IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Activation Tagging In The Switchgrass (*Panicum Virgatum* L.) Plant By A Constructive Vector pSQ5

Vinod Dadarao Parde[1,*], Iqbal Najiroddin Shaikh[2]

[1]Department of Biochemistry, AKI's Poona College of Arts, Science and Commerce, Camp, Pune, India.

[2]Department of Chemistry, AKI's Poona College of Arts, Science and Commerce, Camp, Pune, India.

ABSTRACT: Switchgrass (*Panicum virgatum* L.) is one of the most promising biomass crops for the production of biofuel these days because it is rich in natural diversity, and has adapted to a large portion of the global continents and sub-continents. Despite the increasing importance as a dedicated energy crop, we know little about the basic biology underlying the agronomic traits related to feedstock production. Compared to other crops or high-value forage plants, the genetic and genomic resources suitable for switchgrass molecular breeding, biotechnology, and molecular biology research are still lagging far behind. Our aim to develop functional genomic tools for switchgrass. Switchgrass has large genome and it is not sequenced. Hence it is necessary to develop new molecular markers which will accelerate breeding. Activation tagging involves mutation in the gene which is mutated by transposon. Switchgrass has polyploidy hence it is necessary to have a mutation by overexpressing gene. This can be done by activation tagging which involves use of enhancer in transposon so it can cause overexpression of gene at the site of insertion which can result in morphological or physiological changes.

Keywords: Biomass, Genomic Tools, Activation, Insertion, Gene

I. INTRODUCTION

Transposon system was first discovered by Barabara McClintock in maize and she named it as jumping genes [1]. She discovered Ac(Activator)/ Ds (Dissociator) system in maize. In this system Ds is dependent transposable element and Ac is independent transposable element which codes for transposase i.e. the enzyme required for transposon to cut from one location and paste at another location.

As a self-incompatible and highly heterozygous perennial grass, switchgrass has two major ecotypes, lowland and upland, which are either tetraploid (2n=4x=36) or octoploid (2n=8x=72) with a few exceptions [2, 3, 4]. The genetic complexity of switchgrass largely contributes to the difficulty of direct application of genetic and

genomic tools developed for model plants. Therefore, it is time to develop suitable reverse functional genetic tools that can help annotate the biological functions of switchgrass genes controlling biomass traits. Genetic mutants are critical for functional analysis of plant genes. Transposon tagging is a versatile tool for generating large collections of the gene knockouts in cereal plants like maize and rice, which are inefficient for T-DNA insertion mutagenesis. To harness the benefits of transposon insertion mutagenesis while circumventing the difficulties presented by the polyploid genome of switchgrass, researchers developed the transposon-based activation tagging system in different plant species [5, 6].

Thermally Asymmetric Inter-Laced (TAIL) PCR was first used by Liu for isolation of flanking sequence of T-DNA insertion [7]. It involves use of thermally asymmetric PCR cycles for amplification of flanking sequence. In the pair of primers required for PCR reaction one primer used is short degenerating primers and as other primers vector specific primer is used. Short arbitrary designed primer can be used alone or it can be pulled with other short arbitrary primers and used together. There are three sets of PCR cycles which are carried out in consecutive order. Short arbitrary degenerate primers are arbitrary designed and hence they are universal in application. Specific primers are the ones which are designed from the left or right border of T-DNA of vector and facing towards the site of junction of vector insert and g-DNA. There are total three vector specific primers which are nested. In the pre-amplification reaction one SAD primer is used along with one specific primer. In second PCR we use same LAD primers and second vector specific primer which is nested within the first. In third set of PCR we use again same SAD primer and we use third specific primer which is nested in first two primers. Thus at the end we get amplification of specific product. It can be sent for sequencing. PCR cycles used here are thermally asymmetric since the annealing temperatures are altered in cycles. Lower annealing temperatures are used by arbitrary degenerate primer to bind to genomic sequence and higher annealing temperature are suitable for binding by border specific primers. This was further modified by Liu and they called it as Hi (High efficiency) TAIL PCR [8]. In this modification they used long arbitrary degenerate primer (LAD) in pre-amplification reaction along with first border specific primer. In second set of PCR they used AC primer along with second nested vector specific primer. In third set of PCR they used third nested vector specific primer along with AC primer. LAD primers have been designed arbitrary and they have degenerative sequence at their 3 prime end hence they are not specific to any organism and universal in application. AC (Arbitrary conserved) primer has conserved sequence from LAD primer and by using same primer in second and third reaction we get amplification of specific PCR product.

