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Shalaka Ahale
Department of Plant Pathology Dr. Y S Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh India

Gaurav Rakhonde
Department of Plant Pathology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Sheenam Bhateja
Department of Entomology, Maharana Pratap University of Agriculture and Technology Udaipur, Rajasthan, India

Corresponding Author:
Shalaka Ahale
Department of Plant Pathology Dr. Y S Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh India

A quick review on diagnosis of plant diseases using nanopore sequencing platform

Shalaka Ahale, Gaurav Rakhonde and Sheenam Bhateja

Abstract
Disease diagnosis is the first step in the management of plant diseases. Sustainable management of plant diseases includes efficient diagnostics for surveillance, detection and identification including the development and strategic deployment of resistant varieties. Nanopore sequencing is newly introduced platform which mainly work on sequencing of nucleic acid. An Oxford Nanopore technology (ONT) is a third generation platform used for sequencing of native nucleic acid strands, which is especially well suited to the production of high-quality assemblies of highly repetitive plant genomes. A motor protein was employed to shuttle single-stranded DNA or RNA through a nanopore in Nanopore sequencing device to generate nucleotide dependent electronic current signals which enables the analysis of pathogenic nucleotides. Despite of having several advantages like long read capacity, cost effectiveness and real time analysis, the current scenario of various companies is shifting towards the betterment of the accuracy results by working on the commercially available nanopore.

Keywords: Nanopore, detection, nucleic acid, third generation sequencing

1. Introduction
The rapid development of high throughput and inexpensive DNA sequencing technologies has undoubtedly been the key driving force in the progress of life sciences during the previous decade. Novel technologies that visualise the unseen or detect the undetectable have always contributed to breakthroughs in scientific achievements. The key topic of focus is implementation of these breakthrough technologies in agriculture. Farmers have issues raising and sustaining their crops on a daily basis. Different diseases cause economic losses, which eventually result in food insecurity and a drop in the country's GDP.
Plant disease diagnosis is crucial step in the management of Plant disease diagnostic techniques have limitations in detecting several pathogens simultaneously. They can be detected by direct and indirect methods which includes protein based detection such as ELISA, cell culture and molecular techniques PCR, LAMP (Direct methods) and volatile profiling, Hyperspectral imaging, NIP spectroscopy, thermography imaging (indirect methods) these techniques are based on changes in the several indices such as growth rate, morphology, VOC's (volatile organic compounds) etc. Among different new techniques nanopore sequencing platform, a next generation sequencing platform can be used for reliable detection and identification of plant pathogens.

2. What is nanopore sequencing?
It is a scalable, one-of-a-kind technique that allows for direct real-time examination of lengthy DNA or RNA segments. It operates by detecting variations in an electric current when nucleic acid travels through a protein nanopore and decoding the signal to generate a specific DNA or RNA sequence (Petersen *et al*., 2019) [15].

2.1 What are nanopores and its types?
Nanopore is a small pore of nanometer in size created by pore forming protein or as a hole in the synthetic material such as silicon or graphene. Nanopore are classified into 3 major types namely biological, solid state and hybrid nanopore which are combination of biological and solid state nanopores.

