ORIGINAL RESEARCH ARTICLE OPEN ACCESS

Development of gain of function mutation in rice by tetrad enhancer elements of Cauliflower Mosaic Virus 35S promoter

[1]Krishna Kanth Yadav T, [1]Mahendranath G, [1]Swetha Sharan K, [1]Jagadish P[1], [1]Yamini KN, [2]Muralidhara Rao D, [3]Uma A, [1]Siddiq EA, *[1]Anuradha G

[1]Institute of Biotechnology, Professor Jayashankar Telangana State Agriculture University Rajendranagar, Hyd-30, India
[2]Department of Biotechnology, Sri Krishna Devaraya University, Anantapur-02, India
[3]Center for Biotechnology, Jawaharlal Nehru Technological University, Hyd-85, India
*Corresponding author (email: saps.anuradha@gmail.com)

Received: 6th February 2018, Accepted: 18th May 2018

Abstract

Rice is a primary staple crop for majority of world's population, yet the crop is prone to various biotic and abiotic stresses. Combating these stresses require novel strategies especially for those traits where resistance is not available in the gene pool. Activation tagging (AT), involving random insertion of enhancer elements, is one of the methods to generate mutant lines enhanced for multiple traits. An attempt has been made to develop AT lines in rice by using activation-tagging T-DNA vector (pDEB) carrying a non-autonomous *Dissociation* (*Ds*) element having 4X *CaMV* 35S enhancers and bar gene is used as a plant selection marker. Transformation of popular indica rice cultivar BPT5204 was done using *Agrobacterium*. The putative transformants were selected on (Murashige and Skoog) medium containing phosphinothricin (PPT). The tagged plants (T_1 and T_2) were confirmed by PCR with enhancer and bar gene specific primers. These tagged homozygous plants were advanced to identification of mutants with gain of function for important traits.

Key words: Activation tagging, mutant lines, *Ds* element.

Introduction

Cultivation of high yielding rice varieties and improved cultural practices have imparted to global increase in rice production. The current scenario demands increase in rice production almost to double by the mid-21st century to fulfill the needs of the exponentially increasing world population, expected to cross the mark of 7.7 billion by 2020 (http://www.worldometers.info/).

The main constraints dampening the efforts of improving rice productivity by biotic and abiotic stresses include outbreak of diseases due to infection by various pests, salinity stress, heat stress, scarcity of water, poor irrigation habits, inferior soil quality (Allara et. al., 2012; Deepa Sankar et. al., 2011; Redman et. al., 2011; Peng et. al., 2004).

Rice plant is model crop for functional genomics. Because of its small genome size and genomic resources eventually guided its way to be the first monocot species to undergo whole genome sequence. The release of rice genome sequence has made way to the identification of gene functions (Li et. al., 2018; Zhang et. al., 2017; Singh et. al., 2016; Kim et. al., 2014). Mutant population serves as essential tools for analyzing plant gene functions. Mutants can be generated by chemical, physical as well as biological methods such as ethyl methanesulfonate application, fast neutron irradiation, T-DNA and transposon insertion (Shuyan Wan et. al., 2009). However, these approaches usually cause loss-of-function mutations and not applicable to dissecting the function of redundant genes. Also, it is difficult to conclude the gene function if loss of function mutation results in early embryogenic or gametophytic lethality owing to the involvement of the genes in multiple stages of life cycle (Jeong et. al., 2002; Weigel et. al., 2000).

Out of the various strategies which have surfaced to deal with the aforesaid shortcomings, the enhanced expression of genes through activation tagging system, which provides gain-of-function phenotype, has proved to be a productive identification strategy. *Ds* element has no transposition activity because of absence of transposase enzyme. *Ds* elements are internal deletion derivatives of the *Ac* element. The first ever direct gain-of-function mutation in plants, utilized the enhancer element from

the cauliflower mosaic virus (CaMV) 35S gene (Odell et. al., 1985). T-DNA vectors bearing four copies of this CaMV 35S enhancer element were utilized successfully for generation of activation-tagged lines and mediating transcriptional activation of nearby genes in Arabidopsis and identify a number of novel functional genes (Borevitz et. al., 2000; Li et. al., 2001, 2002; Neff et. al., 1999). This technology is being deployed in diverse plant species such as petunia (Zubko et. al., 2002); tomato (Mathews et. al., 2003) and rice (Jeong et. al., 2002). In this study, an attempt was made towards development of activation tagged lines in elite indica rice variety BPT 5204 by deploying the *Agrobacterium* mediated transformation.

