty two crosses effected, 15 involved the parents with insertional inactivation tagging Ds. (Table 1). F₁s carrying both iAc and Ds (iAc⁺ and Ds⁺) were regarded as mutagenic as they can give rise to Ds tagged mutants. Since the parents used for crossing were not confirmed to be homozygous, the F₁s were tested for the presence of Ds and iAc. From the seven crosses involving pUR224NA Ds parents, 18 F₁ plants showed the presence of both iAc and Ds. Amplification of 497 bp amplicon with iAc (3333:3829) PCR confirmed the presence of iAc. Similarly, the presence of Ds was confirmed with the amplification of a 436 bp product with uidA PCR. Another 18 F₁s originating from eight crosses involving pNU435 parental lines were also mutagenic for insertion inactivation tagging. The F₁ plants showed amplification with uidA PCR and iAc (3333:3829) PCR. Overall, 36 mutagenic F₁s were obtained for insertion inactivation tagging. F₁ plants were bagged to ensure selfing. Seeds were harvested at physiological maturity and the F₂ plants were raised.

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Table 1 Crosses effected between Ds and iAc starter plants

| S. No. | Female parent | Launch pad copy number | Male parent | No. of seed set | Mutagenic F1 | Mutant |
|--------|---------------|------------------------|-------------|----------------|-------------|--------|
| 1      | pUR224NA−T0(40)−T1 | 2                      | pKU352NA−T0(19)−T1 | 12               | 18          |        |
| 2      | pUR224NA−T0(40)−T1 | 2                      | pKU352NA−T0(19)−T1 | 9                | IIM1, IIM2  |        |
| 3      | pUR224NA−T0(40)−T1 | 2                      | pKU352NA−T0(19)−T1 | 9                | IIM1, IIM2  |        |
| 4      | pUR224NA−T0(51)−T1 | 1                      | pKU352NA−T0(25)−T1 | 18               | IIM3, IIM5  |        |
| 5      | pUR224NA−T0(50)−T1 | 2                      | pKU352NA−T0(20)−T1 | 30               | IIM4        |        |
| 6      | pUR224NA−T0(50)−T1 | 2                      | PKU352NA−T0(20)−T1 | 16               | IIM4        |        |
| 7      | pUR224NA−T0(54)−T1 | 1                      | pKU352NA−T0(25)−T1 | 14               | IIM6        |        |
| 8      | pNU435−T0(6)−T1(18)−T2 | 1                    | pKU352NA−T0(20)−T1 | 21               | IIM6        |        |
| 9      | pNU435−T0(7)−T1(5)−T2 | 1                   | PKU352NA−T0(20)−T1 | 14               | IIM7        |        |
| 10     | pNU435−T0(6)−T1(18)−T2 | 1                    | PKU352NA−T0(21)−T1 | 13               | IIM8        |        |
| 11     | pNU435−T0(6)−T1(18)−T2 | 1                    | pKU352NA−T0(20)−T1 | 17               | IIM8        |        |
| 12     | pNU435−T0(6)−T1(18)−T2 | 1                    | pKU352NA−T0(20)−T1 | 9                | IIM3        |        |
| 13     | pNU435−T0(5)−T1(1)−T2 | 1                      | pKU352NA−T0(19)−T1 | 22               | IIM3        |        |
| 14     | pNU435−T0(5)−T1(4)−T2 | 1                      | pKU352NA−T0(25)−T1 | 16               | IIM7        |        |
| 15     | pNU435−T0(1)−T1(9)−T2 | 1                      | pKU352NA−T0(19)−T1 | 23               | IIM8        |        |

Figure 2 Identification of Ds tagged mutants by PCR.
Note: A: uidA PCR for the F2s involving pUR224NA Ds parents; M: 100 bp DNA ladder; 1: DNA of non-transgenic plant; 2: pUR224NA plasmid DNA; 3: IIM1; 4: IIM2; 5: IIM3; 6: IIM4; B: 35Sbar Ds excision PCR among the F2s involving pUR224NA Ds parents; M: 100 bp DNA ladder; 1: pUR224NA plasmid DNA; 2: IIM1; 3: IIM2; 4: IIM3; 5: IIM4

excision PCR. Of the four, three plants showed Ds excision, indicating a high efficiency (75%) of Basta selection. Of the three, one had Ds re-insertion (uidA PCR positive), and no iAc [iAc (3333:3829) PCR negative]. Hence, it was also considered as an insertion inactivation tagged mutant (IIM5).

