Generation of Tnt1 insertional mutants in moss (*P. patens*) and their molecular characterization

A thesis submitted towards partial fulfillment of

BS-MS Dual Degree Program

By

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Under the guidance of

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Certificate

This is to certify that this dissertation entitled "**Generation of Tnt1 insertional mutants in moss (*P. patens*) and their molecular characterization**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by "**Vyankatesh Rajmane**" at Indian Institute of Science Education and Research, Pune under the supervision of Dr. Anjan K. Banerjee, Associate professor, Biology Department" during the academic year 2015-2016.

Signature of the Supervisor
(Dr. Anjan K. Banerjee)

Date: 10/11/2016

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Declaration

I hereby declare that the matter embodied in the report entitled "**Generation of Tnt1 insertional mutants in moss (*P. patens*) and their molecular characterization**" are the results of the investigations carried out by me at the Department of Biology, IISER Pune, under the supervision of **Dr. Anjan K. Banerjee** and the same has not been submitted elsewhere for any other degree.

Vjmane

Signature of the Student **Date:** 10/11/16

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(Name of the Student)

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List of Abbreviation

LTR	Long terminal repeats
GUS	β-glucuronidase
Cyto	Cytokinin
BAP	6-Benzylaminopurine
NAA	1-Naphthaleneacetic acid
IAA	Indole-3-acetic acid
ABA	Abscisic acid
GRN	gene regulatory networks (GRN)
GSP	Gene specific primer
TAIL-PCR	Thermal Asymmetric Interlaced PCR

Abstract

Morphogenesis in plant development has been of great interest for researchers engaged in plant physiological studies. Complex tissue types of higher plants make these studies difficult. A lot of work has been done on the development of plants but, we still lack crucial information on gene regulatory networks (GRN) involved in gametophytic development. *Physcomitrella patens* (moss) is a simple, non-vascular plant belonging to bryophyte family shows relatively simple tissue types but still reflects all major developmental patterns of higher plants. We focused on the GRN involved in bud formation and its transition to gametophore in moss. In this regard, tobacco retrotransposon (Tnt1) and T-DNA was chosen as insertional mutagenesis tool to develop mutants in moss. At the time we started our study, there was no literature available on the protocol for Tnt1 retrotransposon use in moss. We established Tnt1 as mutagenesis tool in moss. In our study, TAIL-PCR analysis showed that Tnt1 retrotransposon has specificity to the gene rich region. A recent study on the use of Tnt1 as mutagenesis tool in moss, supports our data (Vives et al., 2016). We found that LTR promoter of (Tnt1) retrotransposon and modified LTR promoter (mLTR) were inducible by $CuCl_2$, Auxin (IAA), Salicylic acid treatments and temperature gradient. Our forward genetic screen yielded a mutant deficient in bud development (LTR-GUS line 7) and two mutants showing less gametophore formation (LTR-GUS line 4 and Tnt1 line 13). In our analysis, Tnt1 line 13 was found to be deficient in sensing gravity. Overall, this study shows that Tnt1 can be efficiently used as a mutagenesis tool in moss. Further study on these mutant lines will throw light on genes involved in bud and gametophore development.

Chapter 1

1. Introduction

1.1 The model system: *Physcomitrella patens*

Mosses (Bryophytes) are the group of multicellular and non-vascular land plants which share common ancestors with vascular land plants. These plants are of great importance to study evolutionary development of vascular plants. Among mosses, *Physcomitrella patens* is most studied and well established model system (Cove et al., 1993). The important features that make *P. patens* as a desirable model system are its small size, comparatively short life cycle and fully available, well annotated genome sequence. *P. patens* exhibit dominant haploid gametophyte phase which facilitates generation of gene knock out lines along with many other benefits in forward and reverse genetics studies compared to a diploid model system. *P. patens* is the only known plant model system with characteristic of highly efficient gene targeting due to the high frequency of transgene integration in genome by homologous recombination (Cove et al., 2009). This makes it unique model for the study of plant gene functions. Major remaining challenge in the *P. patens* study is the elucidation of functions of genes in plant.

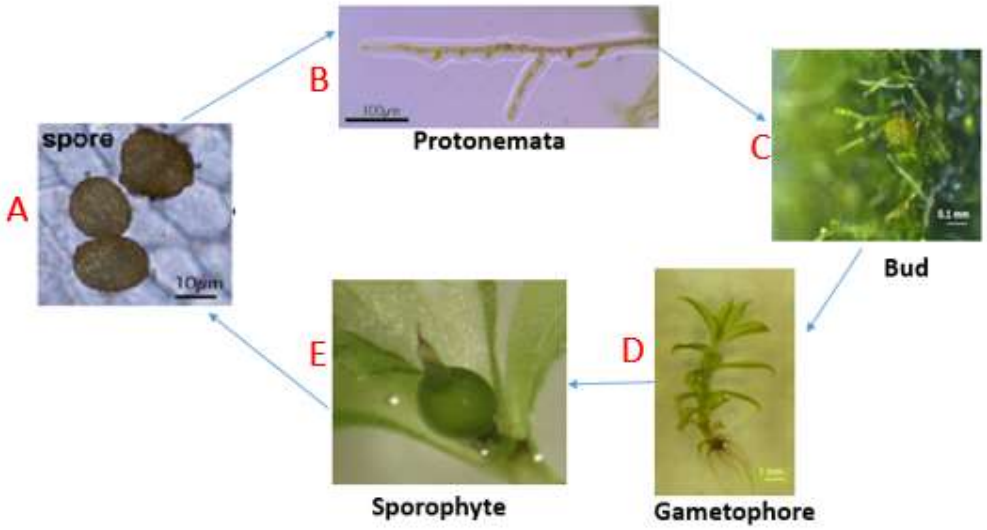

Figure 1. Life cycle of *Physcomitrella patens*. Spore (A) germinates and gives rise to filamentous growth called protonema (B). Bud (C) is developed on protonema which

grows into gametophore (D). Reproductive organs are developed on gametophore and fertilisation of the egg by sperm leads to the development of sporophyte (E).

P. patens has a short life span of about 3 to 4 months and is dominated by haploid generations. As spore germinates single celled filaments starts coming out of it which later undergo serial division at their apical cells (Fig.1 A,B) (Didier et al., 2001). These filaments show dense number of chloroplasts in cells and hence called chloronema cells. Some of these cells differentiate and develop into another type of cells called caulonema cells. Some of the sub apical filaments gives rise to buds (Fig. 1, C) which later develop into leafy shoots which are called gametophore (Fig. 1, D) (Renski et al., 2002). Filamentous root like structure arises from the base of gametophore. On the gametophore apex, development of both male and female organs takes place which are called antheridia and archegonia respectively. Spermatozoids from antheridia swim to eggs in archegonia and a diploid zygote is developed after fertilization which gives rise to sporophyte (Fig.1, E) (Cove et al., 1993). Development of three dimensional bud from protonemal filament and its transition to leafy gametophore is a very complex process and involves regulation of many genes. We are mainly interested in study of genes playing role in bud and gametophore development, since we lack crucial information about these developmental phases.

1.2 Bud development

Formation of three dimensional gametophore from the protonemal filament is complex process involving the switch in the developmental scheme. Transition in this process takes when second subapical caulonemal cell starts dividing asymmetrically to form bud (Perroud et al., 2014). The position of protrusion mediated by asymmetric cell division and development of protrusion into bud is restricted to narrow region of the filament (Yoshida, 1982). Study on development of bud has been carried out by various labs but, molecular machinery underlying this process is still not clear. The role of cytokinin in bud development has been studied in detail (Brands & Kende, 1968, Nair & Raghavan, 1976, Bopp & Jacob, 1986). Changes in gene expression on the application of cytokinin during bud development has been studied in wild type and bud mutants using suppression subtractive hybridization (Bruna et al., 2003). They

found *BIP 1, 2* genes in *P. patens* which were similar to the genes involved in sexual reproduction and cell differentiation genes in the vascular plants. Deletion of Defective kernel 1 (*PpDEK1)* gene in moss resulted in the large number of developmentally arrested buds (Perroud et al., 2014). The mutant showed abnormal cell division and arrest of three dimensional growth of bud. Complementation by overexpression of DEK1 resulted in rescue of wild type phenotype. AP2-type transcription factors orthologous to *Arabidopsis thaliana* were found to be indispensable for the formation of gametophore initial cell from protonema (Aoyama et al, 2012). Since very less information is available on genes regulating bud and gametophore development, we need to search for novel genes and for that forward genetics approach was taken. Generation of large scale insertional mutants and screening for phenotype regarding bud development will help us to get vital information about genes regulating bud development. Here in this study, we are using insertional mutagenesis tools, tobacco retrotransposon Tnt1 and T-DNA insertion to develop insertional mutants.

1.3 Mutagenesis tool: Tobacco retrotransposon Tnt1

Transposable elements are used as mutagenesis tool for insertional mutations in determining gene functions. There are two classes of transposable elements, Class 1 transposable elements which transpose through RNA intermediate like viruses and are called retrotransposon. Class 2 transposable elements transpose through DNA intermediate and are called DNA transposons (Rebollo et al., 2012). Class 1 transposons show copy paste mechanism where original insertion copy is maintained when retrotransposon jumps where as in class 2 transposon part of DNA transposon is excised and inserted in other part of genome. Tnt1 was isolated from *Nicotiana tabacum* (Grandbastien, 1989). Tnt1 is a retrotransposon and transposases through the formation of a daughter copy from an RNA intermediate by reverse transcription. (Boeke and Corces, 1989).

Figure 2: Schematic representation of Tnt1 retrotransposon. Retrotransposon has long terminal repeats (LTR) which flanks gene coding for gag proteins, integrase enzyme, reverse transcriptase and RNase H.

Tnt1 encodes for gag protein and integrase enzyme which help in its insertion, reverse transcriptase and RNase H which help in transposing through RNA intermediate by reverse transcription (Fig. 2).

Tnt1 retrotransposon is from tobacco and has been successfully used in several heterologous hosts. Use of Tnt1 has been reported in higher plants like *A.thaliana*, tomato and soybean etc. (Lucas et.al., 1995, Cui et.al., 2013, C. Mhiri, et.al., 1999). Although retrotransposons are well studied as mutagenesis tool in higher plants, attempts of its use in lower plants were failed in many studies. Here in our lab, we are establishing this mutagenesis tool in moss *P. patens* to understand its developmental program. Earlier experiments in our lab showed that Tnt1 element can be stably transformed into *P. patens*. When we started working on Tnt1, there was no study on the use of Tnt1 in moss. We have used the native Tnt1 retrotransposon cloned in the binary vector. In recent study by Vives et al., two component inducible transposon system was developed, which can transpose only when proteins from Tnt1 are expressed separately. This tool was successfully used in moss for mutagenesis (Vives et al., 2016). Although this study uses modified Tnt1 supports our experiments and results.This study aims for generation of Tnt1 insertional mutant lines in *P. patens* and their characterization through various molecular techniques.

1.4 LTR promoter:

LTR promoter plays important role in Tnt1 expression so, we decided to characterise LTR promoter expression in moss. Tnt1 retrotransposon transcript initiates at U5 region of 5' LTR region and ends at R region of 3' LTR region of Tnt1. The sequence located upstream the transcription start site is called U3, the region between this site and the polyadenylation site is called R, and the LTR sequence downstream the polyadenylation site is designated as U5 (Fig. 3).

Figure 3. Schematic representation of LTR promoter. U3 site, polyadenylation site R and U5 site are located upstream the transcription start site.

Transcriptional regulatory machinery is located mainly in U3 region which contains *cis* acting elements (Casacuberta, Vernhettes & Grandbastien, 1995). This region has short palindromic sequences known as BI element and a domain of 31 base pair tandem repeats present in 3-4 copies in transcriptionally active Tnt1, named as BII element box. This has been shown that BII elements take an active part in induction of Tnt1 transcription (Charlesworth, 1999, Casacuberta et al., 1993). Also, it was observed that in various biotic and abiotic stresses specific proteins bind to BII elements and induces transcription of Tnt1 (Mhiri et.al., 1999, Vernhettes et.al., 1997). For understanding LTR promoter activity in moss, GUS gene was cloned under Tnt1 LTR promoter as well as modified LTR promoter (mLTR) in pBI-101 binary vector and was expressed in *P. patens*. In LTR-GUS construct GUS gene is expressed under native Tnt1 LTR promoter. We wanted to study the LTR promoter expression pattern in moss in the presence and absence of BI and BII box *cis* elements. This experiment will reveal the activity of Tnt1 retrotransposon in moss.

