PLANT RESEARCH INTERNATIONAL
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Eindverslag

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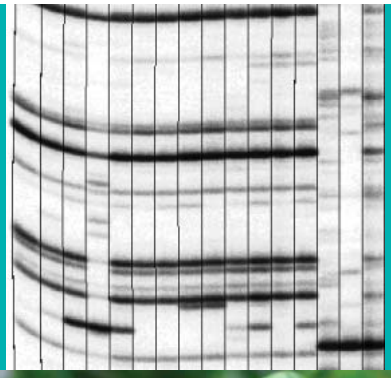
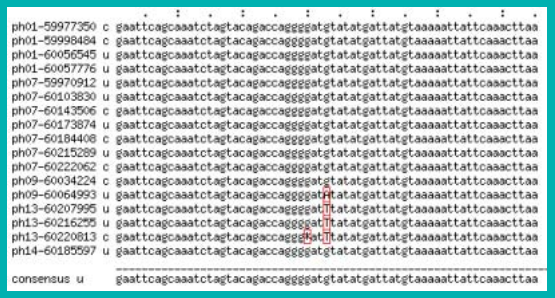
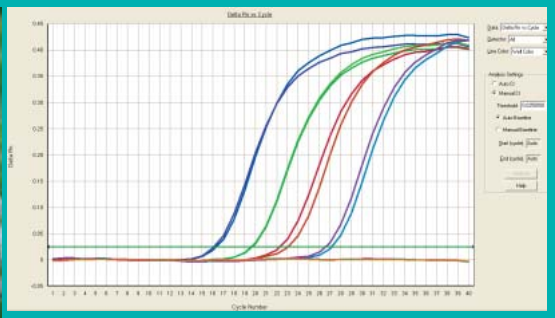
Nummer: ond/2006/04/01

Theo van der Lee, Marga van Gent-Pelzer & Peter Bonants







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T.b.v. Directie Landbouw Ministerie van LNV
Plantenziektenkundige Dienst

Plant Research International B.V., Wageningen
September 2009

Rapport 288

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Inhoudsopgave

1. Management samenvatting

Van de plantpathogene schimmels *Puccinia horiana* (chrysantenroest) en *Synchytrium endobioticum* (wratziekte in aardappel) komen verschillende fysio's voor die bepalend zijn voor de virulentie op een specifiek chrysanten- of aardappelras. De huidige biotoetsen (toetsen op een set van rassen/cultivars) om fysio's te bepalen zijn traag en onzeker en daarom is er behoefte aan een moleculaire toets om fysio's van elkaar te onderscheiden. Voor *P. horiana* is met behulp van CRoPS van 308.000 AFLP fragmenten de sequentie bepaald. Deze sequenties zijn geassembleerd tot 16.196 contigs met in totaal 229.126 sequenties. In 184 van de 16.196 contigs (1 %) werd tenminste 1 SNP gevonden. Na screening van slechte assemblies veroorzaakt door de aanwezigheid van repeats en selectie op basis van onderscheidend vermogen over de fysio's bleven 32 loci over waarvan de meeste een betrouwbaar onderscheid maken tussen één of meerdere fysio's. Zoals verwacht voor klonaal vermeerderende pathogenen gaat het in alle gevallen om merkers die heterozygoot zijn (ab versus aa). Vergelijkbaar kon met behulp van CRoPS voor *Synchytrium endobioticum* de 243.000 sequenties geassembleerd worden tot 14.660 contigs waarvan 85 (0.6%) een of meerdere SNP's bevatte. Na filtering van slechte repetitieve sequenties en selectie op basis van onderscheidend vermogen over de fysio's bleven slechts 9 informatieve loci over. Voor beide pathogenen zijn voldoende polymorfe merkers te genereren om via b.v. een PCR strategie toekomstige isolaten te scoren. Zo kan moleculair vastgesteld worden tot welke klonale lijn de isolaten behoren en kan door associatie het betreffende fysio worden bepaald. De doeltreffendheid van deze voorspelling zal nog wel getoetst moeten worden door nieuw ingebracht materiaal dat gekarakteriseerd is middels een fysio toets. Daarnaast kunnen de gevonden merkers gebruikt worden in populatiestudies die het mogelijk maken de verspreiding van de twee quarantaine pathogenen te monitoren.

2. Inleiding

Van plantpathogene schimmels komen dikwijls verschillende vormen voor, zogenaamde 'fysio's', die al dan niet in staat zijn bepaalde plantensoorten of bepaalde rassen van een gewas te infecteren. Voorbeelden hiervan zijn de quarantaine schimmels *Puccinia horiana* (chrysantenroest) en *Synchytrium endobioticum* (wratziekte in aardappel). De huidige bio-toetsen (toetsen op een set van rassen/cultivars) om fysio's te bepalen zijn traag en onzeker. De behoefte aan (snelle en betrouwbare) moleculaire toetsen om fysio's van elkaar te onderscheiden is daarom groot.

Het in dit project uitgevoerde onderzoek beoogde opheldering omtrent de volgende vragen:

a. Wat is er reeds aan informatie bekend over het optreden van genetische verschillen tussen fysio's bij andere schimmels?
b. Welke schimmelstructuren komen in aanmerking voor DNA-extractie? Welke methoden zijn er voor het verzamelen van zuiver schimmelmateriaal?
c. Welke opties zijn er voor extractie en zuivering van DNA uit schimmelstructuren, vrij van planten-DNA?
d. Welke gebieden op DNA kunnen als mogelijk target dienen voor de ontwikkeling van specifieke toetsen?
e. Welke routinematig toepasbare technieken kunnen worden ingezet om fysio-specifieke merkers op te sporen?

3. Doelstelling

Doel van het onderzoek is het opsporen van stabiele moleculaire merkers voor fysio's voor de ontwikkeling van snelle identificatietoetsen voor de schimmels *Puccinia horiana* (chrysantenroest) en *Synchytrium endobioticum* (wratziekte in aardappel).

4. Verslaglegging

Aan de opdrachtgever is 3 maal een tussenrapportage van het project verstrekt.
Eindrapportage betreft de periode: november 2008 t/m juni 2009.

5. Begeleidingscommissie

De begeleidingscommissie (BGC) van het project is in totaal 4 maal bij elkaar geweest om de voortgang van het project te bespreken: 1 februari 2007, 31 januari 2008, 12 november 2008 en 4 juni 2009
De commissie bestond uit:

- Dhr. G. van Leeuwen (PD)
- Mw. L. Kox (PD)
- Dhr. P. van den Boogert (PD)
- Dhr. H. de Gruyter (PD)
- Dhr. B. Brandwagt (Royal van Zanten)
- Dhr. A. Post (Deliflor)
- Dhr. H. Dresselhuys (Fides)
- Dhr. N. de Vetten (Avebe)
- Mw. J. Peltjens (HLB)
- Dhr. L. Turkensteen (HLB)
- Mw. M. Boerma (HLB)
- Dhr. G. Jongedijk (Naktuinbouw)

Verslagen van deze BGC bijeenkomsten zijn door de secretaris van de BGC (Dr G. van Leeuwen) toegezonden.

6. Praktische vragen

1. De beschikbaarheid en huidige karakterisatie van het quarantaine materiaal van de schimmels *Puccinia horiana* (chrysantenroest of Japanse roest) en *Synchytrium endobioticum* (wratziekte in aardappel).
2. De methodologie voor de isolatie van zuiver DNA voor het uitvoeren van het onderzoek naar moleculaire specificiteit tussen fysio's.
3. De moleculaire basis voor het ontstaan van fysio's en de moleculaire variatie die aanwezig is tussen verschillende fysio's.

