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Future of portable devices for plant pathogen diagnosis

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Abstract

The demand for rapid and accurate diagnosis of plant diseases has risen in the last decade. On-site diagnosis of single or multiple pathogens using portable devices is the first step in this endeavour. Despite extensive attempts to develop portable devices for pathogen detection, current technologies are still restricted to detecting known pathogens with limited detection accuracy. Developing new detection techniques for rapid and accurate detection of multiple plant pathogens and their associated variants is essential. Recent single DNA sequencing technologies are a promising new avenue for developing future portable devices for plant pathogen detection. In this review, we detail the current progress in the portable devices and technologies used for detecting plant pathogens, the current position of emerging sequencing technologies for analysis of plant genomics, and the future of portable devices for rapid pathogen diagnosis.

1 Introduction

Pathogen detection is important in different areas such as clinical research, drug discovery, biological warfare, food safety, and plant health.[1] Among them, plant pathogen detection is important for detecting bacteria, viruses, and fungi in rapid response settings such as greenhouses, country borders, natural landscapes, and mass production facilities. It is essential to detect diseases in trees and shrubs within forests and natural environments, especially for plants that play a major role in the natural landscape and provide habitats for wildlife (Fig. 1a).[2-4] Although the need for plant disease detection in trees is important, it is not as critical as in crops.[5] It was reported that about 20-30% of the field crops are annually lost due to infection to disease.[6-8] In the case of soybean rust, a disease caused by a fungus, it is estimated that removing only 20% of the infection would result in several million-dollars benefit to farmers.[9] Recently, the Journal of Molecular Plant Pathology announced the top ten bacterial plant pathogens based on both their economic and scientific importance.[10] *Pseudomonas syringae* and *Ralstonia solanacearum* ranked first and second respectively, and are economically important diseases that infect crops ranging from potatoes to bananas (Fig. 1b,c).[10] However, the effective techniques for diagnosis of these top pathogens are rare. There is a strong need to develop new detection techniques for pathogen diagnosis. This is essential to enhance treatment and crop resistance against diseases.[11]

Previously, knowing the type of plant provided a list of potential bacterial, fungal, and viral diseases associated with that plant (for example, through the Phytopathological Society resources).[5] Moreover, most of those plant pathogens needed a few days or months to show their symptoms on plant appearance or crop quality. Thus, regular laboratory-based detection techniques were mostly sufficient for pathogen diagnosis and later treatment. Nowadays, rapid pathogen detection is more crucial for several reasons, including recent developments in international trade of

products, increased human mobility and globalization, climate change, emerging pathogens with the resistance to the pesticides, and new regulations limiting the application of toxic chemical materials to prevent the spread of new plant diseases.[12-14] Also, the expenses required for calendar-based spray schedules are no longer tolerable for farmers.[15] Additionally, at international borders, the delays in sending sample products to expert laboratories for pathogen diagnosis – especially quarantine pathogens – is unacceptable as it may result in long-term economic loss. Both importers and exporters want to diagnose pathogens on the field not at the border. Furthermore, pathogens that present a risk of biological attacks to crops and trees must be detected urgency by on-site diagnostic methods.[4, 12, 14] The usage of On-site diagnostic tools will facilitate the design of proper pest distribution strategies to prevent the spread of diseases[7], will improve the design of cultivar choice, and will assist the study of disease epidemiology and the design of new strategies for disease control.[16] This all implies the importance of early detection of pathogens at the point of inspection using on-site detection tools before it causes restrictive import requirements on products.[17]

The general intention is to accurately and rapidly detect specific plant pathogens. However, increasingly the agricultural aim today is to detect multiple potential pathogens or newly emerged pathogens without necessarily knowing the plant species or plant diseases associated with that particular group of crops.[18] For example, *Monilinia fruticola*, *Xanthomonas arboricola pv. pruni*, and *Erwinia amylovora* infections in tree crops are serious threats that cause brown rot, bacterial spot, and fireblight, respectively.[15] However, the main challenge for multiple pathogen detection is that a long list of bacteria, fungi, and viruses may influence the quality of each crop, even though the current technology is limited to detecting only one or a few identified pathogens (Fig. 1d,e).

New technologies should be developed with the aim to rapidly and accurately diagnose both identified and unidentified pathogens, as well as new variants of well-known pathogens.[4, 5] A prerequisite for future pathogen sensors is to have the ability to diagnose greater numbers of pathogens particularly for plant diseases with several known pathogen sources.[1, 19] New plant pathogen sensors should satisfy a number of criteria such as: 1) be competitive with laboratory-scale technologies in accuracy and the cost, 2) involve entire on-chip processing steps from sample preparation, enrichment, amplification, and detection, and 3) have a minimum number of manipulation steps.[20] The sensor needs to be functional under a wide range of operating conditions and would preferably be portable and simple enough to be used directly by growers and producers.[21] The sensitivity required depends mainly on its purpose to either identify the presence of pathogen or to detect the degree of infection.

Many people in different levels would benefit from portable devices with the potential for rapid plant pathogen detection including exporters, individual growers and orchardists, producers, researchers, and regulatory agencies.[16, 22] However, several questions need to be addressed including: 1) whether the current technologies and methods are qualified to satisfy the above requirements, 2) which of the current technologies is predicted to be the leader for future pathogen diagnosis, and 3) what would be the contribution of recent molecular techniques in developing future portable devices? These are challenging questions for the coming years and will define the direction of budgetary spending aimed at developing future diagnostic technologies.[23]

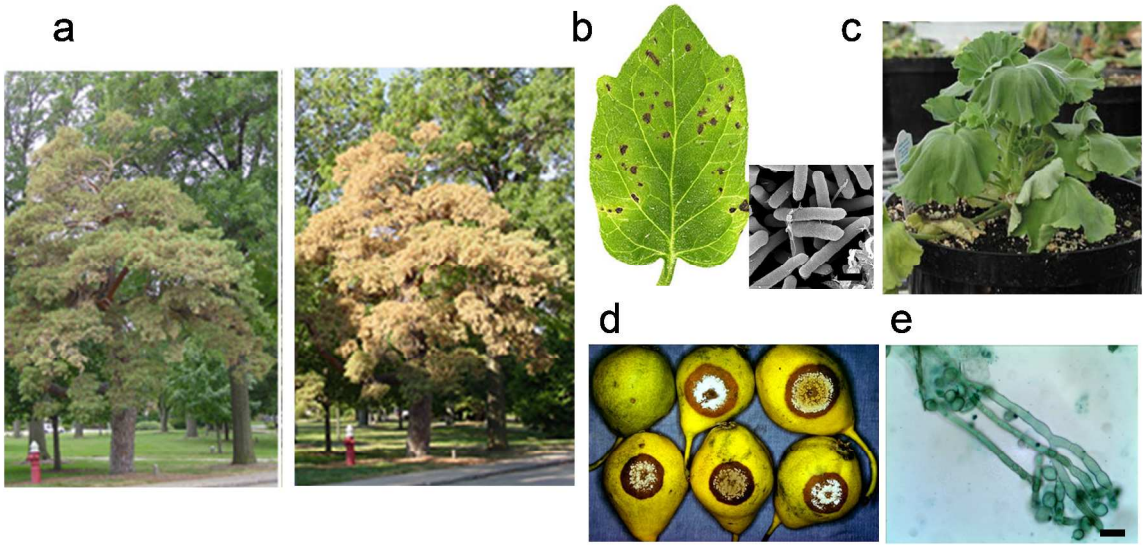

Fig. 1 Examples of plant diseases and pathogens, a) Pine wilt disease progression caused by *Bursaphelenchus xylophilus* within only two weeks, b) Tomato plant leaf infected with *Pseudomonas syringae*, ranked first among plant pathogens based on Molecular Plant Pathology announcement.[10] SEM micrograph of *Pseudomonas syringae* , Source: Gordon Vrdoljak, U.C. Berkeley, Microbewiki, c) *Ralstonia solanacearum* on Geraniums ranked second among bacterial plant pathogens, d) Multiple pathogens on pear fruit due to *M. fructicola*, *M. laxa* & *M. fructigena*. Top left-control; top centre-*M. laxa*; top right-*M. fructigena*; bottom left-M. fructigena; bottom centre-M. fructicola; bottom right-M.*laxa*. H.J. Willetts University of New South Wales (AU), e) SEM micrograph of *Monilinia fructicola*, Source: Microscopetalk, Scale bar: 10 µm.