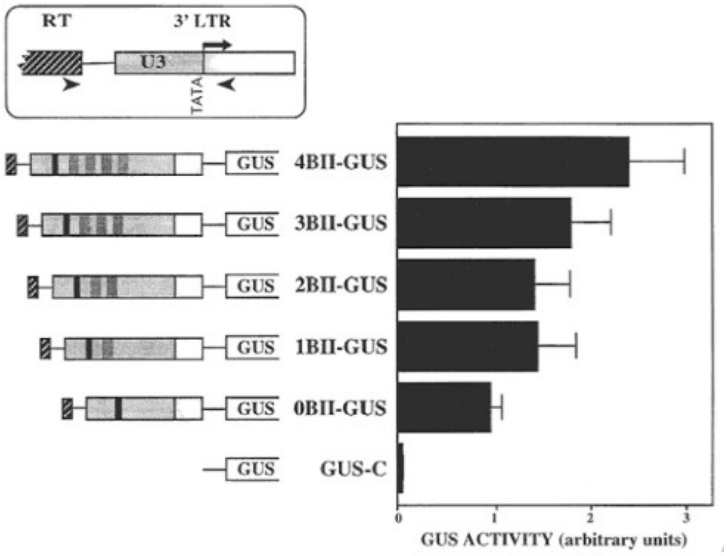

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Figure 4. GUS expression under LTR promoter with various number of BII elements. Left hand side of the figure represents LTR promoter with reducing number of LTR promoter and right hand side represents the corresponding reduction in expression of GUS. Reproduced from Casacuberta, 1995.

1.5 Objectives

Generating large scale mutant with Tnt1 retrotransposon will help us for screening for mutants deficient in bud development. To understand Tnt1 expression and efficient transposition in moss LTR promoter of Tnt1 should be characterised in moss. Based on our literature survey for bud development and our forward genetics approach for searching genes involved in bud and gametophore development, following objectives were undertaken.

1) Generation and confirmation of Tnt1 insertional lines in moss (*P. patens).*
2) Characterization of LTR promoter of Tnt1 in moss.
3) Screening for the phenotype of interest in Tnt1 and T-DNA insertional *P. patens* lines and molecular characterization of putative mutant lines of interest.

Chapter 2

2. Materials and methods

2.1 Moss culture and maintenance

BCDAT medium was used for culture and maintenance of moss protonemata. Glucose (0.5%) was added to the medium to observe bacterial contamination in tissue culture.

BCDAT stock solution preparation

Stock B contains (100 mM) $MgSO_4$ $7H_2O$, Stock C contains (184 mM) KH_2PO_4, pH 6.5 was adjusted with 4 M KOH. Stock D contains 1M KNO_3 and 4.5 mM $FeSO_4$ $7H_2O$. Alternative TES was prepared by mixing $CuSO_4$ $5H_2O$ (0.22 mM), H_3BO_3 (10 mM), $CoCl_2$ $6H_2O$ (0.23 mM), Na_2MoO_4 $2H_2O$ (0.1 mM), $ZnSO_4$ $7H_2O$ (0.19 mM), $MnCl_2$ $4H_2O$ (2 mM) and KI (0.17 mM) in final concentration. Ammonium tartrate stock was prepared in (0.5) M concentration. 1 M $CaCl_2.2H_2O$ was prepared and filter sterilized with (0.22 µm) filter before use. All stocks were stored at 4˚C.

BCDAT medium 1000 ml

H2O	900 ml
Stock B	10 ml
Stock C	10 ml
Stock D	10 ml
Alternative TES	1 ml
500mM Ammonium tartrate	10 ml
50mM $CaCl_2.2H_2O$	20 ml
Agar	8 g (= 0.8%)
	Fill up to 1000 ml with H_2O

For routine culture, *P. patens* grows on solid medium at temperatures below 28˚C. We usually set the temperature at 25˚C with continuous white light.

Routine sub-culture

For routine sub-culture, a small part of protonemal tissue was inoculated on fresh medium to develop a new culture.

To develop homogeneous protonemal culture, the 7-days culture on a petri dish is harvested with forceps and homogenized with polytron homogenizer in 4-5ml liquid BCDAT medium in a scintillation vial. 1.5 to 2ml of this suspension was spread on solid BCDAT medium laid with cellophane disc. The plate was sealed with surgical tape and incubated at 25˚C incubator with continuous white light.

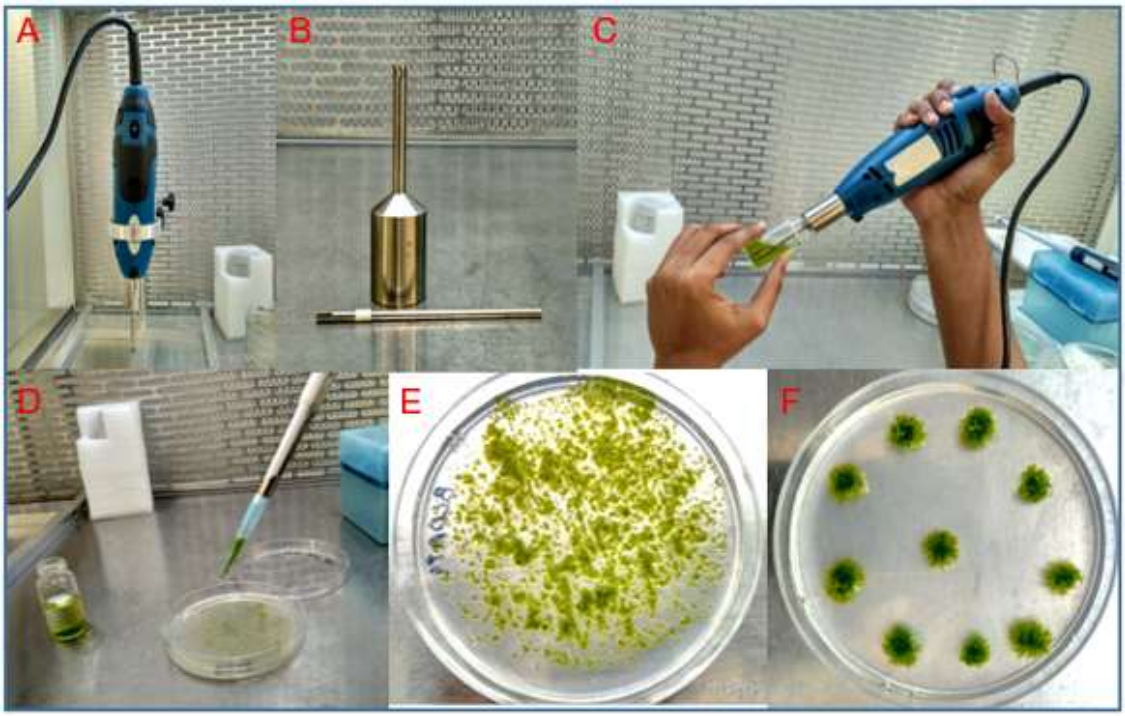

Figure 5. The Routine subculture of moss tissue by homogenization and protonemal inoculation. (A) Polytron Homogenizer. (B). Blade and shaft. Tissue was homogenized as shown in (C), in scintillation vial with polytron homogenizer and spread on plate containing media with pipette (D). Homogenized tissue grown for 6 days (E) is used for regular subculture (F).

2.2 *Agrobacterium* mediated transformation of protonemata

Agrobacterium Culture and Co-Culture

1. An *Agrobacterium* colony with desired plasmid construct was inoculated in 5mL of LB with appropriate antibiotic at 180 rpm and was grown for 24 hours at 28º C temperature.
2. The culture centrifuged at 3,000 rpm for 7 min at room temperature and supernatant was discarded.
3. Pellet was washed by adding 5 mL of BCDAT + 5% Glucose and re-suspend by pipetting up and down with cut tip and centrifuged again at 3,000 rpm for 7 min at room temperature. Supernatant was discarded.
4. Co-culture media was prepared by adding Acetosyringone to 200mM final concentration to the liq. BCDAT + 5% Glucose solution.
5. Pellet was re-suspended in 2 ml of co-culture media and grown at 28º C for 2 hours at 180 rpm shaking
6. OD of bacterial culture was taken on Nanodrop-2000 in UV-visible mode by setting wavelength manually at 600. Path length correction was performed by multiplying the reading by 10.
7. OD of the culture was set to 0.1 by adding co-culture media to the culture and making final volume 10ml in co-culture. Calculation of the volume of bacterial culture in 10ml co-culture was performed by following formula:

Bacterial culture vol. needed= 0.1* 10/ Nanodrop reading after path length correction.

8. 10 ml of co-culture media was pipetted in 9cm petri dish and 5-6 day old protonemal culture from one plate was added to it. The co-culture was swirled to cover the bottom of the dish.
9. Plate was sealed with parafilm and kept in 25 º C growth chamber under continuous light for 2 days.

Washing and 1st Selection Media

Autoclaved BCDAT media supplemented with Claforans (100 µg /ml), Augmentin (50 µg /ml), and appropriate antibiotic was used as selection media. Media was laid with cellophane layer. Autoclaved liquid BCDAT media supplemented with Claforans (100 µg /mL).

Washing and Plating

The washing step is very important because it washes away the excess agrobacteria. If agrobacteria are left with the moss then it will overgrow and kill the moss transformants.

1. Tissue from 2 days co-culture was taken in fresh pertiplate with the help of forceps and cut-tips (for small tissue part) and excess liquid was removed using cut- tip.
2. 15 ml of washing media was added to the plate containing tissue and tissue was re-suspended in media. Tissue was swirled in washing media using forceps gently.
3. Washing media is removed from plate using cut-tips and fresh media is poured in the plate. This action was repeated thrice.
4. After this tissue was spread on selection media overlaid with cellophane using cut tips. Plates were sealed with surgical tape and kept at 25º C incubator for 2 weeks for 1st selection.
5. After two weeks cellophane is transferred to relaxation media without any antibiotic, containing only Claforans and Augmentin, to eliminate transient transformant lines and kept for about 1-2 weeks.
6. Small tissue from lines surviving in relaxation media was transferred to selection plates again. The lines growing on 2nd selection were selected as true line and confirmed by PCR with antibiotic and gene specific primers.

2.3 CTAB method for genomic DNA isolation

1. Tissue was ground in liquid nitrogen with the help of pestle and mortar into fine powder form.
2. The100mg tissue powder was taken into 1.5 ml tube containing 400 µl of 2x CTAB buffer.
3. The mixture was incubated at 60˚C in water bath for 1 hour.
4. An equal volume of Chloroform: Isoamylalcohol was added to the mixture and centrifuged at 15000 rpm for 10 min. The aqueous phase was transferred to new tube.
5. An equal volume of 2-propanol was added to the tube and centrifuged at 15000 rpm for 10 min and supernatant was discarded and the pellet was washed with 70% ethanol by centrifuging at 15000 rpm for 5 min.
6. Supernatant was discarded and pellet was dried at room temperature.
7. The pellet dissolved in 50 µl of TE containing 1 ml of 1 mg/ml RNaseA.

8. The quality of DNA was analyzed by gel electrophoresis and was quantified using nanodrop machine.

2.4 PCR reaction for Taq polymerase

**Annealing temperature was adjusted according to specific primers and extension time was decided on the basis of amplicon size (1 min/ kb).

PCR mix content	Volume (µl)
10x Himedia taq pol. Buffer	2.5
50 mM $MgCl_2$	0.75
Forword Primer(10 mM)	0.5
Reverse Primer(10 mM)	0.5
DNA template (100 ng/µl)	1
Taq polymerase	0.5
water	19.25
Final volume	25

No of cycles	PCR condition
1	94ºC (30 s)
30	94ºC (30 s); Ta** (30 s); 72ºC (** min)
1	72ºC (5 min)
	Hold 4 ºC

Table 1. List of primers

Primer name	Sequence	Amplification size
LTR Forward	TGATGATGTCCATCTCATTGAAG	610 bp
LTR Reverse	TGTTGGGAATAAACCCCTTACCA	
Hygromycin Forward	GATTCCCAATACGAGGTCGCCAACAT	218 bp
Hygromycin Reverse	CCGGATCGGACGATTGCGTCGCATCG	
LTR Forward	TGATGATGTCCATCTCATTGAAG	2272bp
GUS-qRP1	ATCGGCGAAATTCCATACCTG	
mLTR Forward	taggtaccTGATGATGTCCATGTCAAATATTGT	2127bp
GUS-qRP1	ATCGGCGAAATTCCATACCTG	
GSP1- LTR3	AGTTGCTCCTCTCGGGGTCG	Amplicon size varies in TAIL-PCR.
GSP2-LTR 4	TACCGTATCTCGGTGCTACAT	
GSP3-LTR 7	TATTATTCCGCTTTATTACCGTGA	
BM_AD1	NGTCGASWGANAWGAA	
BM_AD2	TGWGNAGSANCASAGA	
BM_AD3	AGWGNAGWANCAWAGG	

BM_AD4	STTGNTASTNCTNTGC	
BM_AD5	NTCGASTWTSGWGTT	
BM_AD6	WGTGNAGWANCANAGA	
LTR_Bisulfite_F:	TATTAATAAAGAGAGAAAGAAAGAGTGAGG	374 bp.
LTR_Bisulfite_R:	ATTAAAAATAAACCCCTTACCAAAATAATA	

2.5 PCR for confirmation of Tnt1 insertional lines

Tnt1 lines genomic DNA was isolated by CTAB method and used for confirmation by LTR gene specific primers (LTR-forward and LTR- reverse) and hygromycin antibiotic gene specific primers (hygromycin forward and hygromycin reverse). For both LTR and hygromycin gene annealing temperature was kept 56 ºC and extension was kept 1 min.

