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Exploiting generic platform technologies for the detection and identification of plant pathogens

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Abstract The detection and identification of plant pathogens currently relies upon a very diverse range of techniques and skills, from traditional culturing and taxonomic skills to modern molecular-based methods. The wide range of methods employed reflects the great diversity of plant pathogens and the hosts they infect. The well-documented decline in taxonomic expertise, along with the need to develop ever more rapid and sensitive diagnostic methods has provided an impetus to develop technologies that are both generic and able to complement traditional skills and techniques. Real-time polymerase chain reaction (PCR) is emerging as one such generic platform technology and one that is well suited to high-throughput detection of a limited number of known target pathogens. Real-time PCR is now exploited as a front line diagnostic screening tool in human health, animal health, homeland security, biosecurity as well as plant health. Progress with developing generic techniques for plant pathogen identification, particularly of unknown samples, has been less rapid. Diagnostic microarrays and direct nucleic acid sequencing (de novo sequencing) both have potential as generic methods for the identification of unknown plant pathogens but are unlikely to be suitable as high-throughput detection techniques. This paper will review the application of generic technologies in the routine laboratory as well as highlighting some new techniques and the trend towards multi-disciplinary studies.

Keywords Plant health · Diagnostics · Detection · Real-time PCR · DNA barcoding · Microarrays · Isothermal amplification · LAMP · Direct tuber testing · Molecular diagnostics · TaqMan · De novo sequencing · Pyrosequencing

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Introduction

A typical diagnostic laboratory usually maintains capacity to perform morphological identification using light or electron microscopy; methods for detecting proteins from the organisms such as enzyme-linked immunosorbent assay (ELISA) or electrophoresis; methods for detecting fatty acids; molecular methods identifying the nucleic acid of the organisms such as polymerase chain reaction (PCR), Reverse transcription PCR, real-time PCR, reverse polyacrylamide gel electrophoresis or nucleic acid hybridisation; and finally, traditional bioassays such as inoculation of test plants, indicators, or isolation on selective media followed by morphological identification. A decline in the availability of trained staff to perform traditional techniques is a significant issue for maintaining the critical mass required to deliver this type of service.

Generic methods that can be used for a number of different pests help with capacity maintenance and sustainability of small diagnostics laboratories. Using a smaller number of techniques in a laboratory means less expertise needs to be maintained and training new staff is more readily achievable; with robust techniques the staff do not need to be highly qualified, and this helps to keep running costs low, allows delivery of a cost-effective service, and makes sustainability more achievable.

Generic platform technologies

Generic methods are those that can be performed using the same basic technology in the same format, albeit with different reagents, to enable the detection of different pathogens. The techniques cut across disciplines whereby the same basic skills can be used for the detection of a range of pests of different types (insect, nematode, bacterial, fungal, or viral). Probably the first and most established of these is ELISA. Assays based on serological detection of virus proteins have been around since the 1950s, but the adoption of a generic platform on which to perform these assays (i.e. the 96-well plate) made the technology, including all the equipment needed, available to a wider market at an accessible cost. In addition, the ELISA test has proven to be very robust; it can be performed in almost any laboratory with a minimum of training. As a result, the technology remains virtually unchanged since it was first mooted for plant pathogens in the late 1970s (Clark and Adams 1977), and is used on a daily basis in almost all diagnostic laboratories world-wide for plant, clinical and veterinary targets.

Methods based on molecular biology have been developed since the early 1980s, usually in situations where ELISA was not suitable due to either low sensitivity, poor specificity, or lack of suitable antibodies. Significant uptake into the routine testing environment has been slow outside of niche applications, but 25 years on they are becoming more readily accepted and are even replacing traditional techniques in some laboratories. The reason for the recent uptake and proliferation of these techniques has been the generic nature and increased robustness brought on by advances in format. Initially, techniques were based on hybridisation or PCR (Puchta and Sanger 1989); the latter proliferated primarily since it did not require probe synthesis or radioactive labels. Conventional gel-based PCR, however, has two major drawbacks; the first is susceptibility to contamination, and the second, perhaps more significantly, is a lack of robustness, especially in the hands of non-specialists in a routine setting.

