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Review

T-DNA direct repeat, vector backbone and gene trap counter selection by a new vector (pNU435) for high throughput functional genomics

R. S. Bhat[1]*, S. S. Biradar[1], R. V. Patil[2], V. U. Patil[1] and M. S. Kuruvinashetti[1]

[1]Department of Biotechnology, University of Agricultural Sciences, Dharwad 580 005, India.
[2]Division of Horticulture, of Agricultural Sciences, Dharwad 580 005, India.

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The nature of T-DNA/*Ds* insertion decides the utility of launch pad lines for i*Ac*/*Ds* based insertional mutagenesis. Direct or inverted T-DNA/*Ds* repeats and insertions with vector backbone lead to poor recovery of flanking sequences; whereas T-DNA/*Ds* insertion leading to gene trap would limit the use of such launch pad lines. A new *Ds* tagging/trapping vector, pNU435 containing two copies of intron-interrupted *barnase* was used in tomato to counter select such T-DAN/*Ds* insertions. T_1 and T_2 plants generated in this study were devoid of direct repeats, vector backbone and gene traps as evidenced by the lack of barnase expression. Further evidence based on the LB flanking sequence recovered through TAIL-PCR did not show any vector backbone or direct repeats. But two out of six plants showed inverted repeats. Genome search with LB flanking sequence indicated that the insertions were not in genic region, and hence not led to gene traps. pNU435 with features for counter selecting undesirable T-DNA/*Ds* insertions can be employed for high throughput functional genomics.

Key words: pNU435 vector, T-DNA/*Ds* insertion, *barnase*, tomato, flanking sequence, functional genomics.

INTRODUCTION

Functional genomics aims at assigning function to genes and their regulatory elements of a genome by various approaches (Hirochika et al., 2004; Krishnan et al., 2009) such as comparative genomics, transcriptome analysis, mutagenesis, gene silencing and FOX (full length cDNA over-Expression) hunting (Nakamura et al., 2007), etc. Mutagenesis is a direct way of discovering novel genes and regulatory elements involved in various biological processes. High throughput functional genomics using maize *Ac*/*Ds* system has been widely employed for insertional tagging in various crops (Bancroft et al., 1992; Enoki et al., 1999). Insertional inactivation with *Ds* requires large scale production of launch pad lines harbouring T-DNA/*Ds*. Further, mobilization of *Ds* is brought about by crossing with i*Ac* lines. Launch pad line with a single copy, clean T-DNA/*Ds* insertion [without vector backbone (VB) and repeats] and insertion not being a gene trap is considered as most useful in insertional inactivation. Earlier efforts have observed that about 30-60% of launch pad lines contain T-DNA repeats (direct and inverted) and vector backbones, and such plants are prone to post-integration rearrangements and gross deletions (Jeon et al., 2000; Upadhyaya et al., 2002; Kim et al., 2003; Eamens et al., 2004; Sallaud et al., 2004). Also rescuing the sequence flanking T-DNA is very difficult or in some cases impossible in such lines.

Since T-DNA has preferential insertion in gene-rich regions when compared with repetitive DNA, significantly large number of launch pad lines can be expected to be gene traps (Jeon et al., 2000; Upadhyaya et al., 2002; Kim et al., 2003; Eamens et al., 2004; Sallaud et al., 2004). In rice, ~23% of launch pad lines were gene traps (Eamens et al., 2004). T-DNA/*Ds* gene traps might result in untagged mutations in the stable mutant derived from such launch pad lines. Though the copy number is not under control, recovering clean integration of T-DNA/*Ds* without any gene traps could be manipulated by improvising the vectors used for developing launch pad

