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High-Throughput Sequencing for Plant Virome Characterization: A Mini-Review

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ABSTRACT

Plant viruses and viroids cause extensive losses with reduction in crop productivity worldwide. The emergence of high-throughput sequencing technologies, commonly referred to as 'Next-generation sequencing' together with the metagenomics approach has led to a rapid increase in our understanding of plant viral communities. The utilization of high throughput NGS technologies has proven to be effective in the detection of previously unidentified disease-associated with new pathogens including viruses. Virome analysis using high-throughput sequencing technologies leads to the exploration of different viruses. These technologies, in combination with automation, artificial intelligence can allow for the efficient utilization of plant disease clinics in virome diagnostics. High-throughput sequencing methods have advantages of identification and genomic characterization of viruses and are important for diversity studies of plant viromes. Plant virome studies have the capability to carry out the detection of unknown viruses in mixed infection to reveal the presence of novel viruses. Further, the new machine learning/deep learning tools have enabled the detection of new viral sequences in already available host nucleotide sequences, enabling us to identify lysogenic viruses. In the era of metagenomics, plant-specific virome studies will help in checking the potential epiphytotic soon. Therefore, the present review highlights the successful utilization of high-throughput sequencing technologies in characterizing plant virome.

Key words: metagenomics, next generation sequencing, viral communities, deep learning

INTRODUCTION

Plant viral diseases are the frontline threat for sustainable agriculture, leading to huge economic losses every year. New infectious viruses are emerging frequently that leads to great demand for the development of new technologies for accurate identification of plant viruses. High-throughput sequencing is progressively gaining attention as a promising tool in the field of plant virology to sequence viral genomes and identify novel viruses. The term 'DNA sequencing' pertains to a methodology employed to ascertain the exact sequence of nucleotides i.e., Adenine, Thymine, Guanine, and Cytosine (A, T, G and C) within a DNA fragment. The 'Next generation sequencing (NGS)' method refers to advanced techniques for DNA sequencing that enable the direct identification and detection of pathogens, without requiring prior knowledge of the pathogens (Prabha et al., 2013). Next-generation sequencing (NGS)-based innovative methods have shown great potential to detect multiple viruses simultaneously; however, such techniques are in the preliminary stages in plant viral disease diagnostics. These NGS technologies possess numerous notable benefits, such as their capacity to generate substantial volumes of data, with the potential to reach up to one billion short reads. The field of plant virology has undergone notable changes in scientific approaches because of the introduction of innovative NGS technologies (Nabi et al. 2021; Studholme et al., 2011). Cloning of microbial DNA was first suggested by Lane et al. (1985), however, the term 'metagenome' was introduced by Handelsman et al. (1998) which refers to the 'genomes of the complete microbiota present in the natural environment'. With the advent of technological enhancements, the use of sequence- and function-based gene analysis tools have resulted in a complete compilation of genetic data for all microorganisms within a certain ecological context. The utilization of screening techniques, high-throughput sequencing, and meta transcriptomics has provided researchers with a remarkable level of understanding (Hess et al., 2011; Qin et al., 2010).

Development of various sequencing platforms has been observed for the past four decades. These sequencing platforms based upon their chemistry and evolution are categorized as First generation, Second generation, Third generation

and Next generation sequencing platforms. The attempts of sequencing the DNA and RNA were carried out in 1964 with the development of chain termination-based sequencing methods by Fredrick Sanger and chemical degradation techniques by Gilbert and Maxam (Sanger et al., 1977; Barba et al., 2014). These first-generation sequencing techniques were based on the termination of DNA strands using deoxynucleotides but only allowed the production of sequence reads up to few hundred in length only. The drawback of first-generation sequencing platforms led to the revolutionized DNA sequencing platforms known as second generation platforms that can carry out the sequencing of thousands to millions of DNA fragments.

The significant second-generation sequencing platforms include Roche's 454 sequencing method, pyrosequencing, Ion Torrent sequencing and SOLiD (Sequencing by Oligonucleotide Ligation and Detection). However, the third-generation sequencing platforms include PacBio Sequencing which is based on single molecule, real time approach known as SMRT and Oxford nanopore sequencing. Oxford Nanopore sequencing methods provide longread lengths thereby enabling them to carry out sequencing of larger DNA. Nanopore sequencing platforms provides portability with real-time analysis. The whole genome sequencing is another powerful sequencing platform that determines the complete DNA sequence of an individual unknown genome. Whole genome sequencing platforms enables the identification of genetic variations ranging from single nucleotide polymorphisms to larger structural changes including insertions and deletions.