In this study, we tried to test if we can establish the transposon activation tagging system in switchgrass. It was also optimized the Hi TAIL-PCR protocol and successfully identified the flanking DNA sequences of seven transposon activation mutants in switchgrass.

II. MATERIALS:

1. Plant material:

Genetic transformation of HR8 variety was done previously by Xu B. in 2011 [9]. He had generated 18 transgenic plants which were used for further analysis in my experiments.

2. Vector for activation tagging:

We used vector pSQ5 which was previously used by Qu in the 2008 in rice for activation tagging [10]. This vector has GFP in cis with Ac and RFP in cis with Ds element. Thus transgenic plants can be screened under fluorescence microscope without need to carry out any assays. It has hygromycin as selection marker for selection after transformation.

III. METHODOLOGY:

1. **Hi-TAIL PCR:**

50 ng DNA in working solution with hygromycin specific primers and LAD or Ac primers were used for PCR. TaKaRA kit with Taq polymerase, dNTP and PCR buffer was used along with PCR cycle as described by Liu [8] . For all PCR machine MyThermocycler by Biorad was used.

For R0 reaction Hyg0A and LAD 1-2 and LAD 1-4 were used. Water was used as negative control and plasmid was used as positive control. PCR products were diluted further with water in the ration of 1:39 and 1 µl from this dilution was used for further PCR reactions. For R1 reaction Hyg1a and AC1 were used. PCR products were further diluted 1:10 with water and 1 µl from this reaction was used for further reactions. For R2 reaction Hyg2a along with AC1 was used. Amplified products of R1 and R2 reactions along with 1Kb plus marker (Invitrogen) were run on the 0.8% agarose gel at 120 V for 30 minutes. It was stained with ethidium bromide and observed under gel doc machine Biorad. These amplified bands which are of more length than 500 bp were supposed to have some flanking genomic sequence and they were send for sequencing. Bands which were less than 500 bp were supposed to have only vector sequence and they were not analyzed further.

2. Gel purification and sequencing:

Bands were purified for DNA before sending for sequencing. Gel purification was done by using kit from Epoch Lie Science. After gel purification quantity of DNA in gel extract was measured by nanodrop at 280 nm. Amount of DNA was measured to be around 8-12 ng/ml. Then samples were sent for sequencing at Core Lab Tech.

3. Analysis of flaking sequences by alignment and NCBI search:

Alignment in Lasergene:

DNASTAR Lasergene was used for alignment with vector sequence. Clustal W method was selected for alignment.

BLAST search at NCBI:

At NCBI blast was used with ‘others (nr etc.) for database selection and we selected optimized program for ‘somewhat similar’ sequence. From the resulting different accessions, Sequence showing highest query coverage and lowest E value were selected as possible match.

IV. RESULTS:

The T-DNA of pSQ5 carries both GFP and RFP genes that could be used as selection markers for identifying transformation events. In this study, 19 pSQ5 T0 transgenic plants were screened by observing GFP expression and PCR assay for detecting presence of transgenes. As shown in Fig. 1, young roots and leaves of each putative transgenic plants were collected and observed under a fluorescent microscope. We screened 19 lines, where 13 lines showed strong GFP fluorescent signals. It was used wild type control plants grown as negative control to correctly distinguish the presence of GFP signal. It was also attempted to observe RFP signal by using the same method; however I failed to observe any RFP signal due to possible masking effect of auto fluorescent signal caused by chlorophyll.