*Corresponding author. E-mail: bhatramesh12@rediffmail.com

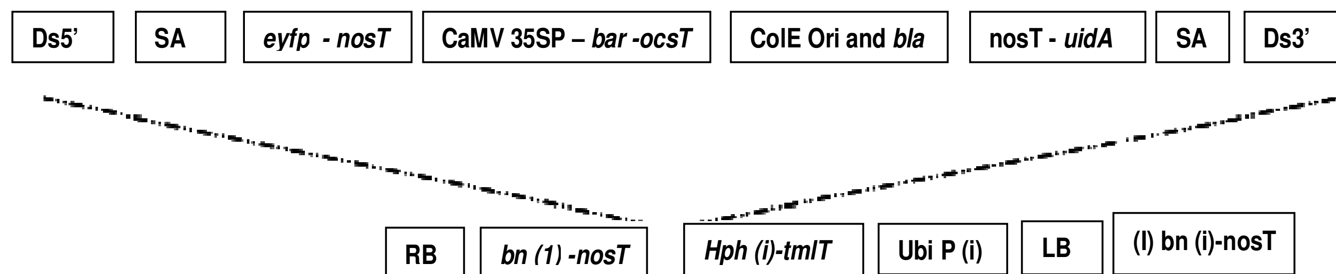

Figure 1. T-DNA/*Ds* of pNU435. 1. A promoterless intron interrupted *barnase-nos*T cassette placed next to RB to serve as T-DNA/*Ds* direct repeat (RB–LB–RB and LB) counter selector and T-DNA/*Ds* gene trap counter selector; 2: maize ubiquitin promoter-first exon-modified intron (with LB repeat sequences incorporated)-intron interrupted *barnase* [bn (i)]-*nos*T, to serve as vector backbone counter selector.

lines. Bidirectional gene trap constructs (Eamens, et al. 2004) utilizing an intron-interrupted *barnase* gene as a VB counter selector outside the T-DNA region of pEU334AN or pEU334BN could reduce the VB containing T-DNA/*Ds* lines (Hanson et al., 1999). However, ~27% lines still contained direct or inverted repeats of T-DNA. Therefore, Upadhyaya et al. (2006) constructed a *Ds* vector, pNU435 (Gen Bank Acc. No. DQ225750) by incorporating features that can counter select lines with T-DNA/*Ds* direct repeat, insertion with VB, and insertion in genic region (gene trap).

However, till date pNU435 has not been tested and validated for its aforesaid activities in any of the systems. In the present study, an effort was made to check T-DNA/*Ds* direct repeat, vector backbone and gene trap counter selection activity of *Ds* vector, pNU435 in tomato. pNU435 (kindly donated by Dr. Narayana Upadhyaya, CSIRO Plant Industry, Canberra, Australia) contained two copies of intron-interrupted *barnase*; one in the VB immediately after ubiquitin promoter-LB sequence and the other immediately after the RB sequence (Figure 1) to counter-select transformants with either VB or direct repeat of T-DNA/*Ds*. *barnase* placed next to RB would also counter select regenerants with T-DNA/*Ds* integration in genic region (gene trap). Seeds of Pusa Ruby cultivar of tomato were sown *in vitro* on half strength MS media (Murashige and Skoog, 1962). Seven-day old cotyledonary leaves were used for co-cultivation by following the standard protocol of *Agrobacterium*-mediated transformation (McCormick, 1991). Co-cultivated cotyledonary leaves were transferred to regeneration medium (MS with 3% sucrose, 2 mg/l zeatin, 0.1 mg l^{-1} IAA, 200 µg ml^{-1} cephotaxime). Shoots were transferred to MS basal medium supplemented with 0.05 mg/l of IBA for rooting. Transgenic plants were confirmed by *gus*-specific PCR (RB59_GUS_F 5' TCACCGAAGTTCATGCCAGTCC 3' and RB59_GUS_R2 5' ACGCTCACACCGATACCATCAG 3') (Figure 2).

Barnase is a bacterial protein with 110 amino acids possesses ribonuclease activity (Hartley, 1988). It is lethal to