The technique of pyrosequencing is based on the addition of nucleotides to a primed template which is regulated by the enzyme DNA polymerase. In pyrosequencing methods, different nucleotides are fluorescently labelled which are detected by the presence of different nucleotides (Ronaghi et al., 1998; Ahmadian et al., 2006). In pyrosequencing, the incorporation of complementary dNTPs by DNA polymerase, pyrophosphate is converted into ATP by using ATP sulphurylase using adenosine phosphosulfate. In the presence of ATP, luciferase converts luciferin into oxyluciferin that generates visible light. The light produced by luciferase reaction which is detected and measured by photodiode. The limitation of pyrosequencing is that it is inaccurate homopolymer sequencing with high cost (Ambardar et al., 2016).

Nanopore sequencing method involves the passing of the DNA through nanopore with an internal diameter of 1 nm because of which its electrical conductance is altered and the signal is detected and measured. Nanopore sequencing technology does not require any PCR amplification as well as chemical labeling. A portable nanopore sequencer known as MinION was developed in 2014 and made available commercially in 2015. MinION nanopore sequencer has been optimized using genomic DNA with 99% of reads mapped to reference genome. This high throughput sequencing method reduces the cost and efforts of traditional sequencing platforms tremendously with portability.

Illumina sequencing platforms involve DNA fragment libraries which are subjected to clonal amplification followed by termination using terminator nucleotide. Addition of reverse terminator nucleotide to the flow cell leads to incorporation of modified nucleotides into DNA strand which is being synthesized.

Helicos sequencing platforms include library preparation ligation and amplification for library preparation. It involves basic steps such as library preparation, transferase and dideoxy nucleotide. This sequencing methodology uses fluorescent tagged nucleotides for sequencing the DNA fragments which are attached to the flow cell through poly T tails. It is a commercial third generation sequencing platform that is based on the principle of use of single molecule fluorescent sequencing. This sequencing platform allows the quantification of RNA molecules involving RNA hybridization.

The third-generation sequencing platforms include the single molecule sequencing technologies which possess several advantages over second generation sequencing platforms. These technologies have several advantages over the second-generation sequencing platforms as they can generate longer reads from the individual samples thereby eliminate the need of assembling contigs from short sequence reads. These third-generation sequencing platforms have fast run time and require low input template with real-time analysis (Lavezzo et al., 2016; Petersen et al., 2019). Among different third generation sequencing platforms, Helicos, SMRT by Pac Bio and MinION nanopore sequencing by Oxford nanopore technologies. MinION as a sequencing platform allows for scalability as well as multiplexing in comparison to other high throughput sequencing platforms (Krehenwinkel et al., 2019; Mushtaq et al., 2020). It has been acknowledged that NGS technology have the potential to serve as effective substitutes for current diagnostic procedures utilized in quarantine facilities for the detection of plant viruses. This is because these

technologies can be used as direct replacements for conventional testing methods and have very low rates of false positive and false negative results. The enhanced sensitivity of NGS technology in identifying small virus amounts is well known. But because of its increased sensitivity, it's also more susceptible to contamination from mycoviruses, insect viruses, and other comparable sources, as well as possible cross-contamination from samples (Rott et al., 2017).

These days, the NGS technology is widely used in the field of Plant Pathology, particularly in the identification and examination of newly discovered diseases (Bag et al., 2015; Villamor et al., 2019; Malapi et al., 2016; Ren et al., 2020). The platforms have several features, such as accuracy, dependability, large-scale parallel processing capacity and output, all of which increase their usability. This groundbreaking project represents the first instance of a eukaryotic phytopathogen's genome sequencing (Studholme et al., 2011; Boonham et al., 2008). The investigation and identification of RNA and DNA viruses, as well as viroids, in plant specimens, is made easier using NGS technology with the support of appropriate computational tools (Wu et al., 2010; Ren et al., 2017; Sukhorukov et al., 2022). In modern agricultural research, RNA sequencing is a commonly used technique for the detection of newly discovered viral infections in a variety of crop species (Figures 1 and 2). According to Boonham et al. (2014), Jones et al. (2017) and Wang et al. (2009), these pathogens include leek yellow stripe virus (Potyvirus), potato spindle tuber viroid, mycoviruses, pepino mosaic virus, and grapevine leaf roll-associated virus (Donaire et al., 2023). Next-generation sequencing approaches offer an alternative diagnostic tool that may enhance the identification of plant viruses in quarantine facilities (Figure 3). This can be attributed by their ability to serve as direct substitutes for traditional evaluation techniques, demonstrating a notably low frequency of false positive and negative outcomes.