Real-time PCR has started to become an established diagnostic technique since the format effectively 'turns PCR into ELISA', making it both robust and accessible. In diagnostics it has often been referred to as 'automated PCR' due to the lack of post-PCR manipulations (which also helps to prevent post-PCR contamination). However, the factor that has more significantly made an impact is the robustness of amplification. A similar level of skill is required to perform real-time PCR as is required for ELISA, and as such, unlike conventional PCR, it is not the preserve of experienced molecular biologists. The adoption of a common format (96-well and latterly 384-well plates), as with ELISA, has made the technology widely available to researchers as well as laboratories working in clinical, veterinary and plant diagnostics. This availability has driven down costs; the price of real-time instrumentation is now almost one tenth of that 10 years ago. These factors have made the technique well suited to high-throughput detection of known target pathogens. In some laboratories where the equipment is available, the generic nature of the technique has started to result in the replacement of ELISA due to the low start up costs when developing new assays, especially, though not exclusively, when antibodies/antiserum are not available. The laborious nature of nucleic acid extraction is now the main factor limiting the uptake of real-time PCR in routine plant pathogen detection.

Simplified detection

A drive to move diagnostics to the point of decision-making, rather than waiting for results returning from a laboratory has also had an impact on the methods being developed in a number of fields besides plant health. The development of in-field, point-of-care, and pen-side methods for plant, clinical, and veterinary diagnostics, respectively, is dominated by a single format: the serologically-based lateral flow device (LFD). Where appropriate reagents are avail-

able, and where serological assays give the level of sensitivity required, the LFD is the method of choice. The tests can be performed with limited training, have very few steps and give results in minutes. In fact, the technique is so dominant that it is often adopted and sold in very large numbers for targets (for example, *Mycobacterium tuberculosis*) where the limited sensitivity will often give false negative results.

To attain greater levels of sensitivity and specificity than can be achieved using LFDs, researchers have again turned to real-time PCR. The drive towards faster and more portable real-time PCR equipment designed with diagnostics in mind has been led by military and homeland security applications, although more recently platforms developed specifically for point-of-care (POC) clinical applications have been developed. The resulting equipment can be robust and rapid, such as the Smartcycler II (Cepheid) and Bioseeq (Smiths Detection); can have a simple user interface, such as the StepOne (Applied Biosystems); or can be completely self-contained, such as the GeneExpert (Cepheid), which is able to sequentially perform both extraction and reaction set up, thus requiring little or no user intervention. Although developed for in-field, remote POC applications, these techniques are also enabling technology for small laboratories, as the techniques are being developed specifically for diagnostics, require little training, and are very robust. This equipment has been deployed in a field situation for plant health applications (Fig. 1) for the detection of several pathogens in support of European Union policy.

Future technologies

There are several technologies on the horizon that might become valuable as generic diagnostic tools; these are, at least in the short term, still in the research stage but worth discussing for the future.

Although the capital cost of real-time PCR has decreased in recent years, newer amplification chemistries may help to further reduce the cost of molecular testing. These include various isothermal amplification methods, such as nucleic acid sequence-based amplification (NASBA) (Leone et al. 1997); helicase-dependent isothermal amplification (Vincent et al. 2004); exponential nucleic acid amplification reaction (Tan et al. 2005); and methods based on rolling circle amplification (Yi et al. 2006; Zhang et al. 2006), all of which have the benefit of not requiring complex thermal cycling equipment, while retaining the specificity and sensitivity advantages of PCR-based methods. Such methods also have the potential to be combined with novel detection strategies such as the use of functionalised gold nanoparticles (Tan et al. 2005), bioluminescence (Gandelman et al. 2006), or biosensors (Jaffrezic-Renault et al. 2007), which could be less costly than the fluorescent detection required for real-time PCR. As an example, probably the most advanced isothermal amplification technology is loop-mediated amplification, which uses a highly processive strand displacing polymerase to separate the DNA strands, and primers that form loops to generate new priming sites (Notomi et al. 2000). In this case, the isothermal reaction generates so much DNA that a simple colour change can be used to discriminate positive and negative results, with a limit of detection approaching that of real-time PCR (Tomlinson et al. 2007).