Here, we review techniques used to identify plant pathogens as well as their challenges and limitations. We further explain the current status of portable devices and commercialized kits used for plant disease diagnosis. Lastly, we anticipate future technology for plant pathogen detection which may satisfy the aforementioned criteria. Present techniques for rapid diagnosis of new or formerly detected diseases show that progress in this area, particularly for unidentified samples, is slow compared to what is needed. However, plant pathogen diagnostics are expected to improve by involving emerging techniques in the coming years. Future devices should not only be able to rapidly detect known plant pathogens but also to detect new pathogens through library generation.

2 Current immunological and molecular techniques for pathogen detection in plants

Traditional means for pathogen diagnosis involve interpreting visual symptoms of disease, followed by pathogen diagnosis using microscopy techniques to confirm the primary data from symptom observation.[16] Although this method may be the cheapest and simplest in some occasions, it cannot diagnose pathogens before the symptoms are observable. Moreover, this approach lacks the accuracy to differentiate the effects of various pathogens based on the observed symptoms. The detection process also needs expertise – plant pathologists and taxonomists – that makes these techniques impractical for on-the-spot diagnosis of plant pathogens. Culturing is another traditional method which usually takes a few days or weeks to indicate the presence of a pathogen in a plant and thus is not practical for rapid pathogen diagnosis. As such, there is a strong interest and need to develop new techniques and on-chip sensors particularly for on-site and rapid diagnosis by unskilled people.

Current methods for rapid pathogen detection with portable devices are categorized into direct and indirect techniques. Direct methods detect properties of the pathogen itself while indirect methods detect the effect of pathogen on the plant behavior. Two main properties of pathogens exploited for direct pathogen detection are the genetic contents and epitopes on the pathogen membrane. Based on these two properties, very diverse methods have been developed for pathogen diagnosis. This diversity implies the strength of current diagnostic techniques for a wide range of plant fungi, bacteria and viruses. In general, these direct techniques are divided into two main subgroups: 1) immunological techniques using antibodies or antibody alternatives and, 2) molecular techniques using nucleic acid-based probes.[24] In the following section we introduce current indirect and direct techniques for plant pathogen detection as well as the relevant portable devices for each case.

2.1 Indirect techniques: detecting the effect of pathogens on physiological response of plant

Indirect techniques are non-invasive methods to identify plant diseases, not through the direct identification of the pathogen, but by detecting the impact of the pathogen on the physiological plant response. Current indirect techniques can be categorized into: 1) spectroscopic and imaging techniques, and 2) volatile organic compounds (VOC) detection methods.[7]

Spectroscopic and imaging techniques include fluorescence spectroscopy[25-28], visible-IR spectroscopy, and hyper-spectral imaging[29, 30] (Fig. 2a) that are mostly used for monitoring fruit quality, photosynthetic activities, tissue structures, disease symptoms, stress levels, and nutrient deficiencies in plants.[25, 27, 28] Fluorescence spectroscopy devices monitor physiological stress levels in plants by analyzing fluorescence at different wavelengths.[31] The response of healthy plants to radiated light varies with the radiation wavelength as a result of light absorption by different plant elements such as photoactive pigments, water, proteins, and other carbon constituents at different wavelengths. The spectral signature is defined as the ratio of the intensity of reflected light to illuminated light for each wavelength in visible (400–700 nm), near-infrared (700–1200 nm), and shortwave infrared (1200–2400 nm) spectral regions. The spectral signature of a diseased plant depends on the influence of that disease on processes such as chlorophyll degradation, photosynthesis, browning, or dryness.[32] Plants also respond quickly to pathogens by changing their thermal properties influencing the thermal infrared band (TIR) in the wavelength range of 8000–14000 nm. This change was used by NMR spectroscopy to detect disease in red raspberries, and by infrared thermography to detect local temperature change due to plant defense mechanisms in tobacco leaves. X-ray imaging, another image-based technique that employs low wavelengths, was used to detect fungal disease in wheat. [7, 33, 34]

The VOC detection technique detects volatile organic compounds (VOCs) produced by plants. VOC profile is affected by the pathogen presence in a plant, as such it can be used as a non-invasive technique for monitoring plant disease. VOCs released from healthy and diseased plants are sensed using electronic nose devices with integrated gas sensors (each sensor is sensitive to a particular organic compound) and a pattern recognition system. [34,35] Volatile components are absorbed on the sensor array surfaces and cause physical changes on the sensors. These changes are typically detected by piezoelectric sensors (gravimetric or acoustic) that propagate acoustic waves of piezoelectric materials, or electrochemical sensors such as potentiometric or conductimetric elements (Fig 2b).[35]

Nose devices have been recently used for diagnosing wheat disease[36] and fire blight in pear plants illustrating the strength of electronic nose-based VOC detection as an effective platform for monitoring plant disease.[37]

Some of the main challenges associated with indirect techniques for plant pathogen detection are: the effects of background data on the VOC measurements due to natural variations in the VOC profile of healthy plants[7], the selection of disease-specific spectral bands, and choice of appropriate statistical classification algorithms for each particular application.[38] As a result, diseases are diagnosable by comparing measurements for healthy and non-healthy plants. This often requires that devices be re-calibrated for each plant. In addition, indirect methods can only indicate the presence of a disease but cannot detect the specific type of pathogen that caused the disease. There are also challenges in hyper-spectral imaging for plant disease detection, especially when data acquisition is carried out under field conditions.[38] Ideally, indirect techniques would be able to detect multiple identified and unidentified pathogens, and identify early onset of disease before severe progression.[32] An autonomous detection system that can detect the presence of disease by on-line monitoring of plant response is desirable.

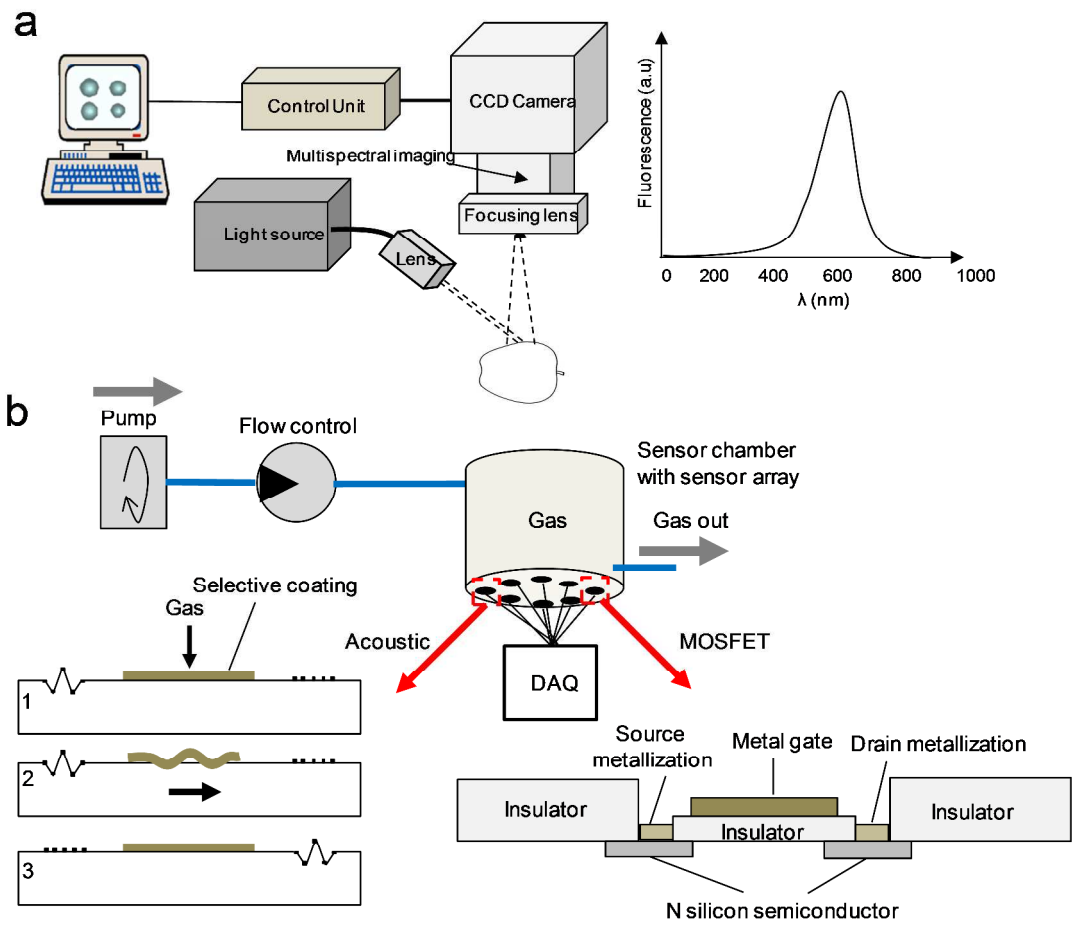

Fig. 2 Laboratory-based tools and portable devices for indirect detection of plant pathogens, **a)** Hyperspectral imaging system and typical fluorescence spectrum of leaf for excitation at 532 nm, **b)** The structure of an electronic nose device for detecting volatile organic compounds (VOCs). The nose system is made of a chamber to store the gas, a detection system composed of an array of gas sensors, and a data analysis section. The detection system is typically based on piezoelectric signals or electronic systems.[39] The sensing principle for acoustic detection systems is that the addition of a mass onto the crystal surface changes the

device frequency. Meanwhile, in MOSFET gas sensors where the source (n-type) transistor operates with three contacts of the gate, the source and the drain, the gas reaction causes a change in the metal gate or insulator properties leading to a change in the MOSFET sensor's electrical properties and thus a change in the drain current.