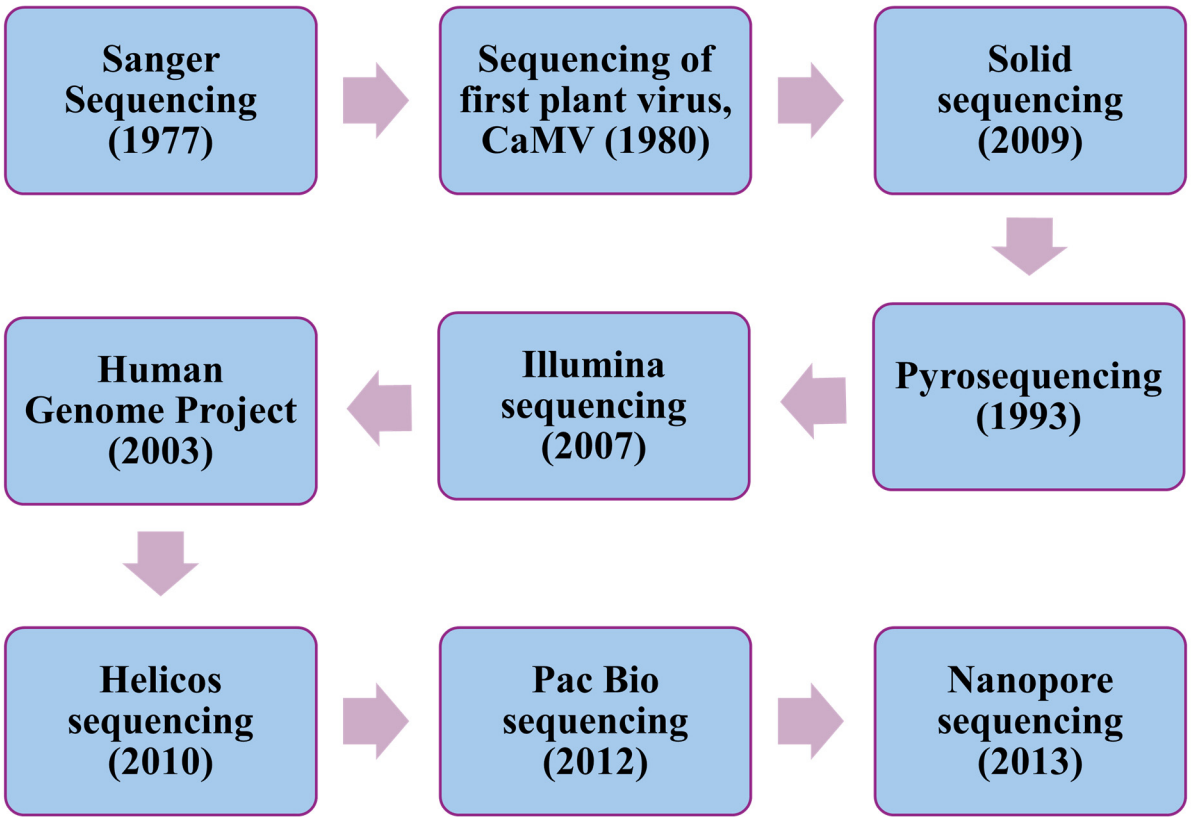

Fig. 1. Sequence of events in development of different NGS platforms.