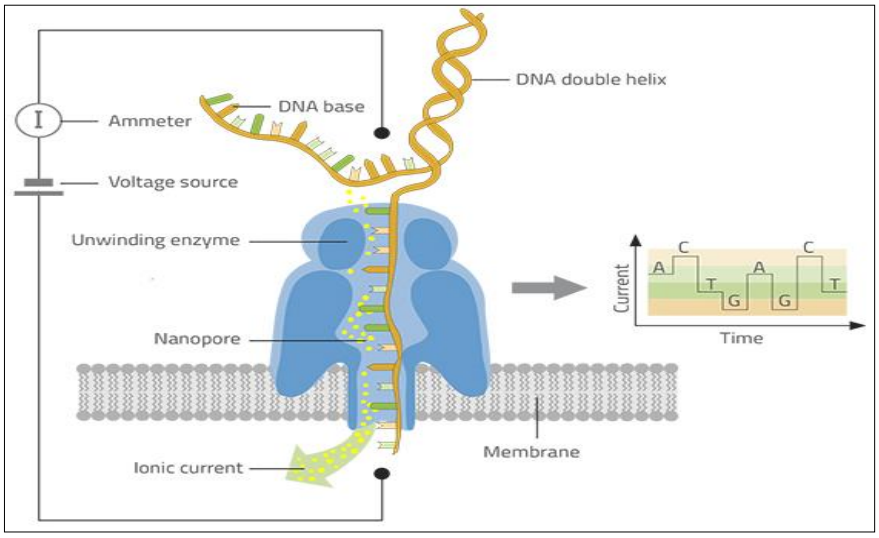

Fig 1: Working flow of a Nanopore sequencing platform (Byrk J., 2017) [4]

2.1.1 Biological Nanopores

Planar lipid membranes, liposomes or polymer membranes enclosed in an electrochemical chamber are the preferred substrates for all biological pores. The characteristics of three well-studied biological pores are briefly mentioned below:

α-Hemolysin channel- The human pathogen *Staphylococcus aureus* secretes an exotoxin called α-Hemolysin. It's a 232.4 kDa heptameric transmembrane pore with a vestibule (3.6 nm diameter; 5 nm length) coupled to a transmembrane-barrel (2.6 nm diameter; 5 nm length) (Song *et al*., 1996) [19]. The pore is narrowest at the vestibule - transmembrane domain junction with a diameter of ~1.4 nm (Fig 2A). α -hemolysin has showed tremendous promise in stochastic sensing of a variety of analytes, including metal ions (Braha *et al*., 2000; Wen *et al*., 2011) [3, 18], tiny organic molecules, DNA, RNA, proteins, and so on, according to its intrinsic nanopore structure. The utilisation of theα -hemolysin channel is limited to the translocation of DNA (1-nm in diameter) due to the pore size limitation. It's thermally stable, working at temperatures as high as 100°C (Kang X *et al*., 2005) [13].

MspA channel- MspA (*Mycobacterium smegmatis* porin A) is an octameric channel pore with a funnel shape that permits water soluble compounds to pass through bacterial cell membranes. It has a single constriction that is 1.2 nm broad and 0.6 nm long (Fig. 2B), which is smaller and narrow compared to that of α -HL, as reported by the Neiderweis lab's crystal structure (Faller *et al*., 2004) [7]. MspA's typical nucleotide translocation rate is less than one microsecond, which is even faster than that of α-hemolysin. As a result, direct strand sequencing of DNA was ruled out. The immobilized ssDNA was retained inside the MspA nanopore, either by a DNA duplex or a biotin-streptavidin complex, to evaluate the nucleotide discriminating ability, similar to the study in α-hemolysin. It has the capacity to read four nucleotides simultaneously.

2.1.1.1 Phi29 connector channel: The bacterial virus phi29 DNA-packaging nanomotor has an exquisite and complex channel made up of twelve copies of the protein gp10 that surround each other to form a dodecamer channel that allows double-stranded DNA to be translocated. The connector is 7 nm long, with a cross-sectional area of 10 nm2 (3.6 nm) at the narrow end and 28 nm2 (6 nm) at the wide end. When ions or DNA flow through the channel, the connector is inserted into a lipid bilayer and the resulting system is demonstrated to have exceptionally accurate, robust, and sensitive conductance signatures, as indicated by single channel conductance measurements. The phi29 connector has a broader channel than other well-studied systems, allowing for the passage of ssDNA, dsDNA, peptides, and possibly small proteins. This is the most significant advantage of the phi29 system over other well-studied systems. The greater pore size also makes channel alterations easier, such as creating a sharper detecting zone for achieving single nucleotide resolution or insertion or conjugation of chemical groups for sensing and diagnostic purposes (Haque *et al*., 2013) [11].