2.6 Preparation of modified LTR (mLTR) promoter

Original LTR promoter has BI box elements and BII box elements as highlighted in following LTR sequence:

>LTR promoter sequence: Total length- 610 bp

TGATGATGTCCATCTCATTGAAGAAGTATTAGGCATGTGCCTAATAAGAGTTTTCTTTGG
TTTGGTAGCCAACCTTGTTGACTTGGTTTGGTTGGTAGCCAACCTTGTTGAATCCTTGTT
GGATTGGTAGCCAACTTTGTTGAATTGTGAAAAATGTGTGTAAATTGTCAAATATTGTAG
GCTTTAGAGGGTGAAGCTTTGGCTATAAAAGGAGAGCTTCAACTCTCATTTCTTCACAC
CAACAAAGAGAGAAAGAAAGAGTGAGGTTTCACAGACAAGGTATAAGAAAATAGTCTGT
GAGGAAAATAGAGAGTGAGCGATATTGTAGTGAGGTGGGAATATCAAAAGAGGGTTAT
TTCTTTTGAGTGTTGTAGTGGTCTTTGGAGTATTTACCTCCGACCTACAAAGTGTAAAAT
TCCTTACTATAGTGATATCAGTTGCTCCTCTCGGGGTCGTGGTTTTTTTTCCCTTATTCA
GAAGGGTTTTCCACGTAAAAATCTTGGTGTCATTGTTACTCTTTTATTCTTGTTAATTACC
GTATCTCGGTGCTACATTATTATTCCGCTTTATTACCGTGAATATTATTTTGGTAAGGGG
TTTATTCCCAACA

BI Box Elements: GAAGAAGTATTAGGCATGTGCCTAATA

BII Box Elements: 1) TTTGGTTTGGTAGCCAACCTTGTTGACT

2) TTTGGTTGGTAGCCAACCTTGTTGAAT

3) TTGGATTGGTAGCCAACTTTGTTGAAT

mLTR promoter was developed by deleting BI box elements and BII box elements from LTR promoter sequence by designing specific primers.

>mLTR promoter sequence: Total length- 456 bp

TGATGATGTCCATGTCAAATATTGTAGGCTTTAGAGGGTGAAGCTTTGGCTATAAAAGG
AGAGCTTCAACTCTCATTTCTTCACACCAACAAAGAGAGAAAGAAAGAGTGAGGTTTCA
CAGACAAGGTATAAGAAAATAGTCTGTGAGGAAAATAGAGAGTGAGCGATATTGTAGTG
AGGTGGGAATATCAAAAGAGGGTTATTTCTTTTGAGTGTTGTAGTGGTCTTTGGAGTAT
TTACCTCCGACCTACAAAGTGTAAAATTCCTTACTATAGTGATATCAGTTGCTCCTCTCG
GGGTCGTGGTTTTTTTTCCCTTATTCAGAAGGGTTTTCCACGTAAAAATCTTGGTGTCAT
TGTTACTCTTTTATTCTTGTTAATTACCGTATCTCGGTGCTACATTATTATTCCGCTTTAT
TACCGTGAATATTATTTTGGTAAGGGGTTTATTCCCAACA

These promoters were cloned in pBI-101 vector between XbaI and XmaI sites. GUS gene will be expressed under LTR promoter in LTR-GUS construct and under mLTR promoter in the mLTR-GUS construct.

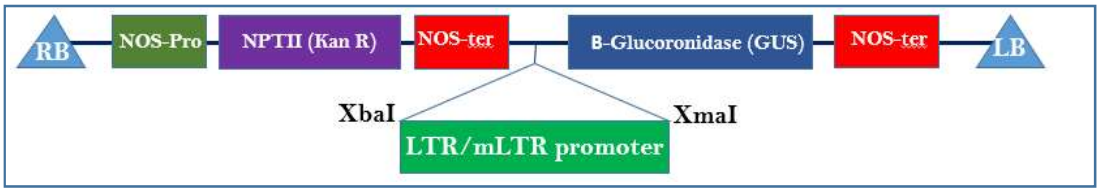

Figure 6. Schematic diagram of LTR-GUS and mLTR-GUS promoter construct. LTR and mLTR promoters were cloned upstream of GUS gene without any promoter in pBI-101 vector.

2.7 Confirmation of LTR-GUS and mLTR-GUS construct

LTR and mLTR promoter cloning in pBI-101 binary vector was confirmed by digestion and PCR with gene specific primers.

Digestion Reaction:

Reaction Mix	Volume (µl)
Cutsmart Buffer	2
Xba-I	1
Xma-I	1
Plasmid	7 (2000ng)
Water	9
Total	20
Kept at 37ºC for 3 Hrs.	

Confirmation of LTR and mLTR by PCR

LTR gene specific primers (LTR-forward and LTR- reverse) and mLTR gene specific primers (mLTR-forward and LTR- reverse) were used for clone confirmation. For both LTR and Hygromycin gene annealing temperature was kept 56 ºC and extension was kept 1 min.

2.8 Confirmation of LTR-GUS and mLTR-GUS insertional lines by performing PCR with gene specific primers

Tnt1 lines genomic DNA was isolated by CTAB method and used for confirmation by LTR and mLTR promoter specific forward primer (LTR-forward/ mLTR- forward) and GUS gene specific reverse primer (GUS-qRP1). For both LTR and mLTR confirmation annealing temperature was kept 56 ºC and extension was kept 2 min 30 sec.

2.9 *Agrobacterium* mediated tobacco transformation

In vitro grown seedling of tobacco plant grown for 3-4 weeks were used for the experiment.

Media:

1. Liquid MS: MS salts (macro + micro + vitamins + sucrose 2%)
2. Co-cultivation medium: MS salts (macro + micro + vitamins + sucrose 2% + agar (0.2%))
3. Regeneration medium: MS salts (macro + micro + vitamins) + sucrose + Phytagel (0.2%) + BAP (1 mg/l) and NAA (0.1 mg/l) + Kanamycin (50 mg/l) and Cefotaxime (500 mg/l), adjusted pH to 5.8. All hormones and antibiotics were added after the medium is autoclaved.

Three days before the transformation experiment:

1. *Agrobacterium* with plasmid construct of interest was grown on LB agar containing Rifampicin and Kanamycin (50 mg/L) at 28º C for two days.
2. On day three, a single colony was inoculated in test tube containing 10 ml of LB medium with required Rifampicin and Kanamycin (50mg/L), overnight.
3. On the next day (day of transformation), the OD_{600} of overnight grown culture was taken and culture was refreshed in 5 ml of culture without antibiotics. The OD was

adjusted to 0.4 and culture was grown for a period of 2-4 hours again. Check the OD again and it should be between 0.8 - 1.0.

4. When the required OD is reached the culture was incubated on ice until your tobacco leaf discs are ready for agro treatment.

On the day of transformation:

1. Tobacco leaf was harvested aseptically and small pieces of 1-2 cm in size were prepared by cutting. The leaf pieces were kept in 40 – 50 ml of liquid MS media in a sterile petri-plate so to avoid desiccation of the leaf sections.
2. To this plate with cut tobacco leaf pieces, 100 µl of agro culture (OD: 0.8 – 1.0) was added. The plate was kept for shaking for 20 – 25 mins to distribute the culture to the cut surface.
3. Collected all leaf disc, blotted in paper towel (sterile) and cultured them in co-cultivation medium for 48 hours in dark.

Figure 7. Tobacco leaf discs on selection media after co-culture with *Agrobacterium.*

After co-cultivation:

4. After 48 hours, harvested all the discs and washed them in sterile water for three times
5. After washing, disks were blotted in sterile paper and cultured in regeneration medium.
6. The plates were incubated in culture room at 25º C for 10-15 days (Fig. 7).
7. Fresh regeneration medium was prepared and all the shoot masses were transferred to it.
8. The lines were confirmed by genomic DNA isolation and PCR with antibiotic and gene specific primers.

2.10 Assays

Tnt1 and T-DNA insertional lines along with wild type plant were grown in different media composition to observe any abnormality in growth compared to wild type *P. patens.* The deviation from wild type plant in mutant growth shows deformation in particular metabolic pathway and indicates the mutation in genes related to that pathway. Moss tissue was homogenized and grown for six days was used for all assays.

1) Hormone assay:

   Tnt1 and T-DNA insertional lines were grown in BCDAT media containing different hormones along with wild type plants for 2 weeks.

   i) Auxin assay : BCDAT+ Indole-3-acetic acid (100 µM)

   ii) Cytokinin assay : BCDAT+ 6-Benzylaminopurine (10 µM)

   iii) Abscisic acid assay : BCDAT+ Abscisic Acid (20 µM)

2) Sugar assay:

   Tnt1 and T-DNA insertional lines were grown in BCDAT media containing different sugars along with wild type plants for 2 weeks.

   i) Glucose assay: BCDAT+ (0.5%) Glucose

   ii) Mannitol assay: BCDAT+ (0.5%) Mannitol

   iii) No sugar assay: BCDAT

3) Depletion of carbon source:

   Wild type and insertional mutant plants were grown on media containing BCD elements only.

4) Gravitropism assay:

   i) Inoculated protonemata on an BCDAT+Glucose (0.5%) plate and cultured for 2 weeks under normal light conditions.

   ii) Then plates were grown in vertical position in dark for 2-3 weeks.

5) Light assay:Moss tissue was inoculated on BCDATG media and grown in tightly packed light protected Red and Blue LED light box for two weeks. After two weeks plates were taken out and branching in the protonema was imaged.

2.11 GUS assay protocol:

LTR-GUS and mLTR-GUS lines were subjected to GUS staining to analyze expression of LTR and mLTR promoter in moss.

Table 2. Reagent mix for GUS assay (Meyerowitz, 1987)

Stock solution	**Final concentration**	**Reagent Mix µl/ml**
1 M $NaPO_4$ buffer, pH 7.0	0.1 M	100
0.25 M EDTA, pH 7.0	10 mM	40
0.005 M K-ferricyanide pH 7.0	0.5 mM	100
0.005 M K-ferrocyanide pH 7.0	0.5 mM	100
0.02 M X- glucuronide	1.0 mM	50
10% triton X-100	0.1%	10
Subtotal		400
Distilled water		600
Final volume		1000

Note: 100ml of 1 M $NaPO_4$ was prepared freshly by mixing 39 ml of Na_2HPO_4 (1 M, pH 7.0) and 61 ml of NaH_2PO_4 (1 M).

Tissue was incubated at 37º C overnight and distained with 30, 50, 70 and 100% in order for 2 hours.

2.12 LTR promoter expression induction:

LTR promoter expression was analyzed by giving $CuCl_2$, salicylic acid, auxin and temperature variation stress. Induction of expression was analyzed by GUS staining of tissue.

$CuCl_2$ treatment: 1 M $CuCl_2$ solution was prepared in sterile water and used in 0.5, 1 and 2 mM final concentration in liquid BCDAT medium. Moss tissue was taken into 24 well plate and 1.5 ml of BCDAT+$CuCl_2$ solution was added to each well and labeled with respective concentration. The plate was incubated at 25º C for 24 hours before GUS treatment. Wild type moss tissue and liquid BCDAT media without any CuCl2 content was used as control for the experiment.

Auxin treatment: 1 M, 1-Naphthaleneacetic acid (NAA) solution was prepared in sterile water and used in 10, 50 and 100 µM final concentration in liquid BCDAT medium. Moss tissue was taken into 24 well plate and 1.5 ml of BCDAT+NAA solution was added to each well and labeled with respective concentration. The plate was incubated at 25º C for 24 hours before GUS treatment. The plate was incubated at 25º C for 24 hours before GUS treatment. Wild type moss tissue and liquid BCDAT media without any NAA content was used as control for the experiment.

Salicylic acid (SA) assay: 1M stock solution of Salicylic acid was prepared in 80% ethanol and was used to prepare liquid BCDAT medium containing 1, 2 and 5mM final concentration of salicylic acid. Moss tissue was taken into 24 well plate and 1.5 ml of BCDAT+SA solution was added to each well and labeled with respective concentration. The plate was incubated at 25º C for 24 hours before GUS treatment. The plate was incubated at 25º C for 24 hours before GUS treatment. Wild type moss tissue and liquid BCDAT media without any SA content was used as control for the experiment.

Temperature treatment:
Moss tissue was incubated at 18, 24, 30 and 37ºC temperature in 1.5 ml tube containing liquid BCDAT medium for 24 hours. Then the tissue was subjected to GUS assay.

Homogenization stress: 6 day old protonemal tissue was homogenized and was plated on BCDAT medium laid with cellophane and grown at 25º C for 7 days. This tissue was used for GUS assay.

2.13 Thermal Asymmetric Interlaced PCR (TAIL-PCR)

TAIL-PCR is useful to know DNA fragments flanking known gene sequences (Liu & Whittier, 1995). Three nested primers were designed from known sequence were used in consecutive reactions together with an arbitrary degenerate primers (ADx) (Fig. 8). Differences in Tm of the primers were used to thermally control amplification frequencies of the fragments. This technique was used to identify genomic region flanking Tnt1 and T-DNA insertion in mutant lines.

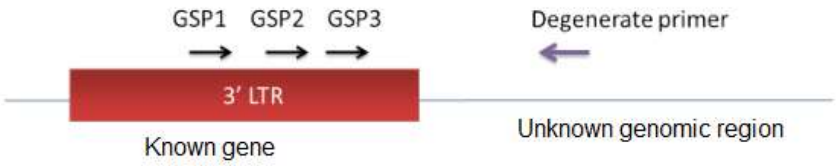

Figure 8. Schematic diagram of TAIL-PCR primer design. Three nested gene specific primers were designed in 3' LTR of Tnt1 retrotransposon and six arbitrary degenerate primers were designed which can bind in genome non-specifically.