7. Resultaten

Onderdeel A: Literatuurstudie

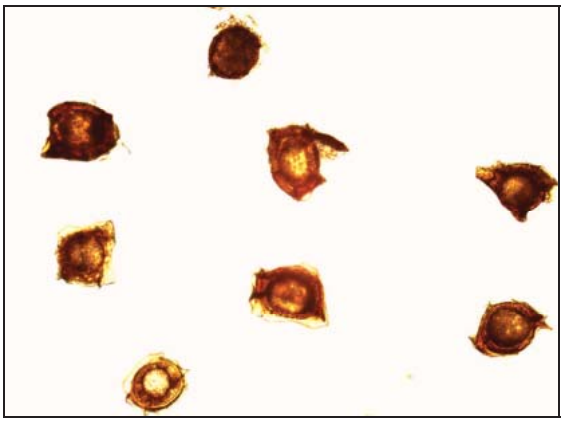
Wat is er reeds aan informatie bekend over het optreden van genetische verschillen tussen fysio's bij andere schimmels?

Voor onderdeel A is een literatuurstudie uitgevoerd die reeds is toegezonden en bij deze eindrapportage is deze met enkele aanvullingen en correcties toegevoegd als bijlage (bijlage A)

Onderdeel B: Materiaal

Welke schimmelstructuren komen in aanmerking voor DNA-extractie? Welke methoden zijn voorhanden voor het verzamelen van zuiver schimmelmateriaal?

Voor *Synchytrium endobioticum* is gebruik gemaakt van vers wratmateriaal uit Spiekerman- of de Lemmerzahltoets of uit wratmateriaal verkregen uit potproeven en van wintersporen verkregen uit gedroogd wratmateriaal. Uit al dit materiaal kan relatief zuiver *Synchytrium endobioticum* DNA verkregen worden zonder remmende factoren in de PCR. De eerder ontwikkelde methodieken voor het opzuiveren van wintersporen uit dit materiaal was zeer effectief. Wel bleek dat elk DNA preparaat ook variabele hoeveelheden aardappel DNA kunnen bevatten en daarnaast eveneens variabele hoeveelheden 'ander' DNA mogelijk afkomstig van bacteriële infecties. Vanwege het heterogene karakter van deze contaminaties is kwantificatie hiervan lastig. Wel is duidelijk dat dit geen belemmering vormde voor de verdere analyses.



Figuur 1. *Fractie met wintersporen van S. endobioticum (200x vergroot) opgezuiverd volgens de procedure van den Boogert et al (2005).*

Voor *Puccinia horiana* is gebruik gemaakt van teliosporen en basidiosporen. Hierbij was een sterk verschil in de AFLP fingerprint patronen waar te nemen. Hoewel het verkrijgen van basiodiosporen bijzonder arbeidsintensief is, is gekozen voor basidiosporen omdat de AFLP profielen van DNA geïsoleerd uit basidiosporen er kwalitatief beter uitzagen.

Onderdeel C: DNA analyse

Welke opties zijn er voor extractie en zuivering van DNA uit schimmelstructuren, vrij van planten-DNA?

Wratziekte

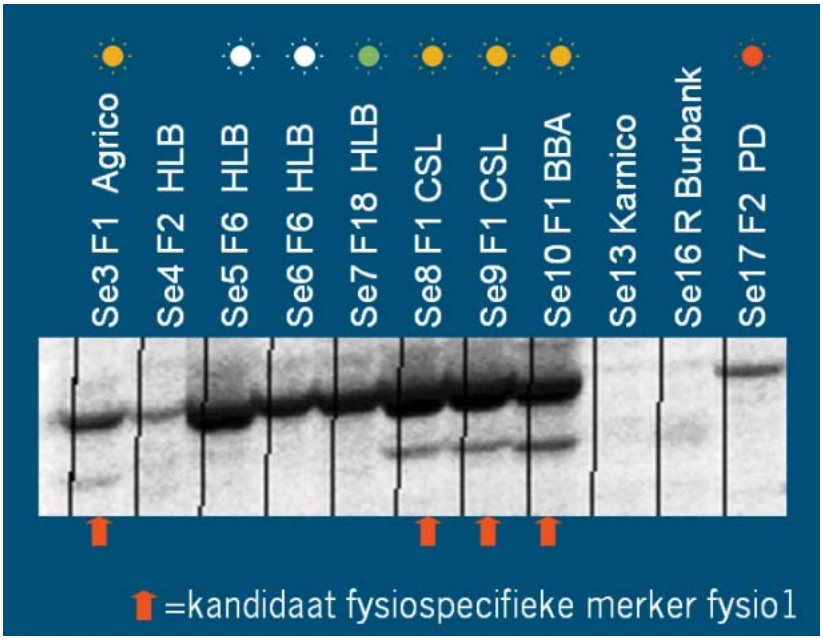
De productie van wratmateriaal werd verzorgd door het HLB (Wijster). Dit laboratorium heeft ook veel ervaring met betrekking tot wratziekte. Gekozen werd voor het vatbare ras Markies, waar een grote hoeveelheid knollen van beschikbaar was. Er zijn 18 verschillende isolaten van wratziekte (zie Tabel 1) ingezet op dit vatbare ras onder geconditioneerde omstandigheden. In mei 2008 is de proef geoogst en er zijn ook ditmaal minimaal wratten gevormd. Om toch aan voldoende materiaal te komen is ook wratmateriaal aangevraagd en verkregen via HLB, Averis, BBA (Duitsland) en CSL (Engeland). Hieruit is volgens een beschikbaar protocol DNA geïsoleerd. In Tabel 1 zijn de hoeveelheden DNA beschreven.

Tabel 1. Beschikbaarheid van DNA hoeveelheden van wratziekte materiaal.

SE	Fysio		Isolaat	Cultivar	Biotoets	Wrat	WS	DNA
	Oud	Nieuw				gram	aantal	ng
1	18	T1	Zweden	Markies	Spieckeman	8.5	5000	150
2	1	D1	Limburg, NL	Markies	Spieckeman	11.3	7500	300
3	1	D1	Agrico	Markies	Spieckeman	12	100000	1000
4	2	G1	BBA F2 09/04	Tomensa	Potproef	41	600000	15000
5	6	O1	HLB P6 (O1) 02-06	Russet Burbank	Potproef	72	300000	7500
6	6	O1	BBA F6 01/05 teelt 8.3	Russet Burbank	Potproef	36	50000	1250
7	18	T1	HLB P18 (T1) -02-06	Kamico	Potproef	44	300000	7500
8	1	D1	Dry Brown wart		?	3.3	600000	800
9	1	D1	Fresh Green	King Edward	?	5	250000	600
10	1	D1	BBA F1		Lemmerzahl?	13	15000	625
11	2	G1	BBA F2		Lemmerzahl?	9.4	20000	250
12	18	T1	BBA F18		Lemmerzahl?	11.5	10000	300
13				Kamico				1000
14				Markies				750
15				Tomensa				750
16				Russet Burbank				1500
17	2	G1	1987 Son4	Deodara 2004	Spieckeman	100	1000000	5000 MQ MQ