Diagnostic microarrays and direct nucleic acid sequencing (DNA barcoding) both offer potential as generic methods for the detection and identification of unknown plant pests. For the diagnostic laboratory, microarrays offer potential as a generic method for detecting large numbers of known pathogens in a

Fig. 1 Real-time PCR being performed 'on-site' at the point of decision making (**a**) showing the SmartCycler II equipment in a car running on a generator and (**b**) for the testing of *Phytophthora ramorum* at a site remote from the laboratory

single test (Mumford et al. 2006; Boonham et al. 2007); in the UK Biochip project (www.bio-chip.co.uk) the generic nature extends beyond plant virus detection to the detection of animal and fish viruses also. Existing microarray methods are, however, complex and relatively insensitive, and a widely accepted diagnostic format has yet to be adopted.

More recently *de novo* sequencing methods have been used to good effect in identifying potential disease-causing agents. Recent reports (Cox-Foster et al. 2007; Ledford 2007) have shown that deep sequencing of cDNA and DNA generated using generic primer sets (e.g. 16S primers for bacteria) can be used to identify the presence of pathogen sequence. These massively parallel sequencing techniques employ sequencing-by-synthesis methods, for example, the Genome Sequencer FLX is based on pyrosequencing (Roche, formerly 454 Life Science) whilst the Genome Analyzer System (Illumina, formerly Solexa) uses a removable fluorescence-based chemistry. Both systems, however, can generate millions of sequences, yet require no *a priori* knowledge of the pathogens present in the host and offer for the first time a completely generic diagnostic tool regardless of host or pathogen. Currently, the sequencing is expensive and the bioinformatics required to deal with the large amount of data is in its infancy, but it is likely that it will be a method widely used in the future.

In the past, successful generic technologies for plant disease diagnosis have been developed based around commercially available platforms or formats; plant health diagnostics itself is not large enough to support novel platform development. This approach effectively 'piggybacks' onto widely available platforms used in other arenas, for example research, clinical diagnostics, or more recently, detection for homeland security. The wide availability of the platform then allows the generation of a commercial market for the specific reagents and consumables needed which helps to achieve both reduced costs and ultimately sustainability.

Applications

Although much has been done in developing modern nucleic acid-based 'molecular' tests over the last 20 years, much of the work has concentrated in niche areas, often where other techniques (such as antibody-based ELISA) cannot be used. In the case of PCR detection, however, real-time PCR has changed this, and routine services based on this technology are not only being developed in niche areas, but are finally beginning to replace established main stream techniques. It is difficult to predict which other molecular techniques in the future will make an impact in the plant health arena, though with developments in sequencing technology moving at pace, the use of direct DNA sequencing almost certainly will. The following are examples of (1) an area in which a molecular biology technique is now established and is displacing the established technology, followed by (2) what will likely become an established technique in the next few years.

Direct tuber testing

The virus indexing of seed potatoes is one of the most widespread testing procedures performed by virologists. In general, a common approach is used based on taking eye cores from dormant tubers and growing these on in a greenhouse for several weeks, before testing the sprouts produced from these cores by ELISA. By testing at least 100 tubers, individually or in small batches, the test can be used to estimate the percentage of virus-infected tubers found in a particular seed stock, hence indicating its suitability for planting or the grade at which it should be classified. Over the last 30 years or more, this method has become almost universally adopted because of the advantages it offers: not only can it be used for most common potato viruses, it is also robust, simple to perform, and is well-suited to high-throughput testing. As a result, testing laboratories can routinely test hundreds or thousands of seed stocks in a season. For example, the Dutch General Inspection Service for agricultural seed and seed potatoes tests an average of 20,000 seed stocks every year, as part of official post-harvest control. This equates to one million ELISA tests for *Potato virus Y* alone (G.W. van den Bovenkamp, personal communication).