TAIL-PCR gene specific primer structure:

AGTTGCTCCTCTCGGGGTCGTGGTTTTTTTTCCCTTATTCAGAAGGGTTTTCCACGTAAAAATCTT
GGTGTCATTGTTACTCTTTTATTCTTGTTAATTACCGTATCTCGGTGCTACATTATTATTCCGCTTT
ATTACCGTGAATATTATTTTGGTAAGGGGTTTATTCCCAACAACTTCTACTCGCGTTACTTTTCTTC
CCTCCGCCTGTTTGTTACACGTTCACAATCAGTATTTCAATCAATACAATGGCTAATTATTCTACA
AGTTTTGTCAGTTCCTCGACGGATCCNNNTCAG

GSP1- LTR3:AGTTGCTCCTCTCGGGGTCG

GSP2-LTR 4:TACCGTATCTCGGTGCTACAT

GSP3-LTR 7:TATTATTCCGCTTTATTACCGTGA

Primary to secondary PCR amplified product difference 98 base pair.

Secondary to tertiary PCR amplified product difference 21 base pair

Protocol

7 reactions were carried out for 6 different degenerate primers, and one for -ve control.

1. Primary TAIL-PCR:

Genomic DNA of Tnt1 insertional line was used as template and gene specific primer-1 (GSP1) and arbitrary degenerate primers (ADx) were used for the PCR.

PCR program is as follows:

No of cycles	PCR condition
1	92ºC (2 min); 95 ºC (1 min)
5	94ºC (15 s); 63ºC (1 min); 72ºC (2 min)
1	94ºC (15 s); 30ºC (3 min); ramp to 72 over 3 min; 72ºC (2 min)
10	94ºC (5 s); 44ºC (1 min); 72ºC (2 min)
12	94ºC (5 s); 63ºC (1 min); 72ºC (2 min) 94ºC (5 s); 63ºC (1 min); 72ºC (2 min) 94ºC (5 s); 44ºC (1 min); 72ºC (2 min)
1	72ºC (5 min)
	Hold 4 ºC

2. Secondary TAIL-PCR

Gene specific primer 2 (GSP2) and arbitrary degenerate primers (ADx) were for the PCR. Four times diluted primary PCR product four times is taken as template for PCR. PCR program as follows:

No of cycles	PCR condition
1	94ºC (30 s)
12	94ºC (5 s); 63ºC (1 min); 72ºC (2 min) 94ºC (5 s); 63ºC (1 min); 72ºC (2 min) 94ºC (5 s); 44ºC (1 min); 72ºC (2 min)
1	72ºC (5 min)
	Hold 4 ºC

3. Tertiary TAIL-PCR

Gene specific primer 3 (GSP3) and arbitrary degenerate primers (ADx) were for the PCR. Four times diluted primary PCR product four times is taken as template for PCR. PCR program as follows:

No of cycles	PCR condition
1	94ºC (30 s)
20	94ºC (10 s); 44ºC (1 min); 72ºC (2 min)
1	72ºC (5 min)
	Hold 4 ºC

4. **Agarose Gel electrophoresis**

PCR product from secondary and tertiary PCR reaction was loaded side by side on the gel and amplified bands were compared for expected size difference.

5. **TAIL-PCR sequencing**

PCR product was cloned into sub-cloning vector pGEM-T easy vector (TA cloning) and sequenced.

Ligation Reaction

2x pGEM-T easy ligation buffer	5µl
pGEM-T easy vector	1µl
pGEM-T T4 ligase	1µl
Tertiary PCR product	3µl
Total	10µl
Kept at 4 ºC overnight.	

This ligation product was transformed into *E.coli.* DH5α competent cells and transformed colonies were screened for expected size insert with the use of M13 primer specific PCR. Plasmids with expected size insert were sequenced using T7 promoter universal primer.

6. **Analysis of TAIL-PCR sequencing results**

Sequencing results were analyzed to obtain Tnt1 flanking region in moss genome. LTR 7 primer, SP6 and pGEM-T vector boundary was annotated on the sequence. The sequence flanked by pGEM-T vector boundary and LTR7 primer was analyzed for similarity with moss genome using BLAST tool on COSMOSS genome browser. The sequence which shows match with moss genome was chosen as Tnt1 flanking region.

2.14 Bisulfite sequencing primer designing

To understand epigenetic regulation of LTR promoter expression and change in methylation pattern of LTR promoter in various tissue and stress conditions. For that methylation specific primers were designed with the help of Methprimer 2.0 software. CpG islands are coloured in yellow in the sequence.

>**LTR promoter original sequence:**

TGATGATGTCCATCTCATTGAAGAAGTATTAGGCATGTGCCTAATAAGAGTTTTCTTTGGTTTGGTAGCCAACC
TTGTTGACTTGGTTTGGTTGGTAGCCAACCTTGTTGAATCCTTGTTGGATTGGTAGCCAACTTTGTTGAATTGT
GAAAAATGTGTGTAAATTGTCAAATATTGTAGGCTTTAGAGGGTGAAGCTTTGGCTATAAAAGGAGAGCTTC
AACTCTCATTTCTTCACACCAACAAAGAGAGAAAGAAAGAGTGAGGTTTCACAGACAAGGTATAAGAAAATA
GTCTGTGAGGAAAATAGAGAGTGAGCGATATTGTAGTGAGGTGGGAATATCAAAAGAGGGTTATTTCTTTTG
AGTGTTGTAGTGGTCTTTGGAGTATTTACCTCCGACCTACAAAGTGTAAAATTCCTTACTATAGTGATATCAGT
TGCTCCTCTCGGGGTCGTGGTTTTTTTTCCCTTATTCAGAAGGGTTTTCCACGTAAAAATCTTGGTGTCATTGTT
ACTCTTTTATTCTTGTTAATTACCGTATCTCGGTGCTACATTATTATTCCGCTTTATTACCGTGAATATTATTTTG
GTAAGGGGTTTATTCCCAACA

>**The expected bisulfite converted sequence:**

TGATGATGTTTATTTTATTGAAGAAGTATTAGGTATGTGTTTAATAAGAGTTTTTTTTGGTTTGGTAGTTA
ATTTTGTTGATTTGGTTTGGTTGGTAGTTAATTTTGTTGAATTTTTGTTGGATTGGTAGTTAATTTTGTTG
AATTGTGAAAAATGTGTGTAAATTGTTAAATATTGTAGGTTTTAGAGGGTGAAGTTTTGGTTATAAAAGG
AGAGTTTTAATTTTTATTTTTTTATATTAATAAAGAGAGAAAGAAAGAGTGAGGTTTTATAGATAAGGTAT

AAGAAAATAGTTTGTGAGGAAAATAGAGAGTGAGCGATATTGTAGTGAGGTGGGAATATTAAAAGAGG
GTTATTTTTTTTGAGTGTTGTAGTGGTTTTTGGAGTATTTATTTTCGATTTATAAAGTGTAAAATTTTTTAT
TATAGTGATATTAGTTGTTTTTTTCGGGGTCGTGGTTTTTTTTTTTTTATTTAGAAGGGTTTTTTACGTAA
AAATTTTGGTGTTATTGTTATTTTTTTATTTTTGTTAATTATCGTATTTCGGTGTTATATTATTATTTCGTT
TTATTATCGTGAATATTATTTTGGTAAGGGGTTTATTTTTAATA

Primers were designed specific to the sequence after bisulfite conversion. Those are as follows:

LTR_Bisulfite_F: TATTAATAAAGAGAGAAAGAAAGAGTGAGG

LTR_Bisulfite_R: ATTAAAAATAAACCCCTTACCAAAATAATA

2.15 Microscopy and image analysis:

Moss plant images were taken on LEICA microscope. For whole gametophore image, gametophore was embedded in 0.8% agar plate. For GUS staining images, white background was used. For protonemal cell subapical images, 6 day old protonema was inoculated on 0.4% phytagel plate and grown for 15 days. This plate contains semisolid media. For taking images, half media was removed by pipetting and images were taken with 8X magnification and black background. For bud count 15 day old single protonema filament was imaged in fragments and buds were counted manually. For whole colony images, 0.32x objective lens was used and magnification was adjusted according to it. Colony area and sub apical cell length was measured using ImageJ software.

Chapter 3

3. Results

3.1. Generation and confirmation of Tnt1 insertional lines in moss *P. patens*

3.1.1 Tnt1 insertional mutant lines generation and confirmation in moss *P. patens.*

34 Tnt1 insertional mutant lines of *P. patens* were generated by using *Agrobacterium tumefaciens* mediated transformation. The lines surviving hygromycin antibiotic selection were subjected to confirmation by PCR from genomic DNA for hygromycin gene and LTR gene insert with respective primers (Fig. 9). Confirmation result shows that all 34 Tnt1 lines are positive.

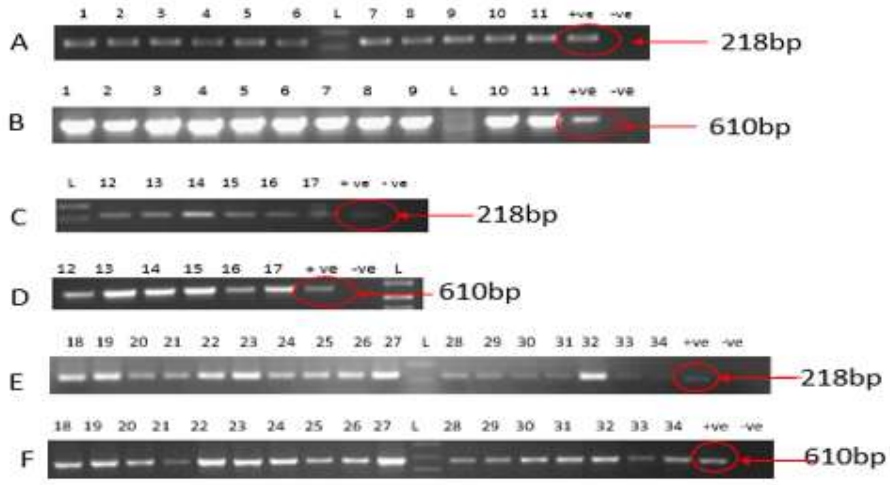

Figure 9. Tnt1 insertion line confirmation by PCR for hygromycin and LTR gene. Expected amplified product for hygromycin gene is 218 bp and 610 bp for LTR gene. Tnt1 vector was used as +ve control and Wild type genomic DNA was used as –ve control. Lane numbers indicate Tnt1 mutant line number. Gel image A, C and E shows hygromycin confirmation of mutant line 1 to 11, 12 to 17 and 18 to 34 respectively. Gel image B, D and F shows LTR confirmation of mutant line 1 to 11, 12 to 17 and 18 to 34 respectively.

3.1.2 TAIL-PCR analysis:

TAIL-PCR was used to trace transposon insertions in Tnt1 insertional lines. Analysis TAIL-PCR sequencing result for Tnt1 line 3 is shown below. All other results were also analysed similarly. pGEM-T cloning site1, LTR 7 ,Tnt1 vector boundary ,Sequence matched with P. patens genome, pGEM-T cloning site2 and Sp6 were annotated in sequencing results. Sequence flanked with GSP 3-LTR 7 primer was analysed as described in material and methods.

A >1st_BASE_2432590_Tnt1 line 3_AD2_1_T7_promoter

GGACGTAGTCGCATGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTATTATTCCGCTTTA
TTACCGTGAATATTATTTTGGTAAGGGGTTTATTCCCAACACCTGGCTGGTTAACAACGATCCAA
ATATGATTTGCGACAATCTTTTGCTAGATTTTTCAGGTAACAACGTTCAAGTGCACAATCTCTGAG
TTTTTCTTGGATCCTTTCAATTAACGGACACCATTCTGAAGTCCATGGGAACAAGACACGCATAT
CCAAGCATATTTCCGGCAGTGATTAGACGTGGTTTAACGTGAATTAAACACAGGTCCCAGTTGCT
GCATCAACACCTCCCGAGTCCCCTACTATTTGCAATGGGAACCTCAATCCACATCCACGAAGAAT
TTGCAGCATCTTGCTGAGAGTGAGTTTTCAGCCCATGATCCAGTCCAGTAAACCGTTCCCGAGA
ATCACTAGCCCTCTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCT
CCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCAT
GGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGA
AGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCT
CACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATCTATAGTGTCACCTAAA
TAGCTTGGCGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTT
CCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAA
AGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGG
CCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCC
CCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAA
AGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTA
CCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTTCCAAAGCTCACGCTGTAA
GGTATTCAAATTCGGGGTAAGGCCGTTCCTTCCAACCTGGGCCGGGGGGCCCAAACCCCCCGT
TTAACCCGAACGCGTGGGCCCTTATACGGGAAATAATTGTCTTGAGTCCACCCCCGGTAGAACC
ACTCA

Figure 10 A. Annotation of sequencing result for TAIL-PCR. Colour code in sequence represents following sequences respectively. pGEM-T cloning site1, LTR 7 ,Tnt1 vector boundary ,Sequence matched with *P. patens* genome, pGEM-T cloning site2 and Sp6.

