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Development of markers for detection of *Fusarium oxysporum* f. spp. *melonis* and *niveum*

Werkbespreking R&D Nakt uinbouw 19.2.15

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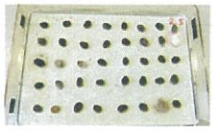
Introduction

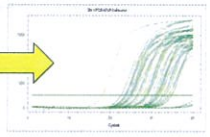
- *Fusarium oxysporum* f.sp. *melonis* and f.sp. *niveum* are pathogens of melon and watermelon respectively.

- Seed-transmitted
  Long term consequences of spread (wider rotation, soil disinfection, grafting)
- Moderate interest in the seed industry for seed testing (Nakt offers test)
- Proper control in seed production?
  - Dry heat treatment of the seeds?
  - Fungicide coating of seed? [benzimidazoles]

Benefits of the PCR tool

- Replacing pathogenicity assay





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Ultimately for use in multiplex detection of pathogens

- Tool for a future all-inclusive PCR for cucurbits
  - *A. citrulli*
  - *P. s. lachrymans/peponis*
  - *Didymella bryoniae*
  - *Fusarium oxysporum*
  - Viruses (CGMMV, MNSV, SqMV)
  - ?
- *Fusarium solani* f.sp. *cucurbitae* out of scope; *F.oxysporum* considered to be of higher concern

Pathogenicity assays

- Lengthy (several weeks), labour intensive; hard to control / reproduce...esp. for watermelon
- But accurate strain characterization prerequisite for marker development

Catch 22:

no good pathogenicity assay without a good marker;

no good marker without a good pathogenicity assay

Published markers

Paper	Target	PCR ID	f.sp.	validation
Zhang et al. (2005) FEMS Microbiology Letters 249 39–47	rDNA, ITS	Fn-1/Fn-2 (SYBR Green)	niveum	7 Chinese strains, large outgroup
Lin et al. (2010) New Biotechnology 27, 4	Anon. (RAPD)	Fon-1/Fon-2 (gel-based)	niveum	Taiwanese isolates, 5 ATCC strains from outside of Taiwan
López-Mondéjar et al. (2012) Crop Protection 33 1-6	Anon. ("SCAR")	FOX S/FOX R (Taqman)	melonis	7 Spanish strains; cross-reaction with other f.spp.
Lievens et al. (2009) FEMS Microbiol. Lett. 300 (2), 201-215	SIX (effector gene)		lycopersici	to be done for melon! (niveum?)

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SIX6 (continued)

- Lievens et al. 2009.
  Of the 7 SIX *Fol* genes homologues only found in several formae speciales for SIX6

SIX6 Pos	Six6 Neg
lycopersici (many)	*radicis lycopersici* (many)
melonis (1)	*niveum* (3) (2/4) Ellis CSIRO Plant Pathol. 60 (2), 232
radicis cucumerinum (23/28)	*radicis cucumerinum* (5/28)
niveum (2/4) Ellis CSIRO Plant Pathol. 60 (2), 232-243 (2011)	*cucumerinum* (many)
vasinfectum (Australian) (19 2 VCGs) Ellis CSIRO Plant Pathol. 60 (2), 232-243 (2011)	*conglutinans* (1) *cubense* (3) *asparagi* (3)
passiflorae (3) Ellis CSIRO Plant Pathol. 60 (2), 232-243 (2011)	*dianthi* (2), *gladioli* (3), *lilii* (2) *luffae* (2), *opuntiarum* (3), *spinaciae* (3), *tulipae* (3)

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SIX6

- 568 bp fragment, primers designed by Harrie Koenraadt
- Lievens et al. (2009) different primers, 973 bp amplicon
- Lievens, B., Houterman, P. and Rep, M. Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discrimination from other formae speciales FEMS Microbiol. Lett. 300 (2), 201-215 (2009)
- SIX6 diagnostic test for Austr. isolates of *F. o.* f.sp. *vasinfectum*
  Chakrabarti, A., Rep, M., Wang, B., Ashton, A., Dodds, P. and Ellis, J. Variation in potential effector genes distinguishing Australian and non-Australian isolates of the cotton wilt pathogen *Fusarium oxysporum* f.sp. *vasinfectum* Plant Pathol. 60 (2), 232-243 (2011)

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Strains for (further) validation of candidate markers

- "AFLP collection"
  - f.sp. *melonis*: 4 AFLP groups: C (12), D (1), F (6), H (1) Σ=20
  - f.sp. *niveum*: 5 AFLP groups: B (4), C (1), E (14), F (2), G (1) Σ=21
- Isolates from commercial melons in Southern Europe
  - 53 f.sp. *melonis*
  - 14 non-pathogenic *F. oxysporum*
- Non-pathogenic *Fusarium oxysporum* from melon seeds
- *Fusarium oxysporum* f.sp. *niveum* being characterized
- Collection strains

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Pathogenicity determination

- Pathogenicity testing on fully susceptible assay plant
- Strains of all known races of f.sp. melon included

