CHARACTERIZATION OF RETROTRANSPOSONS PRESENT IN VIRUS-LIKE PARTICLES EXTRACTED FROM PLANT TISSUES

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By

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Abstract

Transposable elements (TEs) are major component of genome. They are fragments of DNA that can insert themselves into new chromosomal locations, and often make duplicate copies of themselves in the process. Among the other TEs, retrotransposons are most active in eukaryotic genome and significantly contribute to shaping genome evolution. Retrotransposons resemble retroviruses but have complete intracellular lifecycle. Retrotransposons are further classified into two major groups-Long Terminal Repeat (LTR) Retrotransposons and Non Long Terminal Repeat (Non LTR). The LTR retrotransposons complete their lifecycle by forming Virus Like Particles (VLPs). The lifecycle of a LTR retrotransposon starts with the transcription of its mRNA and followed by translation where proteins are processed to complete its lifecycle. The proteins are the capsid protein (GAG), aspartic proteinase (AP), integrase (INT), and reverse transcriptase (RT-RH). Like retroviruses, the GAG forms a shell, the virus-like particle (VLP), which packages the nucleic acids of the element and transports them to the nucleus. The proteinase cleaves the polyprotein into the functional proteins, the reverse transcriptase copies the RNA into complementary cDNA, and the integrase inserts the cDNA back into the genome in a new position.

The goal of my research work was to extract these VLPs from plant tissues to identify the active retrotransposons. To identify the TEs, my work was divided in two steps-first step was to work with model VLP system in *Hordeum vulgare* (Barley) to establish a proper protocol for extracting VLPs and second step was to implement that protocol to other plant species. Through my study, we established a working protocol for extracting VLPs from plant tissues and identify BARE 1 retrotransposons and other elements from both *copia* and *gypsy* super family of LTR retrotransposons. My work also showed several LINE element products of Non LTR retrotransposons group in the VLPs of *Liliaceae* plant family. These interesting findings from my thesis work supports that the VLPs are important stage in life cycle of retrotransposons which are significant contributors to the growth in genome size.

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1. Introduction

1.1 Genome:

Complete set of genetic information in an organism- A genome is an organism's complete set of genetic information. All living being, from unicellular prokaryotes to multicellular eukaryotes have unique genome. The genetic information of genome is stored in DNA or deoxyribonucleic acid. The DNA is constructed with four bases – Adenine, Guanine, Cytosine, and Thymine attached in a phosphate backbone. These four bases follow a particular order or "sequence" in genome, called "DNA sequence". The diverse sequence made up of only these four bases regulates different gene function and biological activities. There are also DNA sequences found in genome with unknown function. These days, sequencing a part or whole genome opened a vast opportunity to explore new and existing genomic information.

1.2 Eukaryotic Genome:

Large and Complex Eukaryotic Genome- The genome of eukaryotic organism is extremely complex and large. It has multiplex cellular compartmentalization and large set of conserved genes (Koonin et al., 2015). Gene regulation is also significantly complicated due to the presence of

multifunctional cells. There are several factors that contribute to the enormous size of the eukaryotic genome. One of the significant contributors is “Intron”. The introns occupy a substantial fraction of DNA but so far their function is unknown to the scientists(Cooper, 2000) Another important factor contributing to the increased genome size is “Gene families and Pseudogenes”. In eukaryotic genome certain genes are present in multiple copies, are called gene families. Gene families are thought to have arisen by duplication of an original ancestral gene. Furthermore, some nonfunctional gene copies, called pseudogenes significantly increase the size of eukaryotic genomes without making a functional genetic contribution (Cooper, 2000).

Genome size in eukaryotes not always proportional to genome complexity- Though the genomes of eukaryotic multicellular organisms are robust but their complexity do not necessarily depend on gigantic size of genome. For example, Genomes of Lilies or Salamander are much larger than human genome but not as complex as that.

The above phenomena can be explained by the fact that a substantial part of eukaryotic genomes contain highly repetitive noncoding DNA sequences. Much of the complexity

of eukaryotic genomes thus results from the abundance of several different types of noncoding sequences present in multiple copies in DNA of the higher eukaryotic cells (Cooper, 2000). The noncoding sequences are either spread throughout the genome or clustered in one particular area. Most of these DNA repeats are Transposable elements. The transposable elements (TEs) or transposons are the fragments of DNA that jump around or transpose in genome. TEs are highly repetitive in nature and with the help of enzyme transposases, they mobilize through the mechanism of transposition or retrotransposition.

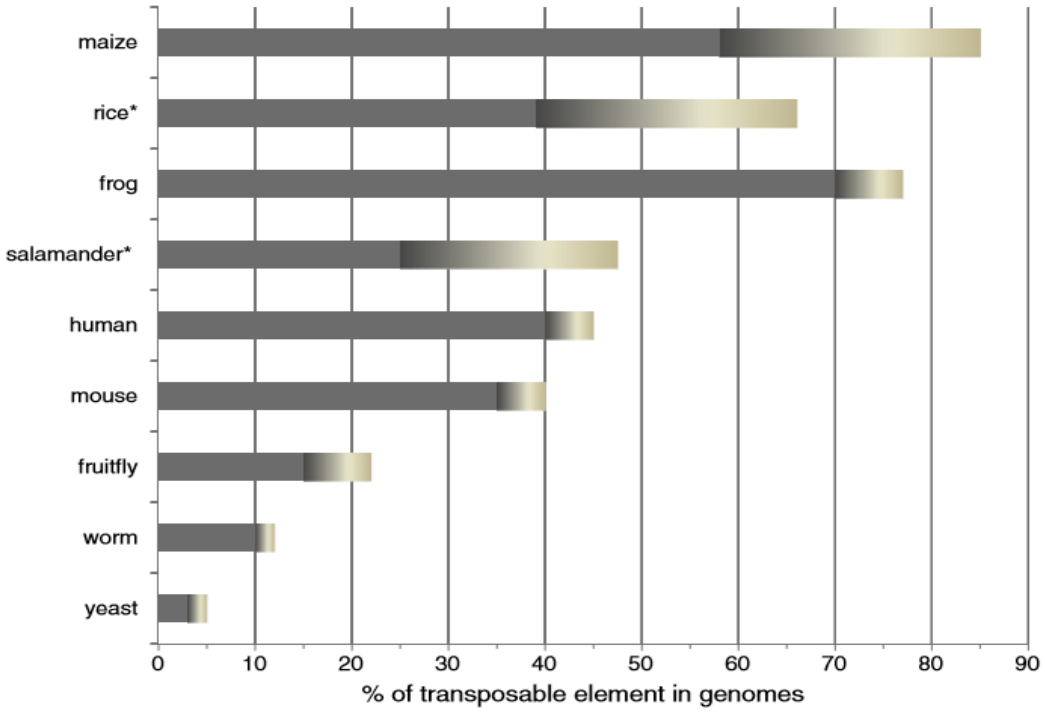


Figure 1: Percentage of Transposable element in genome

1.3 TEs in Eukaryotic genome:

Transposable elements (TEs) are one of the largest part of eukaryotic genome and affect the genome size massively (Figure 1). They account for at least 45% of the human genome and 50–90% of some plant genomes (Wessler, 2006). They are constituted with wide array of DNA sequences with diversified structure and transposition mechanism. Since, transposable elements occupy a large portion of eukaryotic genome, their movement and accumulation represent a major force in shaping the genes and genomes of almost all organisms (Feschotte and Pritham, 2007). The transposons may present in variable sizes in genome but they all exert immense impact on genome. TEs or in other words the mobile DNA fragments have both deleterious and positive affect on eukaryotic genome. The mobility of TEs can induce detrimental effect on genome by mutations, gene disruption and chromosome rearrangements. But transposition activity also has beneficial aspects and the mutational activities of TEs contribute to acquire the genetic diversity in organisms throughout the evolutionary period (Chénais et al., 2012)

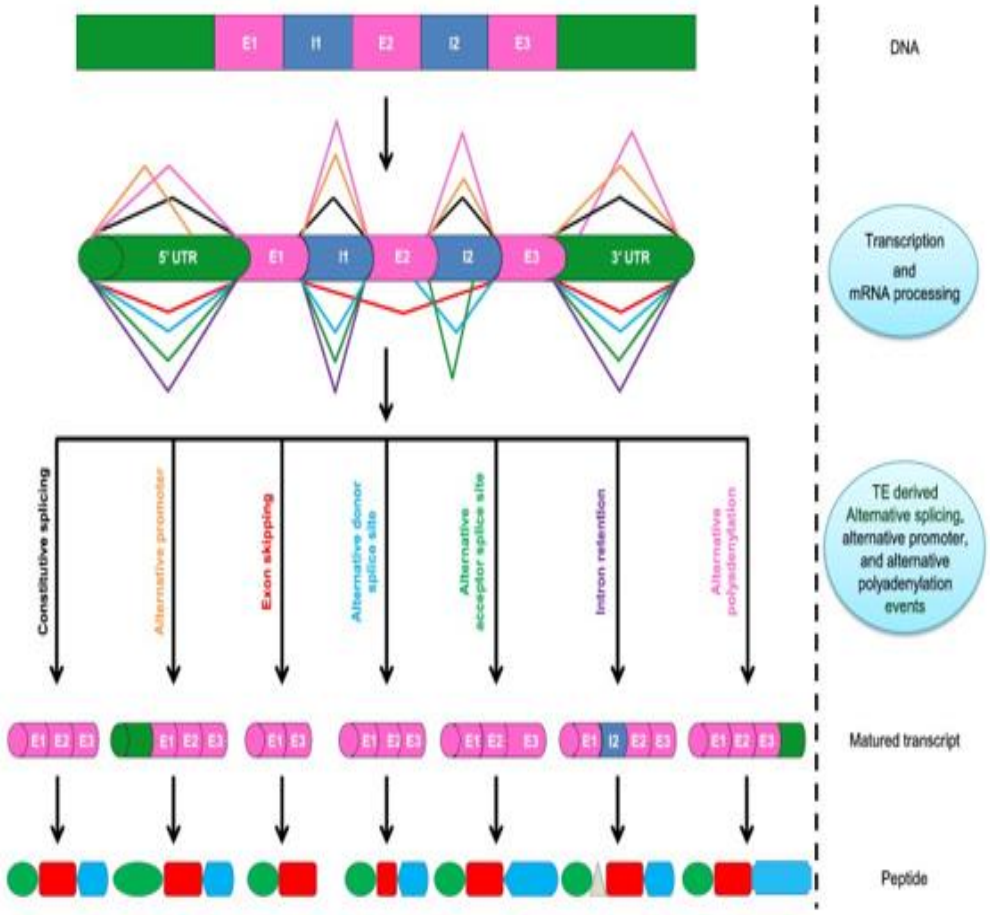


Figure 2: Structure of a Retrotransposon

Transposable elements were discovered by Barbara McClintock in 1940s. While studying chromosome breakup in maize, she discovered a chromosome-breaking locus that could change its position within a chromosome. Later, these mobile genetic elements were named as "transposons" (Ravindran, 2012). McClintock's analysis of chromosomal

breakage in maize led to the discovery of two transposable elements- Dissociation (Ds) and Activator (Ac). "Ds is located at the site of chromosome breakage and can be defined both genetically and cytologically. Ac is dominant and is required for chromosome breakage at Ds" (Ravindran, 2012).

TEs are jumping fragment of DNA- Transposable elements are discrete segment of DNA which transpose in different part of genome with the help of enzyme transposase and the mechanism is called transposition. Based on transposition mechanism, transposable elements are divided in two major classes (Figure 3). Class I or Retrotransposons (Figure 2) which use RNA intermediate for transposition and Class II or DNA transposons. The transposition mechanism of Retrotransposons are called "copy-paste" method where the original element is left on the donor site and a copy is inserted in a new position. On the other hand, DNA transposons use "cut-paste" method where the element leave the donor site and reintegrate in a new position (Lisch, 2013). There is another group of mobile element present in plant genomes in abundance called Helitrons. They transpose via "rolling circle" method (Lisch, 2013).

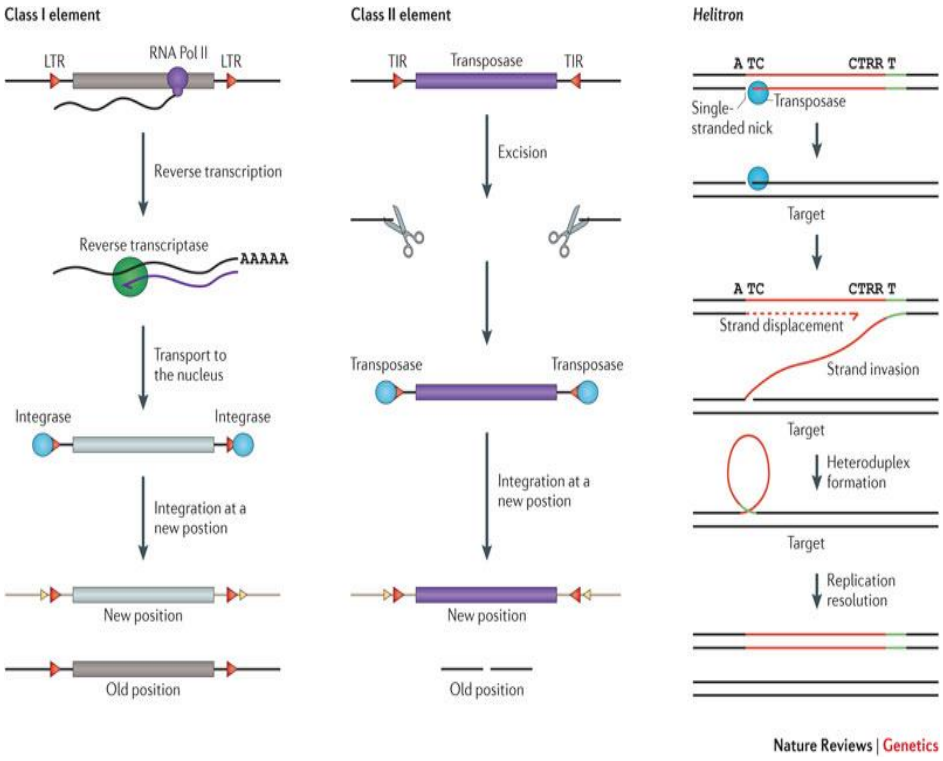


Figure 3: Different Mechanisms of Transpositions

The Class I (Figure 2) and Class II TEs are further divided in several subclasses, orders and super families. The orders for Retrotransposons are LTR, DIRS, PLE, LINE, and SINE, depending on the structures of their genomic DNA copies. The DNA transposons are also divided in two major subclasses depending on their transposition mechanism.

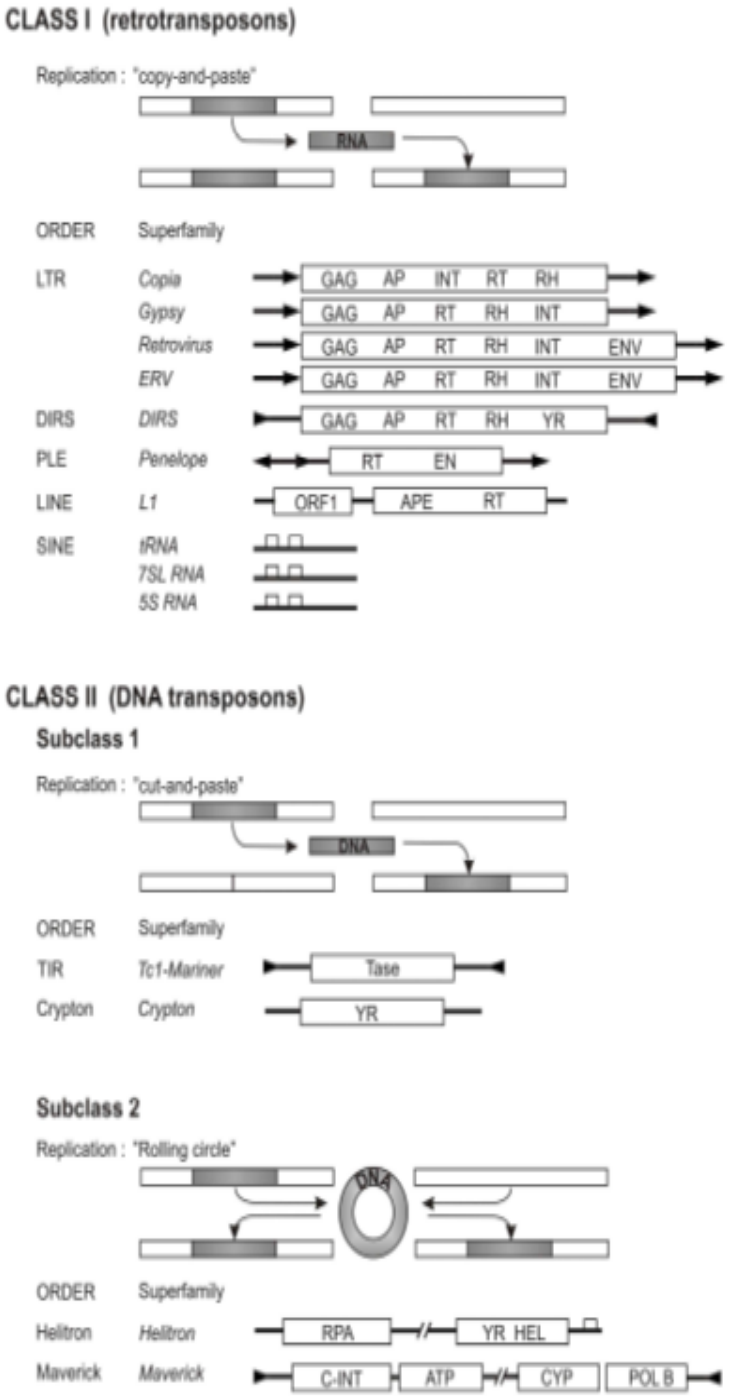


Figure 4: Classification of Transposable Elements

Furthermore, transposable elements are also grouped as Autonomous and Non-autonomous elements (Figure 4). Autonomous elements have open reading frames (ORFs) that encode the products required for transposition. In contrast, non-autonomous elements do not encode transposition proteins but are able to transpose with help of autonomous elements (Wessler, 2006).

Importance of Transposable elements present in genome- These days, whole genome sequencing, transcriptome sequencing and other modern molecular tools have opened the door ajar to study and explore the transposable. Once regarded as "Selfish" or "junk DNA", the TEs are now considered as extremely significant component of genome in controlling gene expressions and shaping the genome over the evolutionary period. Extensive studies in animal and plant models, such as *Drosophila melanogaster* and *Arabidopsis thaliana* state that TEs have considerably shaped the structure, function, and evolution of the genomes and that the regulatory sequences that they possess can interfere with the networks of regulation of many genes, even of genes located at some distance from them (Biémont, 2010). Recent research suggests that their activity is regulated by epigenetic mechanisms, including

methylation of DNA and histone modifications. Moreover, the capacity to induce mutation, regulate genetic system made the idea concrete that TEs not only could generate genetic polymorphism favoring population adaptation, but also could promote speciation (Biémont, 2010). Intensive research is going on to find the relationship between high copy number of transposons in genome causing heavy genetic load and evolutionary benefit in organisms. Specially class I transposons, Retrotransposons in plants are significant contributor to the severe increasing genomic obesity throughout the progression of life.

How TEs restructure genomes? Genome wide TE analysis in eukaryotic organisms showed that insertion and deletion of these DNA fragment can cause major restructure in genome. According to the recent research, transposable elements restructure genome in three different ways (Wessler, 2006) - "TE-mediated Chromosome Breakage and Rejoining, TEs as Insertional Mutagens, TEs and Epigenetic Regulation". Whole genome sequencing of eukaryotic genomes in multiple organisms reveal that transposons act as mutagenes by inserting themselves in different areas of genome and control gene expression. For the above reason, the host genome has developed epigenetic mechanisms in the

eukaryotic cells to silence the expression and mobility of the transposable elements. "Owing to their ability to recruit the silencing machinery, TEs have served as building blocks for epigenetic phenomena, both at the level of single genes and across larger chromosomal regions" (Slotkin et al., 2009). The mechanism of silencing of the TEs is especially important in gametes. Notably, the plants have evolved several interesting pathways to reinforce silencing mechanism. One of the major factors in silencing mechanism is DNA methylation. Methylation of TEs is correlated with a transcriptionally silent state, and impaired DNA methylation often leads to the reactivation of TE activities. For example, *CACTA*-like DNA transposons and several types of retrotransposons are reactivated in the *Arabidopsis ddm1* mutant (Cui and Cao, 2014).

1.4 TEs Plants:

About 85% of Plant genomes are made up of TEs- Among all the eukaryotes, diversify mechanisms and effect of TEs are most effective and abundant in plant genomes. Repeated genome sequencing has revealed that the transposons range from 3% to 85% of plant genomes. Significant genome size variation are common even in a single genera such as rice cotton and sorghum (Chénais et al., 2012). Plant genomes

are also significantly diversified in terms of the distribution of the transposons in the chromosome. Majority of TEs are centered in middle of the chromosome in plants, however, the *Zea mays* (maize) genome has high copy number of transposons distributed throughout the genome. Not only that, 60% of *Glycine max* (soybean) genome is also composed of transposable elements. However, in contrast in rice genome, has several thousand TEs inserted in introns (Hirsch and Springer,2016) which has less clear impact in comparison to insertion of TEs in coding region. Insertion of these mobile elements specially the Long Terminal Repeat (LTR) retrotransposons have affected the C value in majority of plant taxa.

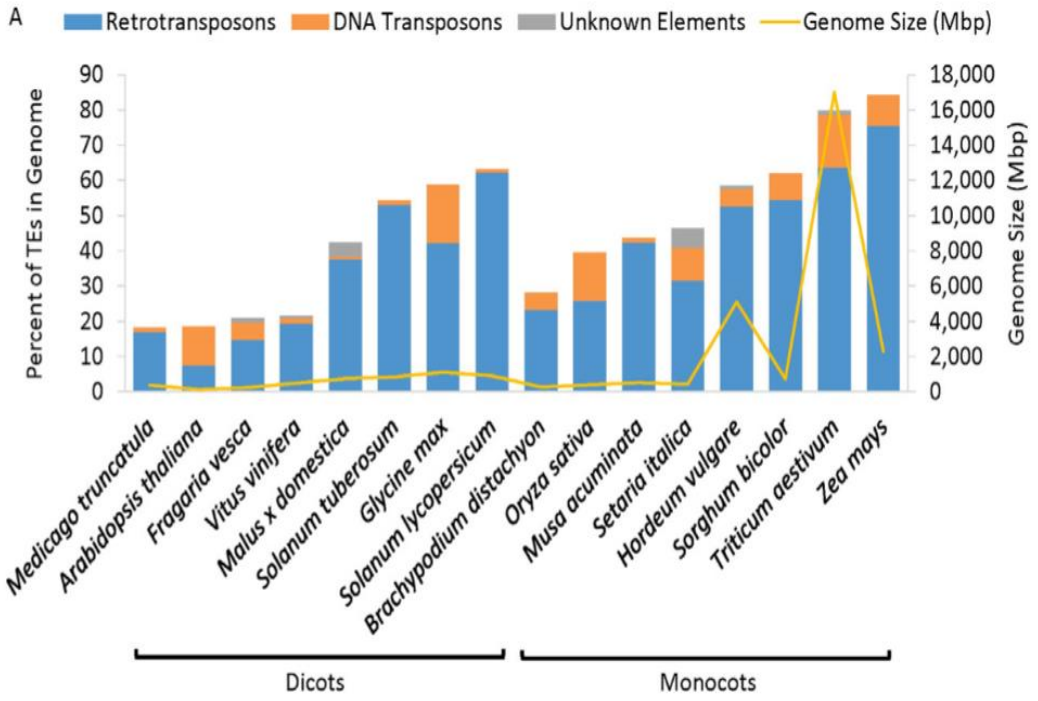


Figure 5: Distribution of Transposable Elements in Genome

C value paradox- C-value' means the 'constant' (or 'characteristic') value of haploid DNA content per nucleus, typically measured in picograms (1 picogram ~1 gigabase) (Eddy, 2012). The general understanding of C: value is that simplicity and complexity of genome varies according to the size of genome. But in reality the C value is highly variable. For example, there are many related species in the same genus that have haploid genome sizes that differ by three to eight fold between them. The plant genomes show more C value variation (Figure 5) than any other eukaryotic

genome. The DNA content in their genomes is highly variable, by as much as 2,056-fold from a 1C amount of 0.0648 pg to 132.5 pg. The mean 1C:value in plants is 2.4 pg, and genome size expansion/contraction is lineage-specific in plant taxonomy (Ayarpadikannan et al., 2015). Recent research suggests that the dynamics of transposition mechanism of class I Retrotransposons are the most significant contributor to the 1C value differences among plant genomes.

