Identification of fungal biotrophy genes by insertional mutagenesis of the crucifer anthracnose pathogen, *Colletotrichum higginsianum*

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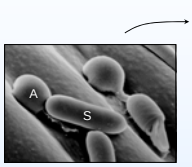
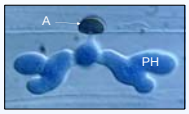
Introduction

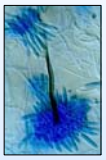
The ascomycete fungus *Colletotrichum higginsianum* invades *Arabidopsis* plants by a two-stage, hemibiotrophic infection process. Our aim is to identify fungal genes required for the initial biotrophic phase. A library of over 8,850 random insertional mutants has been generated in *C. higginsianum* using *Agrobacterium*-mediated transformation. So far, 8,200 mutants have been screened and 48 (0.5%) mutants with defects in pathogenicity have been identified.



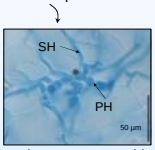
Spore (S) lands on leaf surface, germinates and forms a melanised appressorium (A)



Appressoria (A) penetrate directly to form primary biotrophic hyphae (PH) restricted to one epidermal cell

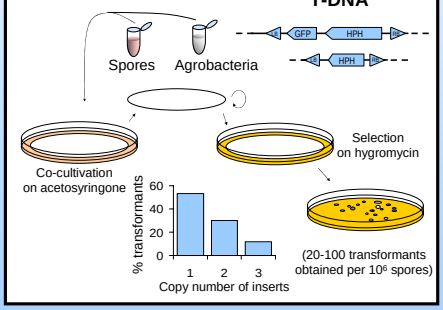


Sporulating acervuli emerge from necrotic lesion



Secondary necrotrophic hyphae (SH) spread to several cells, macerating plant tissue

Transformation



Mutant screen

Primary screen

Epifluoresecence microscopy

droplet-inoculated 10 day old Ler-0 seedlings

Secondary screen

Symptoms and light microscopy

Spray-inoculated (5x10⁵ spores/ml) 4 week plants

Characterisation

Cytological

- Growth rate *in vitro*
- Microscopy of infection
- Invasive growth ability in wounded tissue
- Penetration of cellophane
- Host responses (stain with aniline blue for callose, DAB for H_2O_2)
- Fungal cell wall defect (stain with Congo Red and Calcofluor White)
- Infection of cabbage (true host) and *Arabidopsis* mutants

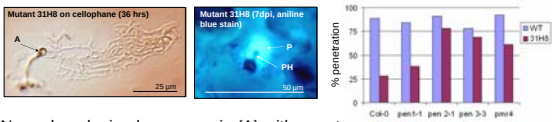
Molecular

- Determine copy number of T-DNA insertions (Southern blot)
- Identify flanking region (TAIL-PCR or Inverse PCR)
- Isolate gene by screening genomic library
- Homology searches and motif prediction
- Expression analyses
- Functional analysis: targeted gene disruption

Cytological characterisation of pathogenicity mutants

Infection stage blocked	Number of mutants
Germination	1
Appressorium melanisation	5
Host penetration (papillae)	2
Host penetration (no visible host responses)	4
Primary hyphae arrested in HR cells	11
Primary hyphae arrested (no host responses)	3
TOTAL	30

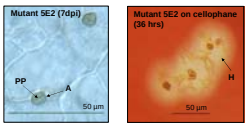
Penetration defect and abnormal papilla induction



- Normal, melanised appressoria (A) with penetration pores
- Penetration and growth inside cellophane membranes
- Rare penetration of leaf, limited to a small peg-like primary hypha (PH)
- Abundant host papilla (P) induction
- Penetration ability partially restored on *pen2, pen3* and *pmr4* mutants

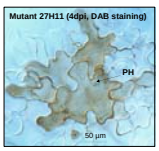
Penetration defect and no host responses

- Normal, melanised appressoria (A) with penetration pores (PP)
- No penetration of host cell wall
- Penetrates cellophane, halo of cellulose degradation (H)



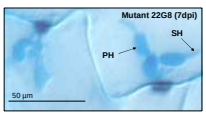
Primary hyphae arrested in HR cells

- Forms primary hyphae (PH) inside an epidermal cell
- Induction of HR in penetrated cell and adjacent epidermal and mesophyll cells



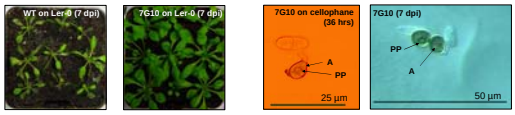
Primary hyphae arrested without host response

- Penetrates cellophane and host cell wall
- Rare and slow development of secondary hyphae (SH), despite normal growth rate *in vitro*
- No obvious callose deposition or HR



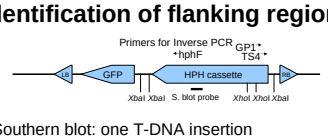
Characterisation of mutant 7G10

1. Characterization of the phenotype



- Non-pathogenic on mature plants
- Normal melanised appressoria (A) with penetration pores (PP)
- No penetration of cellophane or of the plant cell wall

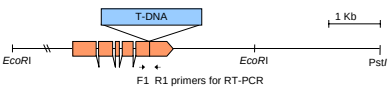
2. Identification of flanking region



- Southern blot: one T-DNA insertion
- 3 Kb sequence of flanking region obtained by Inverse PCR

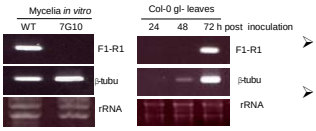
3. Screening of genomic library to identify gene

- Cosmid library (6000 clones with ~30 Kb insertions giving ~4x genome coverage) screened using a 400 bp probe derived from the flanking region gave 4 positive clones



- 786 bp ORF, 4 introns
- Encodes 262 aa
- No homology to known genes

4. Expression analyses (RT-PCR)



- Mutant 7G10 does not express identified gene *in vitro*
- WT expresses identified gene *in planta*

Outlook

- Continue screening up to 10,000 insertional mutants
- Characterisation of mutated genes in pathogenicity mutants
- Study gene function by targeted gene disruption using an approach based on transposon-arrayed gene knock-outs (TAGKO)

Selected references

O'Connell R. J, Herbert C., Sreenivasaprasad S., Khatib M., Esquerré-Tugayé M.-T., Dumas B. *Mol. Plant-Microbe Interact*. **17**, 272 (2004).
Perfect S. E., Hughes H. B., O'Connell R. J., Green J. R. *Fungal Genet. Biol*. **27**, 186 (1999).
Shimada C., Lipka V., O'Connell R.J., Okuno T., Shulze-Lefert P., Takano Y. *Mol. Plant-Microbe Interact*. **19**, 270 (2006)
Tsuji G., Fujii S., Fujihara N., Hirose C., Tsuge S., Shiraishi T., Kubo Y. *J. Gen. Plant Pathol*. **69**, 230 (2003).

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Marie Curie Actions
Human Resources and Mobility Activity

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