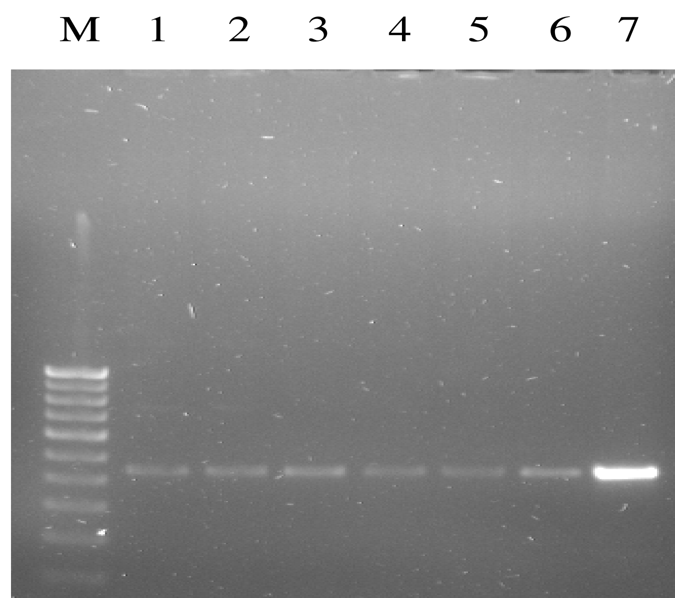

Figure 2. Agarose gel electrophoresis of products amplified by *gus*-specific PCR. Lane M, 100 bp DNA ladder; lanes 1-6, promoter trap lines (PT1, PT2, PT3, PT4, PT5 and PT6); lane 7, Pnu 435 (positive control).

the cell when expressed without its inhibitor barstar. T-DNA/*Ds* integration in genic region leading to either promoter or gene trap would express barnase resulting in cell death. Also in any T-DNA insertion with direct repeat (RB-LB-RB and LB) or with vector backbone (LB-VB), ubiquitin promoter would drive *barnase* gene. But in this study, transgenic plants were normal (without cell/tissue death) and did not show *barnase* expression upon RT-PCR (data not shown) indicating that T-DNA/*Ds* insertion was neither a gene trap, nor associated with direct repeat or vector backbone. Further confirmation that T-DNA/*Ds* insertion was not in geneic region came from right border flanking sequence tag (FST) of T-DNA/*Ds* as recovered by TAIL-PCR (Liu et al., 1995) in randomly selected two plants (PT4 and PT5). These FSTs showed homology to

Figure 3. T_2 plants resistant to Basta. A, Parent (Pusa Ruby); B, Progenies of PT4.

A

1 CAACGACTGA CTGTAGTATT AGGGGATTAG AGTGTCACGT TCCGACACAA TAAGAATAAA GAGAATGAAT

71 CTGGAATTAT GTTAATATAC TCAATTTAAA GAACCTATTT CCCAAGTGAG TATGGTGTGG AGGCTTGAGT

141 CCTCATAGGT GTGCTCGGTG TTGACGCCTA TCCTGAAAAA

B

1 ACGAGCTGAC ATGTAGTATT AGGGGATTAG AGTGTCACGT TCCGACACAA TAAGACTAAA GAGAATGAAT

71 CTGGAATTAT GTTAATATAC TCAATTTAAA GAACCTATTT CCCAAATGAG TATGGTGTGG AAGCTTGTGA

141 CCCCTCATAG GTGTGCTCAA AGTTGACCCC CTATCCTGCA

Figure 4. Left border flanking sequence tag obtained from PT4 (A) and PT5 (B).

RB, indicating the possibility of inverted repeats (LB-RB-RB and LB). This was further tested among the progenies (T_2) of PT4 and PT5. T_2 plants were first confirmed for the presence of T-DNA/*Ds* by spraying BASTA. Majority of the plants could survive the selection (Figure 3). Left border FSTs (Figure 4) recovered using TAIL-PCR in T_2 progenies showed neither repeat nor vector backbone. PT4 and PT5 were therefore confirmed to contain LB-RB-RB and LB inverted repeats without any vector backbone.

Regeneration of such lines with inverted repeats is possible since pNU435 can counter select only direct repeats, but not inverted repeats. BLAST search of these FSTs against tomato genome showed that T-DNA/*Ds* insertion was in retrotransposons like Tork-1 and Jinling-2, but not in any genic region. Therefore, PT4 and PT5, like other plants tested in this study, were confirmed not to be gene traps. Launch pad line with already one of its genes insertionally-tagged/trapped with T-DNA/*Ds*, is not generally employed for generating *Ds* tagged mutants for functional genomics (Sallaud et al., 2004). Reason being, such mutants might carry empty T-DNA (without *Ds*) and *Ds* tags in two different genes leading to untagged mutations. To the best of our knowledge, pNU435 is the only T-DNA/*Ds* vector available to counter select T-DNA/*Ds* direct repeat, vector backbone and gene trap. Majority of the cells upon transformation with pNU435 may not regenerate due to complex insertions, where barnase would express and kill the cell. This was reflected in this study by the relatively low regeneration frequency compared to that obtained with other constructs (data not shown). Thus pNU435 with features for counter selecting undesirable T-DNA insertions at cell level would save time and resources in checking the events for generating launch pad lines for high throughput functional genomics.

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