Development of recombinase polymerase amplification assays to rapidly detect *Phytophthora* species on plant samples

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Abstract

Fluorometric recombinase polymerase amplification (RPA) assays for the genus *Phytophthora* have been developed that provide a simple and rapid method to detect the pathogen (Phytopathology 105:265-278). These assays are extremely tolerant towards inhibitors present in many plant extracts, thereby simplifying DNA extraction procedures. RPA assays have been developed for *Phytophthora* genus specific detection, species-specific assays for 9 taxa including *P. ramorum* and *P. kernoviae,* and a plant internal control. Assays were validated for specificity using DNA extracted from more than 135 *Phytophthora* taxa, 22 *Pythium* spp., and several plant species with a sensitivity of detection approaching TaqMan real time PCR. The assays were validated with 250+ symptomatic plant field samples representing more than 50 hosts. Samples that were positive using the *Phytophthora* genus specific RPA test were also positive using TaqMan PCR and traditional isolation techniques. A technique for the generation of sequencing templates from positive samples to confirm species identification also was developed. Use of species specific TaqMan probe sequences for designing species specific RPA primers provides a systematic approach for assay development. These RPA assays have benefits over PCR because they are rapid (completed in as little as 15 minutes), do not require DNA purification or extensive training to conduct, require less expensive equipment and can be completed directly in the field with portable equipment. The *Phytophthora* genus specific assay has been modified for use in a lateral flow device and is currently undergoing validation, thereby simplifying the ability to complete diagnostics in the field.

TaqMan PCR versus RPA

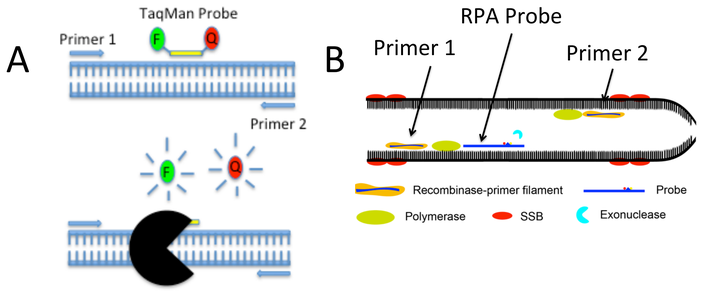

Fig. 1. Graphical representation of TaqMan PCR detection systems (A) and recombinase polymerase amplification (RPA) marker systems (B).

Introduction

Problems for detection

- Traditional plating assays from plant samples can take 4-6 weeks to get results, which can be too long when planting decisions need to be made.
- Enzyme-linked immunosorbant assay (ELISA) is commonly used in the detection of *Phytophthora spp.* but it is important to note that background detection of some *Pythium* spp. may also occur.
- Several PCR and qPCR detection techniques currently exist to detect *Phytophthora* spp. at a genus and species specific level but all require some type of DNA extraction and well trained technical support to perform the assays.
- A simple, rapid, reliable and sensitive technique such as isothermal amplification would be extremely beneficial for small diagnostic labs, regulatory agencies and field applications.

What is isothermal amplification?

- The ability to amplify DNA without the aid of a thermocycling apparatus
- Several types including Helicase dependent amplification (HDA), Loop mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA)
- Very tolerant of inhibitors, so a traditional DNA extraction is not required
- Can grind a plant sample and have results within 10-20 minutes
- The use of fluorescently labeled probes allows for multiplexing
- Results can be read on many different platforms, some of which are field portable

Objectives

1) Develop a RPA *Phytophthora* genus-specific detection assay
2) Identify compatible crude tissue extraction buffers for the RPA system
3) Develop a system to construct species-specific RPA markers,
4) Develop a method to confirm the identification of the species present in a positive RPA method by DNA sequencing
5) Develop a rapid field portable diagnostic assay that could be used directly at the point of sample collection

