Molecular Diagnosis of Plant Pathogenic Bacteria

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Abstract

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Real-time, fluorogenic, PCR assays have recently shown great promise in the diagnosis of many plant pathogenic bacteria. In such assays repeated PCR cycles result in exponential amplification of the PCR product and a corresponding increase in fluorescence intensity, providing "real-time" analysis of the reaction kinetics and allowing quantification of specific DNA targets. As no post-PCR processing steps (such as gel electrophoresis) are required such assays also lend themselves to high throughput screening of samples. Assays that detect *Ralstonia solanacearum*, *Agrobacterium* spp., and *Xanthomonas fragariae* have been developed at CSL, and assays for *Clavibacter michiganensis* subsp. *sepedonicus* and *Erwinia amylovora* have been developed elsewhere. The key in the development of any test is the selection of an appropriate target DNA sequence and the development of a suitable DNA extraction protocol, directly from plant material. A real-time PCR assay (recently developed at CSL) which detects the strawberry angular leaf spot pathogen *Xanthomonas fragariae* (*Xf*) was designed using sequence data obtained from the housekeeping *gyraseB* gene. Although this gene is found in all bacteria, unique sequences were found for use as *Xf* specific PCR primers and probe, when compared to *gyraseB* sequence data obtained from closely related bacteria. In conjunction with a rapid and sensitive DNA extraction protocol this assay can detect the pathogen at 10^3 cells per reaction – a population level associated with latent infections by *Xanthomonas fragariae*.

Key words: Detection, identification, Real Time PCR.

Introduction

In the last 10 years new technologies have revolutionised the diagnosis of plant pathogens. Although classical applications such as standard microbiological techniques, immunological methods and microscopic methods still have a part to play in diagnosis the Polymerase Chain Reaction (PCR), and more specifically real-time PCR, now facilitates high-throughput screening for many plant pathogens, including bacteria, viruses, fungi, nematodes and insects. In this paper we describe some of the assays and extraction techniques developed at the Central Science Laboratory (CSL) for the detection of plant pathogenic bacteria.

Although several real-time PCR chemistries exist at CSL we generally use the TaqMan™ real-time PCR chemistry which exploits the 5′ nuclease activity of *Taq* DNA polymerase (2) in conjunction with fluorogenic DNA probes (3). Each probe, designed to hybridise specifically to the target PCR product, is labelled with a fluorescent reporter dye and a quencher dye (Figure 1). During PCR amplification the probe is digested by *Taq* DNA polymerase, separating the dyes, resulting in an increase in reporter fluorescence. Repeated PCR cycles result in exponential amplification of the PCR product and corresponding increase in fluorescence intensity.

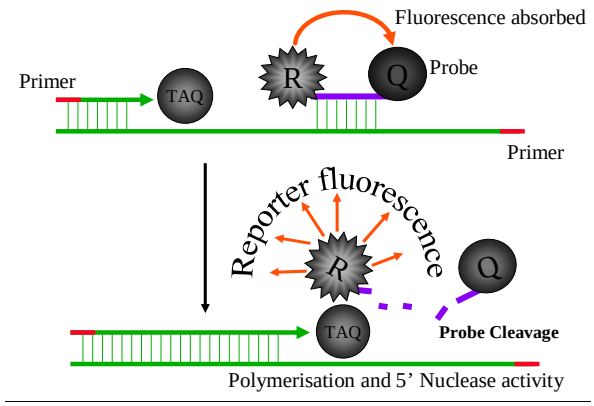

Figure 1. Real-time PCR, TaqMan™, chemistry

Fluorescence can be measured throughout the PCR, providing "real-time" analysis of the reaction kinetics and allowing quantification of specific DNA targets. The measurement of fluorescence throughout the reaction eliminates the need for post-PCR processing steps, such as gel electrophoresis and ethidium bromide staining of target DNA, facilitating automation of the technique and large-scale sample processing. Interpretation of the fluorometric data can be presented during the PCR assay (Figure 2), and also facilitates quantification of the amount of sample DNA present in the reaction by ascertaining when (i.e. during which PCR cycle) fluorescence in a given reaction tube exceeds that of a threshold value (Threshold Cycle (C_T)). Comparison between reaction tubes and/or known standards allows quantification of the amount of DNA template present in a given tube.