BLAST results with *P. patens* genome for cloned sequence

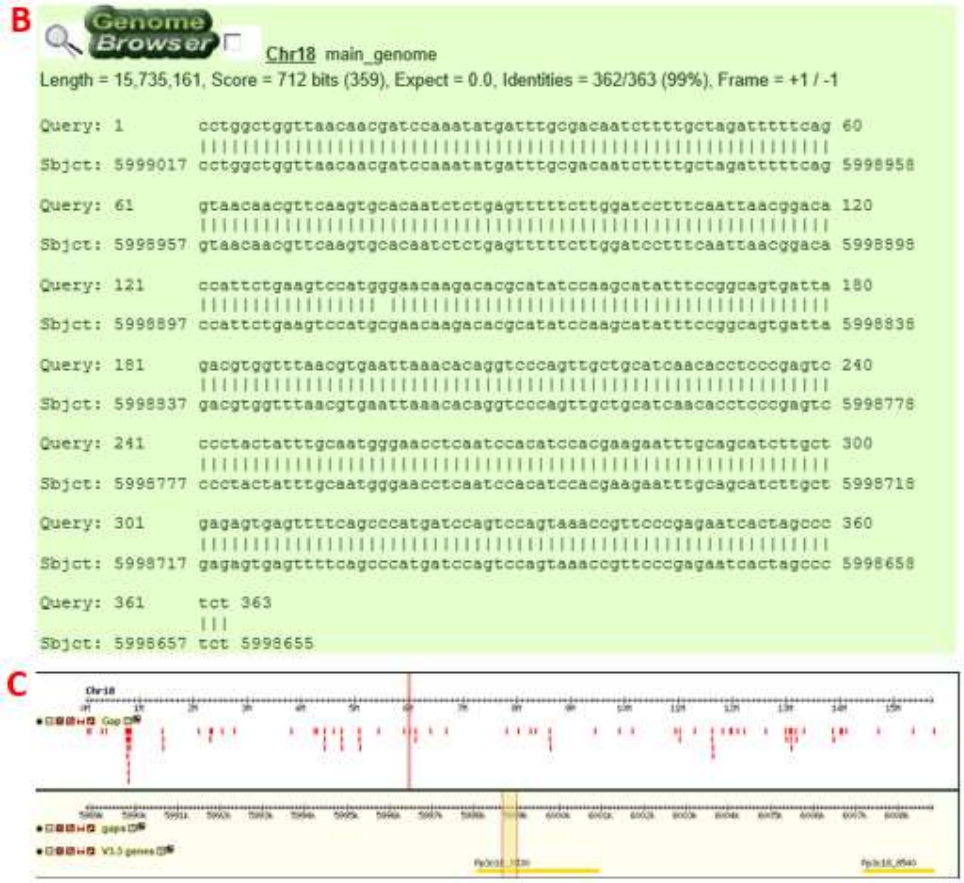

Figure 10. (B) BLAST results for Tnt1 line 3 TAIL-PCR. The sequencing result matched with sequence on chromosome 18 of *P. patens.* (C).The sequence matched with gene coding region Pp3c18_8530.

This BLAST result shows that the Tnt1 retrotransposon has transposed on chromosome no.18 in gene coding region Pp3c18_8530 (Fig. 10, C). This gene is predicted protein coding gene and is positioned at Chr18 from 5998036 to 6000951(- strand). Predicted cDNA length for this gene is 2745 bp. This gene shows 7 introns and 8 exons.Sequence of this gene was retrieved in FASTA format and putative function was analyzed on NCBI genome browser. The gene was analysed for sequence homology with other plant genomes database like NCBI, PlantGDB and TAIR which contains genomes of *Arabidopsis, Z.mays* and rice mainly. The coding sequence analyzed doesn't contain any conserved domain, hence we could not predict a possible function.

All the TAIL-PCR results were analysed as described in materials and methods. Data from TAIL-PCR showed that Tnt1 has specificity to gene rich region for transposition. It was observed that single mutant can have multiple Tnt1 insertions e.g. Tnt1 line 7 shows Tnt1 insertions on chromosome 7, 8, 9 and 18. This shows that Tnt1 is active in *P. patens* and transposing actively. Following table lists mutant lines and respective Tnt1 insertions on different chromosomes.

#	Mutant	Chr #	From base	To base	Remarks/Gene/Loci	type
1	Tnt1 line 13	18	4,343,979	4,343,980	Pp3c18_5860	Three_prime_UTR
2	Tnt1 line 13	13	9539008	9539009	Pp3c13_11600	Non coding
3	Tnt1 line 1	8	17924095	17924096	Pp3c8_25561	Exon
4	Tnt1 line 3	18	5999016	5999017	Pp3c18_8530	Exon
5	Tnt1 line 2	21	13098133	13098134	Pp3c21_20150	Non coding
6	Tnt1 line 7	8	11354571	11354572	After gap_region.891	Non coding
7	Tnt1 line 7	9	15556193	15556194	Pp3c9_22930	EXON
8	Tnt1 line 7	7	108637	108638	Pp3c7_120, Pp3c7_130	EXON
9	Tnt1 line 7	18	12651388	12651389	Pp3c18_17630	Five_prime_UTR
10	Tnt1 line 1	1	28983778	28983779	Pp3c1_41480	CDS

Table 3. List of Tnt1 transposition traced by TAIL-PCR. Tnt1 transposition in the mutant lines were analysed by TAIL-PCR and their position were annotated to chromosome. Insertions were categorised according to type of region it is inserted.

3.2. Characterization of LTR promoter of Tnt1 in moss P. patens

3.2.1 Confirmation of LTR and mLTR promoter constructs

LTR and mLTR promoter characterization constructs were made by Mr. Gajanan in our lab. These constructs were confirmed by restriction digestion (result not shown) as well as by PCR (Fig. 11) and later confirmed by sequencing.

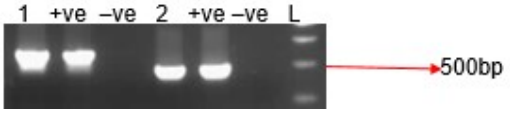

Figure 11: Confirmation of LTR-GUS/mLTR-GUS construct by PCR. Confirmation of LTR and mLTR vectors by PCR with LTR/mLTR F – LTR R. Lane 1 and two represents LTR and mLTR construct respectively. Tnt1 vector was used as positive control and NF water as negative control.

3.2.2 Generation and confirmation of LTR and mLTR promoter characterisation lines in *P. patens*

Agrobacterium tumefaciens mediated transformation was used to insert LTR-GUS and mLTR-GUS construct in *P. patens*. 5 lines for LTR-GUS and 1 line for mLTR-GUS survived antibiotic selection. Mutant lines were confirmed by PCR with LTR forward and Gus Reverse primer for LTR-GUS lines and mLTR forward and Gus reverse primer for mLTR-GUS line (Fig. 12). All 5 LTR-GUS lines and 1 mLTR-GUS line were positive.

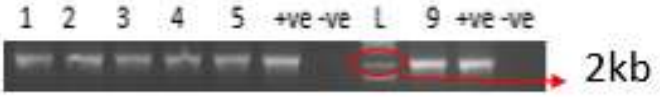

Figure 12. Confirmation of LTR-GUS and mLTR-GUS transformant by PCR. Expected band of 2272 bp for LTR-GUS and 2127 bp for mLTR-GUS lines was observed. In gel LTR-GUS Line 1, Line 4, Line 5, Line 7, Line 9 in lane 1-5 respectively and mLTR Line5 in lane 9. Vector was used as positive control, no template was kept as PCR –ve control and 1kb+ ladder was used in gel.

3.2.3 GUS assay for LTR-GUS and mLTR-GUS promoter lines.

Five LTR-GUS lines and one mLTR-GUS line was subjected to GUS assay and following results were obtained (Fig.13).

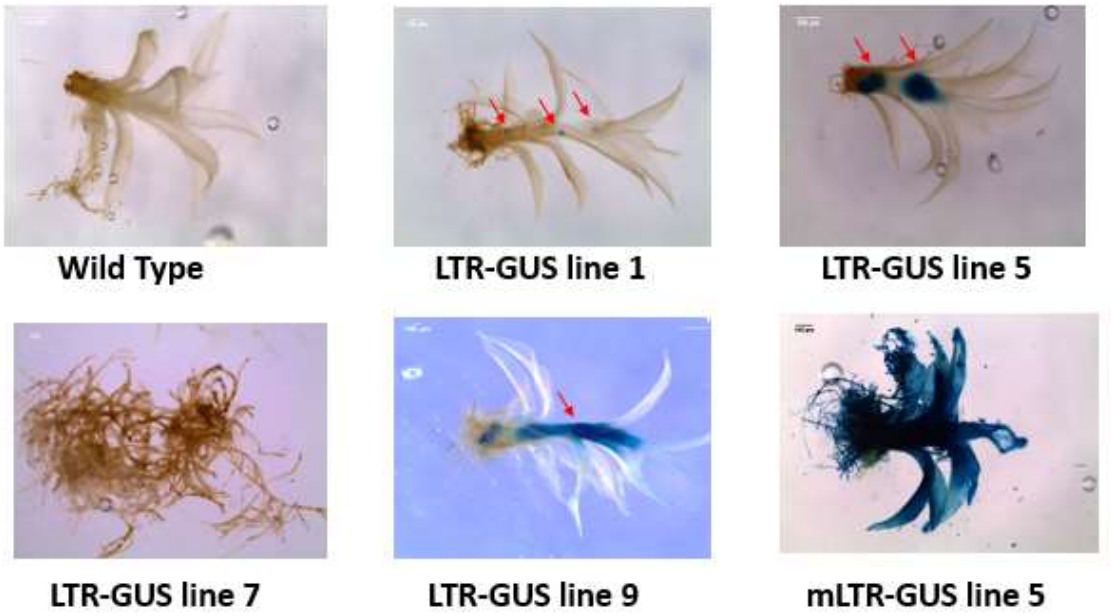

Figure 13. GUS staining of LTR-GUS and mLTR-GUS lines. Red arrow shows places where LTR promoter is expressed and GUS staining was visible.

GUS staining in LTR-GUS lines was mainly found at putative branch points in the stem of the plant. LTR-GUS line 1 shows very less GUS expression and LTR-GUS line 9 exhibits high expression of GUS. Difference in expression level of GUS can be because of different copy number of T-DNA insertions and genome complexity in vicinity of insertion. LTR-GUS line 7 does not proceed to bud and gametophore phase and also lacks GUS expression. Defect in the growth of the plant can be the result of an insertion in some important gene related to development. mLTR -GUS lines showed staining in throughout the plant.

3.2.4 LTR-GUS induction assays:

3.2.4. (A) $CuCl_2$ treatment induces expression of LTR promoter

LTR-GUS lines were subjected to $CuCl_2$ stress and GUS expression was analysed in various tissue types. LTR promoter was responsive to $CuCl_2$ stress and expression of GUS was increasing with increasing concentration of $CuCl_2$.

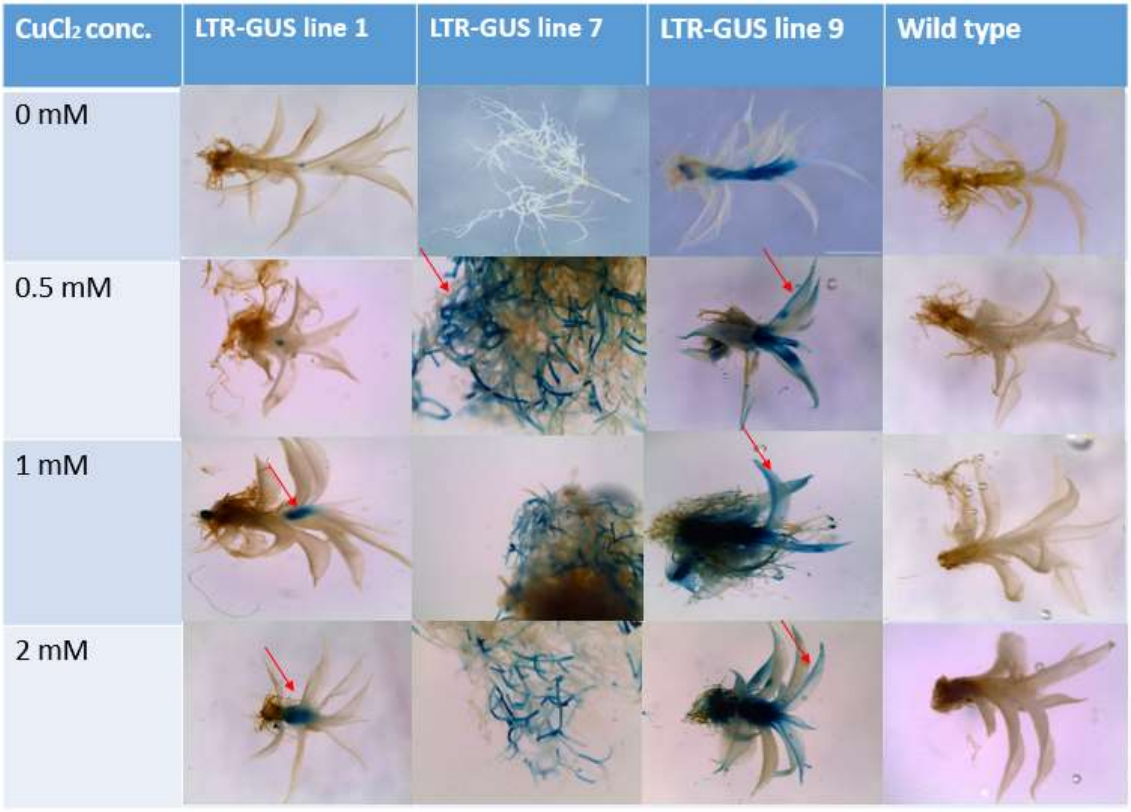

Figure 14. GUS staining of LTR-GUS lines after treatment with different concentration of $CuCl_2$. The red arrow shows places where LTR expression was induced. For LTR-GUS line 1 and LTR-GUS line 9 GUS expression was observed in stem and leaf respectively. For LTR-GUS line 7 GUS expression was observed in protonema.