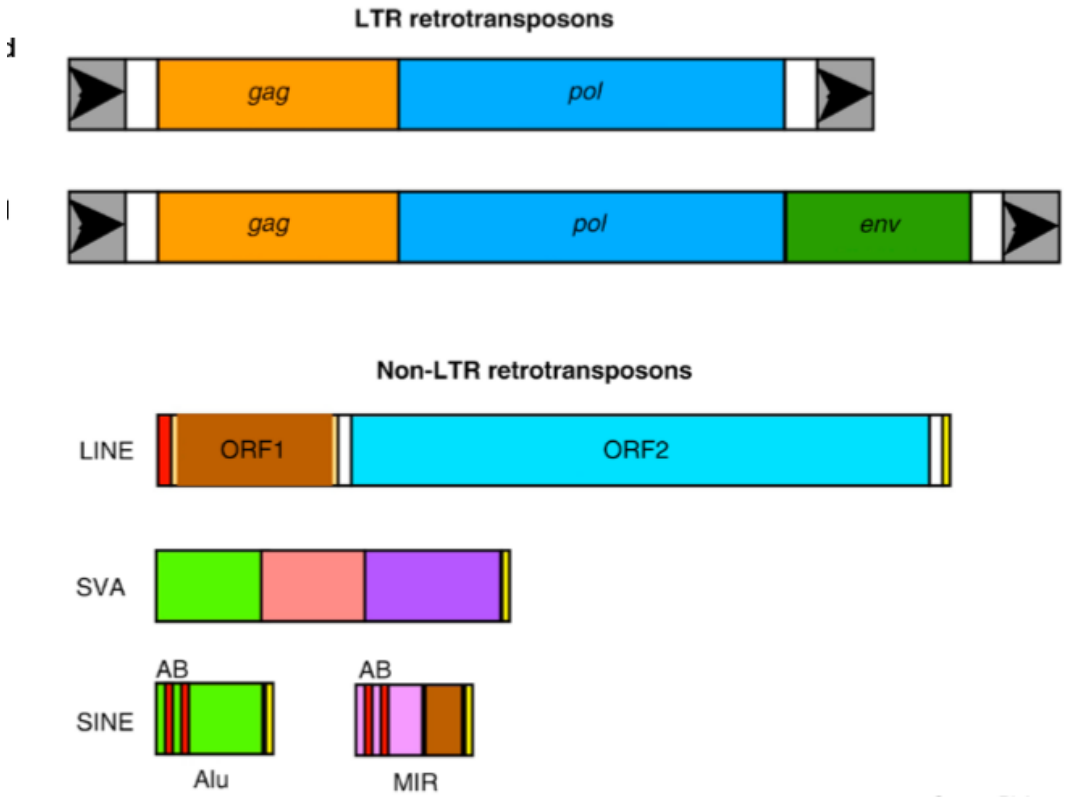
Retrotransposons in Plants- Class I transposable element or Retrotransposons (Figure 6) are prolific in plant genome. Retrotransposons move to new chromosomal location via RNA intermediate which gets converted to cDNA prior to reintegration in new location. This replicative mode of transposition can rapidly increase the copy numbers of elements and can thereby greatly increase plant genome size (Kumar and Bennetzen, 1999). Plant retrotransposons have diverse family member in respect to their sequence and structure. Throughout the evolution, retrotransposons share a dynamic relationship with the host plant genomes. "Because retrotransposons have the potential to dramatically alter gene function and host genome structure, it is not surprising that their transpositional activities

are regulated both by retrotransposon- and host-encoded factors, possibly to avoid deleterious effects on host and retrotransposon survival" (Kumar and Bennetzen, 1999).

1.5 Retrotransposons:

What are Retrotransposons? Transposable elements that contain reverse transcriptase gene are referred to as retrotransposons. These elements use reverse transcription method via RNA intermediate to transpose in the genome. In this process, the DNA strand makes a copy of m- RNA transcript and then transfers the copy of RNA to the target site. Reverse transcription of the RNA sequence makes a c-DNA copy using reverse transcriptase enzyme and get inserted into the new location of DNA strand of the genome. Retrotransposons are classically divided into two segment. Type 1 is Long Terminal Repeat (LTR) and Type 2 is Non-Long terminal Repeats (Non-LTR). LTR retrotransposons, like retroviruses, have a potential t-RNA primer binding site, a polypurinic tract, and genes for to *gag* (a capsid protein), *pol* (a polyprotein) with some having a third gene equivalent to *env* (envelop protein) (Finnegan, 2012). Non-LTR retrotransposons such as LINE, SINE or SVA have two open reading frames. The first encodes a RNA-binding protein while the second codes for a nuclease, usually

related to an apurinic-apyrimidinic repair endonuclease, a reverse transcriptase, and in some cases an RNAse H domain



(Finnegan, 2012).

Figure 6: Typical Structure of Retrotransposons

Effect of Retrotransposons in genome- Insertion of retrotransposons in genome has multitudinal consequences. They goes way beyond the explanation of the larger genome size and its complexity. The retro elements inactivate genes by inserting within them, change gene expression by separating a gene from a regulatory sequence or by bringing

a new regulatory element into its vicinity, alter gene products by modifying patterns of RNA splicing, generate new genes by incorporating an adjacent sequence into the RNA transposition intermediate and inserting it elsewhere in the genome, and allow large-scale genome rearrangement by acting as sites for non-allelic homologous recombination (Finnegan, 2012). Furthermore, these mobile genetic elements are thought have great impact on chromatin instability induced genomic rearrangement and changes in gene topology leading to changes in gene regulation and expression.

1.6 LTR & VLPs:

Major component of Plant Retrotransposons- Long terminal repeat or LTR retrotransposons are major component of the plant genome. They are named for their long terminal repeats flanked on both sides. The length of the LTR can vary between a hundred to several thousand base pairs. Most LTR retrotransposons have common structural features and encode similar genes, still they are diverse in their genomic organization, reflecting the different strategies they use to proliferate within the genomes of their hosts. A typical LTR (Figure 7) contains promoters, terminators and RNA processing signals for the transcription (Figure 8)

of the retrotransposons. Recent studies shows that the LTRs are up- regulated by the different category of external stress and an important player in plant genomic diversification. A significant example is family *Liliaceae*. Multiple studies in species of *Liliaceae* reveal that they have large variations in genome sizes from 12.3 to 127.4 pg DNA in their C value (Ayarpadikannan et al., 2015). In studies of giant genomes of *Fritillaria* lilies, showed that Ty3-gypsy LTR retrotransposons and AT-rich microsatellites were the predominant components of the highly repeated genomic DNA fractions.

Structure of LTR- The Long Terminal Repeats flank the one or two open reading frames (ORFs), respectively as a single gag-pol –frame or as separate gag and pol –frames.

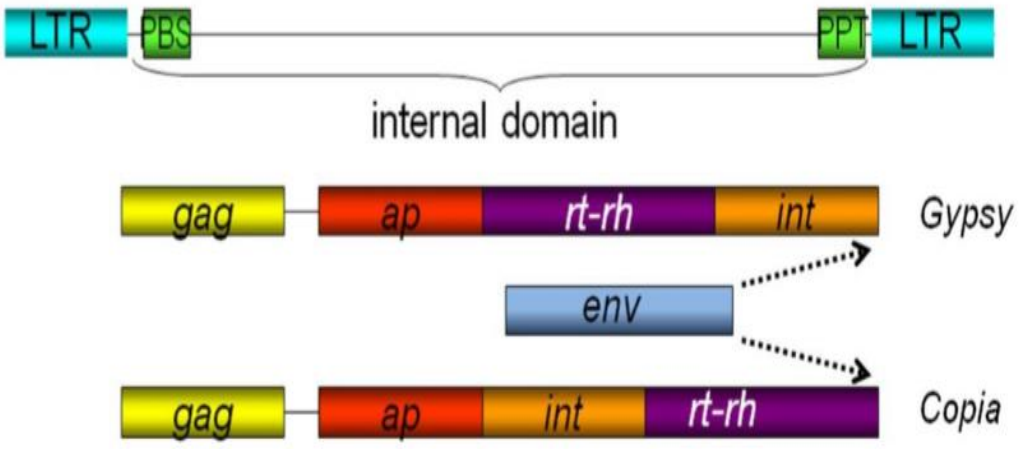


Figure 7: *Copia* and *Gypsy* LTR Retrotransposons

The LTR retrotransposons are further divided into *copia type* (Pseudoviridae) and *gypsy type* (Metaviridae) superfamily based on the gene order in opening reading frame (Grandbastien, 1992)(Granbastein, 2015). Typically the gene order is 5'LTR-gag-ap-rt-rh-int-3'LTR for the Gypsy superfamily and in the Copia superfamily the order is 5'LTR-gag-apint-rt-rh-3'LTR. The gag gene encodes a single structural protein, the GAG, a capsid protein which assembles into the shell of the Virus Like Particles (VLPs), the pol encodes a polyprotein, which comprises three enzymes, the aspartic proteinase (AP), integrase (INT), and reverse transcriptase_RNaseH (RT-RH). The POL and/or GAG-POL polyprotein is cleaved by the AP into functional peptides(Jääskeläinen, 2012). Some plant LTR retrotransposons have both *copia* and *gypsy* types of element with an "env" like protein coding domain similar to retroviral ENV protein. However, there are several types of LTR retrotransposons abundant in host genome and cannot be classified as copia type or gypsy type, due to their lack of coding sequences such as TRIMs, SMARTs and LARDs.

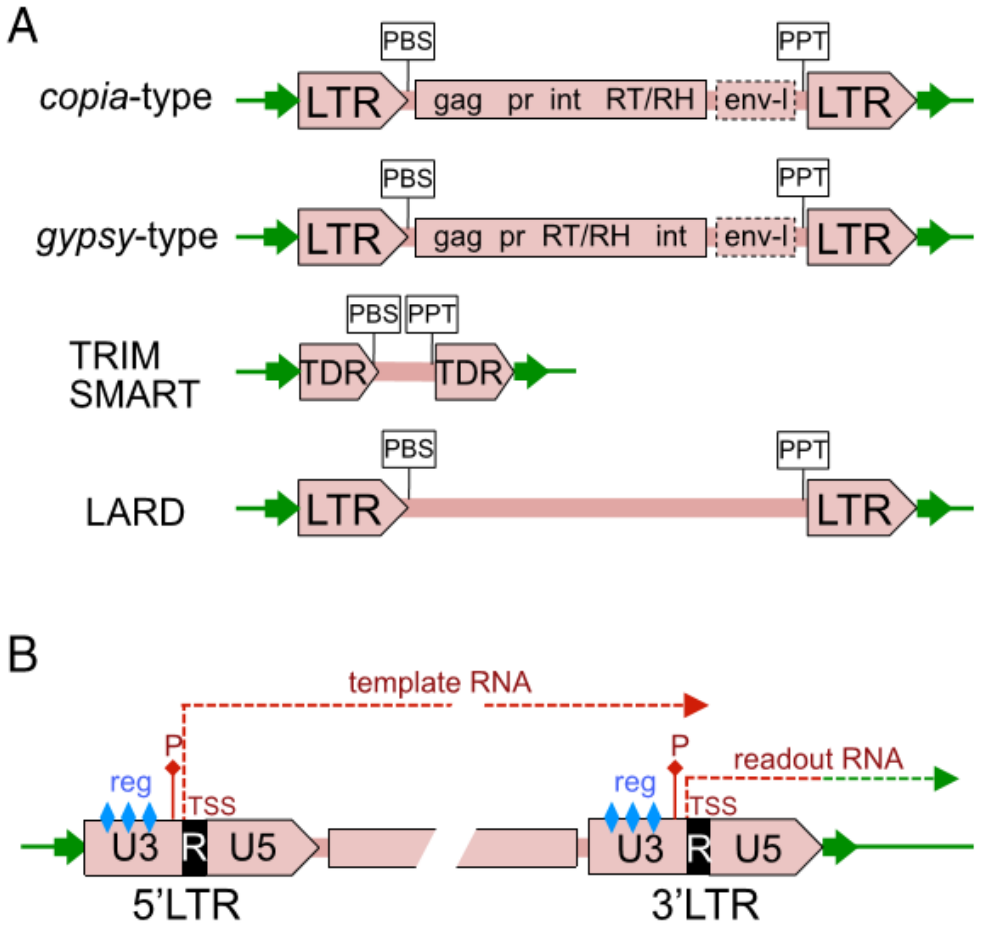
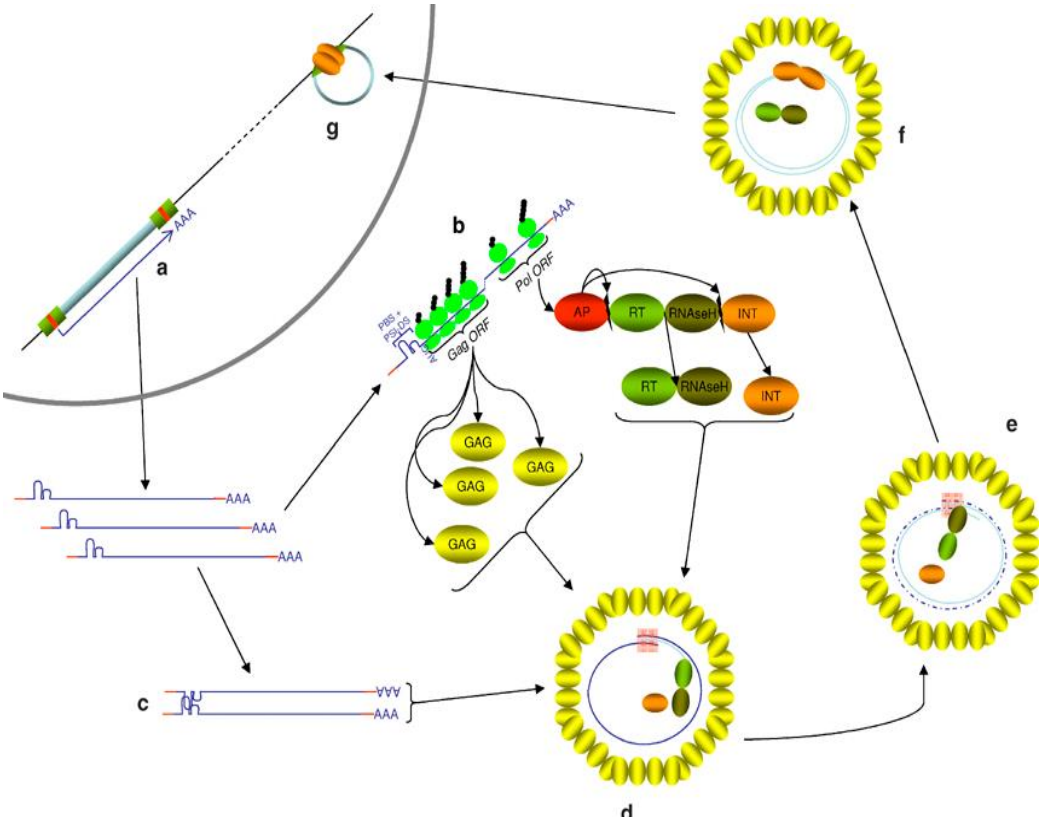


Figure 8: Structural motifs in a typical LTR retrotransposon

Life cycle of LTR- The replication of LTR retrotransposons resembles the intracellular phase of retroviral life cycles. The LTR element replicate through "copy-paste" method. The lifecycle starts with the transcription followed by translocation of the gRNA into the cytoplasm, translation of the proteins, VLP assembly,

replication of the gRNA into cDNA by reverse transcription, transport into the nucleus, and insertion back to the



genome (Jääskeläinen, 2012).

Figure 9: Lifecycle of LTR Retrotransposons

VLPs- Virus-like particles (VLPs) are multi subunit self-assembly competent protein structures with identical or highly related overall structure to their corresponding retroviruses (Zeltins, 2012). The term ‘‘VLP’’ has been used to describe a number of biological objects, such as uncharacterized structures with viral morphology that are

found in biological samples, empty structures of viral origin that are not composed of nucleic acids, infectious viruses with chemically or genetically introduced structure modifications and noninfectious, self-assembled gene products resulting from the cloning and expression of viral structural genes in heterologous host systems (Zeltins, 2012). In LTR retrotransposon life cycle VLPs play crucial role in transposition. In LTR retrotransposon life cycle (Figure 9) VLPs play crucial role in transposition. They are assembled with GAG protein and resemble retroviruses but contains complete intracellular life cycle unlike retroviruses.

VLPs are vehicle to study active transposable elements- The LTR retrotransposons assembles VLPs during their replication. In different stages of their life cycle, VLPs contain gRNA or cDNA depending on the preliminary or end stage of transposition. Therefore, successful extraction of Virus like Particles from plant tissues and subsequent identification of the gRNA or cDNA through Next generation sequencing will help to characterize the active transposons in plant tissues. Thus, VLPs are powerful tools to study existing and discover new transposable elements.

2. **Aim of my study:**

Retrotransposons are prolific in Plants and they play major roles in increasing the genome size, gene silencing, changes in gene expression and introduction of beneficial and detrimental mutation. Therefore, understanding the retrotransposons can hold a key to understand several above mentioned factors. Life cycle of LTR retrotransposons depend on formation of Virus like Particle (VLPs). Therefore, my work includes-

A. Successful extraction of Virus like Particle (VLPs) from plant tissues

B. Characterization of the VLPs by Next Generation Sequencing, to identify retrotransposons as for example, BARE family the major and active retro element in Barley.

3. Material and Methods:

3.1 Isolation of Virus like Particles (VLPs) from Plant tissues:

Objective: Extraction of VLPs from leaves

Materials: Freshly picked leaves of *Hordeum vulgare (Barley), Prosartes smithii, Scoliopus bigelovii, Clintonia uniflora/borealis/umbelullata, Lillium ???Smilax californica,* Cryo tubes (Nunc Cryo tube 1.8 ml vials), chilled autoclaved 0.1M sodium citrate buffer(pH 7) , 0.1M DTT (dithiothreitol) , Bead Beater (Biospec mini bead beater), 2.5mm beads, 1.5mL microfuge tubes, centrifuge (Eppendrof Centrifuge 5424), 33.3% (v/v) Triton X-100, 1.5mL polyallomer ultracentrifugation tubes (Beckman # 357448), chilled 20% sucrose solution in 0.1M sodium citrate buffer, chilled 40% sucrose solution in 0.1M sodium citrate buffer, chilled 60% sucrose solution in 0.1M sodium citrate buffer, six 11mm Delrin adaptors (Beckman # 355919), TL-100 Beckman Ultracentrifuge, chilled 0.5X sodium citrate buffer, chilled-resuspended buffer (with EDTA) and chilled-resuspended buffer (without EDTA).

Methods:

- **(A) Bead beating preparation:**

- Label 6 Cryo Tube from 1-6.
- All the tubes and reagents were kept on ice.
    - 850uL chilled "autoclaved 0.1M sodium citrate buffer" (pH 7) was added into the each cryo tubes
- Then 80uL 0.1M DTT (dithiothreitol) was added into the each cryo tubes
- 100mg ripped plant leaves were added in to the tubes
- Five to six 2.5mm beads in each tube.
    - Tubes were incubated in ice for 10 minutes prior to homogenization.

- **(B) Tissue disruption by Bead Beating**
    - The bead beater was turned on and the machine was set to 5000 rpm (set machine to "50") at 20-second (set machine to 02) intervals for a total of 1 minute.
    - On the interval the tubes were kept on ice.
- **(C) Transferring leaf homogenate.**
    - 6 new sterile 1.5mL microfuge tubes were labelled.
    - The leaf homogenate (liquid) from each of the bead-beated-tubes into the six newly labeled 1.5mL microfuge tubes

- These new six microfuge were centrifuged for 5 min at 15000 rpm.

- **(D) Transferring supernatant**
  - 6 new sterile 1.5mL microfuge tubes were labelled
  - 80uL of 33.3% (v/v) Triton X-100 was added into each tube.
  - The supernatant was immediately transferred to the 6 new sterile 1.5mL microfuge tubes that have the 33.3% Triton X-100 in them.
  - These new tubes (with the Triton X-100 in them and the supernatant) were then inverted a few times and placed on ice.

- **(E) Ultracentrifugation Preparation.**
  - Twelve "1.5mL microfuge Tube Polyallomer Beckman" tubes were labelled 1-6 and 1-6.
  - Now the supernatant + Triton X from step D was estimated before adding sucrose solution as the total volume needs to be 1400uL in each ultracentrifuge tube.
  - The "chilled 20%, 40%, 60% sucrose solution in 0.1M sodium citrate buffer" was added before adding the supernatant on each tube to get a total volume of 1,400μL (supernatant + Triton X + % sucrose)

- Then Barley (1st extraction) leaves extraction (Tubes 1-4) and Prosartes (2nd Extraction) leaves extraction (Tubes 1-6)added on top of 20% sucrose, Scoliopus (3rd Extraction) leaves extract (Tubes 1-6) on were loaded on 20%, 20%,40%,40% and 60%, 60% respectively and Clintonia, Lillium, Prosartes & Smilax (4th Extraction) leaves extracts (Tubes 1-6)were loaded on 40%, 60%, 40%, 60%, 60% and 60% respectively.

- **(F) Ultracentrifugation # 1**

- The six polyallomer ultracentrifugation tubes were weighed meticulously

  - Weight of polyallomer tube # 1
  - Weight of polyallomer tube # 2
  - Weight of polyallomer tube # 3
  - Weight of polyallomer tube # 4
  - Weight of polyallomer tube # 5
  - Weight of polyallomer tube # 6

- A "TLA-100.3 fixed angle rotor" was fitted with six Delrin adapters.

- Ultracentrifugation Machine "TL-100 Ultracentrifugation" was set on following parameters

- "Speed" "- 36,000 rpm

- "Time"- 45 min.
- "Temperature"- 4.0c

o After the 45 min centrifugation the supernatant from each tube were carefully removed by aspiration.

o The pellet (very difficult to see) were resuspended in 1400uL chilled "0.5X sodium citrate buffer" by pipetting.

- **(G) Ultracentrifugation # 2**

o The resuspended pellet (in 1400uL chilled "0.5X sodium citrate buffer") on each tube were centrifuged again at 12,000 rpm and 4.0°c for 10 minutes.

o 1000μL aliquot of the supernatant from the tubes was used for the next step

- **(H) Ultracentrifugation # 3**

o In 6 new 1.5mL polyallomer ultracentrifugation tube, 400uL of chilled "20%, 40%, 60% sucrose solution in 0.1M sodium" were added

o A 1000uL aliquot of supernatant (from the ultracentrifugation #2) was removed from each tube and added into a new 1.5mL polyallomer ultracentrifugation tube with % sucrose in them.

- 6 polyallomer ultracentrifugation tubes (with the 400uL chilled % sucrose in 0.1M sodium citrate buffer + 1000µl supernatant) were weighed again throughly
  - Weight of polyallomer tube # 1
  - Weight of polyallomer tube # 2
  - Weight of polyallomer tube # 3
  - Weight of polyallomer tube # 4
  - Weight of polyallomer tube # 5
  - Weight of polyallomer tube # 6
- The ultracentrifuge machine was set on following parameter
  - Speed"- 53,000 rpm
  - "Time"- 65 min.
  - "Temperature"- 4.0°c

- **(I)** after the final centrifugation supernatants were carefully removed by aspiration and the VLP pellets were resuspended in 30uL of "chilled-resuspended buffer". The VLPs were kept in -80C for long term storage. (*Hordeum vulgare* leaves were resuspended on both with EDTA and without EDTA).

3.2 RT – Assay:

Objective: Verification of Presence of VLP

Materials: RevTrans Asssay RT-1 Primer [5'-d(CATAGGTCAAACCTCCTAGGAATG)-3'], RevTrans Assays RT-2 Primer [5'-d(TCCTGCTCAACTTCCTGTCGAG)-3'], RT assay kit (described below), extracted VLPs from *Hordeum vulgare*, *Prosartes smithii*, Proflex PCR system, PCR reagents, Agarose powder, Blue/Orange 6X loading dye, 100bp Promega ladder.