Het beschikbare DNA is getest met een interne controle PCR en geen van de DNA preparaten vertoonde remming in de PCR. Met behulp van een TaqMan PCR voor aardappel DNA en een TaqMan PCR voor *Synchytrium endobioticum* DNA kon aangetoond worden dat de geïsoleerde DNA preparaten naast aardappel DNA ook verschillende hoeveelheden *Synchytrium endobioticum* DNA bevatten. Van de beschikbare DNA preparaten is door Keygene AFLP analyse uitgevoerd. In eerste instantie zijn van al deze DNA preparaten AFLP patronen gegeneerd met de enzymcombinatie EcoRI-MseI. Deze lieten echter geen verschil zien tussen aardappel en wratmateriaal. Met andere

woorden er konden geen of weinig *Synchytrium endobioticum* banden worden waargenomen. Opmerkelijk omdat vanuit de kwantitatieve TaqMan analyse duidelijk was dat de hoeveelheid aardappel DNA hooguit 20% van het totaal was en in alle monsters *Synchytrium* DNA aanwezig was. Dit betekent dat de enzyme combinatie EcoRI-MseI preferentieel aardappel fragmenten amplificeert. Genoom analyse laat zien dat het aardappel genoom relatief AT-rijk is. MseI heeft een herkennings sequentie die volledig bestaat uit A en T (5'-TTAA-3'). Ook de herkennings sequentie van EcoRI is relatief AT-rijk (5'-GAATTC-3'). Gekozen is voor andere enzymcombinaties de een hoger GC percentage (BamHI, TaqI) hebben en naar enzymen die niet knippen bij methylatie van DNA en waarvan de restrictie sites in planten vaak gemethyleerd zijn (PstI en HpaII). Als beste combinatie kwam HpaII-TaqI naar voren; deze drukt de aardappelbanden vrijwel volledig weg. Deze nieuw AFLP applicatie hebben we target optimized of to-AFLP genoemd en kan ook voor andere obligate pathogenen van grote betekenis zijn om de achtergrond van de host in DNA preparaten te reduceren. Deze methode is uitgetest op de 9 DNA preparaten van wratmateriaal waarvan nog voldoende DNA beschikbaar was en 2 aardappelcultivars (Karmico en Burbank). AFLP patronen gegeneerd op deze 11 DNA preparaten met de enzymcombinatie HpaII-TaqI laten wel duidelijk verschillen zien tussen aardappel en wratmateriaal. Veel *Synchytrium* banden waren aanwezig in alle wratpreparaten. Ook waren er enkele verschillen tussen de fysio's onderling (<1 %) die voor een deel correleerde met bepaalde fysio's en dus potentieel als fysiospecifieke banden kunnen worden geïdentificeerd (zie Figuur 2). Na overleg met leden van de begeleidings-commisie is besloten 8 isolaten te selecteren die met de meer gedetailleerde CRoPS analyse werden geanalyseerd. Voor pathotype 1(D1) waren dit de isolaten SE3, SE8 SE9, SE10; voor pathotype 18 (T1) was dit isolaat SE 7; voor pathotype 6(O1) waren dit de isolaten SE5, SE6; en voor pathotype 2 (G1) was dit SE17. In de CROPS analyse worden de AFLP fragmenten niet gescheiden op grootte met behulp van gel electroforese maar worden alle AFLP fragmenten in een Roche GS-FLEX gesequenced op basis van de 454 technologie. Voor de CRoPS analyse zijn dezelfde templates als voor de AFLP gebruikt zodat (i) geen nieuw materiaal gegenereerd hoefde te worden en (ii) we isolaten met de beste AFLP patronen konden selecteren (iii) de CRoPS direct konden vergelijken met de AFLP analyse. Voor *S. endobioticum* is gebruik gemaakt van de Target optimized enzyme combinatie HpaII-TaqI met een G/T extensie om de complexiteit van het aantal verschillende fragmenten te reduceren. In totaal zijn voor *S. endobioticum* 243.440 goede sequenties gegenereerd met een gemiddelde lengte van 133 bp (voor trimming). Van alle isolaten zijn goede sequenties verkregen met een goede verdeling van de aantallen sequenties over de verschillende isolaten. Tabel 2 laat zien dat per isolaat tussen de 16.760 en 47.920 sequenties werden verkregen. Al deze sequenties zijn van goede kwaliteit en na verwijdering van de barcode/adaptor sequenties zijn deze gegroepeerd in clusters van gelijke sequenties (geassembleerd) waarna per isolaat gezocht is naar kleine sequentie verschillen die gecorreleerd zijn met een bepaald isolaat of groep van isolaten met hetzelfde fysio. In totaal is voor *S. endobioticum* zo'n 25 Mega base aan sequentie informatie gegenereerd. Voor *Synchytrium endobioticum* konden de 243.000 sequenties geassembleerd worden to 14.660 contigs waarvan 85 (0.6%) één of meerder SNP bevatten. Na screening van slechte assemblies, veroorzaakt door de aanwezigheid van repeats en selectie op basis van onderscheidend vermogen over de fysio's, bleven 9 informatieve loci over. Een voorbeeld hiervan is te zien in Figuur 3. Niet alle isolaten hebben echter voldoende sequenties om vast te stellen of in geval van heterozygotie het andere allel niet aanwezig is. Daarnaast dient opgemerkt te worden dat de isolaten die afkomstig waren van het CSL, en volgens de geldende karakterisatie fysio 1 zouden moeten zijn, genetisch meer afstaan van de overige Fysio 1 isolaten.



Figuur 2. *Detail van een AFLP profiel van diverse S. endobioticum isolaten. De isolaatnummers staan boven het profiel en een mogelijk specifieke merker voor fysio1 is aangegeven met de oranje pijl.*

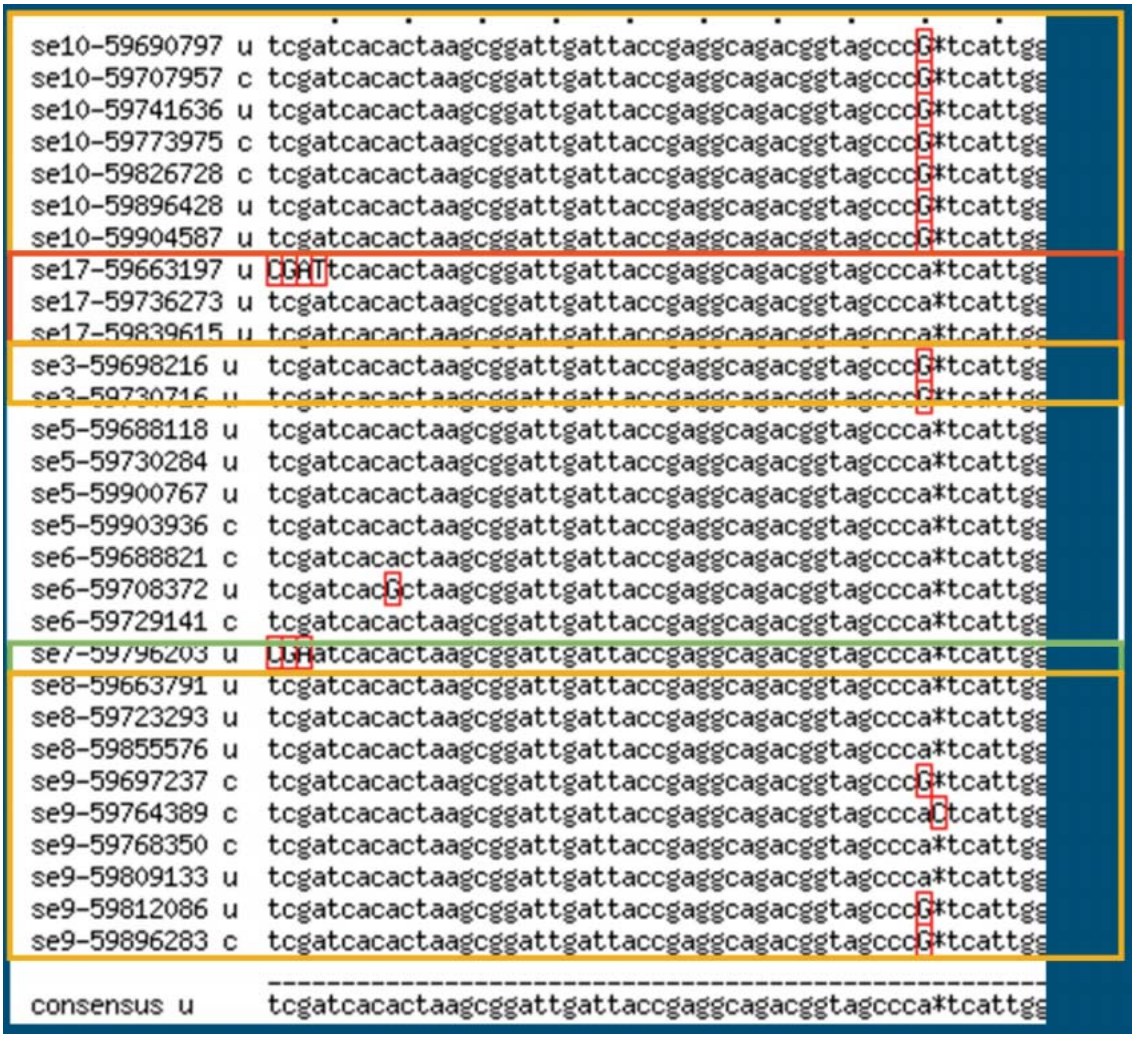
Tabel 2. *Aantal sequenties, kwaliteit en kengetallen van de clustering.*