LTR-GUS line 1 and LTR-GUS line 9 shows GUS expression in stem without any $CuCl_2$ treatment. LTR-GUS line 7 does not show any expression in protonema. GUS expression shows increase after $CuCl_2$ treatment in stem for LTR-GUS line 1 and is induced in leaves of LTR-GUS line 9. LTR-GUS line 7 shows induction of GUS expression in protonema. This shows that $CuCl_2$ can induce LTR expression in moss (Fig. 14). Since some of the regulatory elements for transposon expression shows similarity to defence response regulatory elements in plant in stress condition

transposon expression is induced (Vernhettes & Grandbastien, 1997). In higher plants it has been shown that metal ion stress can induce defence response and also expression of transposons is induced. We have obtained similar results in moss with $CuCl_2$.

3.2.4. (B) Auxin treatment induces expression of LTR promoter

LTR-GUS lines were subjected to auxin (NAA) stress and GUS expression was analysed in various tissue types. LTR promoter expression was responsive to NAA stress and expression of GUS was increasing with increasing concentration of NAA..

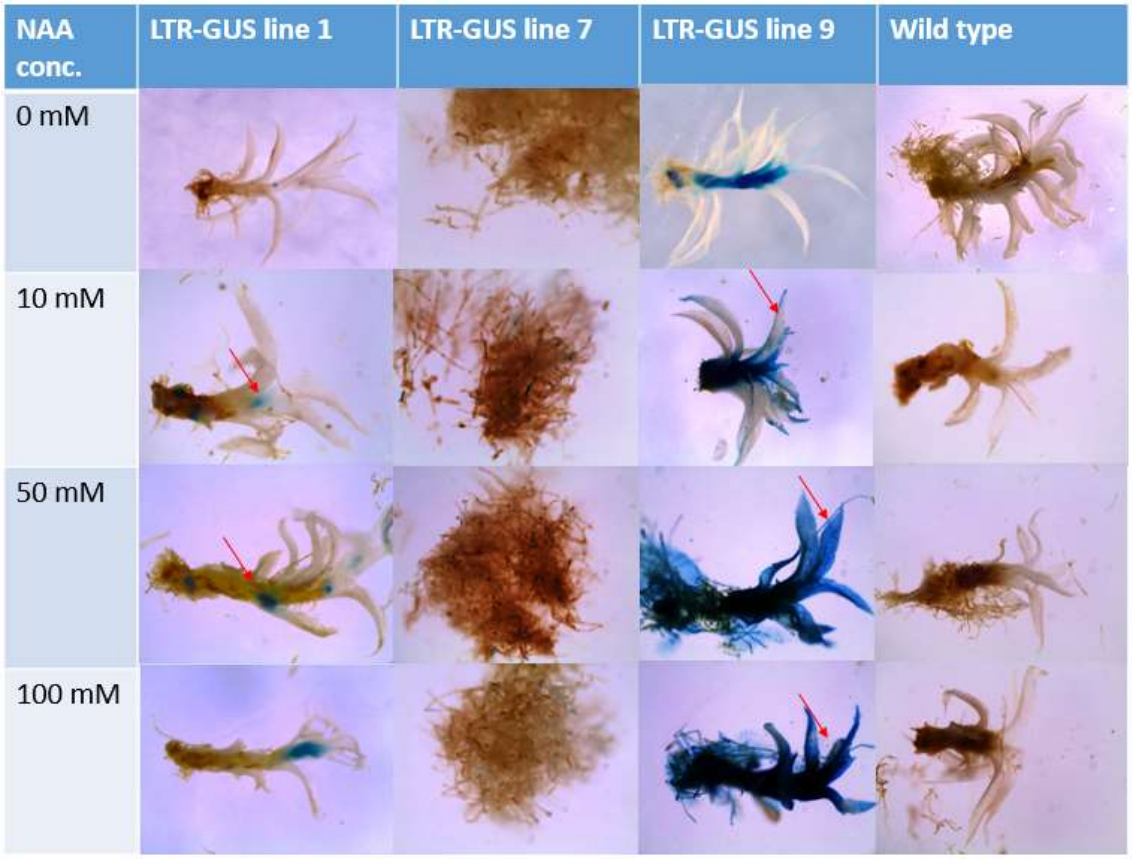

Figure 15. GUS staining of LTR-GUS lines after treatment with different concentration of auxin (NAA). The red arrow shows places where LTR expression was induced. For LTR-GUS line 1 and LTR-GUS line 9 GUS expression was observed in stem and leaf respectively. For LTR-GUS line 7 GUS expression was observed in protonema.

LTR-GUS line 1 and LTR-GUS line 9 shows GUS expression in stem without any NAA treatment. LTR-GUS line 7 does not show any expression in protonema. Wild type

plant did not show any GUS expression.LTR-GUS line 1 showed increase in GUS expression in stem with increasing concentration of NAA. GUS expression was induced leaves of LTR-GUS line 9 (Fig. 15). GUS expression didn't change in protonema of LTR-GUS line 7. This shows that NAA (auxin) can induce LTR expression in moss. In higher plants auxin is shown to induce various abiotic stress response genes (Jain and Khurana, 2009). Auxin treatment may have mimicked stress condition in moss and this can be one of the reason behind the induction of LTR expression.

3.2.4.(C) Salicylic acid treatment induces expression of LTR promoter

LTR-GUS lines were subjected to salicylic acid stress and GUS expression was analysed in various tissue types. LTR promoter expression is responsive to salicylic acid stress and expression of GUS was increasing with increasing concentration of salicylic acid.

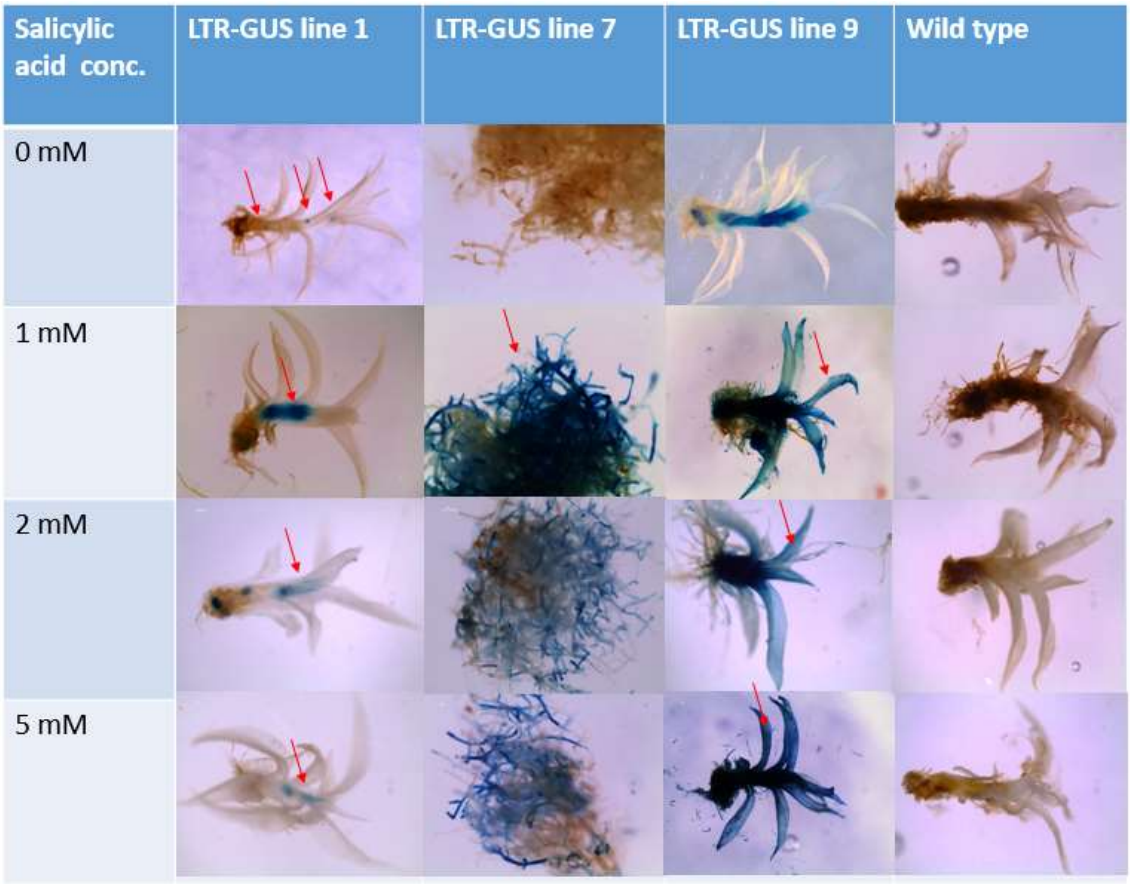

Figure 16. GUS staining of LTR-GUS lines after treatment with different concentration of salicylic acid. The red arrow shows places where LTR expression was induced. For LTR-GUS line 1 and LTR-GUS line 9 GUS expression was observed in stem and leaf respectively. For LTR-GUS line 7 GUS expression was observed in protonema.

LTR-GUS line 1 and LTR-GUS line 9 shows GUS expression in stem without any salicylic acid treatment. LTR-GUS line 7 does not show any expression in protonema. LTR-GUS line 1 showed increase in GUS expression in stem with increasing concentration of Salicylic acid. GUS expression was induced in protonema of LTR-GUS line 7 and leaves of LTR-GUS line 9 (Fig. 16). This shows that salicylic acid can induce LTR expression in moss. Since salicylic acid induces pathogen attack defence pathway in higher plants, it was expected to induce LTR expression in plants (Vernhettes & Grandbastien, 1997). Salicylic acid induces LTR expression in tobacco, the simillar result was obtained in moss.

3.2.4. (D) Temperature gradient induces expression of LTR promoter

LTR-GUS lines were subjected to a gradient of temperature and GUS expression was analysed in various tissue types (Charlesworth, 1999). LTR promoter expression is responsive to temperature variation stress and expression of GUS was increasing after lowering and increasing temperature.

LTR-GUS line 1 and LTR-GUS line 9 shows GUS expression in stem at 24°C (Fig. 17). LTR-GUS line 7 does not show any expression in protonema. GUS expression shows increase in stem for LTR-GUS line 1 and is induced in leaves of LTR-GUS line 9 with the temperature gradient. LTR-GUS line 7 shows induction of GUS expression in protonema at 37°C. This shows that temperature variation stress can induce LTR expression in moss.

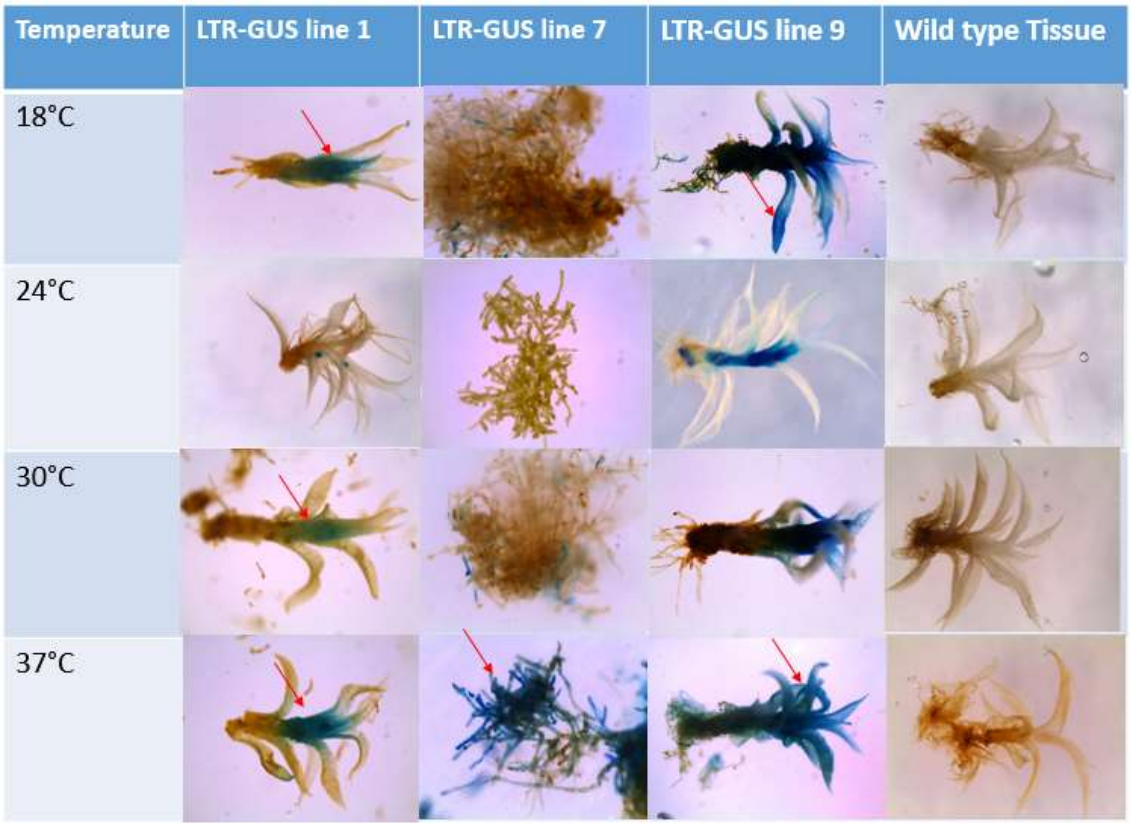

Figure 17. GUS staining of LTR-GUS lines after subjecting to the gradient of temperature. 24°C is regular growth temperature for moss. Red arrow shows places where LTR expression was induced. For LTR-GUS line 1 and LTR-GUS line 9 GUS expression was observed in stem and leaf respectively. For LTR-GUS line 7 GUS expression was observed in protonema.