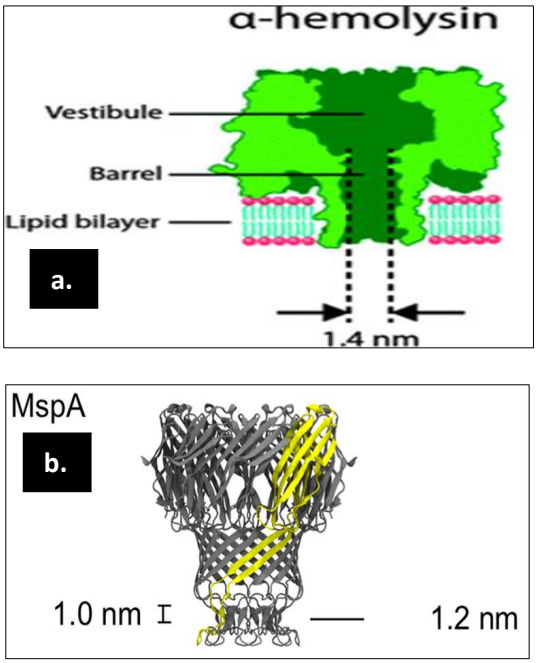

Fig 2: a) α-Hemolysin b) MspA biological Nanopores

2.1.2 Solid state nanopores

A solid-state nanopore is typically a nanometer- sized hole formed in a synthetic membrane (Usually SiNx or SiO2). The pore is usually fabricated by focused ion or electron beams, so the size of the pore can tuned freel, although further development is necessary to reach the atomic precision naturally achieved by protein pores.

2.1.3 Hybrid nanopores

Protein nanopores have chemical selectivity; however solid state nanopores do not. Integrating a protein pore into a solid-state membrane is one way being investigated. By adding α-hemolysin channel in SiN (Silicon nitrite) pores, the idea of hybrid pores was demonstrated (Hall *et al*., 2010) [10]. α-Hemolysin has an atomically precise structure and the ability to be genetically modified or chemically modified at specific sites. However, because α-hemolysin is mechanically supported by a fragile lipid bilayer membrane, it is difficult to integrate into wafer-scale systems. Even so, this limitation can be overcome by encasing a biological pore inside a mechanically robust solid-state nanopore, resulting in a hybrid pore that combines the best of both worlds. The hybrid system was shown to be stable for several days under observation (Hall *et al*., 2010) [10]. However, once in contact with the solid-state pore, the biological pore deforms and loses its ability to distinguish single nucleotides, resulting in massive leakage currents since ions can still flow through the areas between the solid-state and biological pore due to an incomplete seal. This technique or method is influenced by these constraints.

The first NGS technology released in 2005 was pyrosequencing method by 454 Life Sciences (Roche). The 454 Genome Sequencer yielded around 200000 110 base-pair reads (20 Mb) (bp). The Solexa/Illumina sequencing platform was marketed one year later (Illumina acquired Solexa in 2007). In 2007, Applied Biosystems (Life Technologies) introduced Sequencing by Oligo Ligation Detection (SOLiD), the third technology. The Illumina and SOLiD sequencers produced many more reads than the 454 sequencer (30 and 100 million, respectively), although the reads were only 35 bp long (Besser *et al*., 2018) [1]. Three important improvements are shared by these novel sequencing approaches. To begin, they rely on the generation of NGS libraries in a cell-free system rather than bacterial cloning of DNA fragments. Second, instead of hundreds of thousands to millions of sequencing reactions, thousands to millions are created in simultaneously. Third, without the requirement for electrophoresis, the sequencing output is directly detected; base interrogation is done cyclically and in parallel (Rhee and Burns 2006) [16]. Because of the massive amount of reads generated by NGS, whole genomes might be sequenced at breakneck speed (Dumschott *et al*., 2020) [6]. However, one disadvantage of NGS technology was their short read length. As a result, genome assembly became more challenging, necessitating the development of new alignment methods.

The advancement of single-molecule sequencing technologies (commonly referred to as "third generation sequencing") provides several advantages over second-generation sequencing by

1. Producing long read lengths, which are easier to map to a reference genome and to facilitate de novo assembly.
2. Fast run times (within a few hours).
3. Requiring only a small amount of DNA with low cost for a single run (Schadt *et al*., 2010) [17].