However, while the growing-on test has become the established method, it is not without problems. In addition to the requirement for a large amount of greenhouse space, the most obvious issue is the length of time the whole procedure takes. In general it takes at least 4 weeks for the eyes to break dormancy,

sprout and grow sufficiently to be tested. If the growing-on step could be removed, with tubers being tested directly, this would remove the need for glasshouse space and reduce the amount of time taken. However, this cannot be achieved reliably using ELISA. Virus titres within dormant tubers are often very low, especially for late primary infections, and are often below the limit of detection for ELISA. This makes direct tuber testing using this method unreliable (Hill and Jackson 1984), hence the requirement to include growing-on as a bio-amplification step.

Given the limitations of the ELISA-based system, virologists have investigated more sensitive virus detection methods, to see if they might offer a reliable alternative for direct tuber testing. In the 1990s this work mainly focused on molecular methods; during this time, a range of different potato virus assays was designed including assays based on conventional PCR (Spiegel and Martin 1993; Mumford et al. 1994), the ligase chain reaction (O'Donnell et al. 1996), NASBA (Klerks et al. 2001; Leone et al. 1997), and real-time PCR (Schoen et al. 1996; Boonham et al. 2000). While these and other reports demonstrated that such methods could be used for the sensitive detection of potato viruses direct from tubers, it was also clear that much more work was required to turn this early progress into a useable, routine diagnostic service.

The first major issue was the need to validate any new system against the well-established, universally accepted growing-on test, in order to prove that a direct approach could be used as a reliable alternative. In particular, there were major issues related to the cost of performing this type of testing and its overall reliability. In the early stages of development, while it was relatively easy to prove the specificity of a particular assay and demonstrate it was comparable to ELISA, it was far harder to achieve such results when comparing the entire test (i.e. sample processing, RNA extraction, running assays and analysing results) to growing-on. In many cases, sample processing and RNA extraction were identified as the main problems and work focused on these aspects. At Central Science Laboratory (CSL), a combined approach was developed, taking small, uniform potato cores using specially-designed coring devices, homogenising with ball mill grinders and then using automated RNA extraction based on magnetic beads. Using this system, the consistency and reliability of extraction was greatly improved when compared with a more traditional approach using manual methods, which routinely had a failure rate of around 15–20% (Unpublished data). The use of extraction control assays (which detected endogenous plant genes such as *Cytochrome Oxidase 1*) also had a major effect on the overall quality of testing. By routinely using such controls, the performance of extraction, in terms of both quantity and quality of extracted RNA, could be closely monitored, and extractions repeated if necessary. In this way, the risk of false negative results due to extraction failure was virtually eliminated.

The second major issue with direct tuber testing, especially using a PCR-based approach, is the increased cost when compared to an ELISA-based growing-on system. Factors include an increase in the amount of hands-on staff time, the need for RNA extraction, relatively expensive reagents, and high capital equipment costs. Another contributing factor was the requirement to buy licences from the patent holders of the technologies used and the need to charge a royalty payment for every test performed. For example, a 15% royalty was charged for a PCR test. However, while increased costs were indeed a major factor in holding back the new approach, its significance has been reduced over the years. It has been possible to streamline the whole system, to ensure greater efficiency. For example, the use of real-time PCR has removed the need for post-PCR analysis and hence avoids the time and cost of gel running. The use of rapid coring and grinding, with automated extraction has also greatly reduced the amount of staff time required, while the use of liquid handling robotics has further reduced staff input and hence lowered per-sample costs. At the same time, reagent and equipment costs have also tumbled, especially in the real-time PCR arena where increased competition amongst suppliers has pushed down prices. Finally, the expiry of the PCR patents in 2006 has also reduced costs, ending the requirement for specific licences and royalties. While these will still be required for the various real-time technologies being used, the fact that there are different detection systems (e.g. TaqMan, Scorpions, Molecular Beacons, etc) means that competition is likely to keep the costs lower than before, in contrast to PCR where a monopoly existed.