	Puccinia horiana	*Synchritium endobioticum*
Parameters GS-FLX		
Enzyme combination	*Eco*RI/*Mse*I	*Hpa*II/*Taq*I
Primer combination	0/G	G/T
Average obtained read length (before trimming)	144.62	132.89
Trimming		
Total number of reads	304,406	241,290
Good reads*	308,410	243,440
Faulty reads*	6,821	7,727
Singletons reads	77,304	140,633
Chloroplast & mitochondrial reads	1,980	6,254
Good reads		
Sample Ph01/Se3	33,752	40,953
Sample Ph04/Se5	32,592	24,673
Sample Ph07/Se6	50,896	31,760
Sample Ph08/Se7	16,358	16,706
Sample Ph09/Se8	34,820	24,982
Sample Ph11/Se9	40,084	22,525
Sample Ph13/Se10	64,574	33,921
Sample Ph14/Se17	35,334	47,920
Clustering		
Alignments (= number of contigs)	16,196	14,660
Reads in alignments	229,126	96,553
Average # reads per alignment	14.14	6.59

Tabel 3. *Aantal polymorfe loci.*

	loci	SNP's	fysio gecorreleeerde SNP's
Synchytrium endobioticum	85	191	9
Puccinia horiana	184	414	32

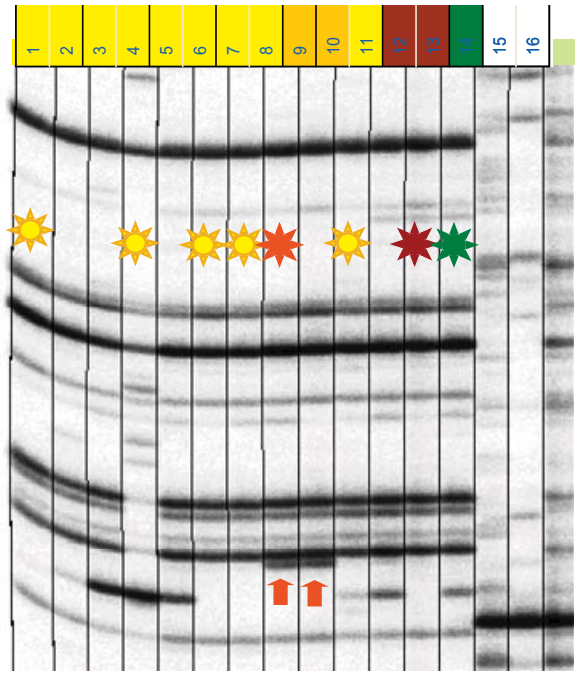
De SNP's in de clusters (contigs) werden opgespoord door specifieke software en algoritme die door Keygene ontwikkeld zijn. Alle SNPs komen van een cluster van op zijn minst twee genotypen met tenminste twee onafhankelijke sequentie bepalingen. De frequentie van een van de allelen moet tussen de 0.1 en 0.9 liggen terwijl voor tenminste één genotype het betreffende SNP homozygoot is.



Figuur 3. *Cluster van sequenties van de verschillende isolaten van S. endobioticum gecodeerd links met hierin aangeven de verschillende fysio's zoals ook te vinden in Figuur 2. De SNP's die het onderscheid maken tussen de isolaten van een verschillend fysio zijn aangegeven met behulp van rode blokken.*

Japanse Roest

Met betrekking tot het tweede pathogeen (Japanse roest, *Puccinia horiana*) zijn DNA monsters verkregen uit proeven van een Belgisch onderzoeksinstituut (ILVO, Merelbeeke). Dit betreft met name het goed karakteriseren van beschikbare Japanse roest isolaten om te achterhalen of er fysio's bestaan bij Japanse roest. Van deze isolaten zijn door ILVO basidiosporen van Japanse roest verzameld vrij van chrysant materiaal. Uit deze basidiosporen zijn dan volgens protocol de DNA preparaten bereid (Tabel 3). De verkregen DNA preparaten zijn vervolgens naar Keygene gebracht voor AFLP analyse. AFLP patronen gegeneerd door Keygene met de enzymcombinatie EcoRI-MseI laten duidelijke verschillen zien tussen chrysant en Japanse roest materiaal. Met andere woorden er konden duidelijke *Puccinia horiana* banden worden waargenomen Er zijn nu vier fysio's bekend van Japanse roest. Van 11 isolaten is voldoende en kwalitatief goed DNA verkregen voor een AFLP analyse (zie Figuur 4). Van een aantal isolaten is de analyse in duplo uitgevoerd. Hierbij bleek dat hetzelfde isolaat in één geval een verschillend bandenpatroon liet zien in twee runs (AFLP). Op basis van de intensiteit, isolatie datum en sequentie analyse is geconcludeerd dat dit werd veroorzaakt door schimmel contaminanten (bijv. een saprofytische gist soort die op chrysant groeit en mogelijk op de postules aanwezig is). Dit soort contaminanten kunnen zich in het niet steriele uitgangsmateriaal ophopen. De fragmenten van deze schimmel contaminanten zijn echter gemakkelijk te herkennen en verstoren de AFLP-analyse niet. Ook in het geval van *Puccinia horiana* is de genetische diversiteit laag en werden enkele polymorfe AFLP fragmenten gevonden die correleren met een specifiek fysio. Veel Japanse roest banden waren aanwezig in alle basidiosporen preparaten. Ook waren er enkele verschillen tussen de fysio's onderling (<1 %) die voor een deel correleerde met bepaalde fysio's en dus potentieel als fysiospecifieke banden kunnen worden geïdentificeerd. Na overleg met leden van de begeleidingscommissie is besloten 8 isolaten te selecteren die met de meer gedetailleerde CRoPS analyse zijn bestudeerd. Voor *Puccinia horiana* waren dit: Fysio 1 Ph 301, Ph 707, Ph SGP UK, Ph Spalding UK, Ph Poland, Fysio 2 Ph PD 20; Fysio 3 Ph Japan 2; Fysio 4 Ph Japan 3. Voor *P. horiana* konden de 308.000 sequenties geassembleerd worden tot 16.196 contigs met in totaal 229.126 sequenties. In 184 van de 16.196 contigs (1 %) werd tenminste 1 SNP gevonden. Na screening van slechte assemblies veroorzaakt door de aanwezigheid van repeats en selectie op basis van onderscheidend vermogen over de fysio's bleven 32 loci over waarvan de meesten een betrouwbaar onderscheidt maken tussen een of meerdere fysio's een voorbeeld is te zien in Figuur 5. In alle gevallen gaat het om merkers die heterozygoot zijn (ab versus aa).