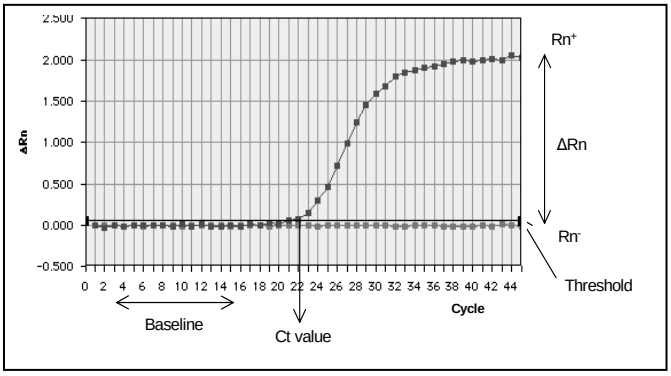

Figure 2. Amplification plot from positive and negative real-time PCR reactions

Real-time PCR protocols have been developed at CSL to detect several plant pathogenic bacteria including the bacterial wilt pathogen *Ralstonia solanacearum* (10), *Agrobacterium* spp. (13, 14) and *Xanthomonas* spp. which cause two diseases of strawberry (15), whilst other assays against pathogens such as *Clavibacter michiganensis* susbp. *sepedonicus* (7) and *Erwinia amylovora* (5) have been developed elsewhere. However, design of a real-time PCR assay is only the first step in developing a successful

application. An efficient extraction of bacterial DNA from plant tissues must also be facilitated. This extract must reduce or eliminate PCR inhibitors present within plant tissues, be quick and cheap to accomplish, but still retain enough bacterial DNA in order to be effective. Knowledge of the pathogen (e.g. normal location on plant, general population numbers) is usually required for a successful extraction technique to be developed.

Real-time PCR assay design

As for all PCR assays a unique DNA sequence (for a given bacterial pathogen) is required for the design of a real-time PCR assay. This can be obtained from sources such as GenBank (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi), sequencing a conventional PCR product, or generating new sequence data. General rules for the design of primers and probes exist (Table 1) with software (Primer Express; Applied Biosystems, Foster City, CA, USA) or internet resources, such as the Perlprimer freeware (http://perlprimer.sourceforge.net/), available to help assay design. The entire target sequence is known as the amplicon and primers and probes, designed by such programmes, should be checked to ensure that the rules stated in Table 1 are being followed.

Table 1. Standard rules to be followed when designing real-time (TaqMan) PCR primers and probes

	Primer Rules
1	Primers with melting temperature (Tm) of 58 – 60 ^{o}C.
2	Primers 15-30 bp in length
3	Run of identical nucleotide should not be allowed, especially G
4	The total no. of Gs and Cs in the last 5 nucleotides at the 3' end should not exceed 2
5	Amplicon length should ideally be 50-150 bp in length and not exceed 400 bp
	Probe Rules
1	Tm of 10^{o}C higher than primers
2	Should not be runs of identical nucleotides, especially Gs
3	GC content 30-80%
4	More Cs than Gs produces a higher DRn
5	No G at the 5' end

If no sequence data or conventional PCRs exist for a particular pathogen then it is necessary to generate unique sequence data. One way this can be achieved is by exploiting non-conserved regions which exist within house-keeping genes possessed by all bacteria (e.g. 16 rDNA, *gyraseB*, *recA* genes). Universal PCR primers have been reported (6) which produce PCR products from some of these genes. These PCR products can then be sequenced. To utilise such an approach in the design of a PCR detection assay, the selected gene from closely related strains to the pathogen of interest must also be sequenced. Once the sequence data has been obtained a simple alignment can be generated and variable regions within which a PCR assay might be able to be designed can be elucidated. An example of how such an approach was followed in order to develop a real-time PCR to detect the strawberry bacterial leaf blight pathogen, *Xanthomonas arboricola* pv. *fragariae* is shown in Figure 3. When following this approach it is sometimes not possible to follow all the standard rules listed in Table 1. This can result in the design of an inefficient PCR which can lead to a reduction in sensitivity of detection. Further information on how variations in target DNA sequence can affect real-time PCR specificity can be found in Boonham *et al*. (1).

X arboricola
cacgtgcccctccaaaacccggaattggcccggaaaccggcaaaaaagggccacccacccg
ggcgccaagccccccggccaaag
X campestris ccagcgtgcgcatcccgcagcc---
gcatggcccgggcgccggcaaga—cggctgcgcaa-----gcaaggccaccggcaacga
X axonopodis ccagcgtgcgcattccgcagcc---
gcatggcccggaagcaggcaagaa----ccaaggccagcggcaagcccaccggtaacga
X fragariae ccagcgtgcgcgtgccgcagcc---ttacggcaccaaggcaggcaaaa---
-gcaactgctggcggcaaacccaccggcaacga
Xf arboricola ccagcgtgc**gcgtgccgcagcc---**
gcatggg**ccggaaaccggcaaga----gca**agcgc**cggcgccaagcccaccgg**caacga