Southern blot with a hygromycin probe was used to further confirm genome integration of transposon constructs and determine the copy number of T-DNAs. As shown in Fig. 2, seven out of eight transgenic lines showed presence of insert. pSQ5-25 does not show presence of any insert. pSQ5-32 and pSQ5-28 are showing presence of single insert. pSQ5-8, pSQ5-26, pSQ5-27, pSQ5-33 show two inserts. pSQ5-33 are show presence of three copies of insert. As we had previously screened plants with GFP marker gene, we know that many plants has Ac element. Presence of the Ac element indicates that it could activate jumping of the DS element. Thus after transformation the Ds element can jump within plants many times. This probably gave rise to the multiple insertions in some plants.

As shown in Fig. 3, it was to identify the flanking DNA sequences of the T-DNA insertions, we used TAIL-PCR primers LAD1-2 and LAD 1-4 to amplify the transgenic genomic DNA. It was tested all transgenic lines by using this primer combination. However, we still could not get specific amplifications. So, it was used again another pair of primers namely LAD 1-1 and LAD 1-3. However, this primer combination could not amplify the products that were not previously amplified by LAD1-2 and LAD 1-4. In the future, products that were not amplified should be tested with new specific primers from either left border or right border. It was found that amplified products, which were more than 500 bp in length, only could give some hits in NCBI search. Amplified products that were less than 500 bp gave vector sequence when blast search of NCBI Genbank.

As shown in Fig. 4, there is phenotype difference in PSQ5 tagged plants and wild type plants. These differences are related to habit type, flowering timer and tiller number. Figure 4 is showing example of difference in tillers. PSQ5-1 is showing reduced number of tillers as compared to wild type.

V. DISCUSSION:

To determine the functions of sequenced genes in an organism, gene is disrupted or mutation is generated in the genes and sequences are analyzed. However, site directed mutagenesis is not possible in all organisms. T-DNA insertion offers some advantages like stability of insertions. However, it is labor intensive for recalcitrant plants [11]. In traditional breeding chemical induced mutagenesis, radiation induced mutagenesis are tools that are used for mutations. However, these approaches are not applicable to switchgrass because switchgrass is recalcitrant for transformation and it is tetraploid so most of the genes are expected to act redundantly. However; T-DNA activation tagging is based on the overexpression of a gene and it can be applied to switchgrass. Plants have some endogenous coloration due to plastids and hence the RFP signal was not observable. Due to lack of RFP, we could not prescreen for presence of Ds in the plants. We could not get amplification in all plants. So it is possible that due to activity of the tranposase Ds has already jumped. Activation tagging is useful as we can observe phenotype changes if plants are mutated. Also due to jumping of transposons we can get seeds with different mutations from single transformation [12]. We can use these plants further for more activation tagging analysis. Plants with single insertion should be crossed with wild type to create seeds of T1 plants. Plants that gave hits in the switchgrass and graminaceae are useful for designing SNP marker for mapping in switchgrass crosses. If some mutation has occurred then we can develop allelic SNP markers and place it on the genetic map of switchgrass [13]. However, we need to get more flanking sequences from transposon tagging so that we can begin this mapping work. For this purpose we need to get more transposon-tagged mutants [128].

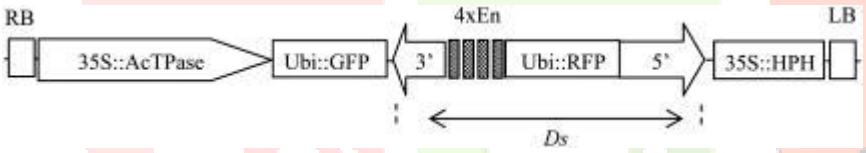

Figure 1.