Overall, these changes have meant that the costs related to a real-time PCR-based test are no longer prohibitive. It should also be remembered that, in

many laboratories, the true cost of performing growing-on tests is unclear, as the real costs involved with growing sprouted eye cores and the maintenance of the glasshouse facilities are often underestimated or totally overlooked. Indeed, if you were to establish from scratch a seed potato testing facility capable of dealing with moderate numbers of stocks, the capital costs of building a glasshouse facility of sufficient size and standard (e.g. insect-proof, with heating and lighting) would almost certainly outweigh the cost of equipping a laboratory capable of testing the same number of tubers by direct real-time PCR.

Ultimately, the success of any new diagnostic system must be measured by its adoption and use in routine testing. This has now been demonstrated for direct tuber testing. CSL has been offering a post-harvest tuber testing service based upon real-time PCR since 2001. Over that time the uptake by growers has grown significantly (Fig. 2), to the extent that in 2006–2007 direct tuber testing accounted for just under half of all the tuber stocks tested. These results clearly show that despite being more expensive, a direct testing approach is attractive to growers and they will be prepared to pay a premium price for a diagnostic test provided that it offers them real benefits (in this case speed). By getting rapid results, growers are able to make better, more timely decisions, for example, deciding which stocks to retain for planting as seed, before grading is carried out. As a result, they can often save large amounts of time, effort, and expense.

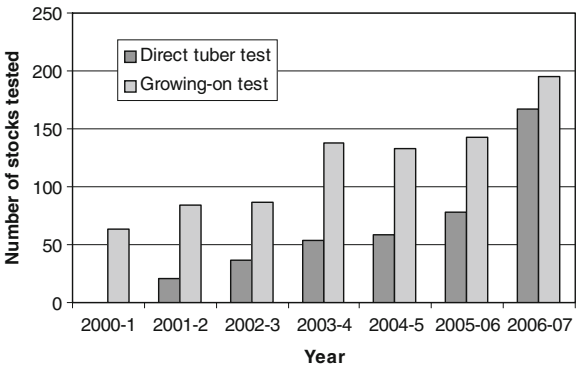

Fig. 2 A histogram showing the number of post-harvest tuber tests performed at CSL using either real-time PCR (direct tuber testing) or ELISA (the growing-on test)

DNA barcoding

DNA barcoding in an academic setting has two aims: firstly to assign unknown individuals to species, and secondly to enhance the discovery of new species (Hebert et al. 2003a; Stoeckle 2003; Blaxter 2003, 2004). The term 'DNA barcoding' originates from the idea of Universal Product Codes for manufactured goods being applied to DNA sequences for different species. Creation of the 'barcode' involves the PCR amplification and sequencing of a conserved gene sequence, typically around 600 bp of the mitochondrial *Cytochrome oxidase I* gene (*COI*). In simple terms, these sequences or barcodes are then aligned and a tree is produced; if a suitable gene has been used, the clusters should identify meaningful groups of individuals as distinct taxa.

Mitochondrial genes are particularly attractive for this application because of their lack of introns. Hebert et al. (2003b) have shown that in arthropods, most species have more than 50 substitutions in each 500 bp of their *COI* gene—more than enough for species identification. However, a common misconception is that each species has its own unique sequence for the entire 650 bp region of *COI*. Most species have a degree of intraspecific variation between individuals or populations, and therefore typically between five and ten individuals from each species are sequenced, in order to define the variation within the taxa studied.