3.2.4. (E) Homogenisation does not induces expression of LTR promoter

LTR-GUS lines were homogenized for three consecutive weeks and effect of homogenization stress on induction of LTR expression was observed by subjecting 7 day old protonema. Homogenisation does not induce LTR expression in protonema.

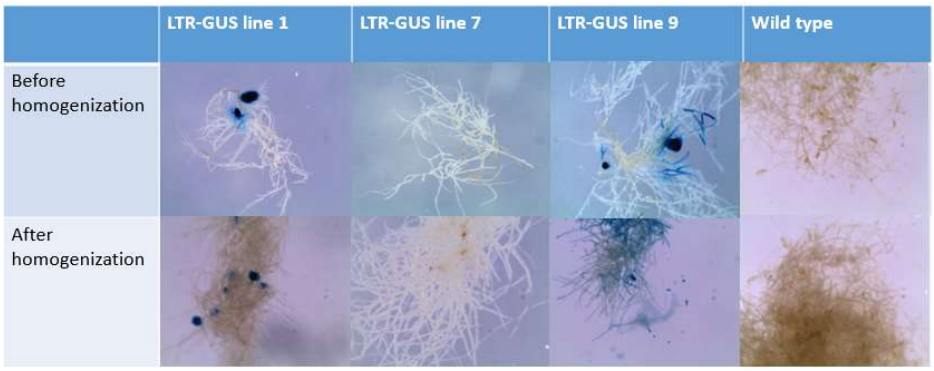

Figure 18. GUS staining of LTR-GUS lines, 7 days after homogenization. Protonemal tissue homogenized for three weeks was analysed for LTR promoter expression induction**.** Homogenisation stress did not show any expression change in protonema of LTR-GUS line 1, 7, 9 and wild type moss.

3.3. Screening for phenotype of interest in Tnt1 and T-DNA insertional *P. patens* lines and molecular characterization

3.3.1 Morphological screening for phenotype of interest in Tnt1 and LTR-GUS T-DNA insertional lines

34 Tnt1 insertional lines and 5 LTR promoter lines were observed under microscope. Following Tnt1 lines and LTR-GUS lines were showing phenotype different from wild type *P. patens*. These lines were selected for further studies (Fig.19).

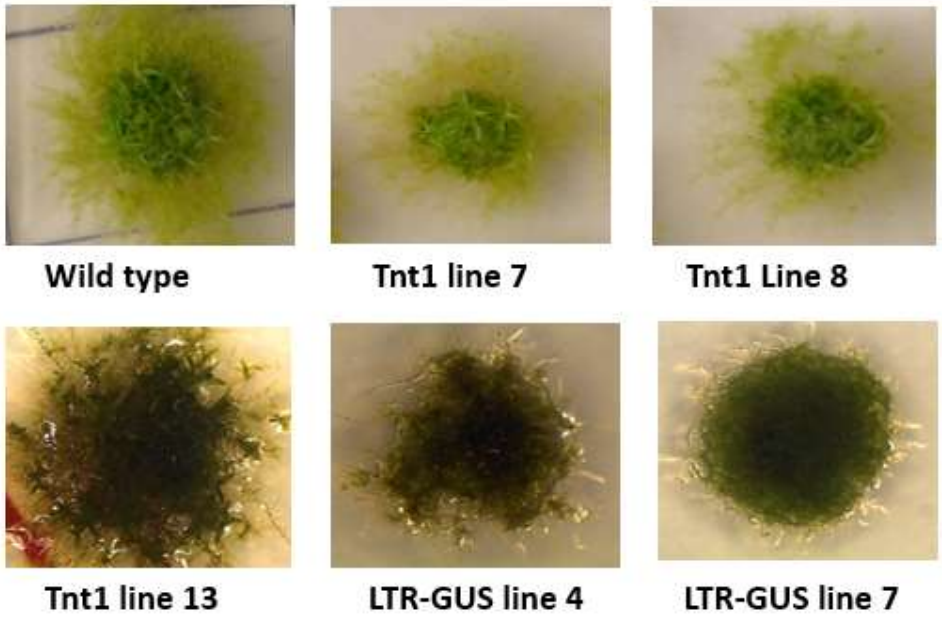

Figure 19. Tnt1 and T-DNA insertion lines which show phenotype different from wild type *P. patens*. Tnt1 line 7 and line 8 shows less dense protonemata growth in colony periphery. Tnt1 line 13 and LTR- GUS line 4 shows retarded growth and less number of gametophores. LTR-GUS line 7 does not proceed to bud development and hence stays in protonemal phase and also shows retarded growth.

3.3.2 Hormone assay for LTR-GUS line7 to characterize phenotype (Bud mutant):

LTR-GUS line 7 tissue was grown on different media supplements to characterize its phenotype. Wild type plant shows early gametophore stage in BCD media which lacks ammonium source, but LTR-GUS line 7 does not proceed to bud stage. In cytokinin supplemented media wild type plant shows high number of bud formation, but LTR line7 does not form bud. In other media types mutant LTR line7 shows retarded growth compared to wild type plant (Fig.20).

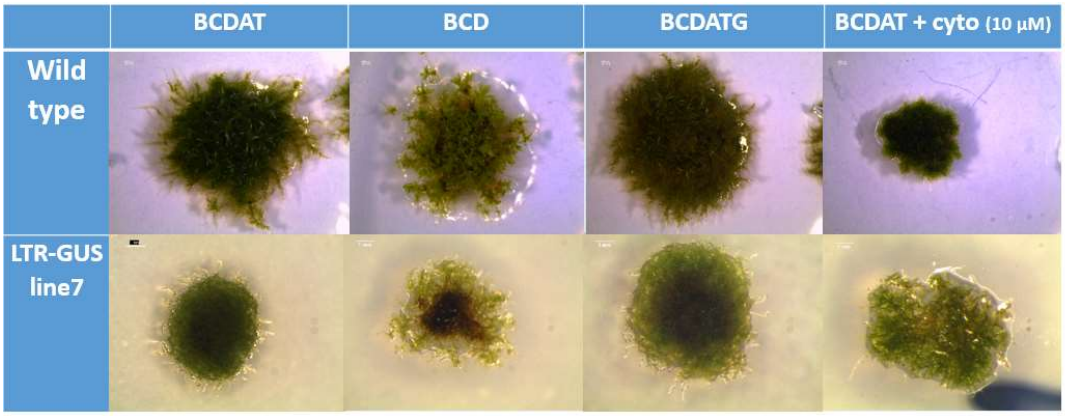

Figure 20. Hormone assay for LTR line 7 with different media supplement. LTR-GUS line 7 was grown on nutrient medium lacking ammonium source, supplemented with carbon, cytokinin hormone and bud development was observed. LTR line 7 did not developed buds in any growth media.

3.3.3 LTR-GUS line 7 shows no bud development compared to wild type plant

LTR-GUS line 7 showed the lack of gametophore development on various nutrient media (Fig. 20). So, bud development in the mutant was compared to wild type. LTR-GUS line 7 was grown on BCDATG (0.5%) medium along with wild type for 21 days and number of bud developed per filament was counted (n=50) (Fig. 21).

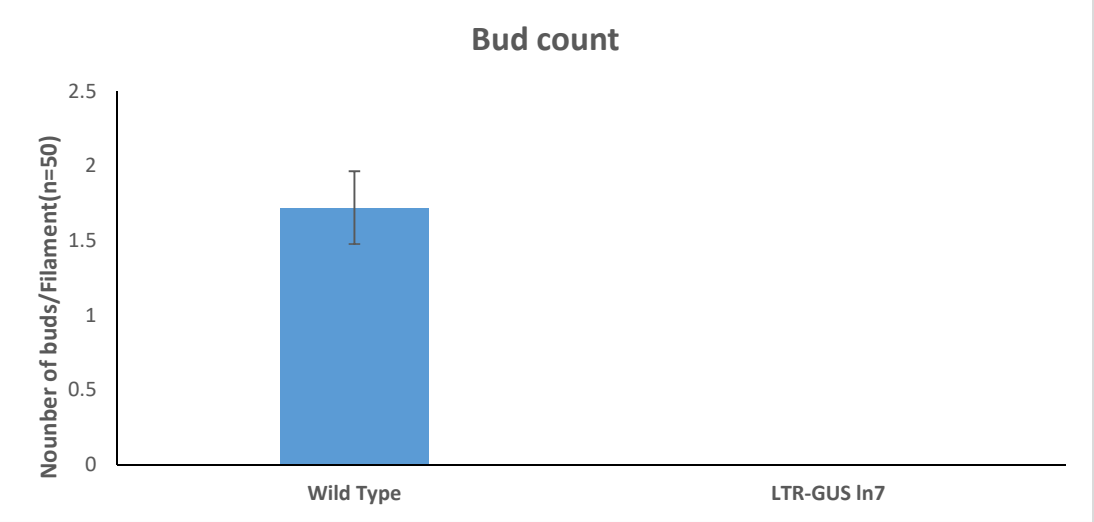

Figure 21. Measurement of number of buds per filaments in wild type tissue and LTR-GUS line7. Error bar represents standard error (n=50).

LTR-GUS line 7 and wild type plants grown on BCDATG were compared for bud number per filament. It was observed that mutant line did not produced any bud. This mutant line was found to be insensitive to cytokinin also. According to previous studies cytokinin plays very important role in bud initiation. The defect in bud development of LTR-GUS line 7 can be because of defect in cytokinin pathway. Whole genome sequencing has been performed for this mutant line, which will reveal genes affected by T-DNA insertion.

3.3.4 LTR-GUS line 4, 7 and Tnt1 line 13 shows reduced colony spread compared to wild type plant:

LTR-GUS line 4, 7 and Tnt1 line 13 showed retarded growth as a phenotype. These mutants along with wild type were inoculated on medium of different nutrient composition and colony spread was measured (Fig. 22). Mutant lines showed reduced colony spread compared to wild type plant on all media except on media containing cytokinin. Tnt1 line 13 does not respond to cytokinin and shows bigger colony spread than wild type.

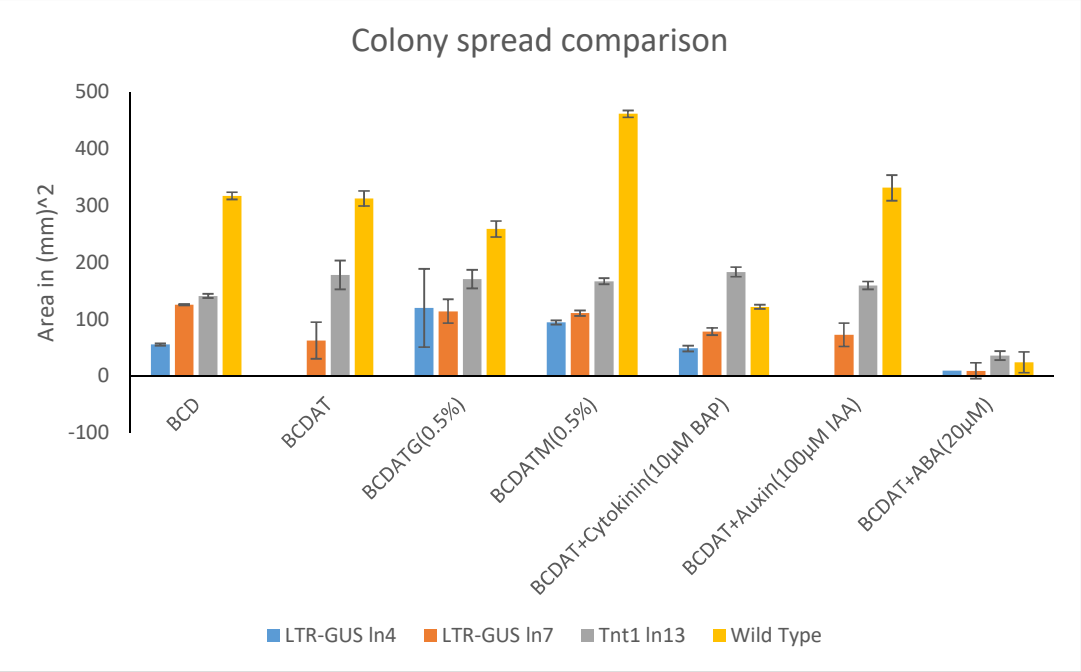

Figure 22. Measurement of colony spread of mutant lines and wild type plants in various medium. LTR-GUS line 4, 7 and Tnt1 line 13 was grown on various medium and colony spread was observed. Mutant lines showed reduced colony spread compared to wild type plant. Error bar represents standard deviation (n=3).