3. Oxford Nanopore Technologies (ONT)

The detection and identification of plant pathogens is possible by the use of sequencing-based methods. The utilisation of Oxford Nanopore's single-molecule sequencing platform as a general tool for diagnosing plant diseases investigated the earliest possibilities of disease diagnosis. Plant pathogen testing begins with the isolation of DNA or RNA (e.g., viruses, bacteria, fungi, phytoplasma) from affected plants, followed by the sequencing of the target pathogens (Gautam and Kumar, 2020) [9]. While Illumina, Thermo Fisher, and Pacific Biosciences' NGS platforms have transformed biomedical research, a unique method to NGS based on nanopore technology has the potential to be used in clinical laboratories in the near future. In 2014, Oxford Nanopore Technologies (ONT) released the MinION to the research market for the first time. The MinION is different from other platforms because it utilizes nanopores for sequencing. It does not take the sequencing by synthesis approach; instead, an ionic current is passed across the flow cell during sequencing and the different nucleotide bases are distinguished by the changes in current as they pass through the nanopores (Laver *et al*., 2015) [14]. When compared to Illumina, Thermo Fisher, and Pacific Biosciences platforms, sequencing with the MinION is less expensive and can be done both in the lab and in the field. The MinION, which fits in the palm of your hand and can be connected to either ONT's "MinIT" computer module or any computer with a USB port, enables for real-time electronic analysis of single molecules. It can be used for the analysis of DNA and RNA for a range of applications including personalized medicine, agriculture and scientific research (Bolger *et al*., 2014) [2]. Other devices available in the market are GridION, PromethION posses more no of flow cells and process several samples at a time. One MinION device is available in the market at a low cost and is much cheaper in cost as compared to other sequencing devices. DNA or RNA from tissue with symptoms from various plants infected with known pathogens (bacteria, DNA and RNA viruses, fungi, phytoplasmas) and unknown samples are sequenced and analysed in real-time with the tools provided by the manufacturer.

Followings steps are followed while sequencing of nucleic acid

- Genomic DNA or RNA extraction
- DNA or RNA library preparation
- MinION sequencing
- Data analysis
- Validation method

4. Applications

The MinION portable pocket DNA sequencer was utilised for the first time in the world to sequence whole plant viral genomes. The begomoviruses that cause the catastrophic CMD (cassava mosaic disease) that is ravaging smallholder farmers' crops in Sub-Saharan Africa are being identified using this technology. Secondly oxford nanopore sequencing (ONT) is used to confirm the presence of *Wheat streak mosaic virus* (WSMV). Symptoms of this virus were discovered in wheat plants carrying the Wsm2 resistance gene (Fellers *et al*., 2019) [8]. Along with WSMV they reported *Triticum mosaic virus* (TriMV), *Barley yellow dwarf virus* (BYDV) which were not detected by the cDNA cloning and Sanger sequencing. Chalupowicz *et al*. 2018 [5] identified several fungal and bacterial pathogens in the seed samples of several host plants such as *Colletotrichum* sp. *Penicillium digitatum*, *Psudomonas syringae* and *Clavibactor michiganensis* subsp. *michiganensis* etc. As a result, this method has broadened the field of rapid detection of fungal, bacterial and viral infections in hosts which will help in management of plant diseases. Additionally, the technique can be expanded to Metagenomics, human genomics such as detection of cancer and plant sciences.

5. Challenges

The rapid translocation of DNA, which is far above the electronic detection limitations of present technologies, is a bottleneck in all nanopore sequencing approaches. Yet, there is still a need to improve the speed of nucleic acid translocation through nanopores. Along with this work in spatial, temporal resolution and accuracy rate much needed.

6. Conclusion

Because many plant genomes are vast and complex, with many repeating sections, generating high-quality assemblies using first-generation or even second-generation sequencing technologies is difficult (Bolger *et al.*, 2014; Jiao and Schneeberger, 2017) [2, 12]. Long-read sequence data from low-cost ONT platforms is increasing in number and quality, which bodes well for future plant genome sequencing studies, which will result in substantial advancements in plant genome and pangenome assembly. The rapid advancements of third generation technology will overcome current obstacles such as read error rates and the merits of ONT already outweigh the demerits. Future breakthroughs in modified basecalling will give a firm groundwork for epigenomic and transcriptome research, while ONT will provide unique insight into the intricacies of plant genomes.

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