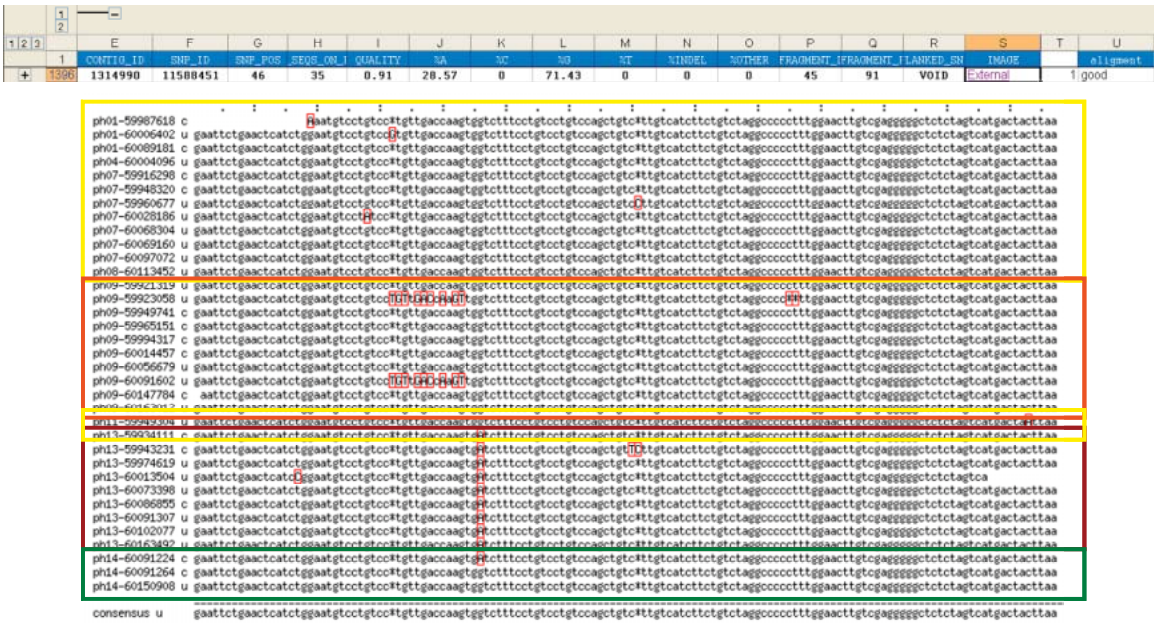


1	Ph 301
2	Ph 308
3	Ph 522
4	Ph 707
5	Ph 707 TS
6	Ph Redhill UK
7	Ph SGP UK
8	Ph Spalding UK
9	Ph PD 20
10	Ph PD 20
11	Ph Poland
12	Ph Japan 2
13	Ph Japan 2
14	Ph Japan 3
15	chrysant Medonia
16	chrysant Taliedo

Figuur 4. *Detail van een AFLP profiel van P. horiana isolaten. De isolaatnummers staan boven het profiel en een mogelijk specifieke merker voor fysio2 is aangegeven met de oranje pijl.*

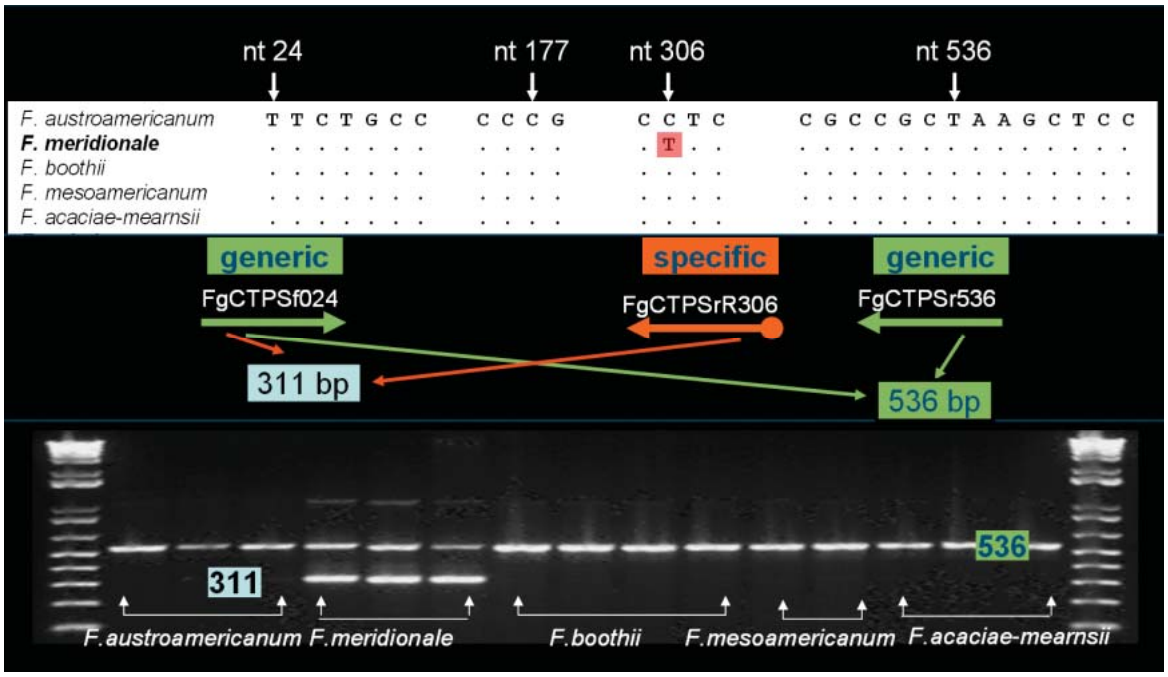
Tabel 3. *Beschikbare DNA preparaten van Japanse Roest. Kleuren geven verschillen in fysio aan.*

1	Ph 301
2	Ph 308
3	Ph 522
4	Ph 707
5	Ph 707 TS
6	Ph Redhill UK
7	Ph SGP UK
8	Ph Spalding UK
9	Ph PD 20
10	Ph PD 20
11	Ph Poland
12	Ph Japan 2
13	Ph Japan 2
14	Ph Japan 3
15	chrysant Medonia
16	chrysant Taliedo



Figuur 5. *Cluster van sequenties van de verschillende isolaten van P. horiana gecodeerd links met hierin aangeven de verschillende fysio's zoals ook te vinden in Figuur 4. De SNP's die het onderscheid maken tussen de isolaten van een verschillend fysio zijn aangegeven met behulp van rode blokken.*

8. Vervolg



Figuur 6. Voorbeeld van de detectie van SNP's met behulp van de duplex competitie PCR strategie.