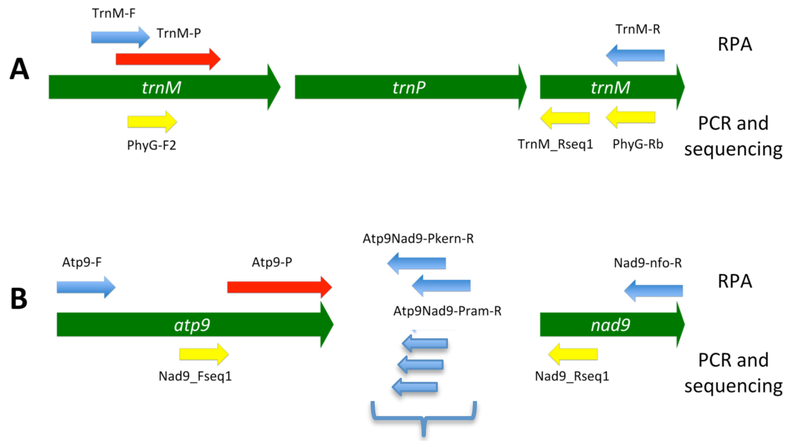

Fig. 2. Mitochondrial loci used in this study for the genus-specific detection of *Phytophthora* (A), the *Phytophthora* species-specific assays (*P. kernoviae* and *P. ramorum*) (B) and other developed species specific markers (Table 1). Also denoted is the location of primers and probes used in RPA detection and the location of nested PCR and sequencing primers used in the confirmation of a positive product.

Fig. 3. The *Phytophthora* genus-specific assay (A) and the *P. ramorum* species-specific assay (C) ranging from 2 ng to 200 fg. The log of the initial DNA quantity of *P. ramorum* against the onset of amplification, where the *Phytophthora* genus-specific assay and *P. ramorum* species-specific assays are denoted by closed and open circles, respectively (B). The log of the initial DNA quantity of *P. ramorum* against the log of the onset of amplification minus the agitation step (OT), where the *Phytophthora* genus-specific assay and *P. ramorum* species-specific assay are denoted by closed and open circles, respectively (D).

Table 1. Some characteristics of species-specific reverse primers for the *atp9-nad9* locus.

Species	Primers required	Size (bp)	Last 6 bases	Unique bases	GC
P. cactorum	1	36	ATGTAA	11	11%
P. cinnamomi	1	30	GATAAT	17	23%
*P. fragariae**	2	31	ATTACG	16	19%
P. kernoviae	15	33	TCACAG	22	15%
P. rubi	2	35	TCTATT	20	25%
*P. sansomeana***	2	35	ATAATA	19	14%
*P. sojae***	10	29	TATCAA	19	17%
P. ramorum	1	30	TAACGT	17	37%

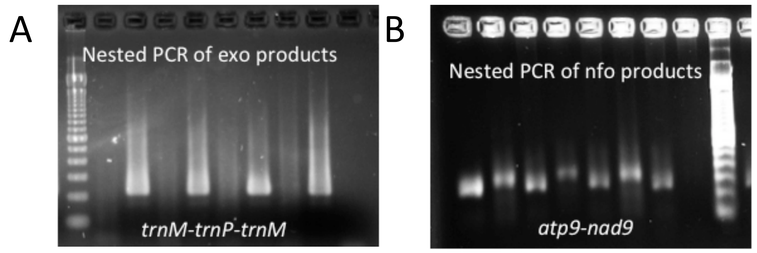

Fig. 4. Ability to amplify and sequence RPA products from both loci after a RPA detection.

Fig. 5. Reactions can be read fluorometrically on a variety of devices (A-D) and can be adapted to lateral flow device technology (E), all of which use extremely crude plant samples (F).

Results

Sensitivity and specificity

- Validated all three tests on over 106 *Phytophthora* species, 22 *Pythium* species and a wide range of plant species
- Identified reliable buffers for grinding plant tissue samples
- Tested sensitivity with several *Phytophthora* species
- Tested the effect of plant material

Field validation

- Tested on many different hosts including strawberry, raspberry, avocado, citrus, bay laurel and several ornamental plants
- 222 symptomatic samples (15 counties in California)
- Validated with Real-time PCR amplification, sequencing and traditional isolation on selective media
- Identified several *Phytophthora* species including *P. cactorum*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. nicotainae*, *P. ramorum*, *P. rubi*, *P. syringae*
- For regulatory purposes a method to confirm a positive using a nested PCR technique followed with sequencing was also developed and validated

Conclusions

1) The described RPA techniques should have a significant impact on our ability to rapidly detect *Phytophthora* **directly in the field** and make management decisions within **20 minutes** of collecting a plant sample
2) Due to the fact that **PCR technologies can be easily transferred to the RPA platform** it is possible that new assays for other species or taxa of plant pathogens could be quickly developed
3) Samples can be sequences following a nested PCR reactions which allows for the **confirmation of a positive detection**.

References/Acknowledgements

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