Methods:

A. Primer Template Reaction

Table 1. Primer/Template Reaction

Primer/Template (1 tube)	**Primer/Template Master Mix** (x9)
2 μL nuclease free water	18 μL nuclease free water
1 μL RT-1 primer (10μM) (primer)	9 μL RT-1 Primer (10μM) (primer)
1 μL MS2 Phage RNA (0.8 μg/μL) (primer)	9 μL MS2 Phage RNA (0.8μg/μL) (template)

Table 2. PCR Tube contents A

Tube#1A +Phage RNA	**Tube#2A** +Phage RNA	**Tube#3A** +Phage RNA	**Tube#4A** +Phage RNA	**Tube#5A** +Phage RNA	**Tube#6A** +Phage RNA	**Tube#7A** +Phage RNA	**Tube#8A** +Phage RNA	**Tube#9A** No Phage RNA
4µL Primer/ Template Master Mix	4µL Primer/ Template Master Mix	4µL Primer/ Template Master Mix	4µL Primer/ Template Master Mix	4µL Primer/ Template Master Mix	4µL Primer/ Template Master Mix	4µL Primer/ Template Master Mix	4µL Primer/ Template Master Mix	—
—	—	—	—	—	—	—	—	1 µL RT-1 primer (10µM)
—	—	—	—	—	—	—	—	3µL Nuclease Free Water
Total Vol 4µL	Total Vol 4µL	Total Vol 4µL	Total Vol 4µL	Total Vol 4µL	Total Vol 4µL	Total Vol 4µL	Total Vol 4µL	Total Vol 4µL

Thermocycler parameters for primer annealing

- Heated at 85°C for 5 minutes
- Annealed at 37°C for 30 minutes.
- Chilled on ice for 5 minutes prior to use.

B. First Strand cDNA Synthesis

Table 3. Master Mix Prep A

cDNA Synthesis (1 Tube)	**cDNA Master Mix A** (x8) Tubes 1A-7A
1 µL dNTPs (10mM)	8 µL dNTPs (10mM)
2 µL 0.1M DTT	16 µL 0.1M DTT
2µL 10X RT Buffer (500mM KCl, 100mM Tris HCL)	16 µL 10X RT Buffer (500mM KCl, 100mM Tris HCL)
4 µL $MgCl_2$ (25mM)	32µL $MgCl_2$ (25mM)
1 µL Nuclease FreeWater	8µL Nuclease Free Water
10µL Total Volume	80µL Total Volume → 10µL in each tube

Table 4. Master Mix Prep B

cDNA Synthesis (1 Tube)	**cDNA Master Mix B** (x3) Tubes 8A-9A
1 μL dNTPs (10mM)	3 μL dNTPs (10mM)
2 μL 0.1M DTT	6 μL 0.1M DTT
2 μL 10X RT Buffer (500mM KCl, 100mM Tris HCL)	6 μL 10X RT Buffer (500mM KCl, 100mM Tris HCL)
4 μL $MgCl_2$ (25mM)	12μL $MgCl_2$ (25mM)
6.5 μL Nuclease Free Water	19.5 μL Nuclease Free Water
0.5 μL SuperScript III RT (200U/μL)	1.5 μL SuperScript III RT (200U/μL)
16μL Total Volume	48μL Total Volume → 16μL in each tube

Table 5. PCR Tube contents B

Tube #1A	**Tube #2A**	**Tube #3A**	**Tube #4A**	**Tube #5A**	**Tube #6A**	**Tube #7A** Negative Control (-RT) (+MS2 RNA)	**Tube #8A** Positive Control (+RT) (+MS2 RNA)	**Tube #9A** Negative Control (+RT) (-MS2 RNA)
4µL Primer/ Template Reaction Already in Tube 1A	4µL Primer/ Template Reaction Already in Tube 2A	4µL Primer/ Template Reaction Already in Tube 3A	4µL Primer/ Template Reaction Already in Tube 4A	4µL Primer/ Template Reaction Already in Tube 5A	4µL Primer/ Template Reaction Already in Tube 6A	4µL Primer/ Template Reaction Already in Tube 7A	4µL Primer/ Template Reaction Already in Tube 8A	4µL RT-1 Primer + NF Water (-MS2 RNA) Already in Tube 9A
10µL cDNA MM A	10µL cDNA MM A	10µL cDNA MM A	10µL cDNA MM A	10µL cDNA MM A	10µL cDNA MM A	10µL cDNA MM A	—	—
—	—	—	—	—	—	—	16µL cDNA MM B	16µL cDNA MM B
6µL VLP Isolate Prep #1	6µL VLP Isolate Prep #2	6µL VLP Isolate Prep #3	6µL VLP Isolate Prep #4	6µL VLP Isolate Prep #5	6µL VLP Isolate Prep #6	—	—	—
—	—	—	—	—	—	6µL Nuclease Free Water	—	—
Total Vol-20µL	Total Vol-20µL	Total Vol-20µL	Total Vol-20µL	Total Vol-20µL	Total Vol-20µL	Total Vol-20µL	Total Vol-20µL	Total Vol-20µL

Thermocycler parameters for primer annealing

- Tubes were incubated at 37°C for 1 hour (with heated lid) and then place on ice for 5 min.
- The cDNA can be used immediately as a template for PCR amplification or frozen.

C. PCR Amplification

Table 6 Master Mix Prep C

PCR Amplification (1 Tube)	**PCR Amplification Master Mix** (x10)
5 μL 2X PCR Master Mix (Promega)	50 μL 2X PCR Master Mix (Promega)
1 μL RNase A (12.5μg/μL)	10 μL RNase A (12.5μg/μL)
1 μL RT- 1 Primer (10μM)	10 μL RT- 1 Primer (10μM)
1 μL RT-2 Primer (10μM)	10 μL RT-2 Primer (10μM)
1 μL Nuclease Free Water	10 μL Nuclease Free Water
9μL Total Volume	90μL Total Volume → 9μL in each tube

Table 7. PCR Tube contents C

Tube #1B	**Tube #2B**	**Tube #3B**	**Tube #4B**	**Tube #5B**	**Tube #6B**	**Tube #7B**	**Tube #8B**	**Tube #9B**
1µL First Strand cDNA from Tube #1A	1µL First Strand cDNA from Tube #2A	1µL First Strand cDNA from Tube #3A	1µL First Strand cDNA from Tube #4A	1µL First Strand cDNA from Tube #5A	1µL First Strand cDNA from Tube #6A	1µL First Strand cDNA from Tube #7A	1µL First Strand cDNA from Tube #8A	1µL First Strand cDNA from Tube #9A
9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix	9µL PCR Amp. Master Mix
Total Vol- 10µL	Total Vol- 10µL	Total Vol- 10µL	Total Vol- 10µL	Total Vol- 10µL	Total Vol- 10µL	Total Vol- 10µL	Total Vol- 10µL	Total Vol- 10µL

Table 8. PCR Thermocycler Parameters

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	94C	5 min	1
Denaturation	94C	30 sec	35
Annealing	55C	45 sec	35
Extension	72C	1 min	35
Final extension	72C	5 min	1
Hold	4C	∞	1

Table 9. PCR Tube contents D

	Tube #2B	**Tube #3B**	**Tube #4B**	**Tube #5B**	**Tube #6B**	**Tube #7B**	**Tube #8B**	**Tube #9B**
1µL First Strand cDNA from Tube #1A	1µL First Strand cDNA from Tube #2A	1µL First Strand cDNA from Tube #3A	1µL First Strand cDNA from Tube #4A	1µL First Strand cDNA from Tube #5A	1µL First Strand cDNA from Tube #6A	1µL First Strand cDNA from Tube #7A	1µL First Strand cDNA from Tube #8A	1µL First Strand cDNA from Tube #9A
5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)	5µL of 2X PCR Master Mix (Promega)
1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)	1µL RNase A(Invitroge, 12.5µg/µL)
1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)	1µL RT-1 Primer (10µM)
1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)	1µL RT-2 Primer (10µM)
1µL Nuclease Free Water	1µL Nuclease Free Water	1µL Nuclease Free Water	1µL Nuclease Free Water	1µL Nuclease Free Water	1µL Nuclease Free Water	1µL Nuclease Free Water	1µL Nuclease Free Water	1µL Nuclease Free Water
Total Vol-10µL	Total Vol-10µL	Total Vol-10µL	Total Vol-10µL	Total Vol-10µL	Total Vol-10µL	Total Vol-10µL	Total Vol-10µL	Total Vol-10µL

D. Visualization of PCR Products

Agarose Gel

- The 3.5% gel was made adding 1.75g agarose + 50mL 1X TAE
- The 10ul PCR Amplification products (from step C) were loaded on the gel with Blue/Orange 6X loading dye.
- 100bp Promega Ladder was used
- The gel electrophoresis was done for an hour at 80mv
- The gel was then stained in ETBR (fresh) for 3 minutes and distained for 35 mins
- The gel was analyzed using Bio Rad image lab.

Table 10. PCR Tube contents E

Lane #1	**Lane #2**	**Lane #3**	**Lane #4**	**Lane #5**	**Lane #6**	**Lane #7**	**Lane #8**	**Lane #9**	**Lane #10**	**Lane #11**	**Lane #12**	**Lane #13**	**Lane #14**	**Lane #15**
2µL LMW Ladder Promega 100bp		10µL PCR Product Tube #1B (GS-17-1)	10µL PCR Product Tube #2B (GS-17-2)	10µL PCR Product Tube #3B (GS-17-3)	10µL PCR Product Tube #4B (GS-17-4)	10µL PCR Product Tube #5B (GS-17-5)	10µL PCR Product Tube #6B (GS-17-6)		10µL PCR Product Tube #7B (GS-17-7)		101µL PCR Product Tube #8B (GS-17-8)		10µL PCR Product Tube #9B (GS-17-	
2µL 6X Loading Dye		2µL 6X Loading Dye	2µL 6X Loading Dye	2µL 6X Loading Dye	2µL 6X Loading Dye	2µL 6X Loading Dye	2µL 6X Loading Dye		2µL 6X Loading Dye		2µL 6X Loading Dye		2µL 6X Loading Dye	
8µL 1X TAE														
Total Vol-12µL		Total Vol-12µL	Total Vol-12µL	Total Vol-12µL	Total Vol-12µL	Total Vol-12µL	Total Vol-12µL		Total Vol-12µL		Total Vol-12µL		Total Vol-12µL	

3.3 PCR with Reverse Transcription Enzyme Primers: GAG, Integrase

Objective: Confirmation of presence of VLPs containing cDNA and rule out genomic DNA contamination in VLP prep by using RBC (Rubisco gene)

Materials: VLP samples from *Hordeum vulgare*, Barley GAG for 5′ –ACTTCAACGTCGCTTTGAGC, Barley GAG rev 5′ – CCCTTCTTTCCTTTGTCGTTT (Sigma), Barley Integrase for 5′ – ACTCCGTTCTCCGGAACAAT, Barley Integrase rev 5′ – AAAACGATAACGGCAAATCG (Sigma), rbc start 5′ – ATGTCACCACAAACAGAAAAC and rbc finish 5′-CTTCACAAGCAGCTAGTTC (Sigma) Primers, PCR mix, Proflex PCR system.

Methods:

Table 11. PCR Tube Contents F

In each PCR tube	**Volume**
Master Mix	10ul
DNA grade H2O	8ul
Primer (diluted R+F)	1ul
DNA grade H2O	8ul
Primer (diluted R+F)	1ul
VLPs	1ul
Total volume-	20ul

Primers dilution

1ul F + 1ul R + 18ul H2O = 20ul for each type

RBC primers were used as control to rule out genomic DNA contamination in VLP preps.

Table 12. PCR conditions: GAG / Integrase

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	95C	2 min	1
Denaturation	94C	30 sec	35
Annealing	60C	30 sec	35
Extension	72C	1 min	35
Final extension	72C	7 min	1
Hold	4C	∞	1

Table 13. **PCR condition: RBC**

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	95C	2 min	1
Denaturation	94C	30 sec	45
Annealing	50C	30 sec	45
Extension	72C	2.30 min	45
Final extension	72C	7 min	1
Hold	4C	∞	1

Table 14. Sample Volume

Primers	Hordeum Extraction #1(No EDTA in buffer)	Hordeum Extraction #2 (No EDTAin buffer)	Hordeum Extraction #3(EDTA in buffer)	Hordeum Extraction #4(EDTA in buffer)
GAG	20ul	20ul	20ul	20ul
Integrase	20ul	20ul	20ul	20ul
RBC	20ul	20ul	20ul	20ul

Agarose Gel:

- The 1% gel was made adding 0.5g agarose + 50mL 1X TAE
- The 3ul PCR Amplification products were loaded on the gel with 1ul of Blue/Orange 6X loading dye.
- 2ul of 1Kbp Promega Ladder was used with 2ul of Blue/Orange 6X loading dye.
- The gel electrophoresis was done for an hour at 80mv
- The gel was then stained in ETBR (fresh) for 3 minutes and distained for 35 mins
- The gel was analyzed using Bio Rad image lab.

3.4 PCR with Reverse Transcription Gene *copia, gypsy* and LINE:

Objective: Verification of presence of retrotransposons in VLPs extracted from plant leaves and to rule out genomic DNA contamination by Rubisco (RBC) PCR

Materials: VLP samples from Prosartes, Smilax, Clintonia, Lillium and Scoliopus, rbc start 5′ – ATGTCACCACAAACAGAAAAC, rbc finish 5′-CTTCACAAGCAGCTAGTTC, Copia for 5′ –CARATGGARGTNAARAC, Copia rev 5′ – CATRTCRTCNACRTA, Gypsy FriesenFor1 5′- MRNATGTGYGTNGAYTAY, Gypsy FriesenRev1 5′-RCAYTTNSWNSRYTTNGCR and LINE1new for 5′- RUNRANTTYCGNCCNATH-AS, LINE4New REV 5′- AGAGAAGGGRGRYAGGGGG (Sigma Primers), PCR mix, Proflex PCR system.

Methods:

Table 15. PCR Tube Contents G

In each PCR tube	**Volume**
Master Mix	10ul
DNA grade H2O	8ul
Primer (diluted R+F)	1ul
DNA grade H2O	8ul
Primer (diluted R+F)	1ul
VLPs	1ul
Total volume-	20ul

Primers dilution

1ul F + 1ul R + 18ul H2O = 20ul for each type

Table 16. PCR condition: *Copia*

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	95C	2 min	1
Denaturation	94C	1 min	45
Annealing	46C	2 min	45
Extension	72C	3 min	45
Final extension	72C	7 min	1
Hold	4C	∞	1

Table 17. PCR condition: *Gypsy*

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	95C	2 min	1
Denaturation	94C	1 min	45
Annealing	53C	1 min	45
Extension	72C	1 min	45
Final extension	4C	7 min	1
Hold	4C	∞	1

Table 18. PCR condition: LINE

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	95C	2 min	1
Denaturation	94C	1 min	45
Annealing	54C	1 min	45
Extension	72C	1 min	45
Final extension	72C	7 min	1
Hold	4C	∞	1

Table 19. PCR condition: RBC

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	95C	2 min	1
Denaturation	94C	30 sec	45
Annealing	50C	30 sec	45
Extension	72C	2.30 min	45
Final extension	72C	7 min	1
Hold	4C	∞	1

Table 20. Sample Volume for VLP extraction #2

Primers	Prosartes 20%	Prosartes 20%	Prosartes 20%	Prosartes 20%	Prosartes 20%	Prosartes 20%
copia	20ul	20ul	20ul	20ul	20ul	20ul
gypsy	20ul	20ul	20ul	20ul	20ul	20ul
LINE	20ul	20ul	20ul	20ul	20ul	20ul
RBC	20ul	20ul	20ul	20ul	20ul	20ul

Table 21. Sample Volume for VLP extraction #3

Primers	Scoliopus 20%	Scoliopus 20%	Scoliopus 40%	Scoliopus 40%	Scoliopus 60%	Scoliopus 60%
copia	20ul	20ul	20ul	20ul	20ul	20ul
gypsy	20ul	20ul	20ul	20ul	20ul	20ul
LINE	20ul	20ul	20ul	20ul	20ul	20ul
RBC	20ul	20ul	20ul	20ul	20ul	20ul

Table 22. Sample Volume for VLP extraction #4

Primers	Clintonia 40%	Clintonia 60%	Lillium 40%	Lillium 60%	Prosates 60%	Smilax 60%
copia	20ul	20ul	20ul	20ul	20ul	20ul
gypsy	20ul	20ul	20ul	20ul	20ul	20ul
LINE	20ul	20ul	20ul	20ul	20ul	20ul
RBC	20ul	20ul	20ul	20ul	20ul	20ul

Agarose Gel:

- The 1% gel was made adding 0.5g agarose + 50mL 1X TAE
- The 10ul PCR Amplification products were loaded on the gel with 3ul of Blue/Orange 6X loading dye.
- 2ul of 1Kbp Promega Ladder/2ul of O Gene Ruler 1kb plus ladder (ThermoFisher) was used with 2ul of Blue/Orange 6X loading dye.
- The gel electrophoresis was done for an hour at 80mv
- The gel was then stained in ETBR (fresh) for 3 minutes and distained for 35 mins
- The gel was analyzed using Bio Rad image lab.

3.5 gRNA Isolation from *Hordeum vulgare* leaves:

Objective: Use as a control in downstream PCR

Material: RNeasy Plant mini kit, Qiagen, Hordeum leaves

Methods:

Before the Extraction:

β-Mercaptoethanol (β-ME) must be added to Buffer RLT or Buffer before use. 10 µlβ-ME per 1 ml Buffer RLT. Buffer RPE is supplied as a concentrate. Before using for the

Buffer RPE first time, 4 volumes of ethanol (96–100%) as indicated on the bottle was added to obtain a working solution.

Procedure

1. Determine the amount of 100 mg Plant leaves were weighed.

2. Leaves were homogenized using mortar and pestle.

3.450 µl Buffer RLT was added to a maximum of 100 mg tissue powder. Vigorous Vortex was done.

4. The lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. The supernatant then carefully transferred of the flow-through to a new micro centrifuge tube (not supplied) without disturbing the cell debris pellet in the collection tube.

5. 0.5 volume of ethanol (96–100%) was added to the cleared lysate, and mix immediately by pipetting. Centrifuge should not be done. Immediately proceeded to step 6.

6. The sample then transferred (usually 650 µl), including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s

at≥8000 x g (≥10,000 rpm). Discard the flow-through was discarded. If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation

7. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifugefor15sat≥8000xg (≥10,000rpm) to wash the spin column membrane. Discard the flow-through.* Reuse the collection tube in step 8. Note- After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifugefor15sat≥8000xg (≥10,000rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9.

9. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifugefor2minat≥8000xg (≥10,000rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

10. Optional- Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at≥8000 x g (≥10,000 rpm) to elute the RNA.

3.6 cDNA Isolation from VLPs of *Hordeum vulgare*:

Objective: To sequence the cDNA to identify the transposable element in cDNA.

Materials: VLPs from Barley leaves, DNeasy blood and tissue kit, centrifuge and micro centrifuge tube

Methods:

Cultured cells- 1. Centrifuge the VlPs for 5 min at 300 x g. Re-suspend the pellet in 200 µl PBS. Add 20 µl proteinase K. Continue with step 2.

2. Add 200 µl Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56˚C for 10 min.

3. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 50

µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

8. For maximum DNA yield, repeat elution once as described in step 7 and added 20ul of AE. Final elution volume is 70ul.

Agarose Gel:

- The 1% gel was made adding 0.5g agarose + 50mL 1X TAE
- The 7ul isolated cDNA sample were loaded on the gel with 2ul of Blue/Orange 6X loading dye.
- 1ul of 1Kbp Promega Ladder was used with 2ul of Blue/Orange 6X loading dye.
- The gel electrophoresis was done for an hour at 80mv
- The gel was then stained in ETBR (fresh) for 3 minutes and distained for 35 mins
- The gel was analyzed using Bio Rad image lab.

3.7. Bio Analyzer (1):

Objective: To quantify DNA and identify the range of base pair length of the fragments

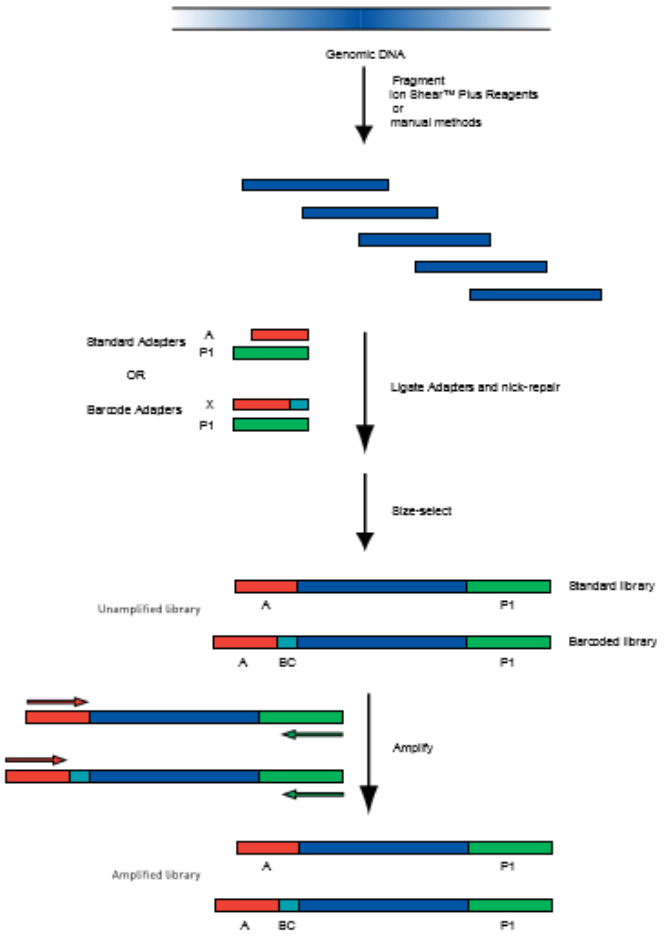
Material: Agilent High Sensitivity DNA Chips, Bio Analyzer 2100

Methods: 1ul of sonicated sample was used. Detail method available on the following link: http://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf

3.8 NEXT GENERATION SEQUENCING of cDNA Extracted from *Hordeum vulgare*

Library Prep: Followed the protocol of Ion Xpress Plus gDNA Fragment Library preparation

Work Flow Diagram:



3.8 A. Sonication: Bioruptor Platform

Objective: To fragment or sheer the template DNA for Library preparation

Materials: cDNA of Hordeum vulgare, Bioruptor® NGS Sonication System with accessories (for 12 × 0.5-mL tubes), Bioruptor® Microtube Attachment and Gearplate (0.5-mL) • Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA)

1. In a 0.65-mL microcentrifuge tube for the Bioruptor® Sonication System, prepare 100 ng or 1 µg of your genomic DNA preparation in 50 µL of Low TE (pH 8). Close the cap with care so as not to damage the lid and to ensure that the lid forms a tight seal with the tube. Keep the samples on ice.

2. (Optional) in a separate tube, prepare a control sample of 100 ng or 1 µg of control DNA in 50 µL Low TE. Keep the sample on ice.

3. Process ≤12 samples at one time with the 12 × 0.65-mL Bioruptor® Sonication System rotor. If there are <12 samples, load tubes with 50 µL of Low TE to fill all empty slots.

4. Unscrew the removable metal ring from the rotor, insert the 12 tubes, and replace the metal ring finger tight. Do not over-tighten the metal ring.

5. Proceed to "Option 1- Sonicate the DNA with the Bioruptor® CD-200 TS Sonication System" on page 25 in the following section or "Option 2- Sonicate the DNA with the Bioruptor® UCD-600 NGS Sonication System" on page 27.

For 100–300-base-read libraries (including the 150- and 200-base-read libraries for the Ion Proton™ System

1. Set the sonication parameters on the Bioruptor® UCD-200 TS Sonication System. Follow the manufacturer's instructions. Time ON/OFF • ON (sonication time, red dial)- 0.5 minutes • OFF (cool-down time, green dial)- 0.5 minutes

Power Level L (low)

2. Fill the Bioruptor® Sonication System UCD-200 to 1 cm below the Fill Line with cold (<10°C) water. Add an even 1-cm layer (250 mL) of crushed ice, ensuring that the water is just at the fill line.

3. Set the timer to 15 minutes and sonicate. 3 cycles of sonication done.

Agarose Gel:

- The 3% gel was made adding 1.5g agarose + 50mL 1X TAE
- The 5ul PCR Amplification products were loaded on the gel with 3ul of Blue/Orange 6X loading dye.
- 3ul of 1Kbp Promega Ladder was used with 3ul of Blue/Orange 6X loading dye.

- The gel electrophoresis was done for an hour at 100mv
- The gel was then stained in ETBR (fresh) for 3 minutes and distained for 35 mins
- The gel was analyzed using Bio Rad image lab.