2.2 Immunological techniques: detecting plant pathogens by affinity binding to the target antigens

Immunological techniques used for pathogen detection are based on specific and high-affinity binding of the target species to a bio-molecule. For complex plant samples, it is crucial to perform sample preparation steps including separation, isolation, and concentration of target species, to enable effective pathogen sensing.[23] Several physical separation techniques including centrifugation, filtration, electrophoresis, and dielectrophoresis have been used. Also, bio-specific antibody-coated paramagnetic particles have been integrated into biosensors to separate or isolate targeted pathogens from the sample matrix.[40, 41] However, the effectiveness of most sample preparation techniques is limited in complex samples. Recently, immuno-adsorption on beads was used to selectively isolate targeted pathogens rapidly from complex samples. However, antibody-coated beads has limitations, namely: the high cost of beads required for effective detection, and the need to find antibodies with specific and high affinity to ensure effective pathogen capture.[42, 43]

After sample preparation to separate pathogens from the sample matrix, targeted pathogens are detected by binding to biomolecules typically in form of antigen-antibody and ligand-receptor interactions. Despite high-affinity of antibodies made *in vivo,* the high cost of *in vivo* extraction from animals[44], low specificity of *in vitro* synthesized antibodies for priority targets[45], limited antibody availability for all potential analytes, and low chemical and physical stability of *in vivo* antibodies, makes their application challenging for portable devices. Recent developments in *in vitro* synthesis of affinity binders for lower cost, reliable affinity, and improved stability resulted in synthesis of aptamers as alternative to antibodies with the potential for modification during immobilization and adaptable for several cycles of regeneration.[46-48] Other alternatives to antibodies are molecularly imprinted polymers,[49] protein-based probes, [50] small molecule probes,[51] and DNA probes for bacterial pathogen detection (Fig. 3a).[52, 53] The final stage of immunological pathogen diagnosis is to detect the binding signal by integrating immunological recognition with numerous detection platforms including antibody-directed epifluorescence microscopy[54], electrochemiluminescence[55], fiber-optic sensing[56, 57] and surface plasmon resonance.[58]

Despite progress on immunological biosensors, their applications for on-site pathogen detection are very slow particularly for plant pathogens. Lateral flow devices (LFD) have been developed for plant pathogen detection, particularly for viruses. In lateral flow devices, antigens from target pathogens bind to pathogen-specific antibodies as liquids are drawn through the device by capillary forces and visibly detected by the accumulation of bound particles (Fig. 3b).[15, 59] Lateral flow devices are often used with a variety of portable readers with different operating mechanisms such as handheld reflectometry, charge-coupled based imaging systems, scanners, and fluorescence systems.[24, 60-63] Nevertheless, the complexity of integrating detection systems for detecting multiple pathogens, as well as the relatively low sensitivity of lateral flow devices limit their widespread use as portable diagnostics for plant pathogens, especially when diseases are in latent stages.[63]

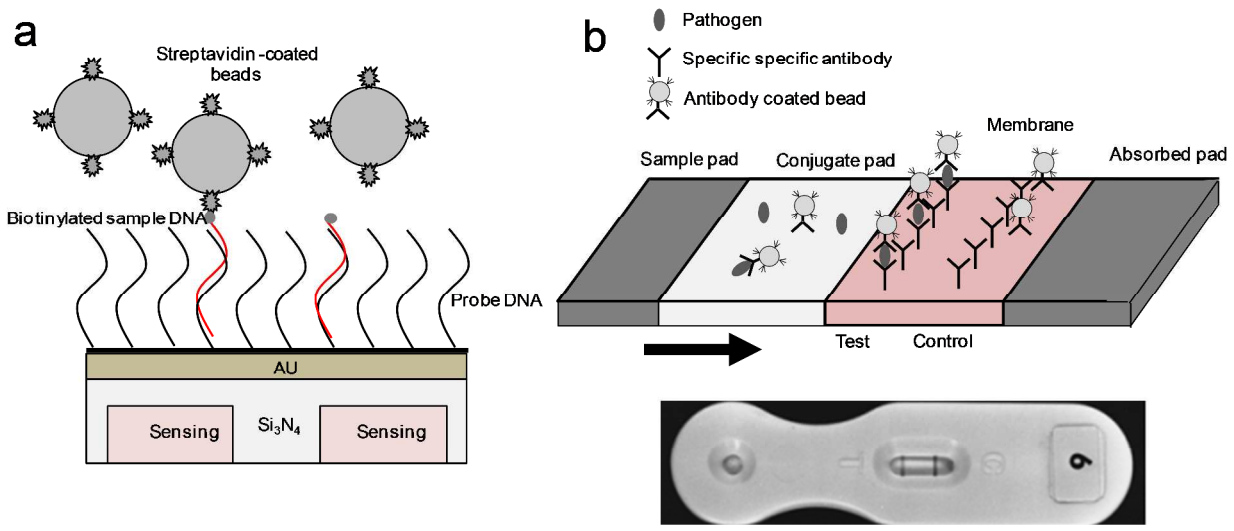

Fig. 3 Plant pathogen detection biosensors that rely on indirect affinity-based techniques, **a)** Schematic diagram of a sandwitch assay, where the target DNA sample is bound to immobilized DNA probe on the integrated sensing area and the magnetic label binds the target by streptavidin–biotin interaction. The binding is detected by sensors measuring induced magnetic field, **b)** Schematic of Lateral Flow Device (LFD) binding interaction of target pathogen antigens with pathogen-specific antibodies.

2.3 Molecular techniques: direct detection of nucleic acid content of the pathogen

Molecular techniques are well established for sensitive detection of many plant pathogenss [7, 59] Current plant pathogen detection systems operating based on molecular techniques are mainly enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR)-based methods. In ELISA-based systems, antigens bind to capture antibodies immobilized on a surface. Detection antibodies conjugated to an enzyme then bind to the antigen, followed by addition the enzyme's substrate or fluorescent dye to detect the antigens. In the presence of the pathogens (antigens), the enzyme linked to the antigen generates a signal, confirming the presence of a particular plant disease. ELISAs have been widely used for detecting plant pathogens and testing multiple samples.[64]

On the other hand, PCR is used to detect the presence of a pathogen's deoxyribose nucleic acid (DNA). DNA first needs to be extracted from the pathogen, amplified to obtain thousands to millions of copies by three temperature cycling steps, and then followed by final detection step.[65, 66] A portable PCR device faces several challenges including: sample preparation, DNA extraction, portable and accurate temperature control system, and sample evaporation concerns in open devices. Also, multiplexing to detect several pathogens poses additional concerns included selecting an adequate detection method as well as complex sequential reagent introduction for multiple pathogen detection. Promisingly, for many of these challenges, we can see progress towards viable solutions either for plant pathogen detection or in related fields.