Materials and Methods

Plant materials: Mature seeds of popular indica rice cultivar BPT 5204 were obtained from the Seed Research and Technology Centre (SRTC), Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad.

Transformation vectors: The Activation Tagging vector pDEB was provided by Dr. K. V. Rao (Centre for Plant Molecular Biology Osmania University, Hyderabad). The pDEB vector carried a non-autonomous *Dissociator* (*Ds*) element, bar gene and tetramer of the transcriptional enhancer of *CaMV* 35S (4XEn) were located between the 5′ and 3′ *Ds* termini. The T-DNA was in the back bone of pCAMBIA-2300 (Fig.1).

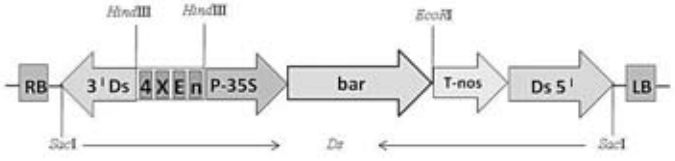

Figure 1: T-DNA of the activation tagging vector pDEB.
RB and LB, Right and Left borders of the T-DNA; Bar gene is selection marker for *Ds* element; 4x En, a tetramer of CaMV35S enhancers; *Hind*III, *E.co*RI and *Sac*I are restriction sites. The T-DNA was in the back bone of pCAMBIA-2300.

Plant Transformation: Regeneration and *Agrobacterium* mediated genetic transformation protocols were described by Manimaran et al., 2013 with some modifications. Mature seeds of BPT5204 were cultured on MS medium supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kinetin for highest callus induction. Proliferated calli was transferred onto MS medium containing 4 mg L^{-1} ABA which produced actively growing embryogenic calli which were used as explants for transformation. After co-cultivation, the calli were rinsed in cefotoxime (250 mg L^{-1}) for 5 min followed by 4 washes with sterile distilled water to remove excess of *Agrobacterium* from calli. Then calli were blot dried and transferred on to MS medium supplemented with 5 mg L^{-1} PPT. After one week, surviving calli were further transferred to regeneration medium (MS containing 2 mg L^{-1} BAP) under light for 2 to 3 weeks which lead to induction of shoots. Shoots were sub-cultured again in the same medium and sufficiently grown shoots transferred to rooting medium (1/2 MS basal salt, 15 g L^{-1} sucrose) to induce roots. Fully rooted plants were transferred to trays contain sterile vermiculate for two weeks, then these plants were transferred to transgenic greenhouse.

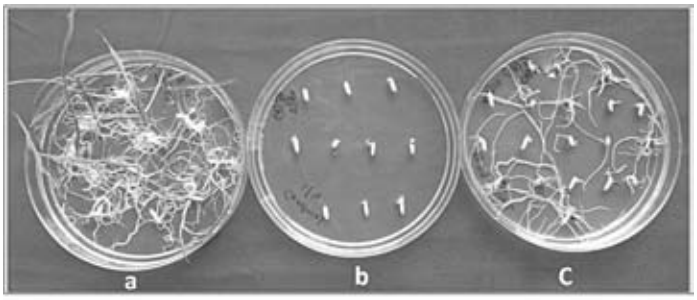

Figure 2: Putative transgenic seeds on selection media containing PPT 5 mg L^{-1} concentration

a. Untransformed control seed without PPT, b. Untransformed control seed with PPT, C. Putative transgenic seed with PPT.