Screening of 80 F2 plants involving four events of pNU435 showed the absence of iAc (iAc), presence of transposed Ds (TDs) in three plants, indicating that they were also IIMs (IIM6, IIM7 and IIM8). Thus eight insertional inactivated mutants were obtained in this study.
Table 2 Genes tagged among the mutants

| Mutant | Chromosome No. | Gene tagged          | Point of Ds insertion (bp) | Gene description                                                                 |
|--------|----------------|----------------------|--------------------------|----------------------------------------------------------------------------------|
| IIM2   | 6              | SORBIDRAFT-06g026720 | 767-768                  | Cyclin family protein -cyclin-T1-4, putative, expressed (Rice)                   |
| IIM3   | 7              | SORBIDRAFT-07g017390 | 1345-1346                | Leucine-rich repeat protein kinase family protein putative, expressed (Rice)       |
| IM6    | 8              | SORBIDRAFT-08g021000 | 1287-1288                | Phytochrome interacting factor helix DNA-binding domain containing protein, expressed (Rice) |
| IIM7   | 1              | SORBIDRAFT-01g008710 | 3033-3034                | Transducin/WD40 repeat-like super family protein putative, expressed (Rice)       |

The new genomic region to which Ds transposed may remain linked or unlinked to the original site where the launch pad integrated. Linked transpositions indicate the transposition of Ds to a nearby region from the launch pad. From the point of view of generating the mutants for all the predicted genes within the genome (genome-wide mutagenesis), it is important to have more of unlinked transpositions, so that a large number of unique mutants covering the whole genome can be generated using a very few (theoretically only one) Ds launch pad plant. In this study, of the four Ds tagged mutants obtained from pUR224NA starter plants, IIM1 and IIM4 gave an amplification of 450 bp with the empty launch pad (T-DNA without Ds) specific PCR (bar PCR), indicating linked transposition of Ds (Figure 3). The other two mutants, IIM2 and IIM3 had unlinked transposition as they failed to amplify 450 bp product with bar PCR. Similarly, of the three Ds tagged mutants obtained from pNU435 starter plants, only IIM6 gave an amplification of 270 bp with barnase PCR (targeting empty launch pad), indicating linked transposition.

In previous studies with a large number of rice mutants, linked transpositions varied from 36%-67% with the majority being within 1 cM of the Ds launch pad (Upadhyaya et al., 2002; Kim et al., 2004). This observation was consistent with the reports both from maize, the original source of Ds (Dooner et al., 1991) and other heterologous systems such as Arabidopsis (Bancroft et al., 1993; Keller et al., 1993; Raina et al., 2002), tobacco (Jones et al., 1990) and barley (Koprek et al., 2000). In this regard, the use of a new transposon system, Spm/dSpm (Kumar et al., 2005) might be more efficient for genome-wide coverage as it could result in 85% unlinked transpositions (Kumar et al., 2005). However, the behaviour of Ds to transpose to a linked site has been exploited for localized mutagenesis in Arabidopsis (Muskett et al., 2003) and rice (Upadhyaya et al., 2006).

Once the Ds tagged mutants are identified, generally they are phenotyped for gross morphological mutations, and analyzed for the tagged gene. Otherwise, the mutants are checked for the site of Ds transposition, and subsequently phenotyped under normal and/or special conditions. In this study, four mutants with unlinked transposition (IIM2, IIM3, IIM6, and IIM7) were analyzed for the site of Ds insertion. It was found that Ds inserted in genic region in all the mutants (Table 2). The high frequency of gene tagging could be due to preferential transposition of Ds into gene coding regions as shown in

![Figure 3 bar PCR specific to the empty launch pad of pUR224NA](image)

Note: M: 100 bp DNA ladder; 1: IIM2; 2: IIM3; 3: IIM1; 4: IIM4
Arabidopsis (Parinov et al., 1999; Raina et al., 2002) and rice (Enoki et al., 1999; Greco et al., 2001; Kolesnik et al., 2004).

The phenotypic evaluation of the mutants (without ascertaining their zygosity) in comparison with M 35–1 for morphological traits like days to 50% flowering, plant height at maturity, number of leaves per plant, number of internodes per plant, panicle length, panicle breadth, number of primaries per panicle, test weight, number of grains per panicle and grain yield per plant showed no major differences. It is too early to speculate on the utility of these mutants for functional genomics. For instance, phenotypic evaluation of insertional inactivation mutants with homozygous mutants under normal or special conditions might give the clear idea about the use of such Ds tagged mutants. However, having demonstrated that both transiently expressed transposase (TET) system and crossing of iAc and Ds lines could yield Ds tagged mutants, now it should be possible to generate more mutants and to analyze them for gene discovery in sorghum.