There have been many attempts at integrating sample preparation with PCR in portable diagnostic devices.[67] Prior to PCR analysis, cells in the sample need to be lysed to extract their DNA content. Various lysis processes have been developed including: physical[68], electrical[69], thermal[70], and chemical methods[71]; however integrating cell lysis often

complicates device fabrication. One particularly promising recent example is the use of Au nanorod for simplified cell lysis. The Au nanorods generated thermal energy that broke down the cells in one step without need for reagent change (Fig. 4a).[72]

To address temperature cycling concerns in portable devices, isothermal amplification methods have been developed.[73] Among various methods of isothermal amplification[74-77], loop-mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification (NASBA) have been applied to plant pathogen detection with potential applications in high throughput and fully integrated PCR systems.[78, 79] To hinder sample evaporation and to stabilize the sample during the DNA amplification process, samples are commonly covered with mineral oil.[80]

There is a strong demand to detect multiple pathogens with PCR systems. One approach to meet this need is label multiplexing, where the sample is exposed to a mixture of different molecular probes and each one is detected in separate channels (Fig. 4b).[81] However, the number of pathogens that can be detected is limited to the number of detection probe channels available.[82] Microfluidic environment can facilitate parallel PCR processing in short reaction times with integrated temperature control units for parallel testing.[80] One of the main perceived strengths of microfluidic platforms is in making multiplex PCR platforms for simultaneous detection of large numbers of complementary DNA sequences in a sample.[23, 83] One challenge of multiplexed PCR is to integrate proper detection technique to monitor the PCR signal for multiple pathogens. Common techniques for acquiring PCR signals are colour changes that can be observed with the naked eye, or fluorescent intensity measurements.

Although laboratory-based PCR systems with reagent spotters and signal readers provide the capability to detect hundreds of pathogens in one test, the cost of reagents and the integration of detection methods are major challenges that prevent development of portable PCR devices.[84] At present, there is a lot of interest in developing affordable and portable PCR-based devices for plant pathogen diagnosis.[5] Isothermal amplification and novel detection systems like bioluminescence and magnetic microbeads align well with the goal of portable microfluidic platforms. Some of the aforementioned techniques have not yet been applied to plant pathogen detection, but would be readily applied to such applications.

Another powerful technique for detecting a large number of complementary DNA sequences in a sample is DNA microarray technology. In DNA microarrays, DNA probes are immobilized or spotted on a fixed matrix and exposed to samples containing various DNA fragments. The strong hybridization of complementary fragments to those probe spots is quantified using diverse detection methods and is used to diagnose pathogens (Fig 4c).[85-87] Nowadays, DNA microarray technology is well developed and has the ability to diagnose most identified sets of distinct genetic sequences. DNA microarrays have been used to diagnose plant pathogens in potatoes, tomatoes, and apples[88, 89], to distinguish between bacterial phytopathogens, and to differentiate species of *Fusarium* and *Pythium*[15, 90, 91].

Due to the competition between primers, the number of target DNA fragments that can be simultaneously amplified in conventional multiplex PCR platforms is limited. PCR and DNA microarray techniques have been composed such that the multiplex PCR amplifies a number of pathogen specific markers that are subsequently detected using DNA microarray.[92] However, there are several challenges associated with using DNA microarrays for pathogen detection such as low sensitivity for use in a portable device or unexpected amplification of contaminating template DNA in PCR-based DNA microarray platforms.[93]

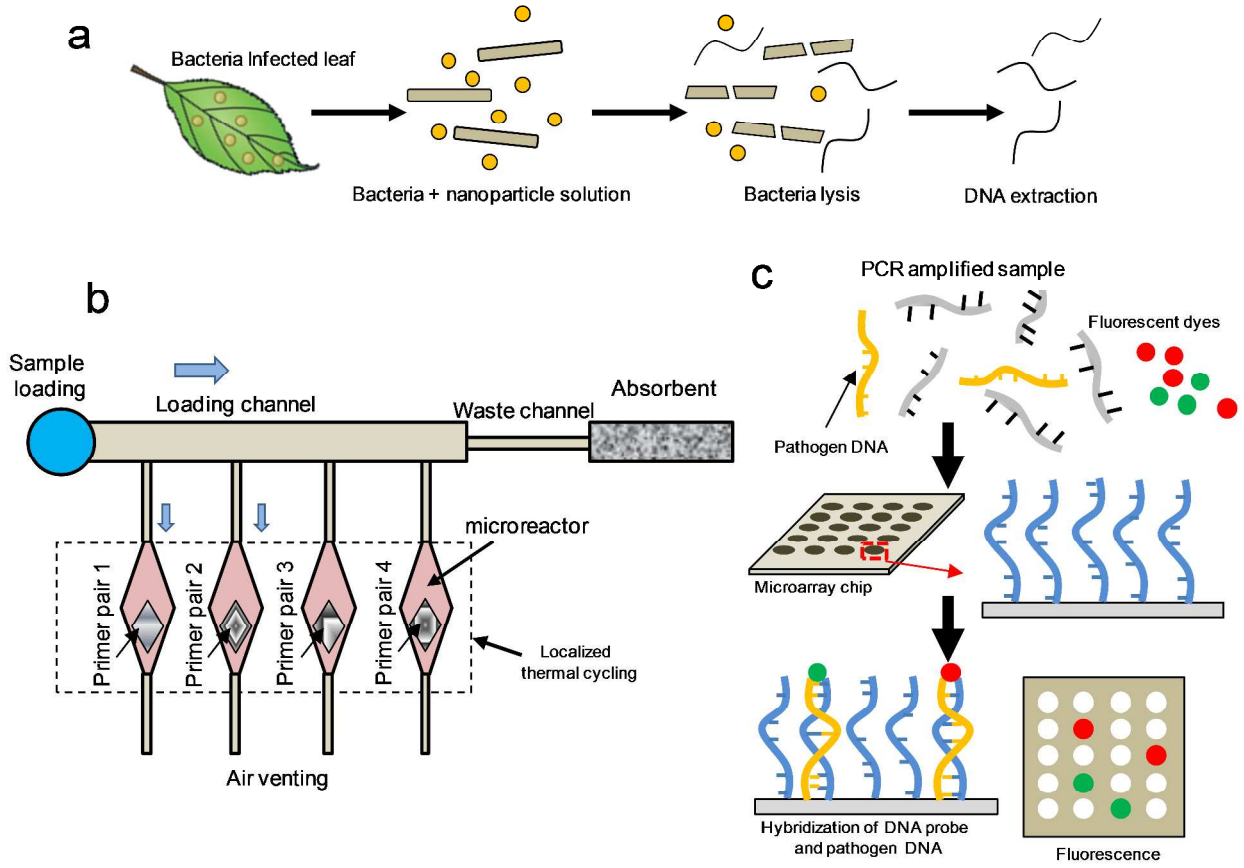

Fig. 4 Techniques with high potential for use in portable devices for pathogens isolation and detection, a) AU nanoparticles as an optothermal medium for one step cell lysis and extraction of DNA useful for portable biosensing. The longitudinal resonance of Au nanorods is utilized to transform near infrared energy into thermal energy. The resulting heat can lyse pathogens without need to removal or changing the reagents, **b)** Schematic of a disposable microfluidic device for simultaneous real-time PCR-based detection of multiple pathogens. The device is pre-loaded with different primer pairs while the PCR mixture containing multiple DNA templates is loaded into the loading channel. The mixture fills the microreactors up by both capillary action and air venting. The reactors are subsequently isolated by a single step capillary-based flow toward absorbent for further process of local thermal cycling and PCR reaction, c) The schematic design of DNA microarray for simultaneously detecting a large number of pre-amplified pathogen DNAs.

Although the sensitivity of molecular techniques for pathogen detection has been significantly improved, the application of these techniques for use in a portable device is restricted to the detection of only a few pathogens. High throughput molecular-based techniques for detecting multiple pathogens are time-consuming and labor-intensive and require elaborate work especially during sample preparation (collection and extraction) to obtain

reliable and accurate results. In addition, these techniques require consumable reagents such as sequence-specific primers for PCR or different DNA probes that must be tailored to detect each specific pathogen. Development of these methods for detecting a large number of identified pathogens and their variants remains unsolved. Also, future goals, such as the desire for a portable device that can detect unidentified pathogens, remain unaddressed.

2.4 Current portable and commercialized devices for plant pathogen diagnosis

On-site detection is increasingly important for rapid plant disease diagnosis. The need for on-site detection motivates the production of test devices and kits for use in-field plant disease detection, even by growers themselves. Majority of the abovementioned techniques have only been used in laboratory settings and are not yet commercially available. Only a few of these techniques are currently available as commercial products or with high potential for commercialization. However, efforts are in progress to develop on-the-spot devices with better performance for plant pathology. The main criteria to select the proper device are the sensitivity, cost, and ease of use.

