A PCR-Based Assay Using Sequence Characterized DNA Markers for the Identification and Detection of *Aphanomyces euteiches*

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*Aphanomyces euteiches*

Plant pathogen that causes severe root rot disease in alfalfa, peas, and beans

Affected Regions

Infected alfalfa

USDA-ARS, Prosser, WA
Limitations of Conventional Methods for the Detection of *Aphanomyces*

- Use of selective media is confounded by presence of other fungicide resistant microbes i.e. *Pythium*.

- “Baiting” soil with susceptible host requires up to three weeks for completion.

- Microscopic detection of oospores is tedious and oospores are only produced at the end of season.
Soil Baiting Technique with Aphanomyces
OBJECTIVES

- Design a system based on PCR that could discriminate *A. euteiches* from other closely related species and genera of soilborne microbes.

- Use the system to detect *A. euteiches* in infected roots.

- Use the system to detect *A. euteiches* in soil.
OBJECTIVES

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Advantages of PCR-Based Detection Assays for Soilborne Microbes

- Rapid
- Selectivity can be broad (genus) or narrow (species, race)
- Not necessary to isolate organisms in pure culture from soil/plant tissue
- Easier for ‘microscopically challenged’ pathologists
We chose to use SCARs for developing the assay

- SCARs = Sequenced Characterized Amplified Regions

- SCARs use a pair of sequenced characterized primers for amplification of target DNA

- SCARs were first developed for mapping resistance genes in lettuce to *Bremia lactucae*
Advantages of SCARs Over RAPDs

- Only detect a single locus (PCR product)
- Highly specific
- More rapid than RAPDs
Method for Designing SCAR Primers

- Identify a RAPD that is only amplified by all isolates of the target species (*Aphanomyces* sp.)
- Clone and sequence the RAPD
- Design primers based on RAPD sequence
- Optimize thermocycling reaction conditions.
  - \([\text{MgCl}_2]\) (1.5 mM-4.5 mM)
  - annealing temperature (60°C-72°C)
RAPDs
PRIMER gcctacactg

CORTAR FRAGMENTO

PURIFICACION DEL FRAGMENTO DE ADN

CLONACION pcrII

AMPLIFICACION DE ADN

DISEÑO DE PRIMERS (20-25-mers)

gcctacactgTTCCATGCATTACGG
gcctacactgGACGTAAGCTGATT

SECUENCIALIZACIÓN DE EXTREMOS DEL FRAGMENTO DE ADN
Developing a SCAR Specific for *A. euteiches*
Effect of $[\text{MgCl}_2]$ on Amplification

1.5 mM $\text{MgCl}_2$  3.0 mM $\text{MgCl}_2$

Annealing Temperature = 60 °C
Optimized SCAR Reaction Conditions
(1.5 mM MgCl$_2$; 70$^\circ$C Annealing)
Effect of Cycle Number on Amplification

Two-Step PCR {94°C (1 min) ↔ 72°C (1 min)}
Developing a SCAR Specific for *A. cochlioides*
A Single PCR Product (SCAR) is Diagnostic of *Aphanomyces euteiches*
Soil microbes tested with SCARs

*Aphanomyces euteiches*  21
*Aphanomyces cochlioides*  8
*Phytophthora infestans*  2
*Pythium ultimum*  3
*Pythium aphanidermatum*  1
*Pythium dissoticum*  1
*Fusarium oxysporum*  4
*Fusarium solani*  2
*Thelaviopisis basicola*  2
*Rhizoctonia solani*  2
*Mycosphaerella pinodes*  1
*Achlyla spp.*  6
Multiplex PCR Demonstrates Species-specific Nature of SCAR Primers

Mixture  A. cochlioides  A. euteiches
SCAR Primers can Detect *A. euteiches* in Infected Roots
Detection of A. euteiches in Field Grown Plants
SCAR Primers can Detect *A. euteiches* in Organic Debris Fraction of Field Soil
Possible Applications for SCARs

- **Qualitative**
  - Detection of pathogen in soil samples
  - Detection of pathogen in infected tissue
  - Discriminate between *Aphanomyces* spp.

- **Quantitative**
  - Compare pathogen colonization/movement between different plant genotypes.
  - Indirect selection for resistance among heterogeneous populations