Materials and Methods

Ds and iAc starter lines

The features of pNU435 and pKU352NA have been previously described (Verma et al., 2011). pNU435 (GenBank Acc. No. DQ225750) has bidirectional gene trap cassettes (eyfp as Ds 5’ trap tracer, uidA as Ds 3’ trap reporter), bar as a transformation/Ds re-insertion marker, hph as an excision marker, and a plasmid rescue cassette. In addition, barnase serves as a counter selector for T-DNA integrations with vector backbone (VB), direct repeat and gene trap. pUR224NA (GenBank Acc. No. DQ225746) has hph as selectable marker, uidA as Ds trap reporter, bar as excision marker and a plasmid rescue cassette. pKU352NA (GenBank Acc. No. DQ225751) has hph as transformation marker, sgfpS65T as an iAc tracer and intron interrupted ubiquitin driven iAc element (Figure 4).

Fig 4 Plants of Mutant and M 35–1 showed no major differences
Note: A: Mutant plants; B: M 35–1 (control)

M 35–1, a rabi genotype of sorghum was used to develop several Ds and iAc starter lines in the previous studies (Verma et al., 2011; Bhat, 2011) at the Department of Biotechnology, UAS, Dharwad, India. Plants transformed with pNU435 and pUR224NA constructs were used as the Ds starter lines for insertional inactivation tagging. iAc starter plants as the source of transposase for insertional inactivation tagging were developed by transforming with pKU352NA. These plants were confirmed for the presence of Ds element by uidA PCR (pUR224NA and pNU435). iAc element was confirmed by iAc (3333:3829) PCR. The primers used for the various PCRs and the expected product size are listed in Table 3. The copy number of the launch pad (T-DNA+Ds)
was checked by Southern blot hybridization using genomic DNA digested with SpI (pNU435), HindIII (pUR224NA) and BamHI (pUbDs). A 436 bp probe corresponding to uidA was labeled using DIG DNA Labeling and Detection Kit (Roche Applied Science, Germany, Cat. No. 11093657910), following its manufacture’s protocol.

Generating mutagenic F1 plants

Ds plants were used as female parent and crossed to iAc plants (male parent) by artificial emasculation and hand pollination to obtain F1 seeds. The mutagenic nature (presence of both Ds and iAc) of F1 plants was confirmed by isolating genomic DNA by following CTAB method with minor modification (Dellaporta et al., 1983) and checking with PCR. Panicles from the PCR confirmed plants were bagged to ensure selfing. Panicles were harvested at physiological maturity and the F2 seeds were collected.

Identifying Ds tagged mutants

The F2 plants were checked for the presence of Ds and iAc using PCR. Ds excision from the launch pad of pNU435 was checked by Ds3′:hph PCR where the forward primer anneals Ds 3′ end and the reverse primer anneals to hph. The amplification of the PCR product would indicate the intact launch pad without Ds excision. Since pUR224NA carried bar gene as Ds excision reporter, the corresponding 10 day old F2 seedlings were sprayed with herbicide Basta (Bayer Crop Sciences Ltd., Mumbai, India) containing glufosinate-ammonium (13.5% w/w) at 12 ppm concentration as standardized previously (Verma, 2010). Plants surviving the herbicide spray were confirmed for Ds excision by 35S:bar PCR, where the forward and reverse primers anneal to CaMV35S and bar flanking the Ds. Amplification of PCR product with 35S:bar PCR indicates the Ds excision.

Checking linked and unlinked transpositions of Ds

Ds tagged mutants were checked for the linked or unlinked Ds transpositions depending on the presence or absence of empty launch pad (T-DNA without Ds), respectively. The presence of empty launch pad of pUR224NA was checked using bar PCR while barnase PCR and uidA PCR were used to check for the empty launch pad of pNU435.

Identifying the tagged gene among the mutants

The gene tagged by Ds among the mutants was identified by recovering the sorghum endogenous sequences flanking the 5′ end of Ds by TAIL-PCR. Ds 5′ specific nested primers; RB94:3–1 (primary TAIL-PCR), RB96:3–2 (secondary TAIL-PCR) and RB98:3–3 (tertiary TAIL-PCR) in combination with arbitrary degenerate (AD) primers were used in TAIL-PCR.

Phenotypic evaluation of the mutants

The Ds tagged mutants were observed in comparison to the control plant for the traits like days to 50 percent flowering, plant height at maturity, number of leaves per plant, number of internodes per plant, panicle length, panicle breadth, number of primaries per panicle, test weight, number of grains per panicle and grain yield per plant as defined by Sorghum Descriptor (IPGRI 1993).

Authors’ contributions

Amrish H. Antre designed and performed research experiments, analyzed the data and wrote the paper; Ramesh Bhat, supervised the project, and Pushpalatha N. generated iAc plants.

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