Among indirect techniques, fluorescence spectroscopy and electronic nose are widely used for plant disease diagnosis mostly to determine the freshness of fruits or detect the plant stress in leafs over a defined course of time. Commercial electronic nose devices are available in different handheld configurations (e.g., 4200 Portable zNose of Electronic Sensor Technology, LP), or laboratory-based devices (e.g., FOX series of ALPHA M.O.S. or LibraNOSE of Technobiochip).[94] Electronic noses differentiate between healthy and infected trees by adjusting odour parameters.[95] Bloodhound® ST214 electronic nose (an array of 14 conducting polymer-based sensors with one sensor as a reference) is a commercial product applied for detecting volatile pest signatures and monitoring disease in cucumber, pepper, and tomato plants.[96] Several other portable and handheld devices are also available to measure the firmness of fruits (Fig. 2d).[97-99]

Amongst direct methods**,** immunoassays make up most of the commercialized portable devices for plant disease diagnosis. Molecular-based devices are increasingly developing and immunoprinting kits and lateral flow devices (LFDs) are well commercialized to detect plant pathogens.[15, 100, 101] The simplicity and low-cost capabilities of microfluidic devices have been motivated development of immunochromatographic strips (ICS) for pathogen detection.[1] Lateral flow devices (LFD) are the dominated commercial tool because facilitate simple, quick, and one-step assays. Many LFDs are also commercially available to detect plant viruses, bacteria, and fungi.[24, 63, 102] EnviroLogix LFDs enable growers to quickly and accurately confirm the presence of tomato apex necrosis virus in the field within 10 min. The performance of LFDs has been enhanced by integrating Bluetooth communication to export data for analysis in a local laboratory (http://www.forsitediagnostics.com/forsite_lfdr101_reader). However, because of the relatively low sensitivity of LFDs, their application is limited to where the sensitivity is adequate for detection. Their commercial use is devoted to pathogens with well-characterized and specific antibodies. In addition, since LFDs are generally designed for single pathogen detection, their practical application for detecting several pathogens is problematic due to the rather high cost per LFD unit.[15] ELISA kits are another commonly

immunological-based detection system for detecting plant pathogens. For example, an ELISA kit (PlantPrint Diagnostics, http://www.plantprint.net) has been developed to diagnose citrus tristeza virus.[15]

In contrast to immunological portable devices, PCR-based tools are used where higher sensitivity is necessary. Cepheid SmartCycler is primary real time PCR-based tools with robust and rapid detection used for detection of oomycete plant pathogen and *Phytophthoraramorum* as the cause of sudden oak death disease.[103] Bioseeq plus is another device used for diagnosis of anthrax and plague as biowarfare agents.[104] PCR-based devices are also integrated with suitable user interfaces such as Stepone (Applied Biosystems) or can perform entirely automatic tasks such as GeneExpert (Cepheid) that does not need to user intervention.[24] Despite their application for on-the-spot pathogen detection, their use still requires expertise for reagent manipulation. Some PCR-based systems such as the Bioseeq Plus are very expensive and their application is limited to dangerous pathogens.

Modified PCR-based methods have provided improved platforms for plant pathogen diagnosis. Rich et al recently reviewed commercial biosensors with the potential for use in "food, agricultural, veterinary, and environmental sciences".[105] A list of companies producing and commercializing microfluidic devices for agricultural applications were also reported by Neethirajan et al.[106] Several other commercially available test kits for use in detecting plant pathogens were reviewed elsewhere.[107, 108]

Besides the commercialized tools, several other research groups have developed highly sensitive handheld devices and fully integrated biosensors with commercialization potential.[15, 109-111] Readily-made modifications would make these new biosensors directly usable for agricultural applications. The combination of a group of bio-recognition reagents and high-throughput time-resolved fluorescence (TRF) readers may facilitate screening of different pathogens in a single run and has the potential to be modified for detecting plant pathogens.[23]

Despite the development of numerous commercialized devices, no device exists yet for on-site diagnosis of multiple plant pathogens. The agricultural industry is seeking rapid on-site, and accurate techniques for detecting plant diseases. The problem of true on-site testing may be untangled by portable devices relying on alternative nucleic acid amplification technologies and not exclusively by using conventional PCR or its modifications.

2.5 Challenges facing microfluidics, biosensors, and portable devices

Some of the drawbacks of microfluidics and biosensor platforms for on-site diagnosis of multiple pathogens are: sample evaporation, limited lifetime of biological reagents, packaging, reagent storage, weak selectivity in complex sample matrices (e.g. plant samples and newly discovered pathogens), complexity of manipulations, and the cost of detecting multiple pathogens per chip.[4, 94, 112] Future application of DNA microarrays in plant pathology are questionable due to the high cost and limited capacity for sample throughput. Progress to develop portable devices for identification of new pathogens using current technology is very slow.[24] However ongoing advances in nanotechnology and biosensor technology are anticipated to improve the sensitivity of sensors and overcome the challenges in the near future.[113]

3 Plant pathogen diagnosis using direct DNA sequencing

In genomic analysis of an organism, the entire genomic information (DNA or RNA) is sequenced. Sequencing refers to determining the order of the nucleotides in the DNA sequencing and arrangement of amino acids in protein sequencing.[114, 115] DNA sequencing dramatically benefits the biological, biomedical, and medical studies. The more advances in DNA sequencing technologies the greater the speed and efficiency of genome sequencing. The success of the 1,000 Genomes Project and announcement of Wellcome Trust project in 2010 targets genome-wide sequence data for thousands of individuals and indicates the significance and benefits of DNA sequencing. Similar benefits are offered to plant science by advances in sequencing technologies. The 1,001 Arabidopsis Genomes Project[116] and 1000 Plant Genomes Project were launched to target the sequence genomes of thousands of plant species.[117] Analyzing genomes of plant pathogens provides new information about the processes and genes involved in the host colonization and pathogenicity that offers identification of unknown plant pathogens.

Old Sanger sequencing technique uses dideoxynucleotide chain termination to determine the sequence of nucleotides in a DNA strand which requires many template fragments of varying sizes. Old Sanger sequencing has been used to sequence the genomes of model plants as well as crop species such as rice, sorghum, soybean and grape.[118-122] While it was successful for a few plants with small or medium genomes, its application for the plants such as wheat is challenging due to the large and complex 16 Giga-bases genomes and resulting high cost and time of sequencing. Development of next generation sequencing (NGS) technologies have cured our knowledge of genomics beyond Sanger sequencing and is expected to secure the future establishment of databases for both human genes and plant pathogens.[5, 123] Rapid sequencing of nucleic acids from infected plants will assist to detect a large number of identified plant pathogens and to diagnose unknown disease agents without need for prior knowledge of its molecular sequence. [124-126] This progress will assist the study of crop genetics, breeding, and plant resistance to disease.[11] Here, we discuss the application of different generations of DNA sequencing systems to plant pathogen diagnosis. The contribution of each system for on-site pathogen detection with future portable devices is explained.

3.1 Application of Second (Next) Generation DNA sequencing to plant genomics and pathogen sdetection

Next-generation sequencing (NGS) is a technology that dramatically facilitated genome sequencing at lower costs for all branches of life sciences. NGS emerged in 2005 using commercial Solexa sequencing technology and expanded rapidly to different systems. NGS techniques are basically grouped into sequencing by synthesis or sequencing by ligation. In synthesis-based sequencing, after fragmentation of DNA samples, the complementary DNA strand is synthesized by DNA polymerase while a chemical or fluorescent signal resulting from the nucleotide incorporation is detected to identify the sequence. The main commercial synthesis-based sequencing technologies are Roche 454 pyrosequencing, Illumina (solid-phase bridge amplification), and Ion Torrent.[123]

Roche 454 pyrosequencing is based on adhesion of single or primed DNA template to a microbead and amplification using emulsion PCR in such a way that each bead is individually placed within a well, subjected to the flow cell and incubated with DNA polymerase, ATP sulfurylase, luciferase, substrates luciferin and adenosine 5′-phosphosulfate (ASP). Following polymerase, dNTP is incorporated into the DNA strand and produces ATP as the

catalyst required for converting luciferin to oxyluciferin in order to emit light. All free ATP and nucleotides are washed and the process is repeated more times until the DNA template is elongated to the desired length (Fig. 5a).[127, 128] In the widely used Illumina system, solid-phase bridge amplification is used where each end of a DNA template is ligated with adapters.[129-132] While one end of adapter-conjugated DNA fragment is attached to a substrate, the other end makes a bridge with immobilized primers and generates clusters of identical template in order to enhance the chemiluminescent signal. This process continues in a cycle in the presence of a mixture of four nucleotides, followed by image capture while each nucleotide is labeled with a different fluorophore. This cycle is repeated until the DNA fragment is synthesized to its target length. In the recent Ion Torrent system, by sequentially adding nucleotides, the incorporated nucleotide is detected by measuring pH change due to the release of H^+ ions[133, 134]. The low cost of sample preparation in the Ion Torrent system has made it an appealing device for sequencing the genomes of various organisms and a promising option for plant genomics and pathogen detection.[119, 133]