3.8 B. Bio Analyzer (2):

Objective: To quantify DNA and identify the range of base pair length of the fragments

Material: Agilent High Sensitivity DNA Chips, Bio Analyzer 2100

Methods: 1ul of sonicated sample was used. Detail method available on the following link: http://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf

3.8 C. GAG & Integrase PCR:

Materials: VLP samples from *Hordeum vulgare*, Barley GAG for 5' -ACTTCAACGTCGCTTTGAGC, Barley GAG rev 5' - CCCTTCTTTCCTTTGTCGTTT (Sigma), Barley Integrase for 5' - ACTCCGTTCTCCGGAACAAT, Barley Integrase rev 5' - AAAACGATAACGGCAAATCG (Sigma), (Sigma) Primers, PCR mix, Proflex PCR system.

Methods:

Table 23. PCR Tube Contents H

In each PCR tube	Volume
Master Mix	10ul
DNA grade H2O	8ul
Primer (diluted R+F)	1ul
DNA grade H2O	8ul
Primer (diluted R+F)	1ul
VLPs	1ul
Total volume-	20ul

Primers dilution

1ul F + 1ul R + 18ul H2O = 20ul for each type

Table 24. PCR condition: GAG / Integrase

Cycle step	Temp	Time	No. of Cycles
Initial denaturation	95C	2 min	1
Denaturation	94C	30 sec	35
Annealing	60C	30 sec	35
Extension	72C	1 min	35
Final extension	72C	7 min	1
Hold	4C	∞	1

Agarose Gel:

- The 1% gel was made adding 0.5g agarose + 50mL 1X TAE
- The 7ul sonicated cDNA were loaded on the gel with 3ul of Blue/Orange 6X loading dye.
- 2ul of 1Kbp Promega Ladder was used with 2ul of Blue/Orange 6X loading dye.
- The gel electrophoresis was done for an hour at 120mv

- The gel was then stained in ETBR (fresh) for 3 minutes and distained for 35 mins
- The gel was analyzed using Bio Rad image lab.

3.8 D. End Repair:

Objective: To repair the uneven ends of DNA fragments and make the ends blunt

Materials: Sheered cDNA, 5X End Repair Buffer, End Repair Enzyme, Nuclease-free Water, 1.5-mL Eppendorf LoBind® Tubes, Agencourt® AMPure® XP Kit, Magnetic rack

End-repair Note- Before use, pulse-spin components of the Ion Plus Fragment Library Kit for 2 seconds to deposit the contents in the bottom of the tubes.

1. Add Nuclease-free Water to the fragmented DNA to bring the total volume to the 40 µL

2. Mix by pipetting in a 1.5-mL Eppendorf LoBind® Tube-

Component Volume- Fragmented cDNA (step 1) 40 µL + 5X End Repair Buffer 10 µL+ End Repair Enzyme 0.5 µL =Total 50ul

3. Incubate the end-repair reaction for 20 minutes at room temperature.

Purify with the Agencourt® AMPure® XP Kit IMPORTANT! Use freshly prepared 70% ethanol (1 mL plus overage per

sample) for the next steps. A higher percentage of ethanol causes inefficient washing of smallersized molecules. A lower percentage of ethanol could cause sample loss.

1. Add the indicated volume of Agencourt® AMPure® XP Reagent beads (1.8X sample volume) to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes. 100 ng Input = 90 µL

2. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the bead pellet.

3. Without removing the tube from the magnet, dispense 250 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

4. Repeat step 3 for a second wash.

5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.

6. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.

7. Remove the tube from the magnet, and add 13µL of Low TE to the sample. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds to mix thoroughly.

8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind® Tube without disturbing the pellet.

STOPPING POINT (Optional) Store the DNA at –30˚C to –10˚C.

3.8 E. Ligate adaptors:

Objective: Ligate adaptors to the both end of DNA fragments for template preparation

Materials: 10X Ligase Buffer, Adapters (for non-barcoded libraries), DNA Ligase, Nick Repair Polymerase, dNTP Mix, Low TE, 0.2-mL PCR tubes, Thermal cycler, Nuclease-free Water, Agencourt® AMPure® XP Kit, Freshly prepared 70% ethanol, Magnetic rack

Methods:

Ligate and nick-repair

1. In a 0.2-mL PCR tube, combine the reagents as indicated in the appropriate table for non-barcoded or barcoded libraries, and mix well by pipetting up and down.

2. Reaction Setup for Non-barcoded Libraries Component Volume by Input gDNA 50-100 ng- DNA ~13 µL + 10X Ligase Buffer 5 µL + Adapters 1 µL + dNTP Mix 1 µL + Nuclease-free Water 25+ DNA Ligase 1 µL + Nick Repair Polymerase 4 µL= Total 50 µL.

3. Place the tube in a thermal cycler and run the following program.32

4. Thermocycler condition- Hold 25°C 15 min, Hold 72°C 5 min, Hold 4°C Hold, continue directly to the next steps.

5. Transfer the entire reaction mixture to a 1.5-mL Eppendorf LoBind® Tube for the next cleanup step.

6. Purify the adapter-ligated and nick-repaired DNA

7. IMPORTANT! Use freshly prepared 70% ethanol (1 mL plus overage per sample) for the next steps.

8. Add the indicated volume of Agencourt® AMPure® XP Reagent to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse-spin

the tube, and incubate the mixture for 5 minutes at room temperature. Library Size Volume of Agencourt® AMPure® XP Reagent 200–300-base-read 60 µL (1.2X sample volume).

9. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet

10. Without removing the tube from the magnet, add 250µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

11. Repeat step 3 for a second wash.

12. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.

13. Keeping the tube on the magnetic rack, air-dry the beads at room temperature for ≤5 minutes.

14. Remove the tube from the magnetic rack and add 30 µL of Low TE directly to the pellet to disperse the beads. Mix thoroughly by pipetting the suspension up and down 5 times, then vortex the sample for 10 seconds.

15. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind® Tube without disturbing the pellet.

16. STOPPING POINT (Optional) Store the DNA at -30°C to -10°C.

3.8 F. Pippin Prep (1):

Objective: Select the correct size fragments

Materials: Pippin Prep (Sage Science) Adaptor ligated DNA, Low TE, Gel Cassette, loading solutions, buffer

Methods: Detail method available on the following link: http://www.sagescience.com/wp-content/uploads/2011/09/quick-guide-final-2percent-1mb.pdf

3.8 G. Amplification of Library:

Objective: To amplify the amount of cDNA

Materials: Platinum® PCR SuperMix High Fidelity, Library Amplification Primer Mix, Low TE, Thermal cycler, 0.2-mL PCR tubes,1.5-mL Eppendorf LoBind® Tubes, Agencourt® AMPure® XP Kit, Freshly prepared 70% ethanol, Magnetic rack

Methods:

1. Adjust the volume of the unamplified library was adjusted to 25ul after Pippin Prep™

2. Combine the following reagents in an appropriately sized tube and mix by pipetting up and down. Component Volume by Input for DNA 50-100 ng Platinum® PCR SuperMix High Fidelity 100 µL + Library Amplification Primer Mix 5 µL + Unamplified library 25 µL = Total 130 µL

3. Split the 130-µL reaction mix equally into multiple 0.2-mL PCR tubes to adjust for the maximum reaction volume recommended by the manufacturer of your thermal cycler.

4. Place the tubes into a thermal cycler and run the following PCR cycling program. Set the number of cycles according to the second table. Note- Minimize the number of cycles to avoid over-amplification, production of concatemers, and introduction of PCR-induced errors. Reduce the number of cycles if concatemers are formed.

Stage Step Temperature Time Holding Denature 95°C 5 min Cycling[1] Denature 95°C 15 sec Anneal 58°C 15 sec Extend 70°C 1 min Holding — 4°C Hold. Set the number of

cycles according to the following table. Not a stopping point; continue directly to the next steps.

1. Combine previously split PCRs in a new 1.5-mL Eppendorf LoBind® Tube.

2. Purify the library

3. IMPORTANT! Use freshly prepared 70% ethanol

4. Add the indicated volume of Agencourt® AMPure® XP Reagent to each sample- Volume of Agencourt® AMPure® XP Reagent- 200–300-base-read library (1.2X Sample volume)- 156 µL

5. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture for 5 minutes at room temperature.

6. Place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

7. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove

and discard the supernatant without disturbing the pellet.

5. Repeat step 4 for a second wash.

8. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.

9. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.

10. Remove the tube from the magnetic rack, and add 30 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.

11. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind® Tube without disturbing the pellet.

12. IMPORTANT! The supernatant contains the final amplified library. Do not discard.

13. To remove residual beads from the eluted DNA, place the tube with the eluted DNA back on the magnet for at least 1 minute, and transfer the supernatant to a new 1.5-mL Eppendorf LoBind® Tube without disturbing the pellet.

14. STOPPING POINT Store the library at −30°C to −10°C. Before use, thaw on ice. To reduce the number of freeze-thaw cycles, store the library in several aliquots.

3.8 H. Pippin Prep (2):

Objective: Select the correct size fragments

Materials: Pippin Prep (Sage Science) Adaptor ligated DNA, Low TE, Gel Cassette, loading solutions, buffer

Methods: Detail method available on the following link: http://www.sagescience.com/wp-content/uploads/2011/09/quick-guide-final-2percent-1mb.pdf

3.8 I. Bio Analyzer (3): To quantify DNA and identify the range of base pair length of the fragments after Pippin Prep

Material: Agilent High Sensitivity DNA Chips, Bio Analyzer 2100

Methods: 1ul of sonicated sample was used. Detail method available on the following link: http://www.agilent.com/cs/library/usermanuals/Public/G2938-90321 SensitivityDNA KG EN.pdf

3.8 J. One Touch:

Objective: To prepare enriched, template positive Ion Sphere Particles

Materials: Ion OneTouch™ Reaction Oil (25-mL size), Nuclease-free Water, Ion PGM™ Hi-Q™ OT2 Reagents (Part no. A27743), Ion PGM™ Hi-Q™ Reagent Mix, Ion PGM™ Hi-Q™ Enzyme Mix, Ion PGM™ Hi-Q™ Ion Sphere™ Particles (ISPs), Ion PGM™ OT2 Supplies (Part no. A27744)- Ion OneTouch™ Reaction Filter (Optional) ,PGM™ Controls Kit v2 (Cat no. 4482010)- E. coli DH10B Control 400 Library, Diluted library, Microcentrifuge, 1.5-mL Eppendorf LoBind® Tubes, Tube rack for 15-mL conical tube, Pipettes, Vortexer

Methods: After setting up the One Touch machine and prepping the amplification solution-

3. Prepare the Ion PGM™ Hi-Q™ ISPs-

a. Vortex the ISPs at maximum speed for 1 minute to resuspend the particles.

b. Centrifuge the ISPs for 2 seconds.

c. Pipet the ISPs up and down to mix.

d. Immediately proceed to the next step.

4. To a 2-mL tube (violet cap) containing 800 µL of Ion PGM™ Hi-Q™ Reagent Mix, add the following components in

the designated order. Add each component, then pipet the amplification solution up and down to mix- Order Reagent Cap color Volume- 1 Nuclease-free Water — 25 µL+ 2 Ion PGM™ Hi-Q™ Enzyme Mix Brown 50 µL+ 3 Diluted library (not stock library) — 25 µL +4 Ion PGM™ Hi-Q™ ISPs Black 100 µL — Total — 1000 µL

5. Vortex the complete amplification solution prepared in step 4 at maximum speed for 5 seconds. IMPORTANT! Start the run on the Ion OneTouch™ 2 Instrument £15 minutes after preparing the amplification solution.

6. Proceed immediately to "Fill and install the Ion OneTouch™ Reaction Filter.

1. Obtain an Ion OneTouch™ Reaction Filter from the Ion PGM™ Hi-Q™ OT2 Kit-

1 Sample port 2 Ion OneTouch™ Reaction Filter 3 Ion OneTouch™ Reaction Tube 4 Short tubing from sample port to Ion OneTouch™ Reaction Tube

2. Place the Ion OneTouch™ Reaction Filter into a tube rack so that the 3 ports of the Ion OneTouch™ Reaction Filter face up.

Fill the Ion OneTouch™ Reaction Filter

3. Locate the sample port on the Ion OneTouch™ Reaction Filter. The short tubing in the Reaction Tube is connected to the sample port-

Note- The color of the short tubing attached to the sample port may vary. 4. Add the amplification solution through the sample port- a. Set a P1000 pipette to 1000 µL, and attach a new 1000-µL tip to the pipette.

b. Vortex the amplification solution at maximum speed for a full 5 seconds, then centrifuge the solution for 2 seconds. Immediately proceed to the next step.

c. Pipet the amplification solution up and down to mix, then fill the tip with 1000 µL of the amplification solution.

d. Insert the tip firmly into the sample port so that the tip is perpendicular to the Ion OneTouch™ Reaction Filter Assembly and fully inserted into the sample port to form a tight seal-

e. Slowly pipet the 1 mL of amplification solution through the sample port. Keep the plunger of the pipette depressed to avoid aspirating solution from the Ion OneTouch™ Reaction Filter. With the plunger still depressed, remove the tip from the sample port, then appropriately discard the tip.

f. If necessary, gently dab a Kimwipes® disposable wipe around the ports to remove any liquid.

5. Add 1.7 mL Ion OneTouch™ Reaction Oil through the sample port-

a. Set a P1000 pipette to 850 µL, and attach a new 1000-µL tip to the pipette.

b. Draw up 850 µL of Ion OneTouch™ Reaction Oil into the pipette tip.

c. Insert the tip firmly into the sample port so that the tip is perpendicular to the Ion OneTouch™ Reaction Filter and fully inserted into the sample port to form a tight seal.

d. Slowly pipet 850 µL of the Reaction Oil through the sample port, then keep the plunger of the pipette depressed.

e. With the plunger depressed, remove the tip from the sample port. Appropriately discard the tip and attach a new pipette tip. Repeat steps b through d once.

f. With the plunger depressed, remove the tip from the sample port, then appropriately discard the tip. g. If necessary, gently dab a Kimwipes® disposable wipe around the ports to remove any liquid.

1. Invert the Ion OneTouch™ Reaction Filter- IMPORTANT! Follow the next steps exactly to minimize contact of the short tubing in the Reaction Tube with the amplification solution.

a. Keep the Ion OneTouch™ Reaction Filter in the tube rack, orient the assembly so that the sample port is on your left.

Install the filled Ion OneTouch™ Reaction Filter

b. Lift straight out the Ion OneTouch™ Reaction Filter from the tube rack. With the short tubing in the Reaction Tube on the left, rotate the assembly to your right until the Reaction Tube is inverted and the 3 ports of the Reaction Plus Filter face down- IMPORTANT!

2. Insert the 3 ports of the Reaction Filter into the three holes on the top stage of the Ion OneTouch™ 2 Instrument, so that the Ion OneTouch™ Reaction Filter is firmly seated on the instrument. The tab protruding from the outer edge of the Reaction Filter fits into the front notch of the stageNote- After inserting the Ion OneTouch™ Reaction Filter, bubbles may shoot up into the Reaction Tube.

Run the Ion OneTouch™ 2 Instrument

1. Ensure that the centrifuge lid of the Ion OneTouch™ 2 Instrument is closed. IMPORTANT! If you raise the centrifuge lid, do not hit the disposable injector against the instrument. You can damage the disposable injector. If you damage the disposable injector, appropriately dispose of the injector, amplification plate, and tubing. Use a new disposable injector and Ion OneTouch™ 2 Amplification Plate.

2. On the home screen, touch Run-

3. Touch the drop-down menu, then select either PGM- Ion PGM™ Hi-Q™ OT2 Kit - 200 or PGM- Ion PGM™ Hi-Q™ OT2 Kit - 400, depending on the read-length of your library-

Note- Do not select PGM- Ion PGM™ Template OT2 200 Kit for Hi-Q from the drop-down menu if you are using the Ion PGM™ Hi-Q™ OT2 Kit. 4. Touch Next.

5. Touch Assisted or Expert-

• Assisted run. Complete each task, then touch next. After you touch next on the last task, you see a progress bar, and the run begins. After the runs starts, you hear clicks from the instrument. This is normal. • Expert run. Empty the waste container and oil waste tray, if necessary,

then touch next. You see a progress bar, and the run begins without the list of task screens.

IMPORTANT! Remember to add 150 µL of Ion OneTouch™ Breaking Solution to each Recovery Tube before starting the run. On the reminder screen, confirm and touch yes to advance-

6. Remove the samples £16 hours after starting the run. If you touched Next on the Centrifuge screen to centrifuge samples at the end of the run, proceed immediately to

Recover the template-positive Ion PGM™ Hi-Q™ ISPs

Materials- Provided in Ion PGM™ Hi-Q™ OT2 Solutions (Part no. A27742)- Ion OneTouch™ Wash Solution, 1.5 mL Eppendorf LoBind® Tubes, Pipettes, Vortexer, Microcentrifuge

Methods-

1. At the end of the run, follow the screen prompts to centrifuge the sample. If you removed the Reaction Tubes at the end of the run before the Ion OneTouch™ 2 Instrument had spun the sample or have not processed the sample after 15 minutes, centrifuge the sample on the instrument-

2. On the home screen of the instrument, touch Open Lid, wait until the lid clicks open, then insert the two filled Ion OneTouch™ Recovery Tubes from the run in the centrifuge rotor. Close the lid until it locks.

3. Touch Options4Final Spin (see figure below), then follow the screen prompts (touch Next on the next 2 screens) until the centrifugation begins. Centrifugation of the samples takes 10 minutes.

4. Immediately proceed to step 2.

5. CAUTION! ROTATION HAZARD. Wait until rotation stops before opening. Rotating parts can cause injury.

6. Immediately after the centrifuge has stopped, on the instrument display, touch Open Lid. Wait until the lid clicks open, then remove and discard the Ion OneTouch™ Recovery Router.

7. Carefully remove both Ion OneTouch™ Recovery Tubes from the instrument and put the two Recovery Tubes in a tube rack. You may see some cloudiness in the tube, which is normal.

8. Remove excess Ion PGM™ OT2 Recovery Solution from the ISPs- Use a pipette to remove all but 100 µL of the Recovery Solution from each Recovery Tube. Withdraw the supernatant from the surface and on the opposite side from

the pellet. Remove any white flocculent material. Do not disturb the ISP pellet-

9. With a new tip and using the same tip for both tubes, resuspend the ISPs in the remaining Ion PGM™ OT2 Recovery Solution. Pipet the pellet up and down until each pellet disperses in the solution.

10. STOPPING POINT Add 500 µL of Ion OneTouch™ Wash Solution to each Recovery Tube and pipet up and down to disperse the ISPs. Transfer the suspensions from both tubes to a new labeled 1.5-mL Eppendorf LoBind® Tube. Store the ISPs at 2°C to 8°C for up to 3 days.

11. Before enrichment, centrifuge the ISPs at 15,500 × g for 2.5 minutes. Carefully remove all but 100 µL of supernatant. With a new tip, pipet up and down to resuspend the ISPs.

12. Process the ISPs- a. Label a new 1.5-mL Eppendorf LoBind® Tube for the ISPs. b. Add 500 µL of Ion OneTouch™ Wash Solution to each Recovery Tube.

13. Pipet the ISPs up and down to disperse the ISPs, then transfer each suspension to the new labeled 1.5-mL Eppendorf LoBind® Tube. Note- If a precipitate is present, incubate the tube at 50°C for 2 minutes to dissolve.

14. Centrifuge the ISPs for 2.5 minutes at 15,500 × g.

15. Use a pipette to remove all but 100 µL of the Wash Solution from the tube. Withdraw the supernatant from the surface and on the opposite side from the pellet. 6. Obtain an 8-well strip from the Ion OneTouch™ ES Supplies Kit. Ensure that the square-shaped tab of an 8-well strip is on the left-

16. Pipet the ISPs up and down 10 times to mix, then transfer the suspension into Well 1 of the 8-well strip. 8. Retain an aliquot of the unenriched Ion PGM™ Hi-Q™ OT2 Kit from Well 1 for quality assessment.

17. Assess the quality of the unenriched, template-positive ISPs using one of the following methods- Qubit® 2.0

18. Protocol available on the following link- https://tools.thermofisher.com/content/sfs/manuals/mp32866.pdf

3.8 K. Enrichment:

Objective: To enrich the Ion Sphere Particles

Materials: Ion OneTouch™ Wash Solution, MyOne™ Beads Wash Solution, Tween® Solution, Neutralization Solution, Nuclease-free Water, 8-well strip, Eppendorf® LoRetention Dualfilter Tips, Ion PGM™ Enrichment Beads (Cat. no. 4478525; Dynabeads® MyOne™ Streptavidin C1 Beads), 1.5-mL

Eppendorf LoBind® Tubes, 0.2-mL PCR tubes, 1 M NaOH, Pipettes, Vortexer, DynaMag™-2 magnet, Microcentrifuge

Methods-

1. Perform the residual volume test on the Ion OneTouch™ ES

2. Prepare reagents then fill the 8-well strip

Prepare fresh Melt-Off Solution by combining the components in the following order-

Order Component Volume- 1 Tween® Solution 280 µL + 2 1 M NaOH 40 µL= Total 320 µL

3. The final composition of the Melt-Off Solution is 125 mM NaOH and 0.1% Tween® 20 detergent.

1. Vortex the tube for 30 seconds to thoroughly resuspend the beads, then centrifuge the tube of Dynabeads® MyOne™ Streptavidin C1 Beads for 2 seconds. 2. Open the tube, then use a new tip to pipet up and down the dark pellet of beads until the pellet disperses. Immediately proceed to the next step. 3. Transfer 13 µL of Dynabeads® MyOne™ Streptavidin C1 Beads to a new 1.5-mL Eppendorf LoBind® Tube. 4. Place the tube on a magnet such as a DynaMag™-2 magnet for 2 minutes, then carefully remove and discard the supernatant without disturbing the pellet of Dynabeads® MyOne™ Streptavidin C1 Beads. 5. Add 130 µL of

MyOne™ Beads Wash Solution to the Dynabeads® MyOne™ Streptavidin C1 Beads. Note- You add the resuspended Dynabeads® MyOne™ Streptavidin C1 Beads in the 130 µL MyOne™ Beads Wash Solution to Well 2 of the 8-well strip.
6. Remove the tube from the magnet, vortex the tube for 30 seconds, and centrifuge the tube for 2 seconds.

Fill the 8- Well strip-

1. Ensure that the template-positive ISPs from the Ion OneTouch™

2 Instrument are in 100 µL of Ion OneTouch™ Wash Solution and are in Well 1 of the 8-well strip.

3. Fill the remaining wells in the 8-well strip as follows - Well number Reagent to dispense in well Well 1[1] Entire template-positive ISP sample [100 µL; prepared in step 1 of this procedure (U)] Well 2 130 µL of Dynabeads® MyOne™ Streptavidin C1 Beads resuspended in MyOne™ Beads Wash Solution] prepared in "Wash and resuspend the Dynabeads® MyOne™ Streptavidin C1 Beads" on page 48 (B)] Well 3 300 µL of Ion OneTouch™ Wash Solution (W) Well 4 300 µL of Ion OneTouch™ Wash Solution (W) Well 5 300 µL of Ion OneTouch™ Wash Solution (W) Well 6 Empty Well 7 300 µL of freshly-prepared Melt-Off Solution [prepared in "Prepare Melt-Off Solution" on page 48 (M)] Well 8 Empty [1] Well

closest to the square-shaped tab 4. Confirm that the square-shaped tab is on the left, then insert the filled 8-well strip with the 8-well strip pushed all the way to the right end of the slot of the Tray.

Prepare the Ion OneTouch™ ES

1. Load a new tip in the Tip Arm-

a. Place a new tip in the Tip Loader- Remove the Tip Arm from the cradle and align the metal fitting of the Tip Arm with the tip. Keeping the fitting on the Tip Arm vertical, firmly press the Tip Arm down onto the new tip until the Tip Arm meets the Tip Loader. Hold the Tip Arm to the Tip Loader for ~1 second to ensure proper installation of the tip. Lift the Tip Arm straight up to pull the installed tip from the Tip Loader tube-

b. Return the Tip Arm to the cradle- Tilt the Tip Arm back. Align the pins with the round notches in the cradle, then lower the Tip Arm into position. Move the Tip Arm forward into the working position.