Figure 3. Alignment of part of the *Xanthomonas* prolyl endopeptidase (*pep*) gene, showing TaqMan primers and probe designed for detection of *X. fragariae* pv *arboricola*

Extraction of bacterial DNA from plant tissues

The extraction of bacterial DNA from plant tissues prior to analysis by real-time PCR is often the most challenging aspect of assay design. Some plant tissues, e.g. strawberry leaves, are known to have high concentrations of compounds which inhibit the PCR reaction. Therefore in any extraction such PCR inhibitors must be removed whilst, at the same time, retaining enough bacterial DNA to facilitate reliable detection. Extraction techniques vary between pathogens and plant type. Examples of three different extraction techniques for various different pathogens / crops follows.

1. **Extraction of bacterial DNA from potato tubers using magnetic beads and the Kingfisher ML extraction system -** At CSL two bacterial pathogens of potato, the brown rot pathogen *Ralstonia solanacearum* (Rs) and the ring rot pathogen *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), are detected from infected potato tissues by using a customised device (Figure 4) which collects small cores from the vascular ring of tubers. DNA is extracted from these tubers using a proprietary kit (Wizard Magnetic DNA Purification System for Food, Promega, Madison, WI, USA). Extraction of DNA from Gram positive Cms cells also requires an enzymatic digestion (lysozyme) to ensure efficient lysis of Cms cells. The kit contains lysis buffers and magnetic beads which bind DNA upon application of an electromagnetic current. Using a magnetic particle processor (Kingfisher ML), DNA is removed from the lysed tuber extract and, after several further processing steps, a purified DNA extract results. The Kingfisher process is summarised in Figure 5. This DNA extract is sometimes required to be diluted to ensure that high concentrations of DNA do not inhibit the resulting Rs and Cms specific PCRs.

2. **Extraction of bacterial DNA by enrichment of plant samples in selective broth -** The use of selective media as a pre-enrichment treatment in PCR protocols has been described in several reports (4, 11). In theory the use of selective media allows an increase in pathogen numbers and reduces levels of PCR inhibitors, and as an aliquot of

lysed broth can be added directly to the PCR reaction no complex DNA extraction techniques are required. For some pathogens / crops this approach can work well. At CSL we use selective Medium 1A broths as a pre-enrichment in the detection, by real-time PCR, of Ri-plasmid harbouring *Agrobacterium* biovar 1 strains, which cause cucumber root mat (12). In this instance nursery run-off water samples are used as the inoculant for the broth cultures.

However, there are several potential disadvantages to this technique. In reality there is no media which is completely specific for a target organism and therefore it is possible that in some sample broths a non-pathogen may out compete the target organism resulting in a false negative result. In addition there are also components of some media that can disrupt the real-time PCR chemistry. Therefore any assay must be carefully validated prior to routine use. We use this approach to detect the root mat pathogen as it is known that high numbers of Ri-plasmid harbouring *Agrobacterium* biovar 1 strains are present in run-off samples, even from latently infected plants. However we would not recommend pre-enrichment in selective broths for the detection of Ti-plasmid harbouring *Agrobacterium* strains, which cause crown gall. In many environments this particular pathogen is often outnumbered by avirulent *Agrobacterium* strains. Therefore the use of an *Agrobacterium* selective broth as a pre-enrichment for detection of this pathogen is likely to lead to many false negative results.

Figure 4. Potato coring device

3. **Extraction of bacterial DNA from strawberry leaf tissues -** To detect *Xanthomonas fragariae* (*Xf*) and *Xanthomonas arboricola* pv. *fragariae* (*Xaf*) DNA can be extracted from 0.7 cm strawberry leaf discs using extraction and dilution buffers from the Extract-N-Amp Plant PCR kit (Sigma) and modifying a previously described protocol (8). This is a quick and easy procedure to follow and although it results in a crude sample (which must be diluted at least 1:20 in molecular grade water) the dilution and extraction buffers negate the influence of PCR inhibitors that are present in high concentrations within strawberry leaf tissues. This technique has enabled detection of 10^3 cells per leaf when utilised with a new real-time PCR protocol which detects *Xf* (15).