In Figuur 6 is een voorbeeld gegeven hoe SNP detectie met behulp van duplex competitie PCR wordt uitgevoerd. Van een genomische regio worden twee geconserveerde regio's gebruikt voor een set van generieke primers welke altijd een PCR fragment zullen geven. Daarnaast wordt voor de SNP een specifieke primer ontworpen die eindigt op de onderscheidende base. Als de specifieke SNP niet matched met de gebruikte primer zal een generiek fragment worden verkregen. Dit generieke fragment dient als PCR controle en verlaagt de kans op aspecifieke priming van de specifieke primer. Als de specifieke primer matched met de sequentie dan zal een tweede korter fragment gegenereerd worden. De score gebeurt vervolgens op basis van de aanwezigheid van een enkele of twee banden. Dit kan op een agarose gels zoals hier weergegeven of met behulp van de smeltcurve in een real-time PCR apparaat.

9. Conclusies

Met betrekking tot *Synchytrium endobioticum* (wratziekte in aardappel) kan worden gesteld dat door goede samenwerking en extra inspanning uiteindelijk voldoende wrat materiaal verkregen is. Wel is het zo dat het materiaal uit het VK mogelijk niet op dezelfde manier gekarakteriseerd is. Dit betekent dat de fysiotypering van de isolaten uit het VK voorzichtig moet worden omgegaan. Uit al dit materiaal is kwantitatief en kwalitatief goed DNA verkregen met een lage contaminatie van aardappel DNA. Met behulp van dit DNA zijn goede AFLP profielen verkregen die het *Synchytrium endobioticum* DNA konden genotyperen. De CRoPS analyse van een subset van deze monsters resulteerde in een groot aantal sequenties die na clustering 9 potentiële sequentie verschillen geven die correleren met de verschillende fysio's. Daarnaast zijn er nog enkele andere sequentie verschillen die mogelijk correleren met specifieke fysio's maar waarvan te weinig sequentie informatie voorhanden is om homo- of heterozygotie vast te stellen of doordat de fysio karakterisatie niet onder dezelfde condities is uitgevoerd.

Met betrekking tot *Puccinia horiana* (chrysantenroest of Japanse roest) is door de goede samenwerking met het ILVO en Nederlandse Chrysanten veredelaars voldoende materiaal van een diverse set van *Puccinia horiana* isolaten verkregen. Van dit materiaal is door het ILVO kwantitatief en kwalitatief goed DNA verkregen zonder achtergrond van chrysant DNA. Met behulp van dit DNA zijn goede AFLP profielen verkregen die het *Puccinia horiana* DNA konden genotyperen. De CRoPS analyse van een subset van deze monsters resulteerde in een groot aantal sequenties, die na clustering 32 potentiële sequentie verschillen geven die correleren met de verschillende fysio's.

Identificatie van merkers

Deze verschillen kunnen door diverse technieken waaronder de duplex strategie (Figuur 6) omgezet worden in merkers voor de praktijk. Hiervoor is het wel noodzakelijk om van meer monsters van gekende fysio de gevonden correlatie met de fysio-specificiteit te valideren. Daarnaast kunnen de gevonden merkers gebruikt worden voor populatie studies. Voor beide pathogenen werden potentiële sequentie verschillen gevonden terwijl dit maar voor één van de twee pathogenen voorzien was. Dit succes is met name te danken aan de goede samenwerking van de partners in het project en de lagere kosten van de moleculaire analyse.

10. Acknowledgements

De AFLP patronen en de CRoPS analyse zijn uitgevoerd onder leiding van Hanneke Witsenboer werkzaam bij KeyGene die in dit project een belangrijke rol vervulde als subcontractor.
Samenwerking met Gerard van Leeuwen (PD), Ronald Bosch, Pim van de Griend en Douwe Werkman (HLB), Kurt Heungens en Mathias De Backer (ILVO, Merelbeelke) werd zeer op prijs gesteld. Voor het verkrijgen van goed uitgangsmateriaal m.b.t. *Puccinia horiana* willen we graag de Nederlandse Chrysanten veredelaars bedanken voor hun inzet. De discussie met leden van de begeleidingscommissie is zeer gewaardeerd en zij worden daarvoor hartelijk bedankt.
Daarnaast willen we Bas Brandwagt en Gerard van Leeuwen bedanken voor hun suggesties, aanvullingen en correcties op dit rapport.

11. Budget project

Er is veel budget en tijd besteed aan het verkrijgen van voldoende materiaal van wratziekte materiaal en Japanse Roest materiaal. De vervolgactiviteiten (DNA isolatie en AFLP) zijn derhalve vooruit verschoven.
Uiteindelijk zijn de zuiverheids analyses, kwantificatie, AFLP en CRoPS analyses en SNP mining voor beide pathosystemen uitgevoerd. Dit terwijl dit in het oorspronkelijke project slechts voor één van beide pathosystemen was voorzien. Deze extra resultaten konden worden verkregen door een efficiënte samenwerking met de leden van de begeleidingscommissie en internationale partners (met name het ILVO) en verlaging van de kosten van moleculair onderzoek. Zo bleef het project met de literatuur studie en de rapportages binnen het gestelde budget.

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Plant Research International BV
25 September 2009

Bijlage I.

Literature Study on the occurrence of races in several pathosystems including *Synchytrium endobioticum* - potato and *Puccinia horiana* - chrysanthemum

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Abstract

This literature study deals with the occurrence of pathosystems and mainly two obligate pathogens: *Synchytrium endobioticum* (wart disease) and *Puccinia horiana* (chrysanthemum rust). Both pathogenic fungi are suspected to consist of several races. Therefore in this study the following topics are addressed:

- What is known on the mentioned pathogens?
- What is race specificity?
- Molecular basis of race specificity: the Avr-R gene model
- Lack of DNA homology but consensus in effector model
- Loss of avirulence / Functional polymorphism
- Association genetics: Tracing identity by descent
- Novel Molecular techniques
- Concluding remarks
- Literature

Synchytrium endobioticum (wart disease)

The chytridiomycete *Synchytrium endobioticum* (Schilbersky) Percival is an obligate plant pathogenic fungus that is able to cause wart disease on tubers stolons and young stems of potato in cool, moist climates world wide. The fungus exhibits an obligate bio-interaction with plant tissue via zoospores for infection and resting sporangia or winterspores for survival. Upon infection, the fungus induces its host to produce wart-like outgrowth (warts) on tubers, stolons and sprouts. Warts consist of hypertrophic host cells each containing a single sporangium which may lead to infectious zoospores (Hampson, 1985; Hampson *et al.*, 1994). In senescing wart tissue infectious zoospores are no longer produced and thick-walled resting sporangia or winterspores are formed in stead. These winterspores are able to survive inter-host periods for up to 20 years (Hampson, 1993).
S. endobioticum was first found in 1876 in the UK (Langerfeld, 1984; Hampson, 1993). It gradually spread over Europe and was first observed in The Netherlands in 1915 (Anon., 1921). Nowadays, potato wart disease is reported in 15 European countries (EPPO, 2005). Originally, only one pathotype of the pathogen occurred in Europe, and a good level of control was achieved using resistant potato cultivars. Since 1941, however, new pathotypes have shown up (Blattny, 1942; Braun, 1942), making efficient control of the disease more troublesome. The first discovery of a new pathotype in Europe occurred in former East Germany (the German Democratic Republic), where new pathotypes were named by the first letter of the locality where they were found, followed by a sequential number (e.g. pathotype G1, found at Giessbel). In former West Germany (the German Federal Republic), however, Arabic numbers were used for new pathotypes (Ullrich, 1958; Langerfeld & Stachewicz, 1993; Hampson, 1993). In obligate pathogens, identification of pathotypes is usually based upon differential reaction to a strictly defined set of cultivars (Wicker *et al.*, 2003; Trimboli, 2004). For *S. endobioticum*, different countries have used different sets of cultivars, making sound comparisons of pathotypes among countries hardly possible (Table 1).
Several pathotypes have a considerably wider virulence spectrum than others and these broadly virulent pathotypes pose a serious threat for potato production in northwest Europe (Baayen *et al.*, 2004).
For pathotyping, different countries have used different sets of differential cultivars, and the usual system of numerical coding of pathotypes has not been consistently followed. In a review by Baayen *et al.* (2006) a new standardized code was proposed to be used for the 43 pathotypes currently known and described in Europe, while in the Netherlands only race 1(D1), 2(G1), 6(o1) and 18(T1) occur.
S. endobioticum is a quarantine organism (EPPO A2 status) and control is achieved by application of race specific resistant potato cultivars or by restricting potato production on contaminated land (Hampson, 1993). Key issue in the application of race specific resistant cultivars is the determination of the race contaminating the field in order to select a resistant cultivar. This race determination is done by a bioassay according to Spieckermann or Lemmerzahl described in the EPPO protocol. These tests are costly, results not always conclusive and can only be performed during the winter season. Therefore new methods using molecular markers are needed to unambiguously determine the race at low cost. The genetic differences between isolates of *S. endobioticum* is expected to be small and relatively few DNA sequences for *S. endobioticum* or related organisms are available.