2. Ensure that the back/bottom end of the Tip Arm is not resting on top of the thumb screw, causing the Tip Arm to tilt forward. This is the correct position of the Tip Arm-

3. Add 10 µL of Neutralization Solution to a new 0.2-mL PCR tube.

4. Insert the opened 0.2-mL PCR tube with the Neutralization Solution into the hole in the base of the Tip Loader, as shown in the preceding photograph.

Perform the run

1. Confirm that a new tip and opened 0.2-mL PCR tube with the Neutralization Solution have been loaded and that the 8-well strip is correctly loaded. Ensure that Well 1 (ISP sample) is the left-most well and that the 8-well strip is pushed to the far-right position within the slot.

2. Pipet the contents of Well 2 up and down to resuspend the beads before starting the run. Do not introduce bubbles into the solution.

3. If necessary, turn ON the Ion OneTouch™ ES and wait for the instrument to initialize. The screen displays "rdy". The Tip Arm performs a series of initialization movements and returns to the home position (~5 seconds).

4. Press Start/Stop. The screen displays "run" during the run. The run takes ~35 minutes. Note- If necessary to stop a run, press Start/Stop. The instrument completes the current step, then stops the run and displays "End". Press Start/Stop again to return the Tip Arm to the home position. It is not possible to restart (where you left off) after stopping a run.

5. At the end of the run, the instrument displays "End" and beeps every 60 seconds. Press the Start/Stop button to silence this alarm and reset the Ion OneTouch™ ES for the next run. The instrument can be left on between runs.

6. Immediately after the run, securely close and remove the PCR tube containing the enriched ISPs.

7. Mix the contents of the PCR tube by gently inverting the tube 5 times. Note- Ensure that the 0.2-mL PCR tube has >200 µL of solution containing the enriched ISPs. After a successful run on the instrument, the sample is in ~230 µL of Melt-Off Solution, Ion OneTouch™ Wash Solution, and Neutralization Solution. If the tube has <<200 µL of solution containing the enriched ISPs, contact Technical Support.

8. Remove the used tip- While you are standing above the Tip Arm, and with the Tip Arm in its cradle, twist the tip counterclockwise and pull it downward to remove and discard the tip-

IMPORTANT! Improper removal of tips can loosen the metal tip adapter fitting on the Tip Arm and affect instrument operation.

9. Remove and discard the used 8-well strip.

3.8 L. Qubit:

Objective: To quantify the ION sphere positive templates

Material: Qubit® 2.0 Fluorometer (Cat. no. Q32866) with the V3.10 firmware and the Ion Sphere™ Quality Control assay, Qubit® Assay Tubes (Cat. no. Q32856), PCR tubes, 0.2 mL (Axygen Cat. no. PCR-02-L-C or BioExpress Cat. no. T-3035-1), Qubit® Easy Calculator Microsoft® Excel® Spreadsheet file containing the instrument specific Calibration Factor, Ion Sphere™ Quality Control Kit (Cat. no. 4468656), Unenriched Ion Sphere™ Particles, GeneAmp® PCR System 9700 thermal cycler (Cat. no. N8050200) or equivalent

Methods: Protocol available on the following link: https://tools.thermofisher.com/content/sfs/manuals/mp32866.pdf

3.8 M. Sequencing: Ion Torrent PGM platform

Objective: To sequence the cDNA extracted from Virus like Particle of Barley leaves to identify and characterize the transposable element BARE family and others

Materials: Materials provided in the kit, Sequencing Primer, Control Ion PGM™ Ion Sphere™ Particles, Annealing Buffer, Ion PGM™ Hi-Q™ Sequencing Polymerase Materials, Ion

PGM™ Ion Sphere™ Test Fragments, Ion Chip kit- Ion 318™ Chip v2, Ion 316™ Chip v2, Ion 314™ Chip v2, Ion 318™ Chip v2 BC, Ion 316™ Chip v2 BC, or Ion 314™ Chip v2 BC, Enriched template-positive ISPs, 0.2-mL PCR tube (non-polystyrene), Rainin® SR-L200F pipette and tips, Vortex mixer, Ion PGM™ Chip Minifuge, Thermal cycler with heated lid (programmed at 95°C for 2 minutes and 37°C for 2 minutes), Barcode scanner (included with the Ion PGM™ System)

Methods- After cleaning and initialization of ION Torrent PGM-

Thaw the Sequencing Primer on ice.

Make sure that you have updated the Torrent Suite™ System and Ion PGM™ System software to Version 4.4 or later.

Note- For each initialization, the first run should be started within 1 hour after initialization, and the last run must be started within 27 hours after initialization.

IMPORTANT! The ISPs are difficult to see. To avoid aspirating the particles- · When centrifuging the ISPs, orient the tab of the tube lid so that it is pointing away from the center of the centrifuge, to indicate where the

pellet will be formed. Always remove supernatant from the tube from the top down.

Optional- Prepare Ion Sphere Test Fragments

If you are performing an installation or troubleshooting sequencing run- 1. Vortex the Ion PGM™ Ion Sphere™ Test Fragments from the Ion PGM™ Controls Kit v2 (Cat. no. 4482010) and pulse spin in a picofuge for 2 seconds before taking aliquots.

2. Add 5 µL of Ion PGM™ Ion Sphere™ Test Fragments to 100 µL of Annealing Buffer in a 0.2-mL non-polystyrene PCR tube. 3. Skip directly to annealing the sequencing primer.

Add controls to the enriched, template-positive ISPs

1. Vortex the Control Ion Sphere™ Particles and pulse spin in a picofuge for 2 seconds before taking aliquots.

2. Add 5 µL of Control ISPs directly to the entire volume of enriched, template- positive ISPs (prepared using your template preparation method) in a 0.2-mL non-polystyrene PCR tube.

3. Proceed to annealing the sequencing primer.

Anneal the Sequencing Primer

1. Mix the tube containing the ISPs (or test fragments) by thoroughly pipetting up and down.

2. Place the tube in a microcentrifuge with an appropriate tube adapter. Orient the tab of the tube lid so that it is pointing away from the center of the centrifuge, to indicate where the pellet will be formed.

3. Centrifuge for 2 minutes at 15,500 × g.

4. Keeping the pipette plunger depressed, insert a pipette tip into the tube containing the pelleted ISPs and carefully remove the supernatant from the top down, avoiding the side of the tube with the pellet (i.e., the side with the tab on the tube lid). Discard the supernatant. Leave ~15 µL in the tube (visually compare to 15 µL of liquid in a separate tube).

5. Ensure that the Sequencing Primer is completely thawed prior to use (no ice crystals should be visible).

6. Vortex the primer for 5 seconds, then pulse spin in a picofuge for 3–5 seconds to collect the contents. Leave on ice until ready to use.

7. Add 12 µL of Sequencing Primer to the ISPs, and confirm that the total volume is 27 µL (add Annealing Buffer if necessary).

8. Pipet the mixture up and down thoroughly to disrupt the pellet. IMPORTANT! Make sure that the pipette tip is at

the bottom of the tube during mixing to avoid introducing air bubbles into the sample.

9. Program a thermal cycler for 95°C for 2 minutes and then 37°C for 2 minutes, using the heated lid option. 10. Place the tube in the thermal cycler and run the program. After cycling, the reaction can remain in the cycler at room temperature (20–30°C) while you proceed with Chip Check.

Perform Chip Check

Chip Check tests the chip and ensures that it is functioning properly prior to loading the sample.

IMPORTANT! To avoid damage due to electrostatic discharge (ESD), do not place the chip directly on the bench or any other surface. Always place the chip either on the grounding plate on the Ion PGM™ Sequencer or in the Ion PGM™ Chip Minifuge adapter bucket. To avoid ESD damage, do not wear gloves when transferring chips on and off the instrument.

1. On the main menu of the Ion PGM™ Sequencer touchscreen, press Run. Remove the waste bottle and completely empty it. Press Next.

2. When prompted to insert a cleaning chip, use the same used chip that was used for initialization. Press Next to clean the fluid lines.

3. When prompted, select the instrument that you used to prepare the template- positive ISPs. Then press Next.

4. Remove gloves, and ground yourself by touching the grounding pad on the sequencer. Remove a new chip from its packaging and label it to identify the experiment (save the chip package). Press Next.

5. Replace the old chip in the chip socket with the new one for the experiment. Close the chip clamp, then press Next.

6. When prompted, use the scanner to scan the barcode located on the chip package, or press Change to enter the barcode manually. Optionally, you can also enter the library kit catalog number. Note- A chip cannot be run without scanning or entering the barcode.

7. Press Chip Check. During the initial part of Chip Check, visually inspect the chip in the clamp for leaks. Note- · If there is a leak, press the Abort button immediately to stop the flow to the chip. Proceed to Appendix A, “Troubleshooting“. · The chip socket can be

damaged by rubbing or wiping its surface. Never rub or wipe the socket to clean up leaks.

8. When Chip Check is complete- • If the chip passes, press Next. • If the chip fails, open the chip clamp, re-seat the chip in the socket, close the clamp, and press Calibrate to repeat the procedure. If the chip passes, press Next. If the chip still fails, press Main Menu and restart the experiment with a new chip.

9. Following a successful Chip Check, completely empty the waste bottle and select the Waste bottle is empty checkbox on the touchscreen. Press Next.

Bind the Sequencing Polymerase to the ISPs

1. Remove the Ion PGM™ Hi-Q™ Sequencing Polymerase from storage and flick mix with your finger tip four times. Pulse spin for 3–5 seconds. Place on ice.

2. After annealing the Sequencing Primer, remove the ISPs from the thermal cycler and add 3 µL of Ion PGM™ Hi-Q™ Sequencing Polymerase to the ISPs, for a total final volume of 30 µL.

3. Pipet the sample up and down to mix, and incubate at room temperature for 5 minutes.

Prepare and load the chip

Remove liquid from the Chip

1. Following Chip Check, remove the new chip from the Ion PGM™ Sequencer. Insert a used chip in the chip clamp while loading the new chip.

2. Tilt the new chip 45 degrees so that the loading port is the lower port.

3. Insert the pipette tip firmly into the loading port and remove as much liquid as possible from the loading port. Discard the liquid. IMPORTANT! For the next steps, if you are preparing one chip at a time, balance the chip in the Ion PGM™ Chip Minifuge rotor with a used chip of the same chip type and orientation. Be careful to balance an upside-down chip with another upside-down chip. Mark the used chip with a laboratory marker to differentiate it from the new chip.

4. Place the chip upside-down in the minifuge bucket and transfer the bucket to the with the chip tab pointing in (toward the center of the minifuge). Balance the bucket with another chip.

5. Centrifuge for 5 seconds to completely empty the chip.

CAUTION! Allow the minifuge to come to a complete stop before opening the lid.

6. Remove the chip from the bucket and wipe the bucket with a disposable wipe to remove any liquid. Place the chip right-side up in the bucket.

Load the chip

1. Place the chip in the bucket on a firm, flat surface. Following polymerase incubation, collect the following volume of prepared ISPs into a Rainin® SR-L200F pipette tip, depending on your chip type- • Ion 316™ or Ion 318™ Chip- Entire volume (~30 µL) • Ion 314™ Chip- 10 µL

2. Insert the tip firmly into the loading port of the chip. Note- When loading the ISPs into the chip, keep the pipette tip at a 90° angle to the chip, press the tip firmly into the circular loading port, and apply gentle pressure between the pipette tip and chip. Centrifuge adapter bucket.

3. With the pipette unlocked, apply gentle pressure between the tip and chip and slowly dial down the pipette (~1 µL per second) to deposit the ISPs. To avoid introducing bubbles into the chip, leave a small amount in the pipette tip (~0.5 µL).

Note- Do not remove the pipette tip from the port during the dial-down process, since this can introduce air bubbles and inhibit loading.

4. Remove and discard any displaced liquid from the other port of the chip.

5. Transfer the chip in the bucket to the minifuge with the chip tab pointing in (toward the center of the minifuge), and centrifuge for 30 seconds.

6. Turn the chip so that the chip tab is pointing out (away from the center of the minifuge), and centrifuge for 30 seconds.

7. Remove the bucket from the minifuge and place it on a flat surface. Set the volume of the pipettor as follows, depending on your chip type- • Ion 316™ or Ion 318™ Chip- 25 µL • Ion 314™ Chip- 5 µL

8. Tilt the chip 45 degrees so that the loading port is the lower port, and insert the pipette tip into the loading port.

9. Without removing the tip, slowly pipet the sample out and then back into the chip one time. Pipet slowly to avoid creating bubbles.

10. Slowly remove as much liquid as possible from the chip by dialing the pipette. Discard the liquid.

11. Turn the chip upside-down in the bucket, transfer it back to the minifuge, and spin upside-down for 5 seconds. Remove and discard any liquid.

12. If some liquid remains in the chip, lightly and rapidly tap the point of the chip tab against the benchtop a few times and remove and discard any collected liquid. Do not flush the chip.

13. When chip loading is complete, press Next on the touchscreen and proceed immediately to performing the run.

Select the Planned Run and perform the run

Select the Planned Run

1. Press Browse next to the Planned Run field and select the name of the plan you created, then touch Next.

2. Confirm that the settings are correct. If necessary, make any changes using the touchscreen controls.

Perform the Run

1. After you enter the Planned Run, press Next to verify the experimental setup. Press OK to confirm the settings or press Cancel to return to the touchscreen to adjust the settings.

2. When prompted by the instrument, load and clamp the chip, then press Next.

3. At the beginning of the run, visually inspect the chip in the clamp for leaks before closing the cover. The instrument will flush any loose ISPs from the chip and begin calibrating the chip.

4. When the calibration is complete (~1 minute), the touchscreen will indicate whether calibration was successful. • If the chip passes calibration, press Next to proceed with the sequencing run. • If the chip fails calibration, see “Error message- Calibration FAILED“on page 48.

5. After 60 seconds, the run will automatically begin, or press Next to begin the run immediately. IMPORTANT! During a run, avoid touching the instrument and any of the attached bottles or tubes, as this may reduce the quality of the measurements.

6. When the run is complete, leave the chip in place, and then touch Next to return to the Main Menu.

3.8.N. Data Analysis- The raw data from Next Generation Sequencing in FASTQ was assembled in SeqMan Ngen and analyzed in SeqMan Pro of DNASTAR software.

4. Results:

4.1 In quest for Barley Retro element (BARE) family and retrotransposons specially LTRs in plant species of Liliaceae family

The *BARE*-1 retrotransposon in *BARE* family is the major and active component of barley (*Hordeum vulgare)* genome. *BARE*-1 is a *copia* like LTR (Long Terminal Repeat) Retrotransposons. In addition, Family Liliaceae has one of the biggest genome on the plant kingdom. Recent studies shows that retrotransposition of the LTRs have significant contribution to the size of the genome. Since the life cycle of LTR, the mechanism of retrotransposition occur through formation of Virus like Particles (VLPs), my major target was successful extraction of VLPs from the cultured barley leaves (1 month old), naturally grown Genus Prosartes leaves (~3months old), Scoliopus leaves (~6 months Old), Clintonia leaves (~12 months), Lilium leaves ((~12 months), Prosartes leaves (~12 months), Smilax leaves (~12 months). The VLPs extracted from plant leaves were mostly invisible or precipitates in a clear pellet.

4.2 The RT assay confirms the presence of VLPs extracted from plant leaves :

The Reverse Transcriptase assay is a good tool to ascertain the reverse transcriptase enzymatic activity, which proves the successful extraction of VLPs from plant leaves. Following is the gel image of RT assay of VLPs. Figure 10. *Hordeum vulgare* (Barley) and Figure 11. *Prosartes smithii.*

In figure 1, a ~ 112 base pair product was visualized. The bands in the gel confirms the presence of VLPs extracted from Barley leaves (N, E, C-, C+, and C-).

However, on the Prosartes VLP preps (1-6) Reverse Transcriptase activity were not detected after the RT assay and that prompted to do gene specific PCR with *copia, gypsy* and LINE.

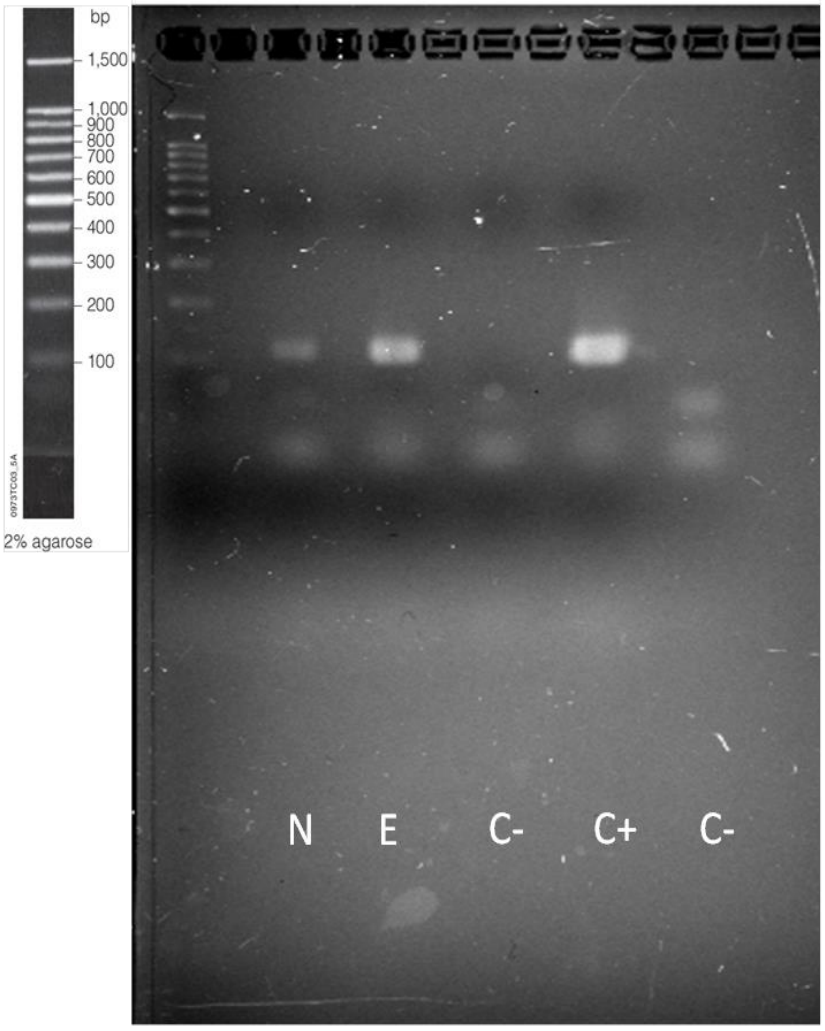


Figure 10(VLP prep # 1): The bands from are left 100bp ladder, VLPs resuspended in buffer without EDTA (N), VLPs resuspended in buffer with EDTA (E) and positive control (+RT and +MS2 RNA)

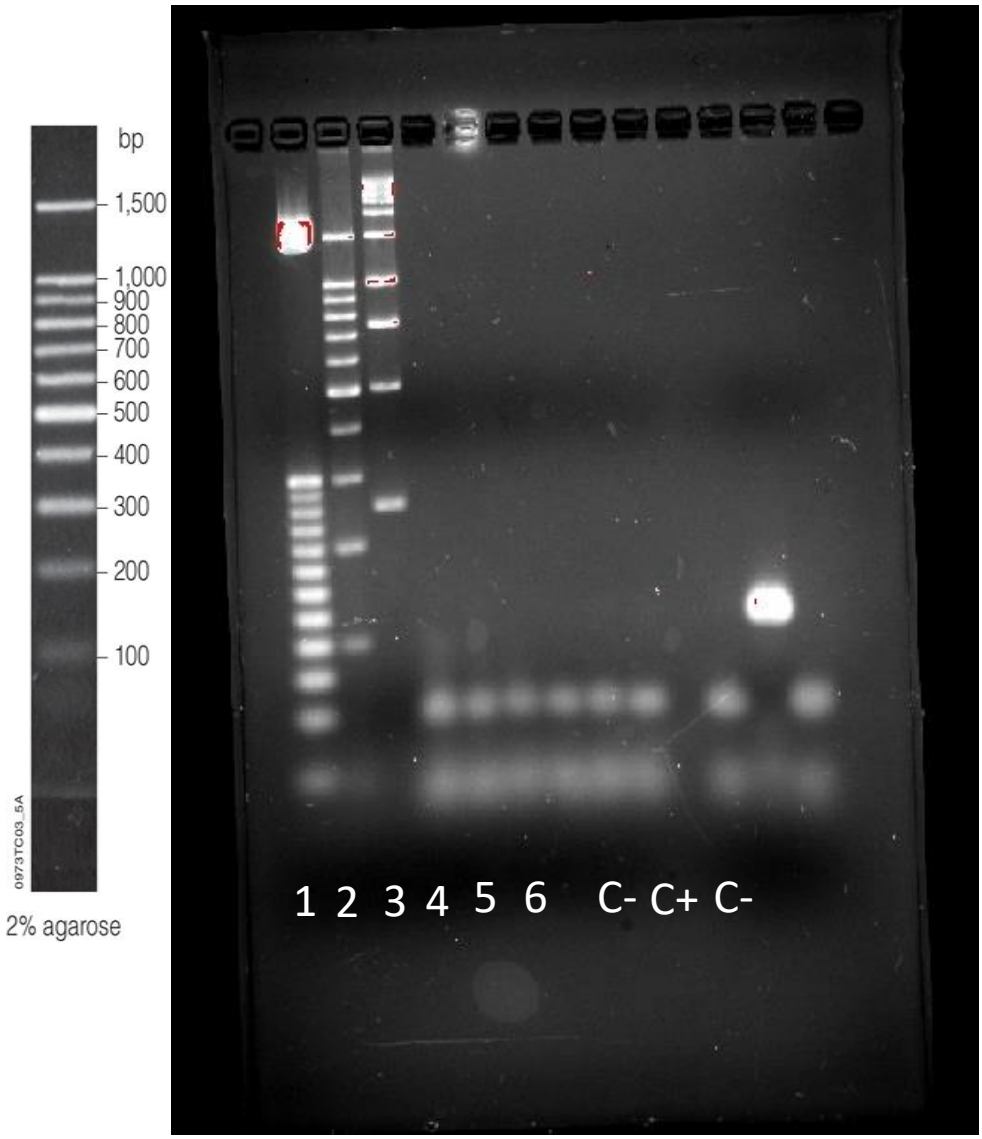


Figure 11 (VLP prep # 2): The bands from left are 25 bp ladder, 100bp ladder, 1kbp ladder and positive control (+RT and +MS2 RNA). Expected band from VLPs of Prosartes (Preps 1-6) were not visualized and both sides of positive control are flanked by two negative controls.

4.3 PCR with Reverse Transcription Enzyme Primers: GAG, Integrase:

In the LTR retrotransposons, the internal domain is located between the Long Terminal repeats. It is the protein coding domain composed of genes for gag (GAG), ap (Aspertate Proteinase), int (Integrase), and rt-rh (Reverse Transcriptase).

PCR involving the gag Primers (R&F) and int primers(R&F) amplifies the GAG and Integrase coding gene and confirms present of cDNA in the VLPs.

In Figure 12, the gel image shows the clear, distinctive bands for (E-G, E-I, N-G, N-I) PCR products of GAG and Integrase primers.

A PCR was also done with Rubisco gene (RBC) primers (start & finish) to rule out genomic DNA contamination (Figure 13).

A repetition of all the PCR products were ran in the same gel shows presence of the cDNA in the Barley VLPs resuspended in EDTA – buffer or non EDTA – buffer (Figure 14).