In ligation-based sequencing such as the Life Technologies system, called Support Oligonucleotide Ligation Detection (SOLiD), the DNA is first fragmented – and depending upon the nucleotide to be determined – primed with a short known anchor sequence. The primed DNA template is added to the flow cell and hybridizes fluorescently labeled probes of varying lengths.[135] The type of incorporated probe is then detected using fluorescence imaging. Polonator is another commercial system relies on ligation sequencing that amplifies DNA templates by emulsion PCR with fully automated polymerase colony of loaded beads and sequencing by ligation.[136]

Next generation sequencing systems are able to simultaneously read the sequence of millions of short DNA fragments (typically 25-400 bps in length).[137] These platforms enable quick, low cost, and comprehensive sequencing of complex nucleic acid populations with huge impact on medical academic research and in particular crop genomes which consist of several thousand million DNA base pairs.[138, 139]

A recent review compares different next generation sequencing techniques based on their benefits and drawbacks.[119] NGS systems vary in terms of the length of each sequence read, the total bases sequenced, and the price of sequence per megabase (Mb). Depending upon the application, one particular type of NGS may be more competent than the others. The cost of sequencing by NGS technologies (US$4–90 per Mb) is much lower than Sanger technology (US$ 1330 per Mb) which has made it the conquering technology for current genome sequencing.[140] In addition, NGS systems have also been used to sequence plant genomes including: genome of the obroma cacao, apple genome, chickpea, and date palm.[119, 141-143] Many other crop genomes and their wild relatives are current being sequenced.[5, 11, 119, 137, 144] Sequencing the prokaryotic and eukaryotic plant pathogens and detection of microorganisms existing in diseased plant tissues are the other applications of NGS in plant health.

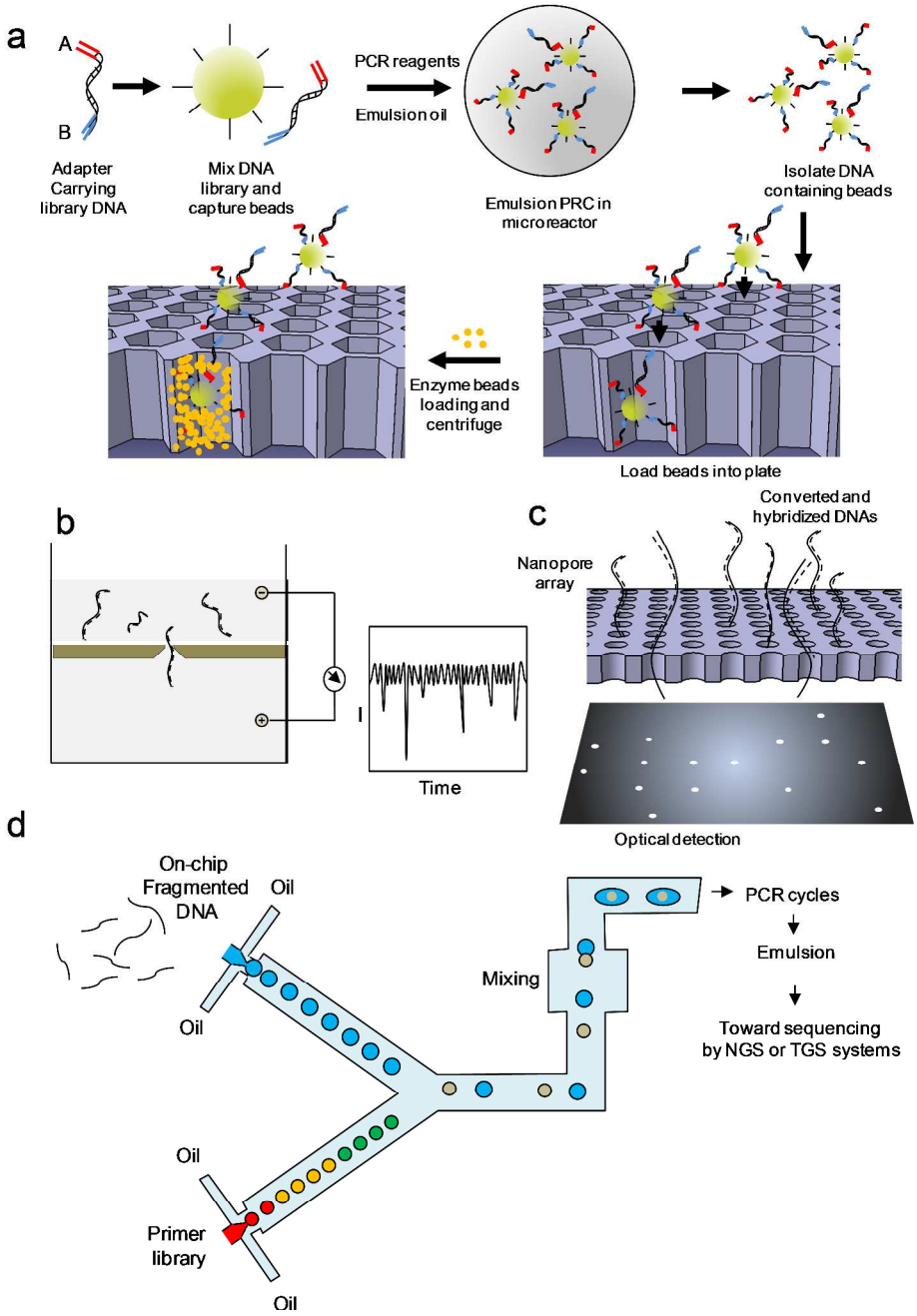

Fig. 5 Sample Next generation sequencing, Third generation sequencing and microfluidic techniques with a high potential for integration and use in a portable device for plant pathogen detection, **a)** The mechanism of the Roche/454 sequencer that operates based on synthesis. The mechanism of Roche/454 sequencer method is based on amplifying single-stranded DNA copies from a fragment library on agarose beads. DNA fragments and agarose beads containing complementary oligonucleotides to the adapters at the fragment ends are mixed. The mixture is subsequently combined with the PCR reactants surrounded by oil and after encapsulation is pipetted into a 96-well microtiter plate for PCR amplification to provide a million copies of the original DNA fragment required for generating strong detection signal, **b)** The basic design of nanopore-based DNA sequencing, where a voltage across the pore causes ion migration towards the membrane and transport through the pore. The generated electric current is measured by high-bandwidth electrometer, **c)** Optical nanopore-sequencing platform. DNA molecular is first converted into oligonucleotides of known sequence representing the four DNA nucleobases and then hybridizes molecular beacons. Passage of hybridized DNA through nanopore unzips the beacons and induces photon burst in one of four colors. Optical system is used to perform parallel detection of emitted photons, **d)** Droplet microfluidic for high-throughput sample preparation.