Table 1.

Pathotype Cultivar	1(D1)	2(G1)	6(O1)	8(F1)	18(T1)
Deodara, Tomensa, eersteling	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Producent, Combi	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
Saphir	Resistant	Susceptible	Resistant	Resistant	Resistant
Delcora	Resistant	Resistant	Resistant	Susceptible	Susceptible
Miriam	Resistant	Resistant	Resistant	Resistant	Susceptible
Belita, Karolin, Ulme	Resistant	Resistant	Resistant	Resistant	Resistant

Synchytrium endobioticum is placed in the following evolutinairy branch: cellular organisms; Eukaryota; Fungi/Metazoa group; Fungi; Chytridiomycota; Chytridiomycetes; Chytridiales; Synchytriaceae; Synchytrium. At Genebank two DNA Sequences are currently deposited (access NCBI December 2007) both are partial sequences of the 18S Ribosomal subunit. The closest relative for which the whole genome was sequenced is *Batrachochytrium dendrobatidis JEL423*. which also belongs to the Chytridiomycetes. The genome was sequenced by the Broad Institute. *Batrachochytrium dendrobatidis* strain JEL423 has a diploid genome and was sequenced at a depth of 7.4X genome coverage using whole genome shotgun (WGS) sequencing. The current assembly (Assembly 1) consists of 348 nuclear genome contigs in 69 supercontigs, and 3 mitochondrial genome contigs in 1 supercontig. The genome assembly will undergo automated annotation. This shows that for *S. endobioticum* new DNA markers and DNA sequences are needed for molecular and evolutionairy studies that focus on the molecular bases of race specificity.

Puccinia horiana (chrysanthemum rust or white rust)

Puccinia horiana is an obligate plant pathogenic fungus that causes rust disease on Chrysanthemum. Chrysanthemum (Chrysanthemum × morifolium Ramat.) is of major importance in the (cut) flower industry worldwide, and its many characteristics have mainly been improved through conventional breeding programs. Within Chrysanthemum x morifolium, some types have been bred that can are commercialized as cut flowers, pot plants or garden mums (multiflora types). For all types, several European breeding companies produce propagating material. They introduce new cultivars on the market on an annual basis. White rust, caused by *P. horiana* Henn, is the most serious fungal disease in the culture of garden mums. *P. horiana* is a quarantine organism (EPPO A2 status) and control is achieved by application of fungicides.

Within the species three races have been identified that show different symptoms on a differential set of Chrysanthemum cultivars (de Backer, pers. comm.). In the Netherlands several races may occur. This race determination is done by a bioassay, which is costly and the results are not always conclusive. Therefore new methods using molecular markers are needed to unambiguously determine the race at low cost. This would allow application of resistance in pest management. *Puccinia horiana* is placed in the following evolutinairy branch: cellular organisms; Eukaryota; Fungi/Metazoa group; Fungi; Dikarya; Basidiomycota; Pucciniomycotina; Pucciniomycetes; Pucciniales; Pucciniaceae; Puccinia. At Genebank two DNA Sequences are currently deposited (access NCBI December 2007) both are partial sequences of the cytb gene located in the mitochondrial genome. The closest relative for which the whole genome was sequenced is *Puccinia graminis* which also belongs to the Pucciniales. The pathogenic fungus *Puccinia graminis* causes stem rust in small cereal crops such as wheat, oat, rye, and barley. Stem rust, also referred to as black rust or black stem rust, is among the most destructive diseases of cereal crops and has resulted in enormous economic and yield losses. *Puccinia graminis* has a complex life cycle that includes five spore stages and two very different hosts, namely, a grass for the primary host and, most commonly, barberry as the alternate host. *Puccinia graminis* has a number of 'special forms', or *forma speciales* (f. sp.), which differ in their primary host specificities. For example, *Puccinia graminis* f. sp. *tritici* infects wheat. The *Puccinia graminis* haploid genome is estimated at 80 Mb, organized in 18 chromosomes.
In a PhD study at ILVO Merelbeeke and University of Gent (Belgium) Anar worked on *Puccinia horiana* and in his thesis (Alaei Shah Vali Anar, 2008) he described different aspects of this obligate pathogen including phylogenetic studies, molecular detection and identification of *Puccinia horiana*, disease symptom development and screening for resistance. Host resistance to white rust was first identified and studied by Baker (1967) in the UK. In addition, De Jong and Rademaker (1986) suggested that the resistance of chrysanthemums to white rust is usually controlled by a single dominant gene. Although a large proportion of the commercial cultivars were sensitive to this disease, several cultivars were reported free of infection while growing near to diseased plants (Baker, 1967; Dickens, 1968; Firman & Martin, 1968; Hahn, 1989; Martin & Firman, 1970; Orlikowski *et al.,* 1982; Sugimura *et al.*, 1998; Wojdyla 1999). It was evident from these studies that a significant difference in interaction phenotype exists between cultivars.
A reliable disease assessment method is crucial not only in the screening of germplasm for resistance, but also in research on pathogen biology and diversity, and in the genetic analysis of host resistance.

Recent data by Mathias De Backer (De Backer, pers. communication), who tested several individual isolates of *P. horiana* of different origin against a set of 36 potentially differential cultivars delivered by the Dutch breeding companies, would suggest that the majority of the current European isolates of *P. horiana* show very little differential reactions. However, at least one isolate was found that is considerably more virulent. This isolate was maintained at the Plant Protection Service in the Netherlands in a mixed *P. horiana* inoculum. This physiological race was able to overcome the resistance in some but not all cultivars. Therefore, it would appear that different versions of the major resistance gene might be present in the chrysanthemum germplasm. Given the international nature of the commercial trade, future breeding efforts should be geared at using the version that can not be overcome by any known isolate. Several other researchers have reported the existence of different physiological races to explain variation in the resistance (Baker, 1967; Dickens, 1971; Firman & Martin, 1968; Kudo & Okamura, 1998; Wojdyla 1999; Yamaguchi, 1981).

What is race specificity?