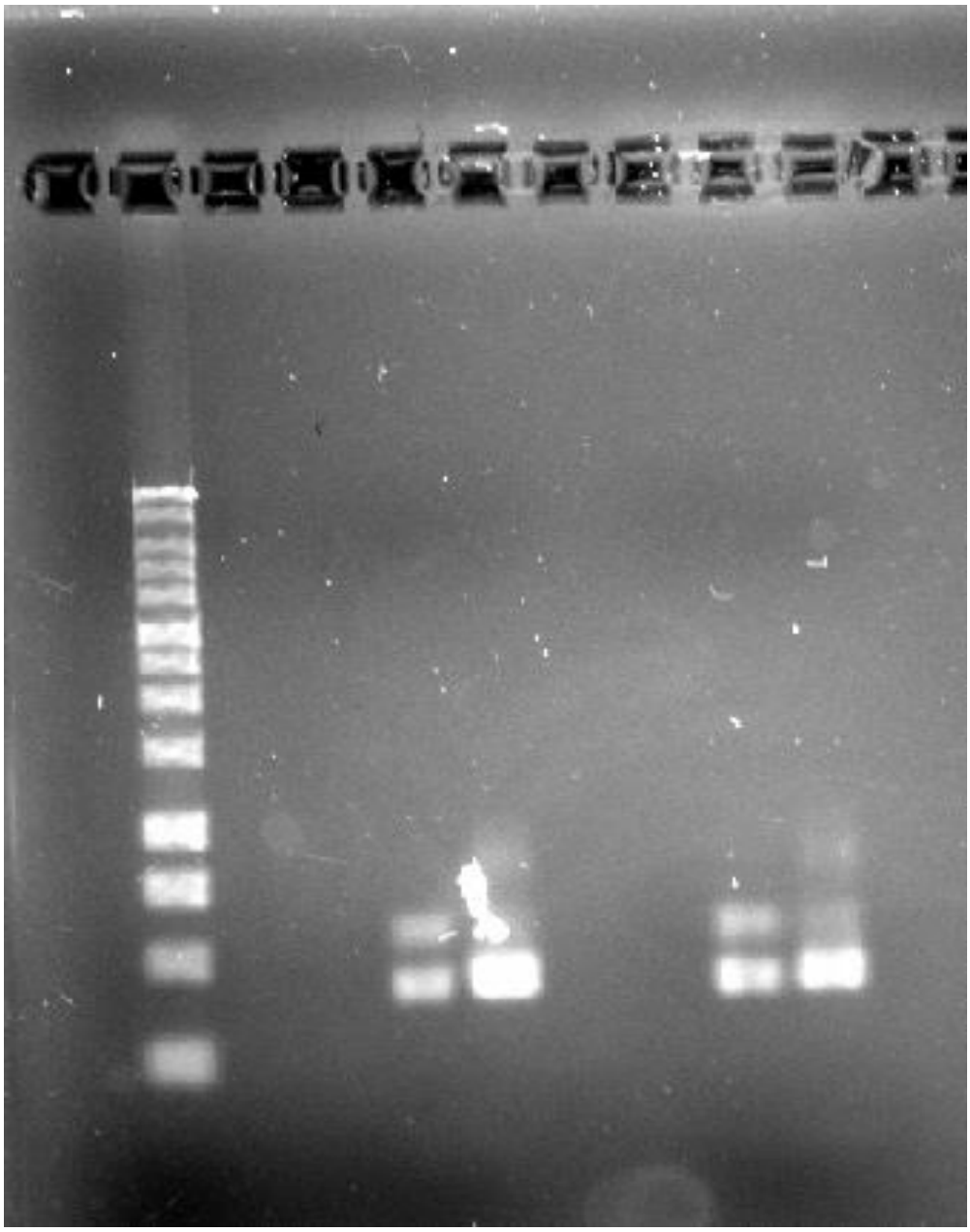


Figure 12 ((VLP prep # 1): The bands from left are 1kbp ladder, VLPs resuspended in buffer with EDTA (E-G) gag pcr product (E-I) integrase pcr product, VLPs resuspended in buffer without EDTA (N-G) gag pcr product, (N-I) integrase pcr product.

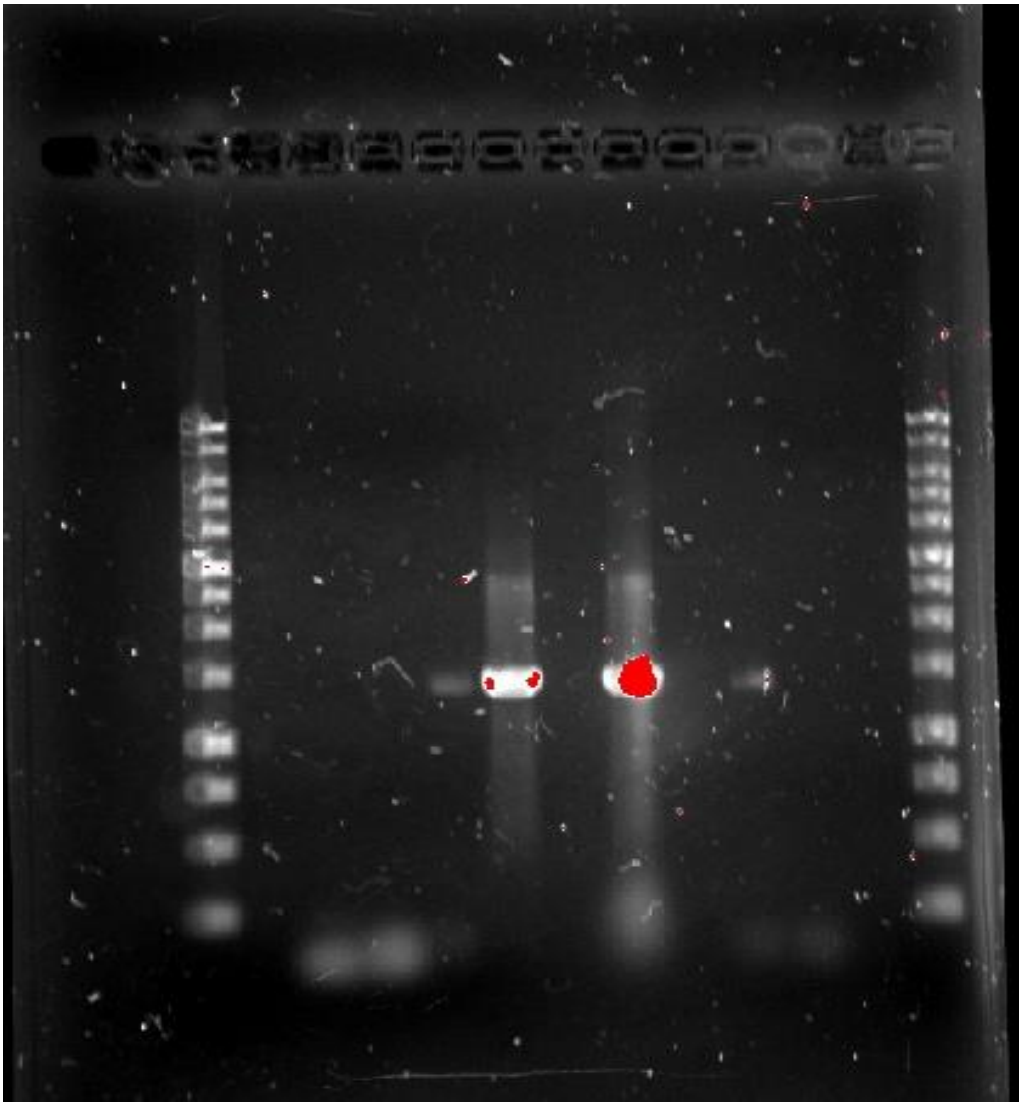


Figure 13 (VLP prep # 1): The bands from left are 1kb (band), no bands { PCR-I} for VLPs resuspended in buffer with EDTA (E-R) RBC pcr product, VLPs resuspended in buffer without EDTA (N-R) RBC pcr product, Control DNA band, control Barley genomic DNA band and no bands {PCR-2} for VLPs resuspended in buffer with EDTA (E-R) RBC pcr product, VLPs resuspended in buffer without EDTA (N-R) RBC pcr product.

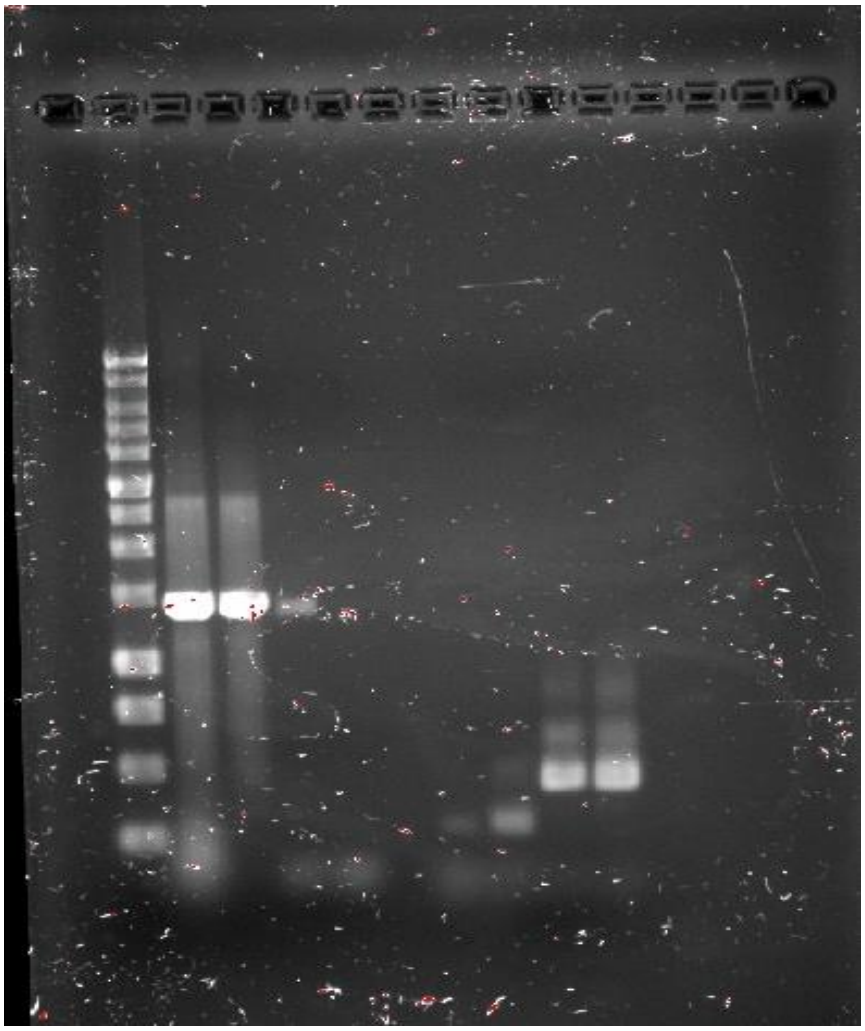


Figure 14 (VLP prep # 1): The bands from left are 1kb , control Barley genomic DNA band, Control DNA band, no bands for VLPs resuspended in buffer without EDTA (N-R) RBC pcr product, VLPs resuspended in buffer with EDTA (E-R) RBC pcr product, VLPs resuspended in buffer without EDTA (N-G) gag pcr product, VLPs resuspended in buffer with EDTA (E-G) gag pcr product, VLPs resuspended in buffer without EDTA (N-I) integrase pcr product and VLPs resuspended in buffer with EDTA (E-I) integrase pcr product.

4.4 PCR with Reverse Transcription Gene *copia*, *gypsy* and LINE: The retrotransposons, transpose via RNA intermediate. Thus, they have reverse transcription gene which encodes for

reverse transcriptase enzyme that converts the RNA into cDNA before integrating back into the genome. The retro elements *copia*, *gypsy* and LINE has reverse transcription gene which are commonly found in plant genomes.

The PCR with these primers ensures the presence of VLPs extracted from the plant tissues.

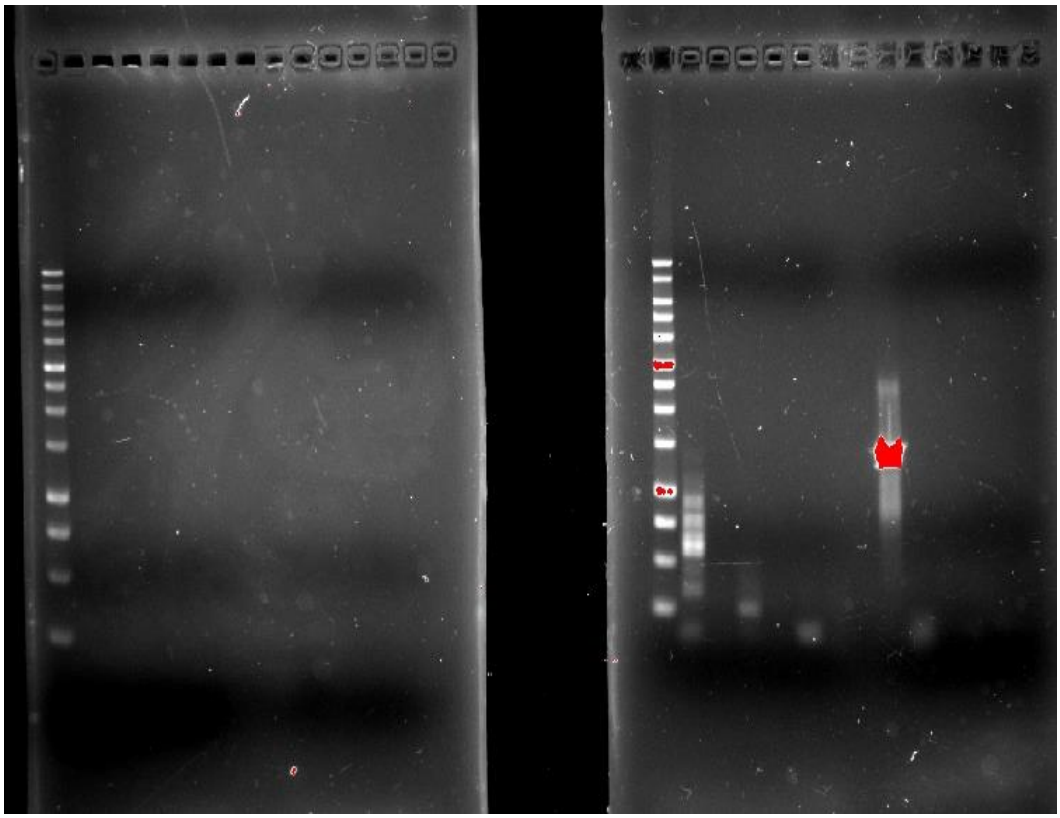
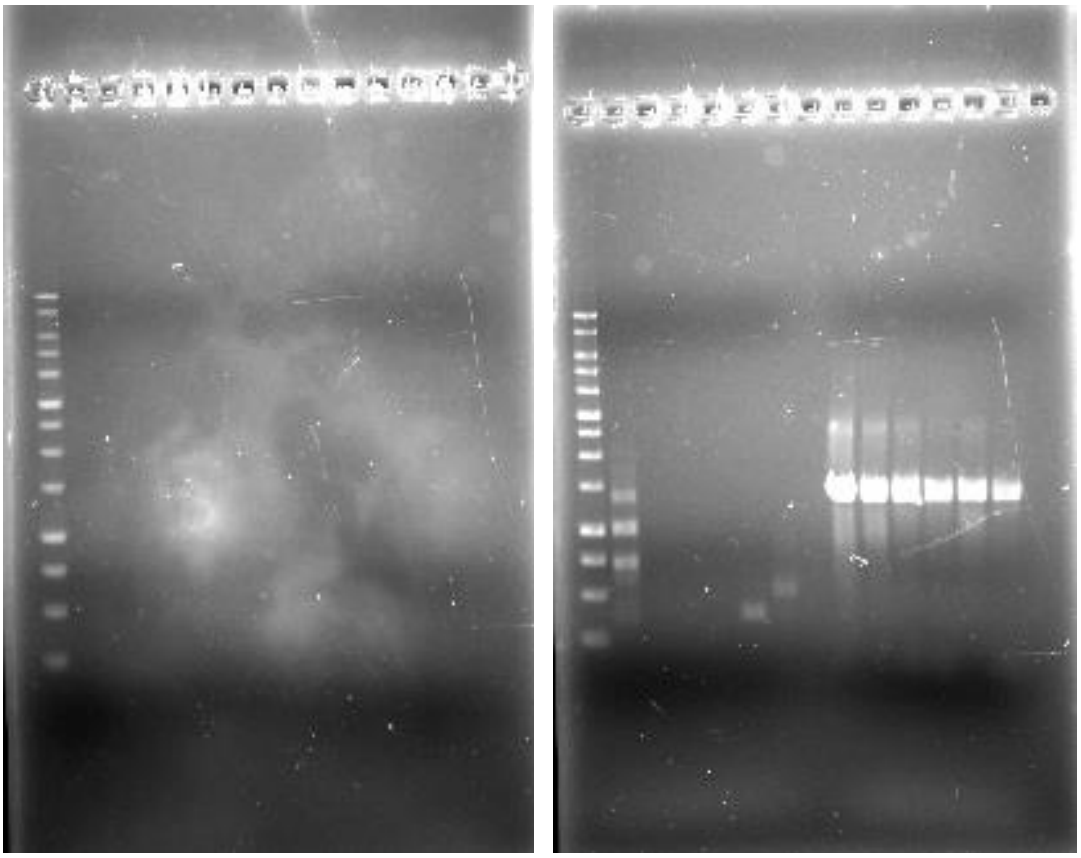


Figure 15 (VLP prep # 2): The bands from left to right are 1kbp ladder, no bands for pcr products for *Prosartes* – copia (1-6), no bands for pcr products for *Prosartes*- gypsy (1-6), 1kbp ladder, band for pcr products for *Prosartes* – LINE –prep 1, no bands for *Prosartes* – LINE (2-6), band for pcr products for *Prosartes* – RBC –prep 1, no bands for *Prosartes* – RBC (2-6).



`Figure 16 (VLP prep # 3)- The bands from left to right are 1kbp ladder, no bands for pcr products for *Scoliopus* – copia (1-20%, 2-20%, 3-40%, 4-40%, 5- 60%, 6-60%), no bands for pcr products for *Scoliopus*- gypsy (1-20%, 2-20%, 3-40%, 4-40%, 5- 60%, 6- 60%), 1kbp ladder, band for pcr products for *Scoliopus* – LINE –prep 1-20%, no bands for *Scoliopus* – LINE (2-20%, 3-40%, 4-40%, 5- 60%, 6-60%), band for pcr products for *Scoliopus* – RBC –prep (1-20%, 2-20%, 3-40%, 4-40%, 5- 60%, 6- 60%).

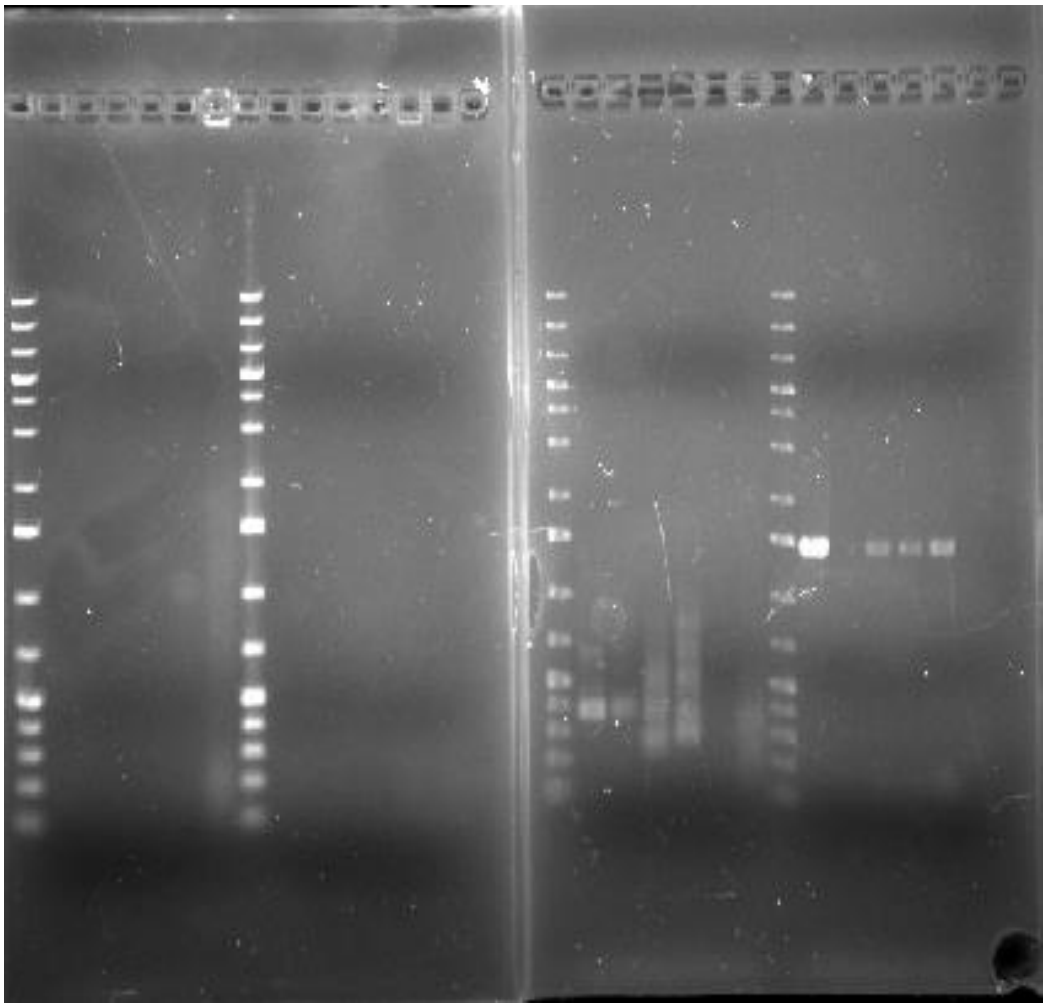


Figure 17 (VLP prep # 4): The bands from left to right are 1kbp ladder, no bands for pcr products for *Clintonia – copia* -40%, *Clintonia – copia* -60%, *Lilium – copia*-40%, *Lilium – copia* - 60%, *Prosartes – copia* - 60%, *Smilax – copia* - 60%,1kbp ladder , no bands for pcr products for *Clintonia – gypsy* -40%, *Clintonia – gypsy* -60%, *Lilium – gypsy*-40%, *Lilium – gypsy* - 60%, *Prosartes – gypsy* - 60%, *Smilax – gypsy* - 60%, 1kbp ladder, bands for pcr products for *Clintonia* – LINE -40%, *Clintonia* – LINE -60%, *Lilium* – LINE-40%, *Lilium* – LINE - 60%, *Smilax* – gypsy - 60%, no band for *Prosartes* – LINE - 60%, 1kbp ladder, bands for pcr products for *Clintonia* – RBC-40%, *Lilium* - RBC-40%, *Lilium* - RBC- 60%, *Prosartes* – RBC - 60%, no band for *Clintonia* – RBC -60% and *Smilax* – RBC - 60%.

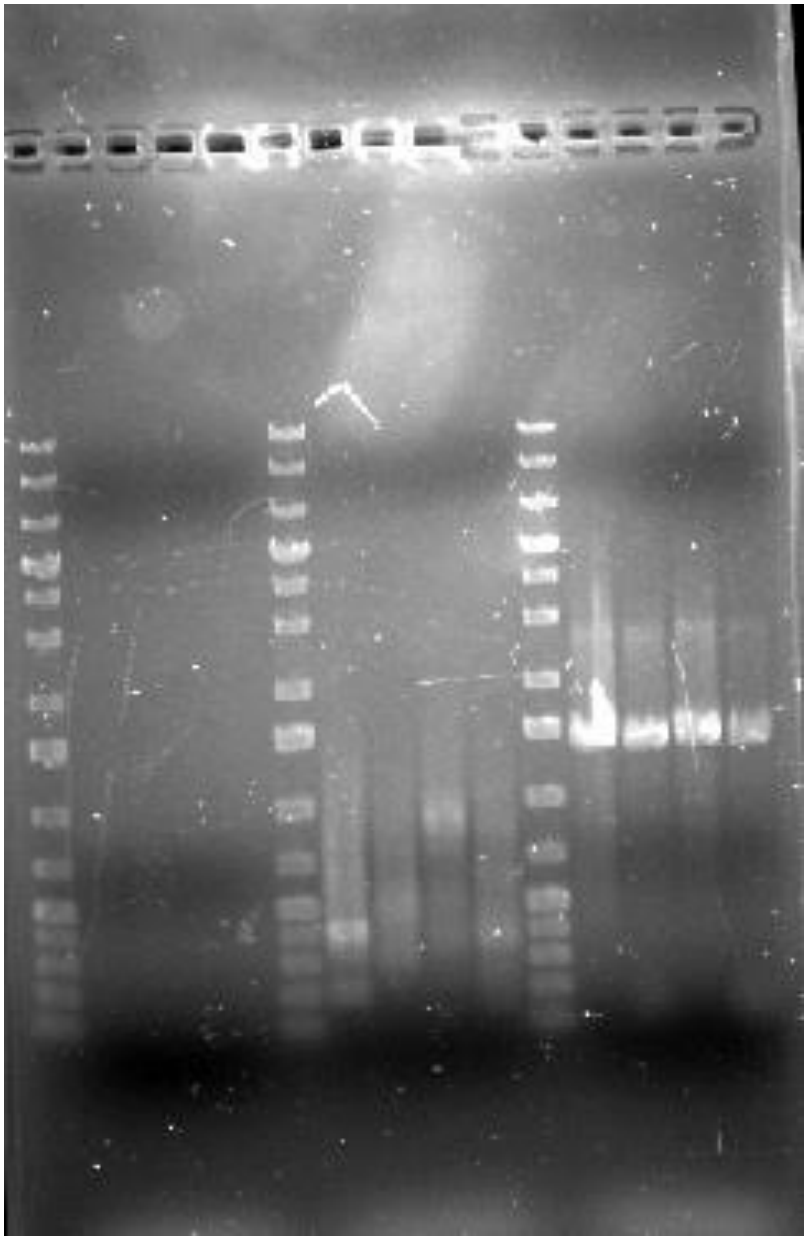


Figure 18: PCR with gDNA extracted from *Clintonia, Lilium, Prosartes* and *Smilax* with *gypsy*, LINE and RBC primers. The bands from left to right are 1kbp ladder, no band for gypsy pcr product, bands for LINE pcr products and bands for RBC pcr product.