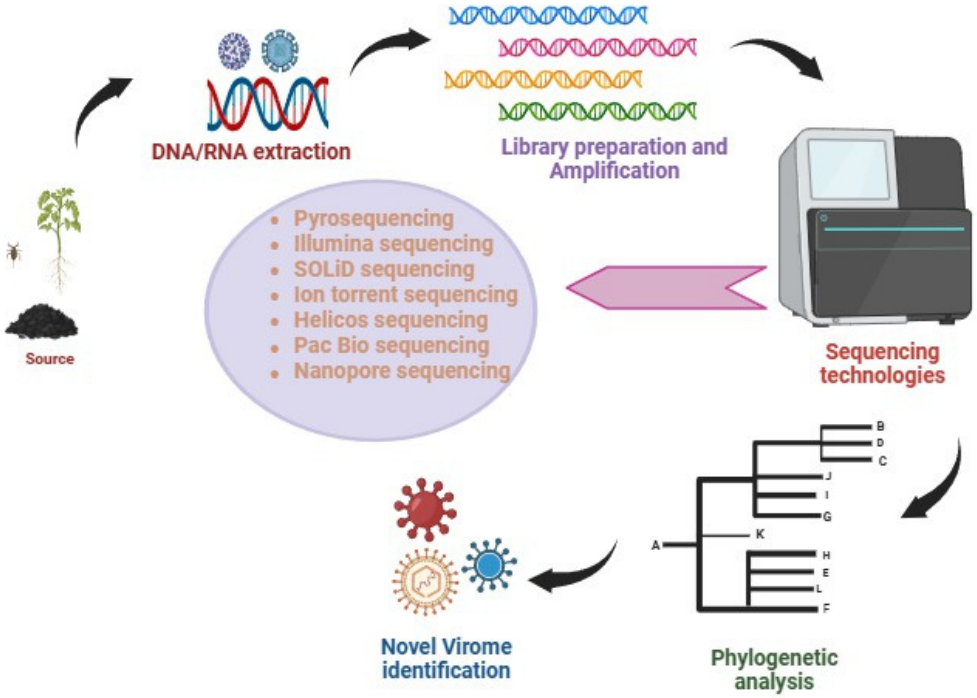

Fig. 2. Steps involved in the exploration of virus genomes through high-throughput sequencing.

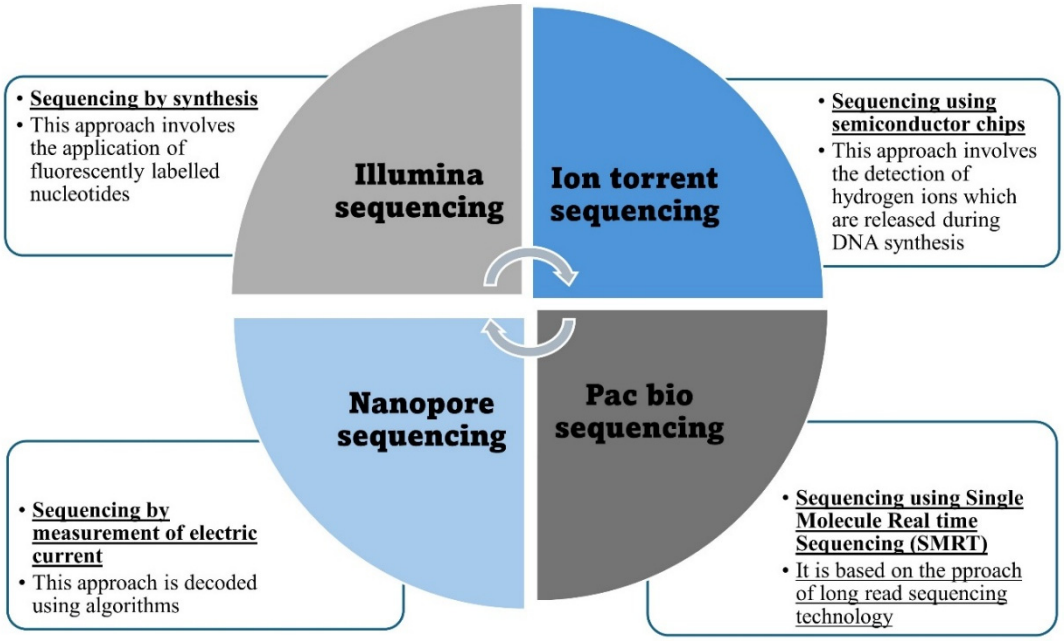

Fig. 3. Widely used high throughput sequencing platforms.

APPLICATIONS OF NGS IN STUDYING PLANT VIROMES

High-throughput sequencing does not require prior knowledge of the virus beforehand, making it easier for the plant biologist without the detailed knowledge of classical plant virology. Further, it reduced a lot of efforts made by plant virologists to characterize each virus individually, therefore it is 'a dream for every plant virologist' to study the complete host plant virome using in-silico analysis of NGS-generated sequences.