DNA isolation and PCR analysis of transformed plants: Genomic DNA was extracted from young leaves of putative transformants and untransformed control rice plants by using CTAB method (Doyle and Doyle, 1990). The DNA was later analyzed by PCR with the gene specific primers. The set of primers for enhancer (En) element and bar gene were; enhancer: En F 5' CAAAGGGTAATATCGGGAAACC 3' and En R 5' TCACATCAATCCACTTGCTT 3' bar F: 5' CGAGACAAGCACGGTCAACTTC 3' and bar R: 5' AACCCACGTCATGCCAGTTC 3'. PCR reaction was set up a total volume of 15 µl containing, 1x PCR Buffer, 0.25 mM dNTPs each, 2.5 mM $MgCl_2$, 0.1 µM forward primer, 0.1 µM reverse primer, 1U *Taq* DNA polymerase and 2 µl of template DNA (50 ng). Thermal cycling conditions were: initial denaturation at 94°C for 5 min then 35 cycles of denaturation at 94°C for 30 sec, annealing at 58° C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 7 min. The plasmid DNA was used as positive control and non transformed rice plant genomic DNA was used as negative control for the PCR reaction. The PCR amplified products were electrophoresed on 1.5% agarose gel and analyzed on Gel Documentation system (Bio-RAD).

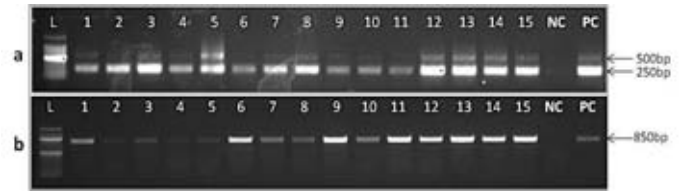

Figure 3: Molecular confirmations of putative transgenic plants.

a. Confirmation of 4XEn in lanes 1-15 plants with en F and R primer, b. Confirmation of bar gene in lanes 1-15 plants with bar F and Nos R primer, PC; Positive control (pDEB plasmid), NC; Negative control (untransformed BPT 5204).

Phenotypic evolution of mutated lines: Approximately 700 transformed tagged plants (T_1) were used to screen the phenotypic characteristics. The data was recorded systematically for all these T_1 transformed lines. These transformed lines were separated into different categories based on phenotypic observations like plant height, number of tillers, leaf length, leaf width, heading date and variations in root length. From each T_1 mutated tagged lines, 10 seeds were propagated for T_2 generation and selected for phenotypic similarities with previous generation. The selected similar T_1 and T_2 phenotypic mutants were further propagated to next generation.

Results

Generation of activation tagged lines

More than 3000 embryogenic calli were co-cultivated with *Agrobacterium (*strain, *EHA*105) containing DEB vector. After co-cultivation, all these calli were subjected to selection medium supplemented with 5 mg L^{-1} PPT. After repeated subculture, Approximately 700 plantlets (T_0) were produced after survived on plant selection medium supplemented with 5 mg L^{-1} PPT. Among them, only 165 plants had shown expected PCR amplification with gene specific primers of bar gene and enhancer element. From the selected PCR positive T_0 plants, 20 seeds (T_1) were subjected to selection medium containing 5 mg L^{-1} PPT (Table.1). Out of 165 lines only 105 lines shown Mendelian segregation ratio of 3:1. All survived plants were transfer to transgenic green house out of these only 66 lines shown the expected amplicon size. All the PCR positive transgenic plants were maintained in transgenic green house and phenotypic variations was recorded followed by seeds were collected from these positive transformants. 20 seeds from each 66 lines were subjected to selection medium containing 5 mg L^{-1} PPT and these plants were maintained in transgenic green house followed by observing phenotypic similarities with previous generations.

Table 1: Transformation efficiency based on PPT selection and PCR confirmation

Vector used for transformation	Generation	No. of calli co-cultivated	No. of explants survived on PPT selection media	No. of calli regenerated	No. of plants produced in regenerated media	No. of plants shows PCR +ve
pDEB	T_0	3,000 (calli)	700	490	680	165
	T_1	3,300 (seed)	480	-	480	66
	T_2	660 (seed)	220	-	220	38

Phenotypic evolution of mutated tagged population

The phenotypic evolutions of tagged lines were screened based on different morphological characteristics. For this study we used approximately 700 putative T_0 transformed plants and these plants were phenotypic screened for further two generations (T_1 and T_2). The frequency of the phenotype being carried to next generation (T_2) was very low in T_1 mutated lines. Mutated lines of STD3, STD8, STD17 and STD23 showed a dominant plant height ranging from 72.37-82.27 cms with productive tillers ranging from 13-17.67 cms compared to untransformed control plants (Table. 2). These mutated lines are valuable resources for functional genomics studies.