4.5 cDNA isolation from VLPs extracted from *Hordeum vulgare* (Barley):

The Virus like particle (VLPs) extracted from barley contained cDNA which was confirmed by gag (gene encodes for capsid protein GAG) and int (gene encodes for protein Integrase) pcr. After extraction, gel electrophoresis was done to visualize the cDNA. The gel image shows no bands for cDNA (Figure 19).

The result of gel electrophoresis prompted to perform the BioAnalyzer to identify the number of basepairs and quantify the cDNA (Figure 20). The BioAnalyzer results shows that the cDNA is present in fragment size between 990bps to 7800bps.

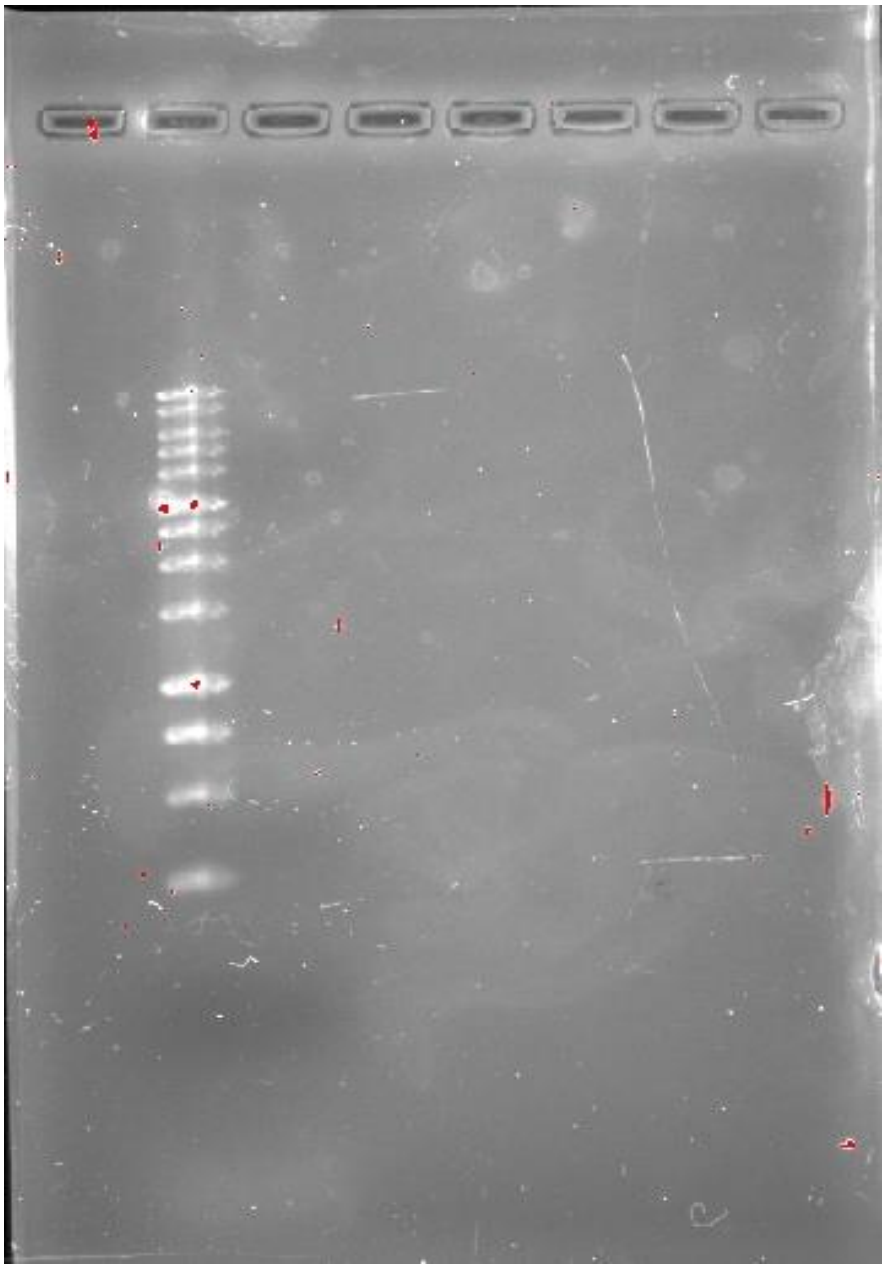


Figure 19: The gel electrophoresis shows only the 1kbp ladder but no bands for cDNA

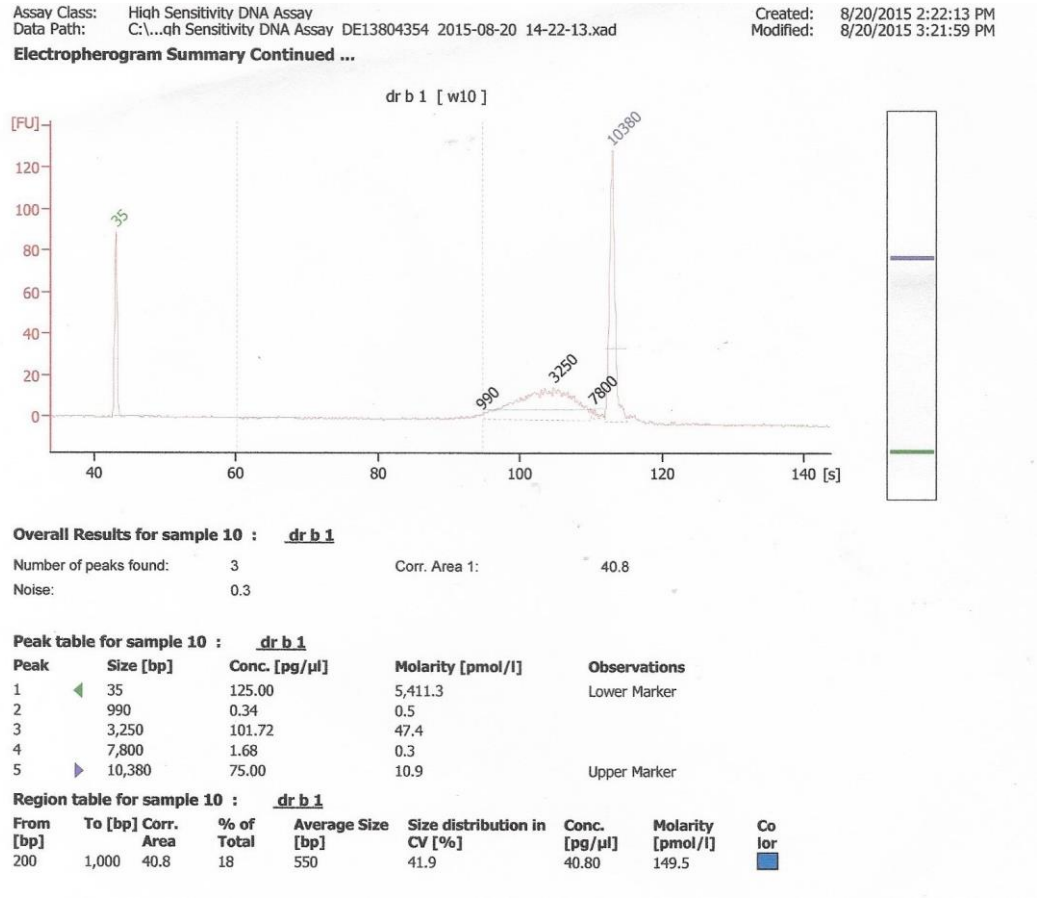


Figure 20: Bioanalyzer (1) result shows that base pair sizes ranges between 990- 7800. The concentration is highest (101.72pg/ul) for the base pair size of 3250

4.6 NEXT GENERATION SEQUENCING of cDNA Extracted from *Hordeum vulgare* (Barley):

The 1st step of Library prep in NGS is fragmenting the DNA with sonication. The sonication result is visualized by the running gel electrophoresis, which shows smears of DNA instead of band. But the gel (Figure 21) did not show any

smear image. So the Bioanalyzer was done to identify the sonicated cDNA. The BioAnalyzer result (Fig 16) showed there is a 286 base pair size fragment present. The concentration of the cDNA was significantly low. So a PCR was done with gag and int. primers to confirm the presence of fragmented cDNA. The gel image (Fig 17) of pcr product showed band for both GAG and Integrase. On the following steps of library prep end repair and adaptor ligation were done. After the first size selection (Pippin Prep) of the cDNA fragment, a pcr process was done to amplify the cDNA and the Bioanalyzer was repeated. The third Bioanalyzer result showed a significant increase in cDNA concentration (Figure 18). The Qubit 2.0 fluorometer results (Figure 19) summarizes the percentage of templated Ion Sphere Positive Particles (ISPs) before loading them for sequencing in Ion Torrent PGM. Finally the Figure 20A and Figure 20B summarizes the sequencing run of Barley cDNA isolated from Virus Like Particle of Barley.

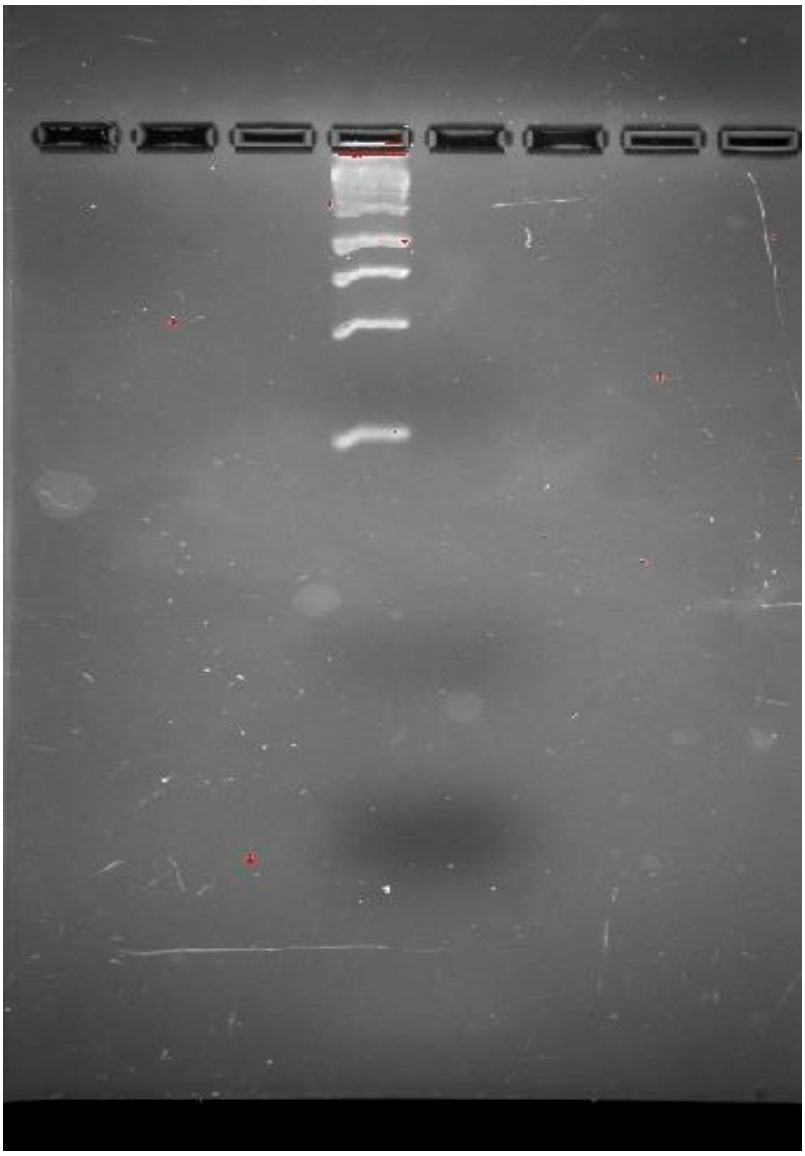
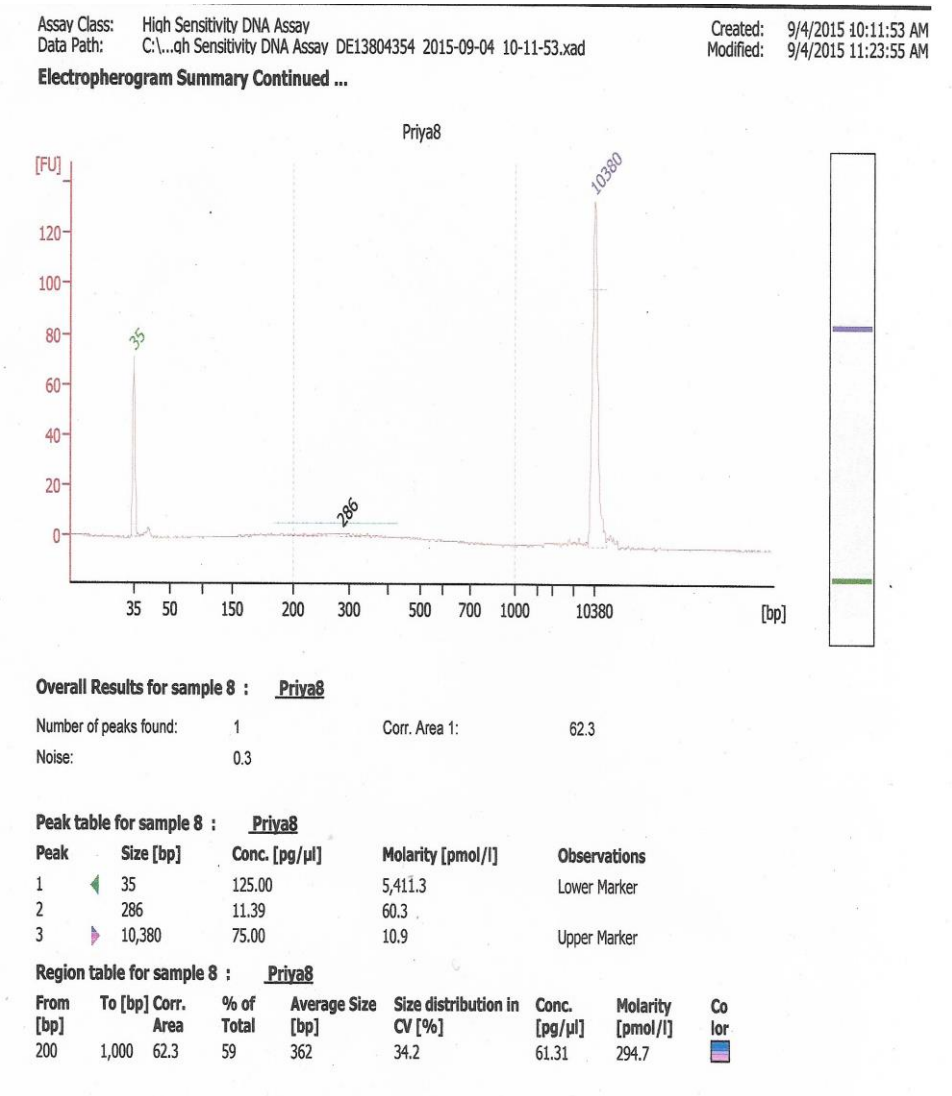


Figure 21: The gel electrophoresis shows only the 1kbp ladder but no smears of cDNA after sonication.



Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay DE13804354 2015-09-04 10-11-53.xad

Created: 9/4/2015 10:11:53 AM
Modified: 9/4/2015 11:23:55 AM

Electropherogram Summary Continued ...

Overall Results for sample 8 : Priya8

Number of peaks found:	1	Corr. Area 1:	62.3
Noise:	0.3		

Peak table for sample 8 : Priya8

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	286	11.39	60.3	
3	10,380	75.00	10.9	Upper Marker

Region table for sample 8 : Priya8

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	62.3	59	362	34.2	61.31	294.7	

Figure 22: Bioanalyzer (2) done after sonication showed a trace amount of cDNA

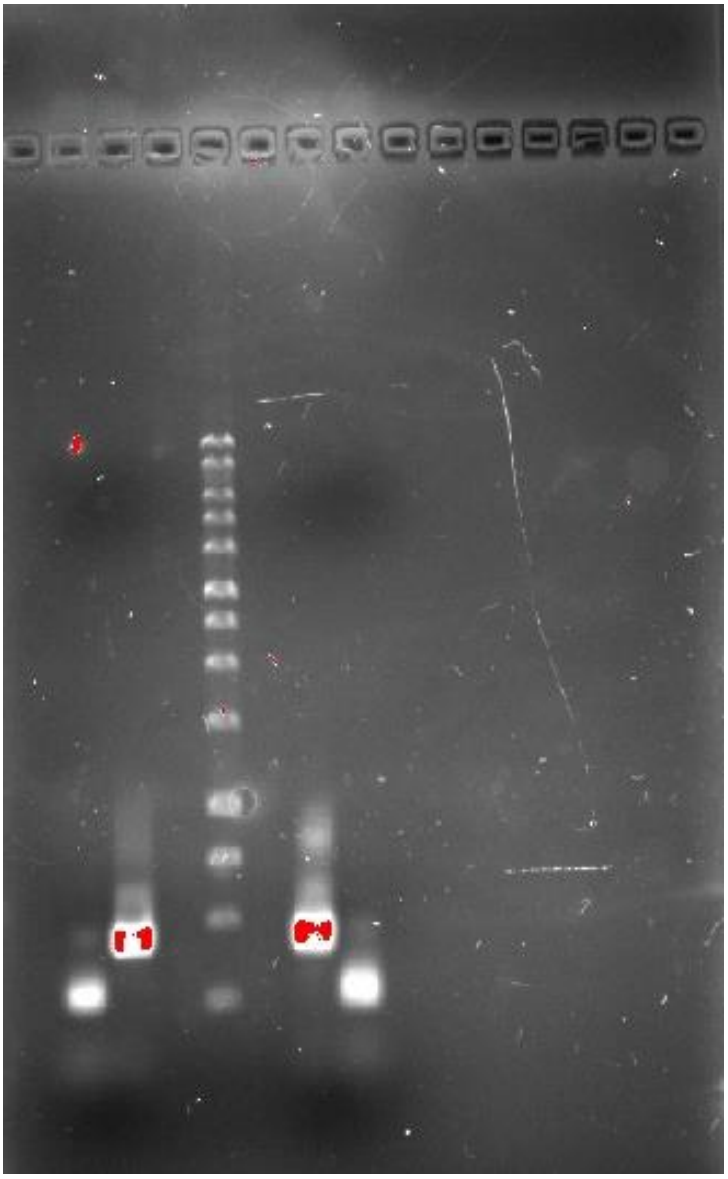


Figure 23: The bands from left to right are pcr product of gag, pcr product of integrase, 1kbp ladder, pcr product of integrase, pcr product of gag.

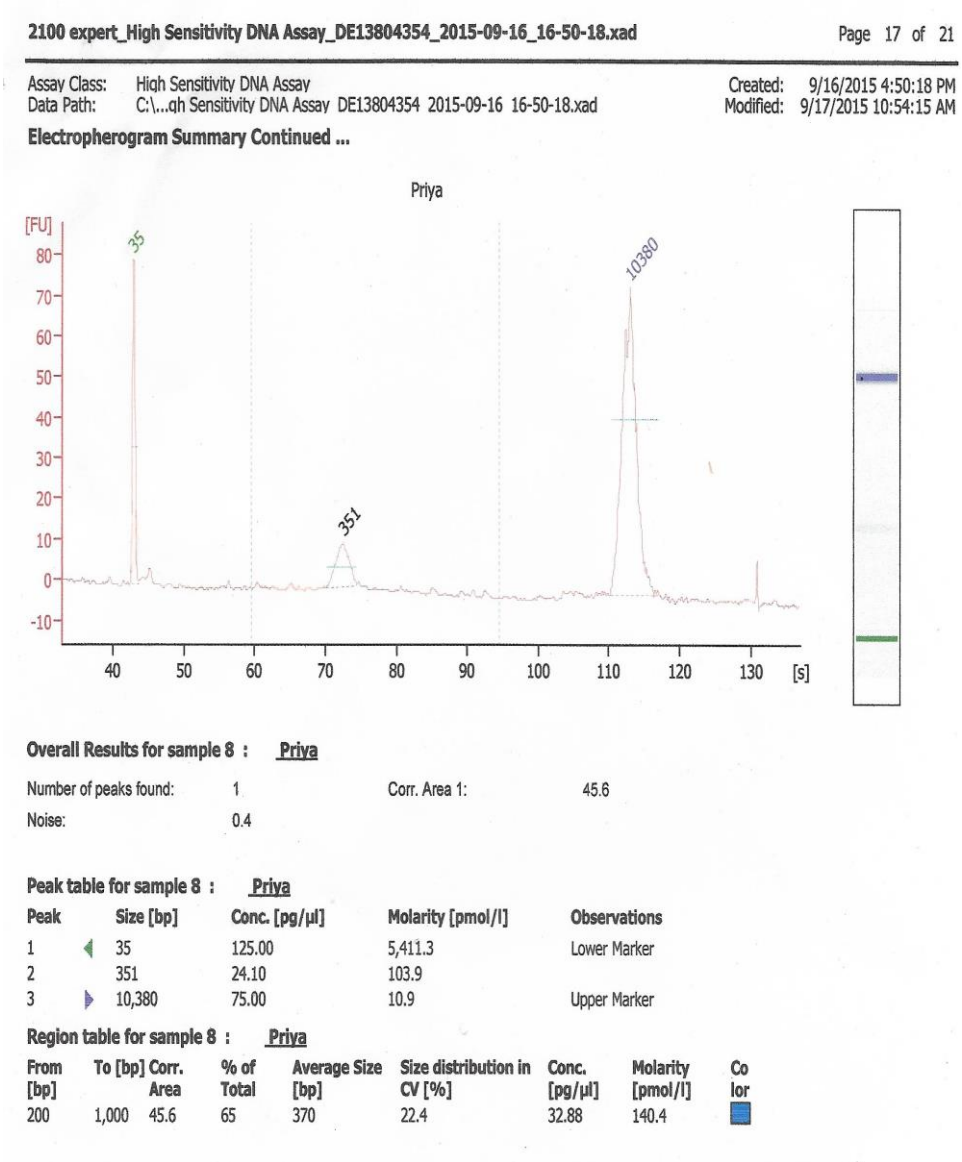


Figure 24: Bioanalyzer (3) Result after 2nd Pippin showed increase in concentration of cDNA and desirable base pair size.

Qubit Calibration Factor Calculation

Calibration Standard	RFU	Calibration Factor
Alexa Fluor® 488 Calibration Standard	29000	0.29
Alexa Fluor® 647 Calibration Standard	92200	

Percent Templated ISPs

	Raw RFU Value		Background RFU (Negative Control Tube)			
Sample ID	AF 488	AF 647	AF 488	AF 647	Conversion Factor*	Percent Templated ISPs
314	1118.0	300.0	55.0	13.0	0.7	5%
barley VLPs	527.0	464.0	55.0	13.0	0.7	19%
						#DIV/0!
						#DIV/0!
						#DIV/0!
						#DIV/0!
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						#DIV/0!
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						#DIV/0!
						#DIV/0!