3.3.5 LTR-GUS line 7 shows the reduction in cell length compared to wild type plant:

LTR-GUS line 7 showed reduced colony spread in various kind of media (Fig. 22) so, cell length comparison between wild type and mutant was done. LTR-GUS line7 and wild type tissue was grown on BCDATG medium and sub apical cell length (n=100) of 15 day old culture was measured (Fig. 23). One-way ANOVA test was performed on the data to calculate the significance of difference in cell length and the reduction in cell length is significant ($p<0.0001$).

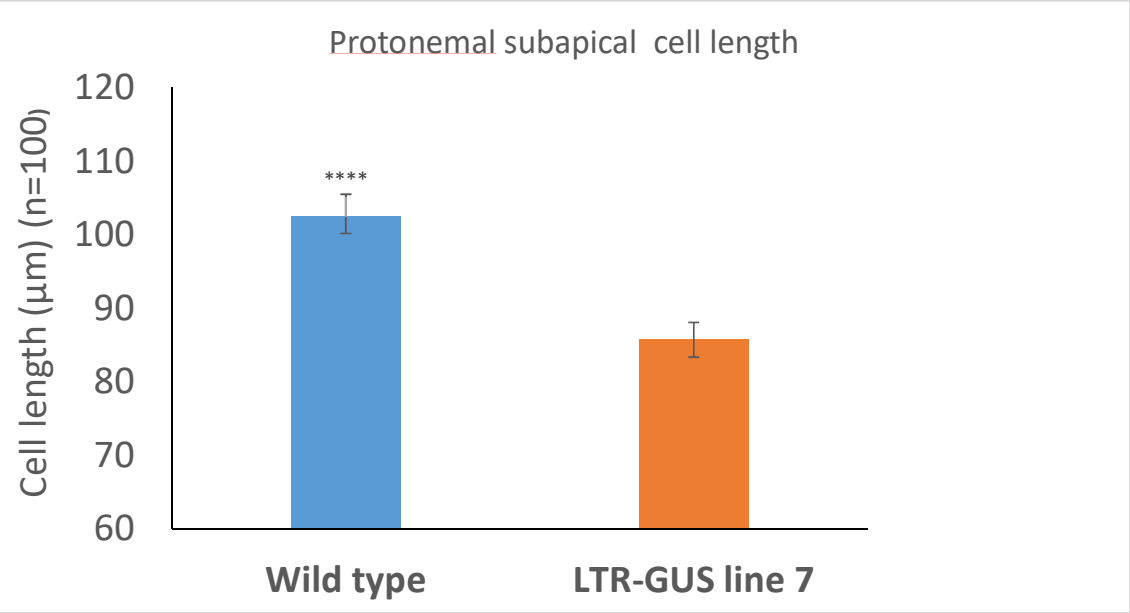

Figure 23. Measurement of sub apical cell length of LTR-GUS line 7 and wild type plant. Error bar represents standard error (n=100). The reduction in cell length is significant ($p<0.0001$)**.**

3.3.6 Gravitropism assay for Tnt1 and T-DNA insertional mutants

Mutant lines of interest showed less colony spread compared to wild type tissue. Caulonemal cell filament growth is necessary for colony spread. Moss tissue grown in dark shows growth of only caulonemal cells. To know if there is any defect in development of caulonema, gravitropism experiment was carried out (Cove, 2006). All mutant lines showed less number of caulonemal filaments compared to wild type tissue. In gravitropism experiment caulonema filaments grow against gravity vector in

dark conditions. This phenomenon is called negative gravitropism. Tnt1 line13 didn't show negative gravitropism and so it is gravitropism mutant (Figure.24). This mutant phenotype is rare in nature. This phenotype may have exhibited because of abnormal development of statocytes or defect in sedimentation of plastids (Cove, 2006). To know exact reason behind this response, Tnt1 insertional analysis is in progress.

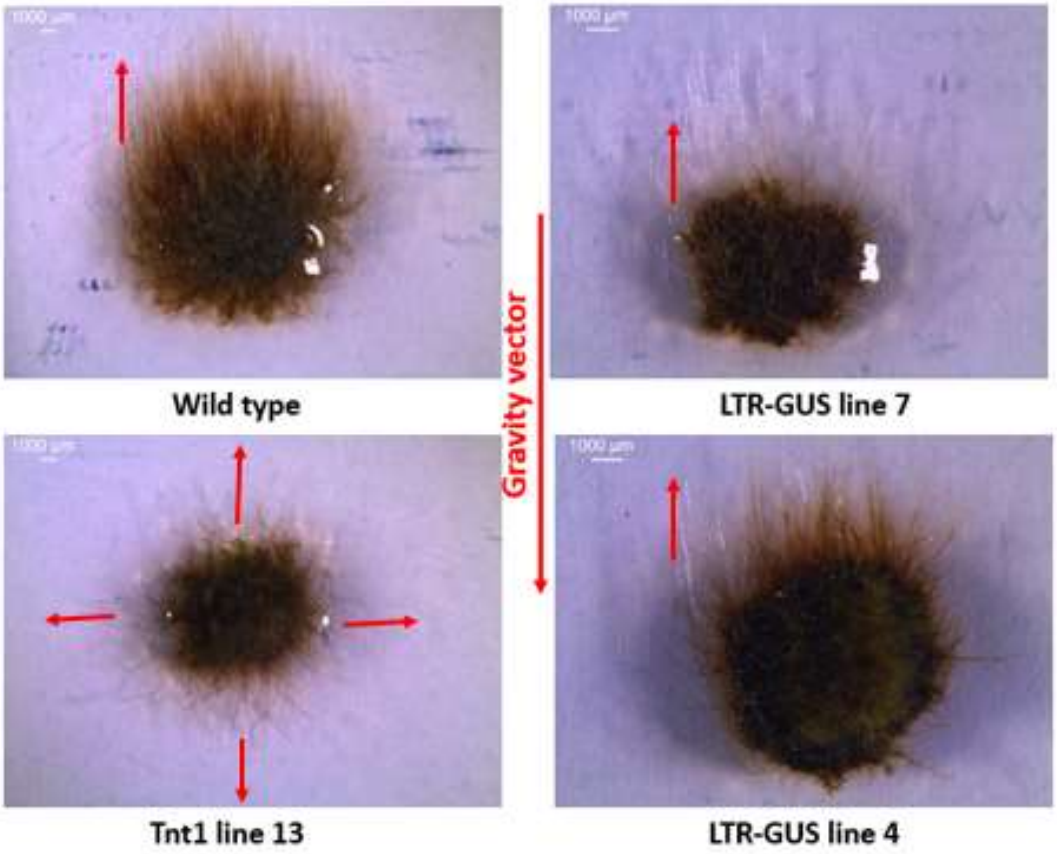

Figure 24. Gravitropism experiment for mutant lines of interest. LTR-GUS line 4, LTR-GUS line 7 and Tnt1 13 were subjected to gravitropism experiment. Red arrows show caulonema filaments growth direction in mutants. Scale bar represents 1 mm.

Chapter 4

Discussion

Retrotransposons are well studied as mutagenesis tool in higher plants (Lucas et.al., 1995, Cui et.al., 2013, C. Mhiri, et.al., 1999), but attempts of its use in lower plants were not much successful. Here in this study, we have established Tnt1 as mutagenesis tool in moss *P. patens*. Stable Tnt1 and T-DNA insertional mutants in *P. patens* were successfully developed .Tnt1 was active in moss and transposition was traced by TAIL-PCR with Tnt1 mutant lines. TAIL-PCR results in this study along with previous data in our lab show that Tnt1 transposition has the high preference to gene rich region (Table 3). This shows that Tnt1 can be used as mutagenesis tool in moss *P. patens*. To understand the expression of Tnt1 in moss, GUS gene was expressed under LTR promoter. We found that, LTR promoter is expressed mainly in bud and stem of *P. patens* (Fig. 13). Previous studies show that, Tnt1 transposition can be induced by subjecting plant to various stress conditions (Charlesworth, 1999). Here, it was observed qualitatively that, $CuCl_2$, salicylic acid, auxin treatment and temperature v stress induced expression of LTR in moss (Fig. 14, 15, 16 and 17). So, stress mediated expression regulation of Tnt1 was conserved in moss. This is qualitative analysis of LTR induction, to understand quantitatively MUG assay can be performed. To understand role *cis* regulatory elements of LTR expression in moss, BI and BII elements were removed from LTR promoter and mLTR promoter was developed. Removing BII elements was shown to cause the reduction in expression of LTR (Casacuberta et.al., 1995). Here in this study, we found that removing BI and BII elements from LTR leads to uncontrolled expression of mLTR and expression was observed throughout the plant (Fig. 13). This result was contrasting to the previous results in higher plants. This can happen if *cis* regulatory elements in higher plants do not play same role in *P. patens.* Generation of more mLTR-GUS lines and replication of similar expression pattern is necessary to support this result. Generation of more mLTR-GUS lines is in progress. We are developing mLTR-GUS promoter lines in tobacco, to understand how LTR promoter expresses in tobacco in absence of both BI and BII elements. This will also tell us about the importance of BI elements in LTR expression. Generation of mLTR-GUS lines in tobacco is in progress. To understand epigenetic regulation of LTR expression in moss bisulfite sequencing can be

performed. This will help us to understand tissue specific expression regulation of LTR in moss.

Tnt1 and T-DNA insertional mutants were screened for mutants deficient in bud and gametophore development. We found that LTR-GUS line7 lacks bud development and never proceeds to gametophore development. Other mutants, LTR-GUS line 4 and Tnt1 line 13 showed reduced number of gametophore. All three mutants showed retarded growth and less colony spread (Fig. 21). To understand reduced colony area we compared sub apical cell length in LTR-GUS line 7 and wild type. Mutant showed the significant reduction ($p<0.0001$) in cell length compared to wild type (Fig. 23). Caulonemal filament growth was analysed with gravitropism experiment to understand, if mutants show any defect in caulonema development. It was observed that, all three mutant show reduced number of caulonemal filaments (Fig. 24). Caulonema filaments are important to spread colony and major filaments playing role in bud development. Also, we found that Tnt1 line 13 is gravitropism mutant and does not show negatively gravitropic behaviour (Cove, 2006). To track T-DNA insertion in LTR-GUS line7 we are performing whole genome sequencing. This will help us to understand genes responsible for no bud phenotype.

Summary

Tnt1 retrotransposon was used for generation of insertional mutants in *P. patens* (moss). Tnt1 was successfully established as mutagenesis in moss and stable insertional lines were developed. Tnt1 insertions were traced by TAIL-PCR for mutant lines. Our TAIL-PCR analysis for Tnt1 transposition showed specificity to the gene rich region. LTR promoter of Tnt1 was characterised for induction of expression with stress conditions like $CuCl_2$, salicylic acid, auxin treatment and temperature gradient qualitatively. Insertional mutants were screened for bud and gametophore development defects. One Tnt1 (Tnt1 line 13) mutant and 2 T-DNA (LTR-GUS line 4 and 7) insertional mutants were selected as an abnormal bud and gametophore mutants. Our results show that LTR-GUS line 7 mutant exhibiting no bud phenotype is insensitive to cytokinin and shows significant reduction in protonemal cell length and colony spread. LTR-GUS line 4 showed buds defective in progression to

gametophore phase. Tnt1 line 13 showed less number of gametophore compared to wild type and is also agravitropic mutant. All three mutants exhibited very less number of caulonema filament development in gravitropism experiment, which justifies reduction in colony spread of the mutants. Whole genome sequencing was performed for LTR-GUS line 7, to understand T-DNA insertions in the genome. This will help us to know genes involved in bud development.

Salient features of the study

1. Tnt1 retrotransposon was successfully established as mutagenesis tool in moss.
2. Our TAIL-PCR analysis for Tnt1 transposition showed specificity to the gene rich region.
3. Modified LTR promoter (mLTR) was developed to study role of BI and BII *cis*-regulatory elements in LTR expression.
4. LTR promoter was characterised for induction by stress condition. We found that LTR promoter expression can be induced by giving $CuCl_2$, salicylic acid, auxin treatment and also temperature gradient affect the promoter activity.
5. Insertional mutants defected in bud (LTR-GUS line 4 and 7) and gametophore (Tnt1 line 13) development were developed and characterised.
6. Tnt1 insertional mutant (Tnt1 line 13) deficient in sensing cytokinin and gravity was screened characterised.

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