Results Fn1-Fn2 primers

- Many non-pathogenic *F. oxysporum* strains react with Fn-1/Fn-2 PCR
- This was observed by Lin et al. in their study as well
- Validation of Fn-1/Fn2 PCR will no longer be pursued
- Multicopy target attractive

Results AFLP collection with 3 PCRs

AFLP group	# total	# FOX S / FOX R	# SIX6	# Fon-1 / Fon-2
F. o. melonis				
C	12	12	11	0
D	1	1	1	0
F	6	0	6	0
H	1	0	1	0
F. o. niveum				
B	4	0	0	0
C	1	1*	1*	0
E	14	0	12	14
F	2**	0	1**	1**
G	1	0	0	0

* Original strain from CBS negative for both

**Same strain is negative for both SIX6 and Fon-1/Fon-2

Result "melon collection" plants Southern Europe and seed isolates

Source and identity	# total	# FOX S / FOX R	# SIX6	# Fon-1 / Fon-2
Plants				
F. o. melonis	53	51	53	ND?
F. oxysporum	14	1	0	ND?
Seed				
F. oxysporum	112	1	0	ND?

SIX6 (+ FOX S/FOX R) promising for melonis, but all diversity covered?

- Kerry O'Donnell et al. (2009)
  A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex
  Fungal Genetics and Biology 46, 936–948
- 850 isolates typed, 101 EF-1a, 203 IGS rDNA, and 256 two-locus sequence types (STs)
- *melonis* 18 isolates, 5 haplotypes (STs)
- *niveum* 5 isolates, 4 haplotypes (STs)
- 3 *melonis*, 4 *niveum* DNA preps received
  - one *melonis* DNA prep negative with SIX6; corresponds to CBS strain

F. oxysporum f.sp. *melonis* CBS strains

- All SIX6 negative (and FOX S FOX R negative)
- All non-pathogenic....
- Four SIX1 positive

ST	NRRL					
165	36472	CBS 420.90	F. oxysporum f. sp. melonis race 0		Cucumis melo	Israel
167	36473	CBS 421.90	F. oxysporum f. sp. melonis race 0		Cucumis melo	Israel
165	36474	CBS 422.90	F. oxysporum f. sp. melonis race 0		Cucumis melo	Israel
167	36475	CBS 423.90	F. oxysporum f. sp. melonis race 2	ICMPP 10416	Cucumis melo	Israel
167	36476	CBS 424.90	F. oxysporum f. sp. melonis race 1.2		Cucumis melo	Israel

Pathogenicity assay CBS strains (De Ruiter Seeds)

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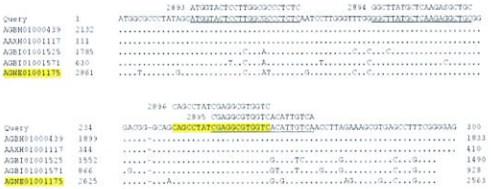
Primers for SIX1 targeting conserved region



Query: F. o. f.sp. lycopersici SIX1

AGNED1001175: F.o. f.sp. melonis

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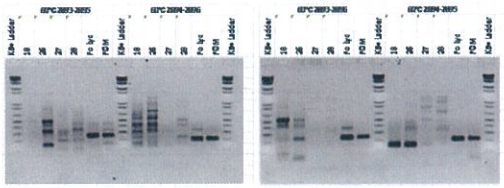
SIX1 primer screening of F. o. f.sp. niveum (SIX6 neg.)



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Conclusions and discussion

- SIX6 is a very promising candidate marker for *F. oxysporum* f.sp. *melonis* (confirmed by M. Rep, unpublished)
- FOX S / FOX R PCR does not pick up *F. oxysporum* f.sp. *melonis* belonging to two AFLP groups, but may have additional value for SIX6 negatives in other groups (one strain)
- Is SIX6 unstable if *F. oxysporum* f.sp. *melonis (and niveum)* is propagated outside the host? Or is additional marker (SIX1) needed?
- Fon-1 / Fon-2 and SIX6 reactions are correlated in *F. oxysporum* f.sp. *niveum* AFLP groups, but *F. oxysporum* f.sp. *niveum* group(s) appear to exist that lack both markers.

Conclusions and discussion

- Are SIX genes present in non-pathogenic F. oxysporum? (False-positives.)
- A conclusion on acceptability of marker will have to account for some risk: pathogen/non-pathogen distinction (assay plant treatment, assay plant type, incubation conditions/humidity, effect of strain culturing on media); collection always limited.
- But balanced by benefits of screening larger numbers of seed/suspects.

Acknowledgements

- All suppliers of DNA to the AFLP project
- Jessica Oosterhof (Rijk Zwaan) for mediating and providing DNA
- Remco Hulst for running PCRs and pathogenicity assays

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Schmidt et al. *BMC Genomics* 2013, **14**:119

- While some ff. spp. are monophyletic, others are composed of several clonal lineages that appear to have independently acquired the ability to infect the same host plant [4-6]. This polyphyletic origin was likely caused by horizontal transfer of chromosomes encoding host specific virulence genes between *Fo* lineages, thereby allowing the distinction of members of a f. sp., not by overall genetic relatedness, but by the presence or absence of certain LS chromosomes [1].

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