Further, in the case of viral co-infections (many viruses infecting the same host at a time), the virus with the highest titer value (more viral particles) is likely to be characterized, whereas the low titer viruses are excluded from characterization attempt. Particularly, the occurrence of different viruses sharing common regions of high homology can also affect the

proper reconstruction of viral genomes leading to erroneous genome assembly or an erroneous conclusion (Maclot et al., 2020). The NGS-based methods of plant virome characterization have led to the identification of new viruses, new hosts of the already known viruses, and the intra-specific diversity of viruses; besides elucidating the viral dynamics in case of mixed infections (Table 1).

Further, it may lead to the study of viral population genetics in a broader sense. The grapevine virologists at initial stages, could anticipate the potential of this technology and tried to analyze the virome of diseases similar to grapevine viruses, but with unclear etiologies. After analyzing sRNA libraries of grapevine exhibiting vein clearing and vine decline signs, Zhang et al. (2011) discovered conflicting associations between grapevine fanleaf virus (GFLV), tomato ringspot virus (ToRSV), and grapevine rupestris stem pitting associated virus (GRSPaV). Unexpectedly, a novel badnavirus with a double-stranded DNA genome was found as grapevine vein clearing virus (GVCV). This discovery validated multiple infections of vitiviruses, marafi viruses, macula viruses, and nepo viruses in these vines and also showed the potential of using sRNA libraries in detecting DNA viruses because of the RNA silencing activity of degrading overlapping viral messenger RNAs (Seguin et al., 2014; He et al., 2017). The development of a metagenomic approach for the detection of virus was facilitated by advancements in next generation sequencing technologies. RNA isolated from garlic leaves was subjected to Illumina sequencing, to check for the presence of various viral isolates, including potyviruses, allexiviruses, and carlaviruses (Table 2).

Using HiSeq 1000 sequencing technology, a novel pepper virus known as pepper virus A was found in a subsequent investigation (Jo et al., 2015; Jo et al., 2017). Specifically, grapevine pinot gris virus and grapevine yellow speckle virus 1 were the novel viroids. Using Illumina HiSeq 2000 technology, the strains of a novel potyvirus called PepMV were identified, and its entire genome was assembled (Matsumura et al., 2017).

Table 1. Identification of plant viruses using high throughput genome sequencing platforms in various crops.

	Sequencing technology	Virus(s)/viroid(s)	Reference(s)
1.	RNA Sequencing	Grapevine latent viroid	Zhang et al., 2014
2.	Illumina sequencing	Citrus exocortis viroid	Poojari et al., 2013
3.	Illumina sequencing	Rice stripe virus	Yan et al., 2010
4.	Illumina deep sequencing	Sweet potato feathery mottle virus	Kreuze et al., 2009
5.	Illumina Hi-Seq sequencing	Bean yellow mosaic virus	Kehoe et al., 2014
6.	Illumina Hi-Seq sequencing	Citrus vein enation virus	Vives et al., 2013
7.	Pyrosequencing	Tomato necrotic stunt virus	Li et al., 2012
8.	Pyrosequencing	Vanilla virus X	Grisoni et al., 2017
9.	RNA sequencing	Grapevine vein clearing virus	Zhang et al., 2011
10.	RNA sequencing	Lettuce necrotic leaf curl	Verbeek et al., 2014
11.	SOLiD sequencing	Pepper yellow leaf curl virus	Dombrovsky et al., 2013
12.	SOLiD sequencing	Eggplant mild leaf mottle virus	Dombrovsky et al., 2012
13.	MinION Nanopore sequencing	Wheat streak mosaic virus	Fellers et al., 2019
14.	MinION Nanopore sequencing	Zucchini yellow mosaic virus	Chalupowicz et al., 2019
15.	MinION Nanopore sequencing	Potato virus Y	Della et al., 2020
16.	MinION Nanopore sequencing	Plum pox virus	Bronzato et al., 2018
17.	Ion Torrent sequencing	Little cherry virus 1	Katsiani et al., 2018

Table 2. Characterization of Virome in different host plants.