Table 2: Phenotypic data of mutated tagged lines

Tagged line	Plant height (cm)				No. of productive tillers			
	T_1		T_2		T_1		T_2	
	UC (mean±SE)	ML (mean±SE)	UC (mean±SE)	ML (mean±SE)	UC (mean±SE)	ML (mean±SE)	UC (mean±SE)	ML (mean±SE)
SDT 3	71.5±0.87	72.36±0.88	73.9±1.02	76.66±0.67	9.66±0.33	14.33±0.88	11±0.58	16.66±0.88
SDT 8	72.8±0.12	76.73±2.26	75.13±0.64	82.26±0.67	9.00±0.58	13.66±0.88	10±0.58	17±0.58
SDT 17	75.46±0.66	80.96±0.90	76±0.23	81.5±0.71	10.33±0.88	16.66±0.88	12±0.58	17.33±0.88
SDT 23	75.6±1.44	77.33±0.88	76.83±1.20	79.16±0.44	11±0.58	14±0.58	10±0.58	13±0.58

UC: untransformed control, ML: mutated line, T_1 and T_2: first and second generations. The data was considered statistically significant at $P< 0.05$ using one way ANOVA.

Discussion

The present study utilized an activation tagging vector pDEB in which a T-DNA carries a *Ds* element having a 4X *CaMV* enhancers, while the bar gene act as selectable marker. Even though large T-DNA insertion libraries have been generated in rice, effective T-DNA transformation system is present only for japonica subspecies (Hiei et. al., 1994). However difficulties are encountered during T-DNA transformation of the widely cultivated indica rice subspecies (Lin et. al., 2005). Nevertheless, transposon mutagenesis is a useful approach for plant functional genomics (Qu et. al., 2008). In T-DNA vector, *Ds* element carries four copies of *CaMV*35S enhancer elements. These tetrameric enhancers could mediate the activation of genes by interacting with their transcriptional factors. The activation of genes is based on the proximity of genes to transgene insertion site. Previous reports studied the activation of genes in different genetic distances (7 kb and 12.5 kb) from the insertion site (Hsing et. al., 2007, Wan et. al., 2009). The plants generated by using this vector were stable, because no transposition occurred in *Ds* element.

Some of these transformants showed phenotypic variations compared to the untransformed plants. These variations would be further confirmed for inheritance by studying these traits in further generations. We observe more number of phenotypic mutants in T_1 transformants like plant morphology, leaf morphology, root length difference, seed morphology, number of productive tillers and number of days to flowering. Among these phenotypic mutants plant height and number of productive tiller mutants were inherent to further generation. Some studies reported that phenotypic variation in activation tagged lines of javanica rice (Fladung et. al., 2012), japonica rice (Yang et al., 2013) and indica rice (Reddy et. al., 2018). Zhu and co-workers (2017) studied mutant phenotypes and identified a new uncharacterized gene involvement in spikelet heading date in rice. Dominant mutants were observed at a frequency of 0.3% (Tani et. al., 2004). Similar percentage of dominant mutations was observed in our study. Among all, four mutant lines showed dominant mutations in T_1 and T_2 generations. These mutant lines were confirmed by PPT selection and also positive results were obtained in PCR with transgene specific primers. These AT mutant lines developed in this study would be propagated further for screening of important traits. AT lines with enhancement in specific traits of importance could be a storehouse of vast and valuable informations which will be useful to the rice growers and researchers.

Acknowledgements

The authors gratefully acknowledge the financial support from Department of Biotechnology (DBT) through a collaborative project and we thank to P. J. T. S Agriculture University for providing facilities and support to carry out this work at Institute of Biotechnology (IBT).

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