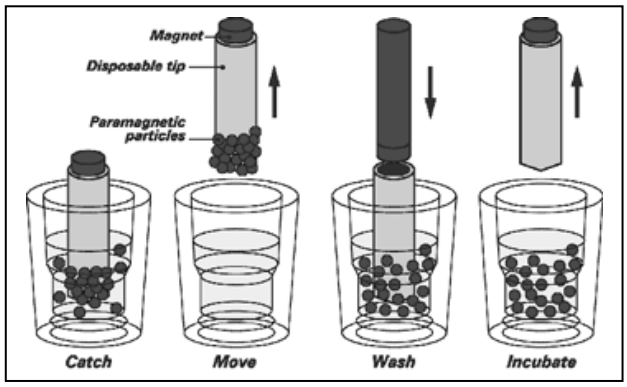

Figure 5. Schematic diagram showing extraction of DNA from potato tuber cores, using magnetic beads and the Kingfisher ML machine

Conclusions

Real-time PCR offers significant advantages over conventional PCR, and other techniques, in the diagnosis of plant pests and pathogens. High-throughput screening of plant samples is facilitated by shorter PCR run times and also by the fact that no post-PCR processing steps are required. As different reporter dyes exist, which fluoresce at different wavelengths, it is also possible to include more than one reaction in one tube. Known as multiplex PCR this allows the incorporation of internal controls [e.g. the COX assay of Weller *et al*., (10)] into an assay. However, it is vital to validate and optimise multiplex PCRs. Often successful DNA amplification by one PCR can prevent the other reaction in the tube from working.

Perhaps the major disadvantage of real-time PCR is the initial cost of the equipment though significant reductions in unit cost have occurred over the past few years. Real-time PCR machines have recently been bought to the market which can be used on-site (9). As such machines become smaller and less expensive their use in locations such as custom depots and docks, where rapid clearance of plant cargos is required, is likely to become more prevalent.

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الملخص

وولر، سيمون، جون إلفينستون، نيل باركينسون وريتشارد ثوايتيس. 2006. التشخيص الجزيئي للبكتيريا الممرضة للنبات. مجلة وقاية النبات العربية. 24: 143−146.

لقد أعطت تجارب التفاعل التسلسلي للبوليميراز PCR المعتمدة على تحليل مخطط الزمن الحقيقي وبوجود (صبغات أو ملونات) متوهجة وعوداً كبيرة لتشخيص البكتيريا الممرضة للعديد من النباتات. تؤدي عملية تكرار دورات التفاعل التسلسلي للبوليميراز في هذا النوع من التجارب لمكاثرة نواتج هذا التفاعل (والتي هي الـــDNA) بشكل كبير يترجم بزيادة كثافة التوهج ومن ثم يتم التقدير الكمي لقطعة الـــDNA المتخصصة (النوعية) والتي هي الهدف من خلال تحليل مخطط الزمن الحقيقي لمراحل التفاعل. يسمح هذا النوع من التحاليل بغربلة وتحليل عدد كبير من العينات وذلك بسبب عدم الحاجة لخطوات تتبع عملية الـــPCR (مثل استخدام

الهلامات في عملية الرحلان الكهربائي). تم تطوير التجارب التي أجريت على *Ralstonia solancearum*، *Agrobacterium* spp. و *Xanthomonas fragariae* في مخبر العلوم المركزي (CSL)، في حين طُوّرت التجارب المتعلقة بـ*Clavibacter michignensis* ssp. *sepedonicus* و *Erwinia amylovora* في مكان آخر. إن الخطوة الأساسية (المفتاحية) في تطوير أي اختبار هي الاختيار المناسب لمقطع الـDNA الذي سيستخدم كهدف وكذلك تطوير تقنية مناسبة لاستخلاص الـDNA من المادة النباتية مباشرة. لقد تم حديثاً في CSL تصميم تجربة التفاعل التسلسلي للبوليميراز بالزمن الحقيقي التي سمحت بكشف وتحديد بكتيريا التبقع الزاوي على أوراق الفريز (*xf*) *Xanthomonas fragariae* ذلك باستخدام معطيات لمقاطع DNA مأخوذة من المورثة *gyrase B*. على الرغم من وجود هذه المورثة في جميع أنواع البكتيريا، إلا أن الدراسة التي أجريت لمقارنة مقاطع من هذه المورثة في أنواع بكتيرية قريبة من بعضها البعض قد سمحت بالعثور على مقاطع معينة يمكن استخدامها كمسابر نوعية أو كبادئات PCR متخصصة بالبكتيريا *Xf*. لقد سمحت عملية الجمع ما بين هذه التقنية RT-PCR والطريقة السريعة والحساسة في استخلاص الـDNA بكشف الكائن الممرض عند وجوده بمعدل 10^3 خلية في وسط التفاعل– على مستوى مجتمع بمرحلة كمون العدوى بالبكتيريا *Xanthomonas fragariae*.

كلمات مفتاحية: تعريف، تشخيص، Real Time PCR.

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