T-DNA of the activation-tagging *Ac-Ds* vector pSQ5. RB and LB, Right and left borders of the T-DNA; Ubi, maize ubiquitin 1 promoter; GFP, GFP gene as a negative selection marker; RFP, RFP (or DsRed) gene as selection marker for the *Ds* element; 4xEn, a tetramer of CaMV 35S enhancers; HPH, hygromycin phosphotransferase gene as plant transformation selection marker. The T-DNA is in the backbone of pCAMBIA-1300.

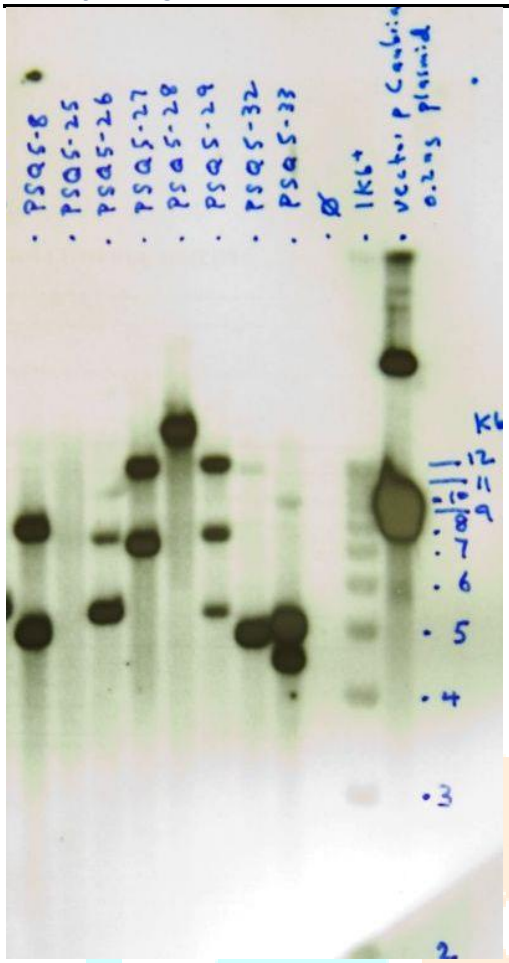

Figure 2.
Southern blot analysis with hygromycin gene probe: Genomic DNA extracted from transgenic plants maintained in greenhouse was digested with Hind III restriction enzyme. Restriction sites for hind III are absent in hygromycin gene. Lanes- 1:pSQ5-8, 2: pSQ5-25, 3: pSQ5-26, 4:pSQ5-27, 5:pSQ5-28, 6:pSQ5-29, 7:pSQ5-32, 8:pSQ5-33, L: Ladder, -C: water as negative control, C: pCambia1305 plasmid DNA that carry a Hygromycin gene was used as the positive control.