Sequences produced by NGS technology from a diseased plant include sequences from any pathogens present. However, this sequence also includes the large genome size of the host plant, complicating DNA sequencing of the targeted pathogens. Hence, depending on the pathogen concentration and sample matrix, an extra enrichment step may be necessary to purify the nucleic acids and to reduce the complexity and the cost of sequencing.[74, 145]

Among various targeted enrichment techniques in plant biology[146], the standard PCR-based, hybridization-based, and sequence-capture based techniques have been widely used along with NGS systems. Standard PCR was used as effective enrichment technique with the earliest NGS systems, but due to the high cost and the challenges for integrating with the new high-throughput NGS technologies its application is only limited to the old NGS devices.[146] Along with the standard PCR, the technique of multiple displacement amplification (MDA) was developed using random hexamer primers to amplify a relatively complete genome. This can significantly increase the quantity and chance of pathogen detection.[93] In the hybridization-based enrichment technique, when the complex DNA is applied to the array, the target fragments of DNA hybridize the probes while the non-desired regions are washed away. However, the hybridization techniques needs usage of blocking DNA which restrain non-specific DNA binding leading to the capturing of "off-target fragments".[147] Sequence-capture technology is the alternative to the hybridization-based enrichment which resequence targeted regions of genome, where the library of repetitive sequences is depleted first, while the target region is secondly enriched. [146]

Applications of these enrichment techniques in plants are still in their infancy, but have been used for different plants such as Sorghum bicolor , Bread wheat, Triticum aestivum, black cottonwood, pine genome, and particularly for crops of maize, conifers, and wheat with large genomes and complicated high-copy repeat interspersion. [146, 148-152] Through isolation of total DNA or RNA from disease plant, elimination of host nucleic acid, enrichment of pathogen DNA, and exploitation of different NGS technologies, [29, 11, 145] a variety of known and unknown plant pathogens have been detected. Pathogens detected with NGS technologies include Pyrenophora teres f. teres[149], Phytophthora infestans[150], viruses in sweet potato[124], rice stripe virus[125], tomato necrotic stunt virus[126], Caulimoviridae clearing virus[153] and Grapevine red-blotch-associated virus[154]. The expansion of the identified gene pool available for plants will assist to re-sequence whole crop genomes, to improve the crop genome diversity, and to discover new genetic markers useful in breeding.[137]

3.2 Third generation sequencing (TGS)

In contrast to NGS techniques where the sequencing reaction is detected on amplified clonal DNA templates by emulsion or solid phase PCR methods, single molecule sequencing (SMS) uses single DNA molecules for sequence reactions without need for DNA template amplification. Accordingly, NGS are referred to as second-generation sequencing (SGS) technologies and more recent single molecule sequencing technologies are known as third-generation sequencing (TGS).[119] Although the technology of SMS systems has been employed mostly for human genomics so far, a few applications to plant genomics and pathogen detection have reported[114, 155] TGS are superior to SGS as they simplify the sample preparation, increase the detection accuracy by eliminating PCR-caused errors,

and generate longer sequence reads by better throughput platforms.[119, 156] It is anticipated that TGS technology will not only be used for whole genome sequencing of diverse plants, but will also be used with degrees of modification in future portable devices for rapid plant pathogen diagnosis. Accordingly, the current state of TGS research for plant pathogen diagnosis is worth discussing.

Currently, academic or commercial efforts on single molecule DNA sequencing can be grouped into 1) fluorescence-based methods such as true single-molecule sequencing (tSMS) and single-molecule real-time sequencing (SMRT) and; 2) non-fluorescent sequencing systems like nanopore-based sequencing, pyrosequencing, motion-based sequencing and scanning tunneling spectroscopy-based sequencing. In True single-molecule sequencing, the sequence of DNA strands immobilized in a flow cell is detected by adding DNA polymerase and fluorescently labeled nucleotides, added one at a time in a cyclic manner.[157] In single-molecule real-time sequencing techniques, the chip is made of thousands of zero-mode waveguides (ZMW), where a single DNA polymerase molecule is immobilized on each ZMW. When nucleotides labeled with different fluorophores are applied to the chip, DNA synthesis occurs followed by emission of base-specific fluorophores. This continues by cleavage of phosphogroups and incorporation of the next nucleotide.[158]

Nanopore technology provides an extremely confined gap within which single nucleic acid polymers are analyzed. Under a small voltage imposed across a nanopore sandwiched between two aqueous electrolytes chambers, DNA is directed through the pore and the resulting ionic current is measured (Fig. 5b). Polymers with kilobase length (sstDNA or RNA) or small molecules (e.g., nucleosides) can be identified without need to amplify or label the original DNA. The DNA or RNA can be sequenced using inherent electronic or chemical properties of each nucleotide once the DNA is conducted through the nanopore by cleaving or using an enzyme (Fig 5c).[159-161]

Several companies have brought commercial TGS systems to the market to perform sequencing with dramatic low cost compare to SGS methods.[137] Helicos Biosciences is the first commercial product that relies on true Single-Molecule Sequencing (tSMS).[162] Pacific biosciences developed a Single Molecule Real Time (SMRT) sequencing system with the ability of sequencing the length of several Gbp with rather long reads of 1 kbp.[139, 163] Oxford Nanopore technology and recently IBM's plan of silicon-based nanopores are the recent devices developed for third generation DNA sequencing.[164] It is hoped that the advances in different SMS techniques will ultimately lead to even more commercial platforms.[165] Nanopore technology with a high-throughput system is considered promising as the future technique for rapid DNA sequencing.[166] However, several challenges have to be addressed especially to slow down the speed of passage through nanopores in a manner that the detection method would be able to detect each nucletoide at the time.[159-161]

3.3 Challenges facing DNA sequencing techniques for plant pathogen detection

Since NGS and TGS technologies read the sequence of a large number of fragmented DNAs and generate an extensive amount of data using high-throughput systems, computational biology and bioinformatics have crucial roles to play for data storage, transfer and analysis.[167, 168]

In comparison with the other NGS techniques, the Roche 454 technology is more compatible with a portable device for pathogen detection. It produces the longest reads amongst the currently available NGS technologies which clearly simplifies the cost of data assembly and analysis (Fig. 5a). In addition, the bead-based and microwell design of Roche 454 simplifies the integration of this technique with available high-throughput microfluidic platforms such as droplet microfluidics for sample preparation (Fig. 5d) and enrichment before sequencing.[123]

The other NGS technologies are more challenging for integration with an integrated sample preparation step and for use in a portable device. The situation gets worse for technologies like the Illumina that have shorter reads.[114, 169] Novel computational techniques are established to elicit the genome of sample from a large number of short lengths. Well-founded software and informatics algorithms to manage the assembly and analysis of sequences currently exist or are in development.[138, 170, 171], although their integration with portable devices is yet debatable due to costly analysis and low accuracy of quality control. The challenge of developing a portable DNA sequencing technology is greater for plants with very long and complex genomes like wheat (16 Gbp), in contrast to the less complex genomes of cereal (5 Gbp) and maize (2.3 Gbp).[172] Because of this complexity, the identification of the genetic variations in the genomes of closely related plant species is cumbersome.[114]

For plant species without sequenced genomes, the alternative technique for pathogen detection is to identify new markers and to use the aforementioned molecular and immunological-based techniques.[139] Attempts at data analysis for plant sample sequencing are currently in progress. For example, iPlant Collaborative company presents infrastructure and tools to address the problems of data analysis of NGS technology for plant samples.[171] New strategies have improved the assembly algorithms used for genomics analysis of several plants.[114, 122]

Although DNA sequencing is a promising approach for future portable devices for plant pathogen detection, there are several limitations that prevent widespread on-site adoption of sequencing technologies. Data analysis remains a hurdle that prevents usage in developing countries where internet and communication networks are not readily available for sending data from a sequencing device to a central database or bioinformatics lab for further studies.[137, 173] Another challenge is the interval needed to read the required DNA lengths for on-site pathogen detection. Although using technologies that generate shorter sequence reads lessens the time required for extracting data, shorter sequence reads deteriorate the bioinformatic analysis and accuracy of genome analysis. Both the interval of data extraction and the accuracy criterion need to be considered when developing future portable devices. The read lengths of TGS technologies are approaching 1 kb facilitating bioinformatics approaches relevant to de novo assembly, while maintaining reasonable data extraction times that are reasonable for a portable device.[119]

4 Future of on-site portable devices for plant pathogen detection

Based on the necessity of rapid detection of multiple pathogens, the diversity of plant variants, and the constraints of current immunological and molecular-based portable devices, it is anticipated that direct DNA sequencing will be the dominant technology for future portable devices over the next decade.[15] The main reasons for the delay in implementing DNA sequencing techniques for routine use are technical challenges and high cost. These two concerns need to be addressed when developing DNA sequencing-based detection techniques for plant health

applications. The technical challenges are related to the limitations of current DNA sequencing technologies in detecting complex and long sequences of plant genomes. Meanwhile, the challenge of high cost is focused on deciding which of the available technologies will have the highest impact on future portable devices and how those technologies could be miniaturized in an inexpensive format for on-site use by non-experts.

Although, it is predicted that direct DNA sequencing will likely be the conquering technique for on-site plant pathogen detection, a few important steps are still currently missing before portable devices can be developed. Two main steps (one pre-processing step and one post-processing step) should go along with plant DNA sequencing. Unlike human samples, plant samples contain intricate communities of microorganisms. As a result, effective sample preparation techniques need to be tailored for plant samples.[11] In addition, the bioinformatics post-processing step is critical to discriminate between host and pathogen sequences. Because biological knowledge might be necessary for proper data interpretation, accurate and reliable gene sequence databases will be essential for sequence-based diagnosis of plant-associated microorganisms at the international level. New pathogens and plant variants should be rapidly detected. This requires high accuracy in both DNA sequencing and plant genomic analysis to distinguish known pathogens and their similar variants. Future platforms should be able to diagnose unknown pathogens from DNA sequencing databases.[11, 174] Based on these requirements, the key solution for the future portable devices will be to integrate the capabilities of microfluidic platforms with one of the present direct DNA sequencing techniques.