	Crop(s)	Tissue involved	Sequencing method	Virome characteristics	Reference(s)
1.	Chilli (*Capsicum annuum* L.)	Leaf	Illumina NOVASEQ 6000 platform	Chilli leaf curl virus (ChiLCV), Cucumber mosaic virus (CMV), Pepper cryptic virus-2 (PCV-2)	Reddy et al., 2023
2.	Wheat (*Triticum aestivum*)	Leaf	Oxford Nanopore Sequencing	Wheat leaf yellowing- associated virus (WLYaV)	Lee et al., 2023
3.	Alfalfa (*Medicago sativa* L.)	Leaf	Illumina sequencing	Alfalfa Mosaic Virus (AMV)	Samarfard et al., 2020
4.	Sweet potato (*Ipomoea batatas* L.)	Vine cuttings	Illumina sequencing	Sweet potato chlorotic stunt virus (SPCSV)	Nakasu et al., 2022
5.	Tomato (*Solanum lycopersicum* L.)	Fruit	HiSeq sequencing	Tomato latent virus (TLV), Tomato vein clearing deformation virus (ToVCD), Tomato brown rugose fruit virus (ToBRFV)	Rivarez et al., 2021
6.	Pepper (*Capsicum annuum* L.)	Fruit	RNA sequencing	Bean broad wilt virus 2 (BBWV2), Cucumber mosaic virus (CMV)	Jo et al., 2022
7.	Potato (*Solanum tuberosum*)	Leaf samples	RNA sequencing	Potato leaf roll virus (PLRV)	Elwan et al., 2023

Novel Virus Identification

Different NGS platforms were utilized for the identification of novel viruses in stones fruits like nectarines responsible for stem pitting disease. Peach leaf pitting associated virus (PLPaV) causing severe leaf symptoms, Peach associated luteovirus and peach virus D (PeVD) are the novel viruses of peach identified using NGS (Villamor et al., 2016; He et al., 2017; Wu et al., 2017; Igori et al., 2017).

Among the stone fruits, peach and nectarines are majorly infected by several viruses and viroids like plum pox virus (PPV), Prunus necrotic ringspot virus (PNRSV) and Nectarine stem pitting-associated virus (NSPaV). The identification and characterization of these novel viruses was carried out using the high-throughput sequencing technologies. (Maliogka et al., 2018). The presence of novel viruses like peach associated luteovirus and nectarine stem pitting associated virus was detected in peach cultivars in Hungary (Barath et al., 2022; Krizbai et al., 2017).

Novel viruses and viroids associated with Apple mosaic disease in symptomatic apple plants were characterized using RNA sequencing by Illumina Hiseq 2500 in two cultivars of apple i.e., Oregon Spur, Golden Delicious and Red Fuji (Nabi et al., 2022). These novel viruses detected using sequencing were Apple necrotic mosaic virus (ApNMV), apple mosaic virus (ApMV), apple stem grooving virus (ASGV) and apple stem pitting virus (ASPV), apple chlorotic leaf spot virus (ACLSV) and viroid, Apple hammerhead viroid (AHVd). This reports the first viral genomic analysis of viruses associated with apple mosaic disease from India. Using Illumina platform of high throughput sequencing, apple crinkle disease associated with different viruses like Apricot latent virus, peach chlorotic mottle virus in apples and peaches from China, Korea and Japan (Noda et al., 2017; Cho et al., 2017).

Multiplexing PCR with small amplicons of size 120–135 bp, targeting about 27 viruses and 7 viroids followed by single high-throughput sequencing run led to the detection of different viruses and viroids from about 123 pome and stone fruit samples (Costa et al., 2022). However, the metagenomic sequencing is a powerful tool for plant virus detection, the major drawback lies with the fact that the detection of plant viruses from metagenomic sequencing datasets involves the overabundance of unnecessary plant host reads in low titre of plant viruses. The cost of metagenomics based high throughput sequencing has become too high which imposes a drawback for the utilization of these advanced technologies at root level (Maina et al., 2021; Gaafar et al., 2021).

Comparison of different multiplex PCR based amplicon sequencing with RNA sequencing methods have showed substantial results for detection of viruses and viroids in different fruit tree samples as the RNA sequencing method has been increasingly used in Plant Virology for critical detection of new plant viruses (Liu et al., 2018; Costa et al., 2022; Villamor et al., 2016).