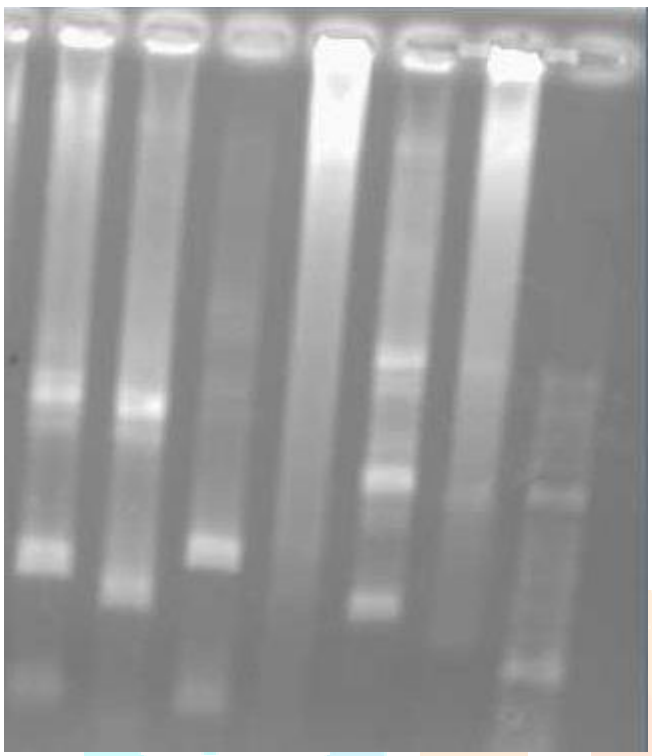

Figure 3.
HI TAIL-PCR analysis: The flanking DNA sequences of selected pSQ5 lines were amplified by HI TAIL-PCR. Transgenic plant names ID: pSQ5:26, pSQ5:32, pSQ5:5

R1: primary HI TAIL-PCR amplification, R2: secondary HI TAIL-PCR amplification, L: 2 Kb marker

A B

Figure 4.
Phenotype of one pSQ5 transgenic plant. (A): Wild type control plant grew normally in greenhouse, (B): Transgenic plant pSQ5-1 grown under the same conditions produced much less tillers in comparison to control plants.

ACKNOWLEDGMENT:

We are thankful to AKIs Poona College of Arts, Science & Commerce teaching, non-teaching and technician staff for providing adequate support.

REFERENCES

[1] McClintock, B. 1950. Origin and bahavior of myatable loci in maize. Proc. Nat. Acad. Science. USA, 36:345-355.

[3] Talbert, LE. TD., Burns, J.C., Rawlings, J.O. and Moll, R.H. 1983. Estimates of genetic-parameters in switchgrass. Crop Sci. 23,725-728.

[4] Parrish, DJ. FJ. 2005. The biology and agronomy of switchgrass for biofuels. Crc. Rv. Plant Sci., 24:423-459.

[5] Fladung, M. and Polak, O. 2012. Ac/Ds-transposon activation tagging in poplar: a powerful tool for gene discovery. BMC Genomics, 13(61), 1113-1161.

[6] Schneider, A. Kirch. T., Gigolashvili, T., Mock, H.P., Sonnewald, U., Simon, R., Flugge, U.I., Werr, W. 2005. A transposon-based activation-tagging population in Arabidopsis thaliana (TAMARA) and its application in the identification of dominant developmental and metabolic mutations. FEBS letters, 579(21), 4622-4628**.**

[7] Liu, Y. MN., Oosumi, T. and Whittier, R. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal assymetric PCR ibterlaced PCR. The Plant Journal, 457-463

[8] Liu, Y. CY. 2007. High-efficiency thermal assymetric interlaced PCR for ampliication of unknown flanking sequences. Bio.Techniques, 43,649-656

[9] Xu, B. HL., Shen, Z. Welbaum, G., Xang, X. and Zhao, B. 2011. Selection and characterisation of a new switchgrass (*Panicum virgatum*) line with high embryogenic capacity for genetic transformation. Scientia Horticulturae, 129,8854-8861

[10] Qu, S. DA., Wing, R. and Sundersan, V. 2008. A versatile transposon based activation tag vector system for functional genomics in cereals and other monocot plants. Plant Physiology, 146, 189-199

[11] Ramchandran, S. SV. 2001. Transposons as tool for functional genomics. Plant Physiol. Bioch., 39, 243-252.

[12] Fitzmaurice, W NL., Wernsman, E., Thompson, W. and Conkling, M. 1999. Transposon tagging of the sulfur gene of tobacco using engineered maize Ac/Ds elements. Genetics, 153, 1919-1928.

[13] Qu, S. DA., Wing, R. and Sundersan, V. 2008. A versatile transposon based activation tag vector system for functional genomics in cereals and other monocot plants. Plant Physiology, 146, 189-199.

[14] Fladung, M. PO. 2012. Ac/Ds-transposon activation tagging in poplar: a powerful tool for gene discovery. BMC Genomics, 13, 1-15.