4.1 Contribution of microfluidics for the future of DNA sequencing-based portable devices

Microfluidic systems have several advantages, including: the intrinsic feature of laminar flow, miniaturization, portability, high surface to volume ratio, fast rate of mass transfer, and the ability to handle very small volumes of sample, that make them an appropriate platform for sample-to-result devices such as for sample preparation required prior to DNA sequencing.[175] Current progress in biosensors for sample preparation, DNA extraction, and development of parallel microarrays and high-throughput designs can be applied to DNA sequencing technologies.

Sample preparation is one major area where the contribution of microfluidic devices for future portable DNA sequencing devices would be significant. For plant samples, this step is extremely essential to enrich the sample, remove inhibitors, and concentrate pathogens from biological samples or tissue lysate. Dielectrophoresis (DEP), diverse metallic and magnetic micro/nano particles as well as different physical, chemical or membrane-based filters are broadly used approaches for DNA and RNA sample preparation in microfluidic devices.[74, 176-186] The potential for multilayer structures in microfluidic platforms is another specific feature that can be applied for sample preparation and filtering purposes, for instance using integrating micropillars.[187]

Another area where existing microfluidic and lab-on-chip platforms can be applied to DNA sequencing technologies is in the detection of multiple pathogens. High-throughput designs and microarray-based methods based on microfluidic platforms have been broadly reported.[188] High-throughput platforms were implemented by integrating parallel microchannels, micro-pumps, and micro-valves as well as exploiting various techniques for cell/molecule trapping and transport (Fig. 4b).[189-192] Typically fluid handling for these high-throughput systems is provided by 1)

simple fabricated low-cost pumps such as electro-hydraulic pump (EHP) and Lab-on-a-disk devices[76, 193] or 2) by pump-less and valve-less systems relying on gravity-driven flow, electrokinetic, electroosmotic, and capillary-based systems provide superior platforms for portable devices.[194-197]

Among various techniques for cell/DNA transport within microfluidic chips, microdroplet-based devices and digital microfluidics (DMF) are well-suited to high-throughput systems for single molecule or cell isolation, single cell genetic analysis, PCR, cell lysis, and DNA hybridization.[70, 106, 198, 199] Droplets trap target DNAs and conduct them toward array-based detection regions for DNA sequencing.[200] In digital microfluidic (DMF) platforms, droplets are individually transported over 2D high-throughput arrays of electrodes based on the electrowetting on dielectric (EWOD) principle. Digital microfluidic platforms have been applied to DNA hybridization and PCR.[201-203]

Despite current progress in the field, an integrated microfluidic device that performs all or most of the steps for automated DNA extraction, amplification, and sequencing has rarely been implemented.[164, 204-207] Development of new microfluidic techniques for separating the DNA content of the plant pathogen from the host origin is very promising.[11, 145, 208, 209] Without this separation, the heavy contamination with sequences of host origin results in limited sensitivity of portable device and also complicates bioinformatics analysis for pathogen detection.

4.2 Contributions of DNA sequencing technologies to future portable devices for plant pathogen detection

Once the DNA content of a diseased plant is filtered from the sample using microfluidics, it should be directed toward the detection site in order to identify its DNA sequence. Existing NGS and TGS technologies were discussed in section 3; however, the successful adoption of any of these techniques into a portable device for plant pathogen detection depends mainly on the capability for fast, accurate, and high-throughput sequencing, compatibility with microfluidic pre-processing environments, and the merits of detecting complicated genomes to study diversity and genetic variations.[114, 210] An appropriate sequencing technology for plant pathogen detection should be able to sequence large DNA fragments in a parallel sequencing process with thousands or millions of sequences to efficiently reduce the cost of sequencing for use in a portable platform. Some of the current DNA sequencing technologies such as the Roche/454 sequencer (Fig 5a) are tolerably high-throughput and more compatible with a portable microfluidic-based device with the ability of sequencing tens of millions bases per few hours with the length of a few hundred bps. However, for all DNA sequencing technologies discussed in section 3.1, the DNA is sequenced using PCR or hybridization, where nucleotide formation is needed one by one necessitating a large number of reagent introduction, detection, and washing steps. This complicates the sequencing process, making its practical use in portable devices questionable.

Recent progress on single DNA sequencing techniques have shown promising results for rapid and accurate DNA sequencing in comparison with the NGS methods, although there are several challenges yet to be addressed.[166] For instance, for nanopore sequencing system, despite its potential for robust, inexpensive, and fast sequencing in portable devices, there are challenges such as: 1) optimizing its performance to better contrast the bases, 2) controlling the unidirectional translocation to stably sample over noise and molecular motion, 3) controlling DNA velocity and orientation during translocation and 4) distinguishing each base from the next base in the unknown

DNA sequence.[166, 211-214] In addition, to use nanopore technology in a high-throughput portable device, it should be implemented in high-throughput arrays of nanopores for simultaneously reading a large number of short DNA fragments. Two optical detection techniques for high-throughput reading of the translocating DNAs sequence and the electrostatic DNA detection based on a metal-oxide-silicon capacitor integrated into the nanopore have brought hope for future use of nanopore technology in whole genome sequencing.[215] Despite its potential for high-throughput sensing, the fabrication of arrays of uniform solid-state nanopores with diameters between 1.5 and 2.0 nm with integrated capacitors remains a huge challenge.[216, 217] The integration of solid state nanopores with microfluidic platforms for DNA enrichment and manipulation toward the nanopores is another challenge that should be addressed. Also, the microelectronic detection system integrated in the nanopore membrane is suggested as one high-throughput detection system to read the nucleotides sequence. Although nanopore technology is still naive for single DNA sequencing and pathogen detection, the recent portable device by MinION Oxford Nanopore released in 2014 is expected to be directly used for DNA sequencing with minimum need for sample preparation.

Although there is still no proof of concept portable DNA sequencing device for whole genome sequencing and plant pathogen detection, the combination of NGS techniques and microfluidic devices can be used in future for detecting a large library of plant pathogens. At the moment, it is not clear which target enrichment techniques will be adopted for future portable devices, however it is expected that the use of these techniques will be essential for effective pathogen detection for a lower sequencing cost. PCR-based enrichment coupled with high-throughput NGS platforms is expected to be an alternative practical technique for a portable device. In addition, microdroplet PCR techniques can be used to manipulate high-throughput droplets containing fragmented genomic DNA and PCR reagents for targeting different regions of genome followed by DNA sequencing using the techniques used in Roche 454 or Illumina platforms. In addition to proposed DNA sequencing techniques, real-time PCR is a sensitive and rapid technique useful for high throughput screening and will continue to be used for plant pathologists in the near future. Although the development of microarray-based plant pathogen detection is slow mainly because of low sensitivity and high reagent cost, it has promising potential especially when coupled with multiplex-PCR. This combination may need several years to be recognized as a practical portable tool for on-field detection of plant pathogens.

4.3 The bioinformatics problem: the ultimate challenge of future portable DNA sequencing devices

Even though future portable devices would likely be able to sequence complicated plant genomes, the analysis of huge sequence databases presents the most significant challenge to future portable devices. Plant genome sequences need to be compared against databases of known pathogen sequences by pair-wise sequence-similarity search methods. These techniques are not sufficient to diagnose new pathogens, thereby there is an essential need to develop new techniques to diagnose unknown pathogens or variants of a known pathogen. In this context, new pathogens should be detected by comparing the genome of a diseased plant and a healthy reference plant – this is currently challenging due to poor alignment of short sequence reads.[123, 131]

Conclusion

Based on the urgent need for rapid detection of multiple known or unknown pathogens, the performance of currently available portable devices, and the recent progress in third generation sequencing technology, it is concluded that that future portable devices for plant disease diagnosis will integrate ultra high-throughput direct DNA sequencing with microfluidic and microelectronic platforms. These field-based portable devices are anticipated to be useful for laboratory-based experiments to generate databases and protocols for protecting plants. Furthermore, these devices would also be used for developing new markers with important agricultural implications in breeding programs for crop improvement.

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