Virome in wheat cultivars from three different geographical regions were analyzed using the Oxford Nanopore sequencing technology. As per the exploration of Virome, about five viral strains i.e., Barley virus G, Hordeum vulgare endornavirus (HvEV), Sugarcane yellow leaf virus (SCYLV), Wheat leaf yellowing associated virus (WLYaV) were identified in Korea (Lee et al., 2023). They concluded that the application and utilization of Oxford nanopore technology served as a reliable platform for detecting and identifying the wheat viruses. Nanopore technology enables the sequencing of long length reads in real time with an accuracy of over 99.9 per cent. The technology has been utilized in the past for detection of viruses in wheat like Wheat virus Q, Wheat yellow stunt associated betaflexivirus, Wheat Umbra like virus (Lu et al., 2016; Van Dijk et al., 2018; Valenzuela et al., 2022). Oxford nanopore technologies provides portability, cost effectiveness and continued accuracy for virus diagnostics (Liefting et al., 2021). These sequencing platforms improved the accuracy and efficiency of plant virus diagnostics with emphasis on detecting mixed infections. Elaborative overview on the developments and challenges in plant viral diagnostics have been discussed by Mehetre et al. (2021) pertaining to the utilization of oxford nano technology as most readily applicable plant viral diagnostic.

Diversity and Dynamics of Plant Viromes

Viruses can cause epidemics on all significant crops of agronomic importance across the world. They can infect all the cultivated and wild plants species, threatening global food security. However, this 'invisible foe' has a variable host range. For instance, the Indian citrus ringspot virus infects few species in the genus citrus, whereas the cucumber mosaic virus infects over >1200 species in 100 plant families. For best or for worst, plant viruses have a remarkable capacity to reprogram plant growth and development. With the advent of next-generation sequencing technologies and bioinformatics analysis, the discovery, abundance, and species richness of plant viruses, more aptly 'the plant virome' has become easier to decipher.

Identification of Viral Sequences in Metagenomics Data

Recently, classical machine learning-based, k-mer frequency matrix-based and deep learning-based approaches have been used to identify the viral sequences from plant nucleotide sequences for the contig-based study of virome. The identification of novel plant viruses like Tomato severe rugose virus (ToSRV) in leaf samples of Physalis angulata using the Oxford Nanopore Technology. The wheat plant containing Wheat Streak Mosaic Virus, Barley Yellow Mosaic Virus were identified in four different varieties of wheat. The results of the study revealed that nanopore technology can more accurately carry out the identification of pathogens in the infected samples in real time with less run time. Similar results were recorded by Filloux et al. (2018), in yam plants with detection of viruses like Dioscorea bacilliform virus (DBV), Yam Mild Mosaic Virus (YMMV) and Yam Chlorotic Necrosis Virus (YCNV) in yam plants. In pepino (*Solanum muricatum*), Pac Bio sequencing was used to assemble their circular mitogenome and it represents the first report of research on evolutionary biology, the results will assist in the development of molecular breeding strategies for the beneficial agronomic traits of species. The complete mitochondrial genome of pepino was assembled using the PacBio sequencing to develop phylogenetic relationships (Li et al., 2024).

Conclusions and Future Prospects

Plant viruses are a major limiting factor for crop production. In the past few decades, the mechanisms of viral pathogenicity have been revealed to an appreciable extent, however, the genomic information regarding viruses was limited to the extent of a few viruses for which the whole genome sequences were available in the public domain. The advancement in metabolomics tools and integration of deep learning with classical bioinformatics has led to the characterization of viral metagenomics generally referred to as the 'virome' approach. The Next-Generation Sequencing (NGS) technology was initially introduced in 2000, and the first sequencing platform to be sold commercially was released in 2004. Development of different innovative techniques should be competitive with respect to accuracy with cost-effectiveness. Different portable devices were developed among which Oxford nanopore technology is more promising due to its ability to remain functional with portability. Although, great advancement has been made in these techniques but still, need for the different computer intensive technologies is there for mapping and identifying novel viruses (Guo et al., 2021). Among, all the advanced technologies nanopore sequencing is a promising tool with portability however, one key limitation of the technology lies to its higher error rate with low signal-to-noise ratio.
Consequently, NGS has become widely available in labs, opening new avenues for genomes and diagnostics research. Although genome sequences are now more widely available, however, our understanding of genomics is still hampered by the limited data processing and biological interpretation tools. High-throughput sequencing techniques have shown to be very useful tools for finding new viruses and detecting previously unknown disease-associated viruses. Further mining of the sequence data using such tools has revealed the viral nucleotide sequences in nucleotide datasets, already available in the public domain. Plant virology overall has made a fairly good use of NGS technology, particularly in the areas of transcriptomics, genome sequencing, detection and identification, and discovery of plant viruses. Deep sequencing technology has great potential to improve plant protection tactics by the identification of new viral sequences, their host range and potential threats, which may turn into epidemics if remain unattended. Therefore, such potential epidemics may be avoided by adopting appropriate plant protection interventions in advance.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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