* Conversion factor can be found on Ion Community website (http://ioncommunity.lifetechnologies.com/docs/DOC-2665) and is Template Kit lot speci

Figure 25: Qubit Result before the sequence run

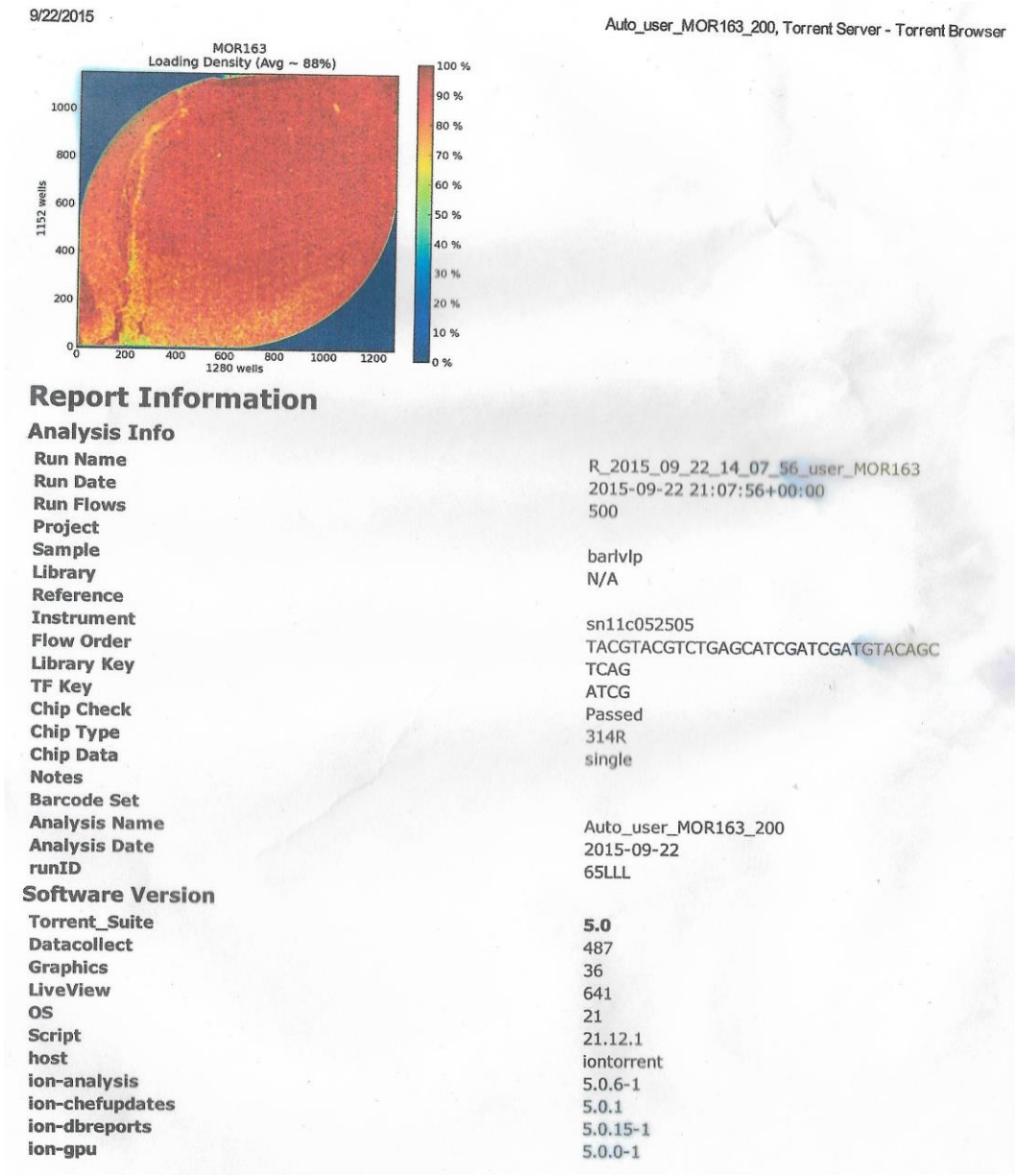
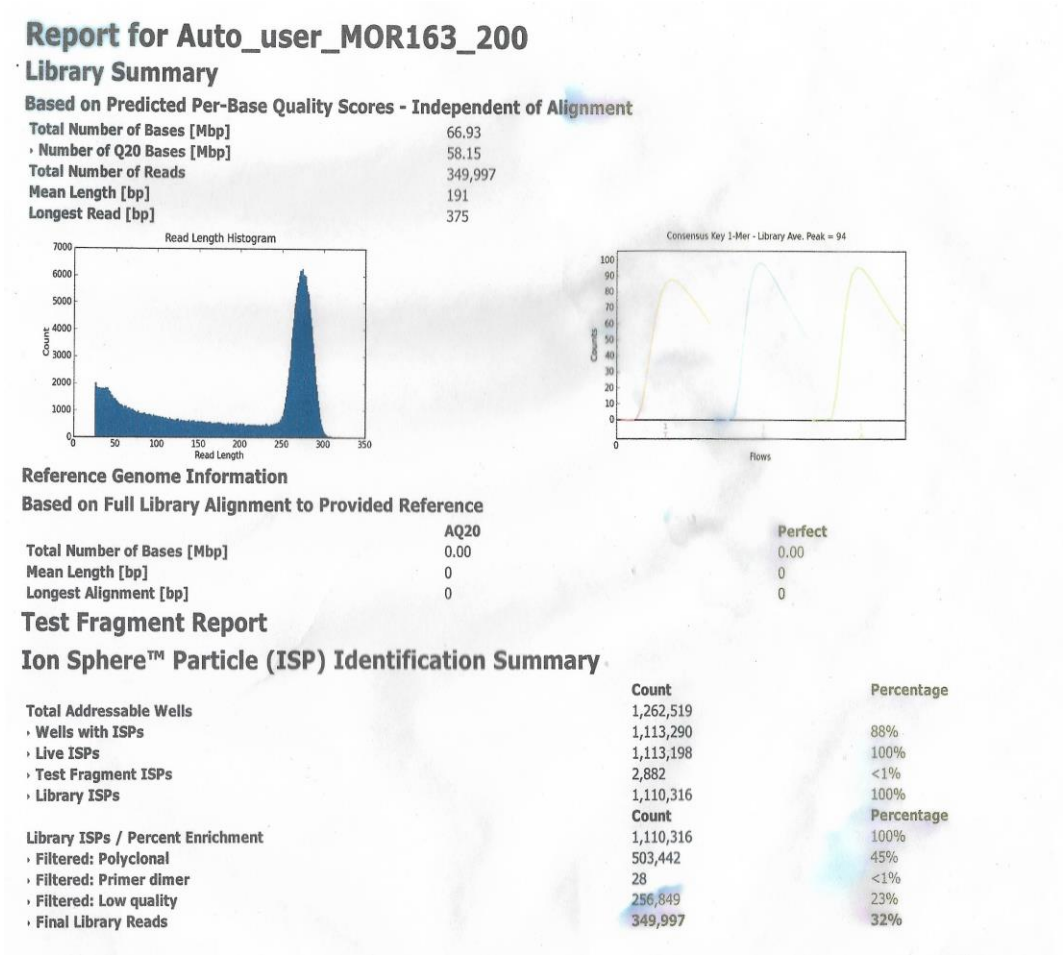


Figure 26: Library summary of the sequence run on Ion Torrent PGM



Report for Auto_user_MOR163_200

Library Summary

Based on Predicted Per-Base Quality Scores - Independent of Alignment

Total Number of Bases [Mbp]	66.93
Number of Q20 Bases [Mbp]	58.15
Total Number of Reads	349,997
Mean Length [bp]	191
Longest Read [bp]	375

Read Length Histogram

Count

Read Length

Consensus Key 1-Mer - Library Ave. Peak = 94

Counts

Flows

Reference Genome Information

Based on Full Library Alignment to Provided Reference

	AQ20	Perfect
Total Number of Bases [Mbp]	0.00	0.00
Mean Length [bp]	0	0
Longest Alignment [bp]	0	0

Test Fragment Report

Ion Sphere™ Particle (ISP) Identification Summary

	Count	Percentage
Total Addressable Wells	1,262,519	
Wells with ISPs	1,113,290	88%
Live ISPs	1,113,198	100%
Test Fragment ISPs	2,882	<1%
Library ISPs	1,110,316	100%
	Count	**Percentage**
Library ISPs / Percent Enrichment	1,110,316	100%
Filtered: Polyclonal	503,442	45%
Filtered: Primer dimer	28	<1%
Filtered: Low quality	256,849	23%
Final Library Reads	349,997	32%

Figure 27: Library summary of the sequence run on Ion Torrent PGM

4.7 Data Analysis: The data achieved from sequencing of Barley cDNA isolated from Barley VLPs were assembled using SeqMan Ngen and then visualize and analyze with the help of SeqMan pro.

The contigs generated from the sequence assembly were used in BLAST search. As for example the contig # 3 (Figure 28) in BLAST search yielded several close match with the anotated (Figure 29) sequence like the following one with 97% similarity.

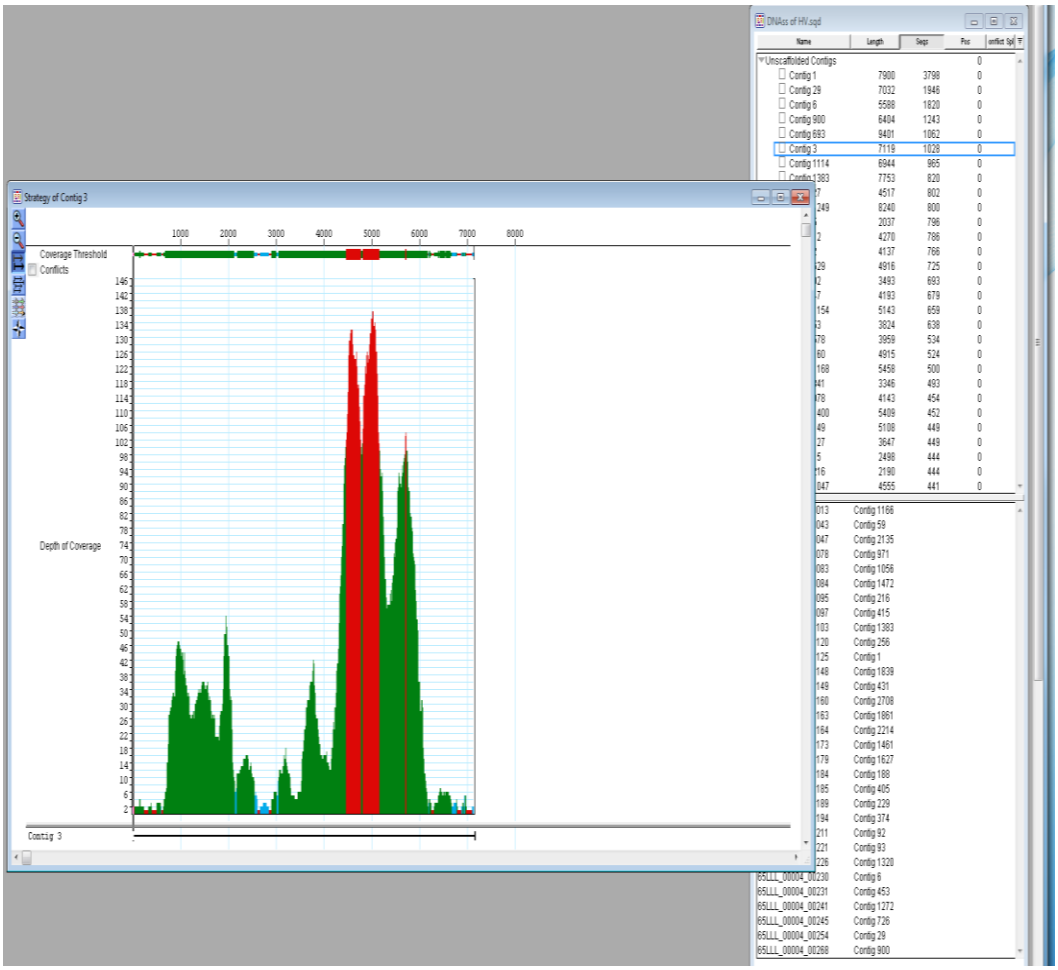


Figure 28: Contig # 3 with depth coverage

Contig3:

Hordeum vulgare subsp. vulgare cultivar Morex BAC clone 745c13, complete sequence

GenBank- AF474071.1

between Sabrina LTR-2 and LTR-3 in AF254799"

repeat region	19516..28357
	/standard_name="BARE-745c13-1"
	/rpt_family="LTR retrotransposon"
repeat region	19516..21298
	/standard_name="BARE-1"
	/rpt_type=long_terminal_repeat
repeat region	26584..28357
	/standard_name="BARE-1"
	/rpt_type=long_terminal_repeat
repeat region	29326..30900

Figure 29: Similarities with one of the matches

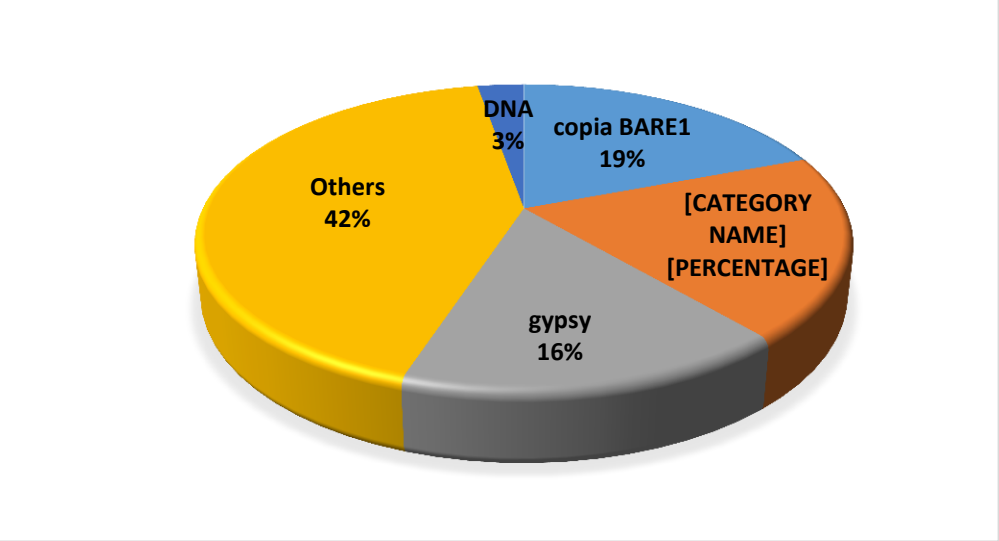


Figure 30: Analysis of top 100 contigs to find various Retrotransposons

5 Discussion:

The transposable elements earlier tagged as "junk DNA", now considered as key player in shaping the genome throughout the evolution. Among the transposable elements, the retrotransposons are ubiquitous and prolific in plant genomes. The retrotransposons, especially the LTRs resembles the lifecycle of retroviruses. The Virus Like Particle or VLPs are formed during the life cycle of LTRs are intracellular unlike the retroviruses due to lack of a function envelop (env) gene. The objective of my thesis work is to characterize the transposable elements by extracting the VLPs and sequence the retrotransposons within.

5.1 BARE -1:

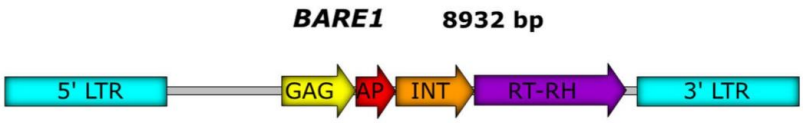


Figure 31. Structure of BARE-1

My first goal was to identify the Barley Retro element or BARE in Barley (*Hordeum vulgare*) plant. BARE -1 (Figure 31) is the major, autonomous, actively transcribed and

translated retro element in BARE family of Barley genome. This copia like retrotransposon has 1.8-kb long terminal repeats bounding an internal domain of 5275 bp which encodes a predicted polyprotein of 1301 residues (Schulman et al, 2000). The assembly of VLPs is the precursor of translation of retrotransposons. VLPs have been shown to contain RNA or cDNA, RT, and INT within a capsid consisting of GAG. So the VLPs extracted from Barley leaves were run through Reverse Transcriptase assay to confirm the successful extraction and positive RT activity. In the gel electrophoresis, a ~100bp product (Figure 10) which corresponds to the positive control (MS2 Phage RNA) was visualize for all four of my samples. The VLPs resuspended in EDTA buffer showed more prominent bands than the non EDTA. But overall, there were no major difference between the bands. Since the Reverse Transcriptase is a major enzyme in life cycle of LTRs, positive RT activity in my samples confirms presence of a type of retroelement. Once the presence of VLPs were confirmed on the Barley Plant tissues samples, a PCR was done with gag and integrase primers to identify the VLPs with RNA and VLPs with cDNA as the VLPs might contain either or both due to the different stages of life cycle (Figure 9). The ‘gag’ gene code for

the GAG protein which is a capsid protein responsible for the assembly of the outer shell of the VLPs in LTR retrotransposons and Retroviruses. The gag gene is significantly diverse in the different LTR retrotransposons. The integrase protein coded by 'int'gene is the enzyme responsible for integration of the cDNA to the genome at the end of the life cycle. The PCR result was visualized in 1% agarose gel and the all the samples showed ~ 300bp bands for both gag & int (Figure 12). The gel electrophoresis confirmed the presence of cDNA- VLPs in Barley VLP prep. Since the genomic DNA or gDNA also has these genes, a PCR was done using RuBisCo (RBC) primers. The enzyme Rubisco, short for ribulose-1, 5-bisphosphate carboxylase/oxygenase, is the enzyme that incorporates CO_2 into plants during photosynthesis. RBC is the most abundant protein in plants. The RBC-PCR was visualized in 1% agarose gel and had only control bands for Barley gDNA (Figure 13). Therefore, the VLPs extracted from Barley definitely contain cDNA containing genes encoded for classical retroelement polyprotein. The presence of RNA-VLP was ruled out due to the presence of bands in the gel. Repeated gag-PCR and int-PCR (Figure 14) confirms again the same result of earlier PCR. Based on all the gene specific

PCR results, I decided to sequence the cDNA present on the Barley VLPs.

The cDNA, extracted from VLPs were ran through 1% agarose gel electrophoresis but not yielded any band (Figure 19). So I ran the Bio Analyzer, with high sensitive DNA chip and the results showed the trace amount cDNA (Figure 20). The result also explains that the amount of cDNA was insufficient to get a band for gel electrophoresis (Figure 21). The sonication of the cDNA during library prep also showed no smear for gel electrophoresis and the Bio Analyzer result (Figure 22) was also inconclusive. To confirm the presence of cDNA still present, a GAG and Integrase (INT) PCR was repeated. The bands (Figure 23) were visualized in gel image proves the presence of cDNA though it was in trace amount. The library prep was then continued with the cDNA extracted from the Barley (*Hordeum vulgare*). The intermediate results (Figure 24 &25) from different steps of library prep were contiguous with the cDNA (Figure 26 & 27). After the successful sequencing of the cDNA (Figure 28 & 29), the data analysis clearly and evidently shows that it is a LTR retrotransposons and contains a

part BARE – 1 retrotransposons (Figure 30). Rigorous BLAST analysis of the other contigs obtained from sequencing shows 98%-100% similarities between query sequences and matches for “gypsy” and “copia” retrotransposons. Moreover, analysis of top 100 contigs based on abundance shows Barley VLPs harbor significant amount of copia, gypsy and other LTR retrotransposons besides BARE 1. Therefore, above data indicate that I identified the BARE 1 and other retrotransposons capable of accomplishing their lifecycle, expressing proteins, assembling VLPs, replicating, and inserting back into the genome.

5.2 Retro element in family Liliaceae:

In plant kingdom, most angiosperms have small genome but some plant species shows huge variation in genome sizes. The plant species of *Liliaceae* are one of them. Studies showed that the plant species harbor active retrotransposons which might be a significant contributor of increased genome size. In this work, successful extraction of VLPs from above mentioned Plant species of Lily family showed several LINE PCR product (Figure 15,16& 17) , when universal LINE primers are used. The

above finding was considerably important as LINE retrotransposons are part of Non LTR family which do not form VLPs during their life cycle. In light of these new findings, the LINE PCR products were sequenced through Sanger Sequencing method. The result came up as multiple overlapping peak which made it tough to identify each, single LINE product. So, a further analysis required using Next Generation Sequencing. Moreover, earlier gDNA analysis of plant species showed abundance in copia and gypsy retrotransposons. Therefore, a further PCR is required with new set of copia and gypsy Primers.

6 Conclusion:

Retrotransposons are ubiquitous in eukaryotic genome. Over seventy years of research shows that they have significant contribution in increased genome size and shaping the genome through the evolution. Among the different kinds of retrotransposons, LTR Retrotransposons occupy a large fraction of the genome in most eukaryotes. To identify, understand the active LTR retrotransposons, it is important to study the life cycle of them. In my research work, the aspects of transcription, translation and VLP formation was studied using model VLP system of *Hordeum vulgare* and different plant species of *Liliaceae* family.

My work resulted in successful extraction of VLPs from *Hordeum vulgare* Barley and from plants of the *Liliaceae* family. We identified the BARE1 as active retrotransposon and interestingly several active retrotransposons from copia and gypsy family other than BARE 1in Barley VLPs. The above finding is instrumental for the further study in Barley and other grass family. Furthermore, we also identified some active Retrotransposons in Lily VLPs and investigation is going on for proper identification through Next Generation Sequencing.

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Appendix

Buffer Recipes

(1) 0.1M Citric Acid Stock

- Transfer 2.10g of citric acid to 100mL volumetric flask.
- Bring up the final volume to 100mL with Milli Q water

(2) 0.1M Sodium Citrate Stock

- Transfer 2.94g sodium citrate to 100mL volumetric flask.
- Bring up the final volume to 100mL with Milli Q water.

(3) 0.1M Sodium Citrate (pH 6.2)

- Transfer 7.2mL of 0.1M "Citric Acid Stock" to a 50mL bottle.
- Bring up the final volume to 50mL with "0.1M Sodium Citrate Stock".
- Transfer this to a 100mL bottle.
- Add 50mL Milli Q water.

Autoclave the above buffers and put them in the 4°c refrigerator.

(4) Resuspension Buffer with EDTA

- In a 15mL conical tube add the following-
  - 25mM Tris HCL (250μL of 1M Tris HCL) (aka Trizma-HCL)
  - 50mM KCL (500μL of 1M KCL)
  - 5mM DTT (500μL of 0.1M DTT)
  - 0.25mM EDTA (5μL of 0.5M EDTA) Not TRIS-EDTA
  - 0.025% Triton X-100 (2.5uL of Triton x-100)
  - 50% glycerol (5mL of glycerol)
  - Bring final volume up to 10mL with DNA grade water.
  - Keep in the refrigerator.

(5) Resuspension Buffer without EDTA

- In a 15mL conical tube add the following-

- 25mM Tris HCL (250μL of 1M Tris HCL) (aka Trizma- HCL)
- 50mM KCL (500μL of 1M KCL)
- 5mM DTT (500μL of 0.1M DTT)
- 0.025% Triton X-100 (2.5uL of Triton x-100)
- 50% glycerol (5mL of glycerol)
- Bring final volume up to 10mL with DNA grade water.
- Keep in the refrigerator.

- **0.1M DTT** (dithiothreitol)
- *MW- 154.25 g/mole*
- *We want 0.1M solution*
- *Weigh out 0.15g of DTT and bring up the final volume to 10mL.*

- **33.3% Triton X**
- *Need to make 33% Triton X by adding 330uL Triton X-100 + 670uL DNA grade water.*
- *Store at room temperature.*

- Prepare chilled **20% sucrose solution in 0.1M sodium citrate buffer.**
- *Need to make this by adding- 2g sucrose into the conical tube and bring up the final volume to 10mL with "0.1M sodium citrate buffer" (in the fridge drawer)*

- Prepare chilled **0.5X sodium citrate buffer**
- *Need to make this by adding- 7mL of 0.1M sodium citrate buffer + 7mL water.*

Diluting Primer Stock to get 10uM:

- RevTrans Asssay RT-1 Primer [5'-d(CATAGGTCAAACCTCCTAGGAATG)-3']
- *10uM (1-10 dilution- 10X concentration)= 2uL RT-1 primer (100uM) + 18uL DNA grade water to get a total of 20uL.*
- RevTrans Assays RT-2 Primer [5'-d(TCCTGCTCAACTTCCTGTCGAG)-3']

- *10uM (1:10 dilution- 10X concentration)= 2uL RT-1 primer (100uM) + 18uL A grade water to get a total of 20uL.*

Agarose Formula-

- [(% agarose)/(100)] x (vol)
- [(3.5)/(100)] x (50mL)= 1.75g + 50mL 1X TAE

TAE Buffer Formula-

- (c1)(v1)=(c2)(v2)
- (50x)(x)= (1x)(1000mL) = 20mL of 50X TAE + 980mL of DI Water

Detailed Pippin Protocol- http://www.sagescience.com/wp-content/uploads/2011/09/pippin-prep-operations-manual-rev-g-39-7_7_11.pdf