The concept of sequencing a region of DNA and using it to identify species is not new; however, in recent years the scope and utilisation of DNA barcoding has expanded rapidly. The creation of the Consortium for the Barcode of Life (www.barcoding.si.edu) has led to the standardisation of protocols for DNA barcoding and many projects are ongoing. Recent projects have targeted birds (Hebert et al. 2004; Kerr et al. 2007), fish (Ward et al. 2005), bats (Clare et al. 2007), fungi (Seifert et al. 2007), and invertebrates (Ball et al. 2006).

DNA barcoding and plant health

Identifying invertebrate pests and fungal/bacterial pathogens to species-level using morphology alone can be time-consuming and requires specialist skills and knowledge. Many invertebrates can be morpho-

logically cryptic in their juvenile stages and may require culturing to gain a positive identification, a process that can take many weeks. Furthermore, an identification cannot be made if these samples are dead on arrival at the laboratory or die during culturing. In addition, there is a well-documented decline (e.g. Coomans 2002; Hopkins and Freckleton 2002) in the availability of experts in the field of morphological taxonomy, due at least in part to changing trends in teaching at Universities. Thus, maintaining a critical mass of expertise in these fields in order to provide a service is often difficult.

DNA barcoding could become a valuable tool in this arena. In addition to assigning unknown individuals to species and enhancing the discovery of new species, the technique can be used to identify unknown specimens. Given a validated dataset of sequences obtained from morphologically identified species, an unknown individual or juvenile may be identified by placing its sequence in the tree and seeing which species it clusters with. In contrast to the more traditional molecular diagnostic 'tests' (usually based on PCR) that only produce a yes/no answer (where the latter is often an unhelpful result) for the specific assay used, DNA barcoding can be thought of as a molecular identification tool. The technique also has a number of technological advantages since it is relatively simple, requiring only PCR (and access to the relevant primer sequences) and sequencing (a readily available service which is both rapid and inexpensive). The future of DNA barcoding as an application in the plant health arena will ultimately be determined by the availability of validated databases of sequences.

Many projects are currently underway to produce validated datasets of DNA barcodes. Their applications include forensics (Nelson et al. 2007) and elucidating cryptic species (Hulcr et al. 2007), in addition to identifying economically important species for biosecurity (Armstrong and Ball 2005; Ball and Armstrong 2006; Brunner et al. 2002). The Consortium for the Barcode of Life is also coordinating many bar coding projects. Of notable interest are the Mosquito Barcode Initiative, Tephritid Barcode Initiative and the International Network for Barcoding Invasive and Pest Species.

A further application of DNA barcoding data that is currently emerging is the use of short sections of the barcode as probes on microarrays (Pfunder et al. 2004; Hajibabaei et al. 2007). This has the potential for large-scale microarrays containing probes for thousands of species on one slide; the future of this technique, however, lies in the development of inexpensive and validated arrays. The comparable cost of direct sequencing to even modest size arrays is weighted heavily in favour of the sequencing approach. Furthermore, whilst the sequencing approach generates the actual sequence data from which the identification is determined, the microarray approach effectively infers short stretches of the sequence on the basis of a hybridisation pattern. This in itself could be seen as a retrograde step.

One of the most important aspects of a DNA barcoding effort is the initial identification of material to be sequenced. The downstream identification of any unknown specimen is only as good as the data used for comparison. Web-based software produced by Ratnasingham and Hebert (2007) provides an identification engine that contains the DNA barcodes held to date, all of which were positively identified by morphology prior to sequencing. As the number of species, and the number of barcodes per species, in the public domain becomes larger, the power of the technique increases. However, beyond the academic aspects, it is important to consider the likely applications and potential practitioners of the method, and to engage with this community at an early stage. This community is the source of identified and validated material, without which the barcoding effort will be worthless. It is important that all the required information is captured for the material to be bar-coded and that this information is both relevant to the person using the approach to achieve an identification, and links to voucher specimens. The concept of DNA vouchers and also digital vouchers from which the DNA was extracted also need to be addressed.

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