Many plant-pathogen interactions are governed by specific interactions between pathogen avirulence (*Avr*) genes and plant disease resistance (R) genes. Through products of R genes host plants are capable to recognize specific, pathogen-derived molecules and launch a defense response against the invader, including the hypersensitive response, in which a few plant cells at the site of infection die. These elicitors of resistance reactions are direct or indirect products of a microbial *Avr* gene. Lacking or disfunctioning of either *R* gene or *Avr* gene results in a compatible interaction (Keen, 1982). This cross talk between host and pathogen was assembled in the gene-for-gene model by Flor (1942), who derived the concept from his genetic work on the interactions between flax and flax rust. This high specificity appears to be prevalent in plant-pathogen interactions because the gene-for-gene model has explained the genetics of diseases caused by viruses, bacteria, fungi, oomycetes nematodes and insects.

Molecular basis of race specificity: the Avr-R gene model

In its simplest form, this model predicts the product of the *R* gene to be a receptor that perceives the product of the *Avr* gene. The number of host-pathogen relationships however, for which a direct interaction between R and *Avr* gene products has been detected, is still very limited. In fact, for most gene-for-gene relationships studied so far, experimental evidence is more consistent with indirect perception of an AVR protein by an R protein than with a direct physical interaction between these two proteins. This indirect perception implies that at least a third component is required for specific recognition of an avirulence factor by its resistant host (Luderer & Joosten, 2001).
In recent years, the guard model that was put forward by Van der Biezen & Jones (1998) has won ground particularly by studies on a few model pathosystems such as the interaction between tomato and the bacterial speck pathogen *Pseudomonas syringae*. This model proposes that the third component that is required for perception of an Avr protein is represented by the virulence target of the Avr protein. Binding of the Avr protein to its virulence target is perceived by the matching R protein, which is guarding this virulence target (Dangl & Jones, 2001; Luderer & Joosten, 2001; Van der Hoorn *et al.*, 2002; De Wit, 2002).
In the tomato-*Pseudomonas syringae* interaction, the avirulence gene *AvrPto* conditions avirulence on tomato carrying resistance gene Pto (reviewed by Luderer & Joosten, 2001). However, this latter encodes for a 164aa protein kinase (Martin *et al.*, 1993) and does not possess the main features of R genes like the LRR structure. The gene Prf, which codes for a cytoplasmic protein, NBS-LRR is required for the AvrPto/Pto interaction (Salmeron *et al.*, 1996) and it was suggested that Pto would be the third component that will form a complex with *AvrPto*. This latter will afterwards be recognized by the R gene which is Prf (Van der Biezen & Jones, 1998).

During the past years, a great number of R genes have been cloned. Most of them encode related proteins that are classified based on a set of characteristic structural features such as leucine-rich repeats (LRRs), nucleotide binding sites (NBS), leucine zipper or Toll/Interleukin-1 receptor-like domain and a transmembrane regions (Martin *et al.*, 2003). The predominant class of R proteins in gene-for-gene plant resistance belongs to the NBS-LRR type (Van Der Biezen & Jones, 1998; Dangl & Jones, 2001, Chrisholm *et al.*, 2006). The prevalence of NB-LRR proteins in various

plant species is consistent with their proposed function as adaptable surveillance molecules for rapidly evolving pathogens (Hammond-Kosack & Jones, 1996).
On the other hand, many bacterial *Avr* genes and a growing number of fungal and oomycete plant pathogens have been sequenced (Van't Slot & Knogge, 2002; Chrisholm *et al.*, 2006). Unlike the structural similarity of *R*-gene-encoded proteins, the pathogens Avr proteins or effectors are highly divergent (Luderer & Joosten, 2001; Van't Slot & Knogge, 2002) and no proper classification can be made according to common functional domains (Laugé & De Wit, 1998).
Nevertheless*, Avr* genes from eukaryotic plant pathogens exhibit a limited number of common structural and functional features. The majority of fungal *Avr* genes described to date encode for extracellular small proteins of unknown function containing a signal for secretion into the apoplast (Chrisholm *et al.*, 2006) and representing in total 15 *Avr* genes. Moreover, these *Avr* genes were isolated from no more than six fungal pathogen species (Gout *et al.*, 2006).
Many eukaryotic *Avr* genes, such as *Avr2*, *Avr4, Avr4E, Avr9 Ecp1, Ecp2, Ecp3, Ecp4, Ecp5* of *Cladosporium fulvum*, *Nip1* of *Rhynchosporium secalis* and *Avr-Pita*, *Avr-CO39*, *Pwl2* and *Ace1* of *Magnaporthe grisea* (Sweigard *et al.*, 1995; Dioh *et al.*, 2000; Jia *et al.*, 2000 and Farman *et al.*, 2002) and *AvrLm1* of *Leptosphaeria maculans* (Gout *et al.*, 2006) encode for small secreted proteins, with an even number of cysteine residues.
From oomycetes, 4 avirulenence genes are described *Avr3a* from *Phytophtora infestans* (Armstrong *et al.*, 2005), *Avr1b* from *Phytophtora sojae* (Shan *et al.*, 2004) and several from *Peronospora parasitica* such as *ATR13* and *ATR1* (Chrisholm *et al.*, 2006). For *P. infestans* several additional genes are identified or currently characterized (Avr1, Avr2, Avr4 ipi-O,. So far all *Avr* genes from oomycetes identified lack cysteine residues but are secreted and contain a RXLR-DEER motif which may help targetting of these proteins into the plant cell. The RXLR-DEER motif resembles the host targeting motif used by the malaria parasite Plasmodium (Rehmany *et al.*, 2005). The occurrence of a common motif in unrelated pathogens with a very different host range, plants and humans suggests a shared novel mechanism to interact with the eukaryotic host cells.
Several strategies have been used for cloning fungal and oomycete avirulence genes. As an example, the reverse genetics strategy using purified elicitor preparations as starting material was used in *C. fulvum* (*Avr2*, *Avr4*, *Avr4E*, *Avr9)* (Laugé & De Wit, 1998). For genetically more tractable fungi, such as *M. grisea* (Dioh *et al.*, 2000; Farman and Leong 1998), positional cloning appeared to be a more suitable approach. Such an approach has been also developed for major pathogens such as *P. infestans* (van der Lee *et al.*, 2001a) and *L. maculans* (Cozijnsen *et al.*, 2000; Gout *et al.*, 2006) and for cloning bacterial *Avr* genes, classical bacterial genetics such as genetic complementation proved to be very efficient (Van den Ackerveken & Bonas, 1997).

- **Interaction *Cladosporium fulvum* and tomato**

The interaction between tomato and the strictly apoplastic biotrophic pathogen *Cladosporium fulvum* complies with the gene-for-gene model (De Wit, 1992). To date, nine genes for *C. fulvum* have been cloned that encode for elicitor proteins. Four of these elicitors, Avr2, Avr4, Avr4E, and Avr9, are race-specific and induce a HR, visible as necrosis, following injection in tomato lines harbouring the corresponding *C. fulvum* (Cf) resistance gene (Luderer *et al.*, 2002).
In addition to the race-specific *Avr* factors, many other fungal low molecular weight peptides have been purified from apoplastic fluids from infected tomato leaves. Several of these corresponding Ecps (Extracellular Proteins, including Ecp1, Ecp2, Ecp3, Ecp4 and Ecp5) have been isolated and, except for Ecp3, the encoding genes have been isolated (Van den Ackerveken *et al.,* 1993; Laugé *et al.*, 2000). During pathogenesis, all strains of *C. fulvum* produce and secrete these Ecps abundantly.
All Cf genes are predicted to encode a receptor-like protein with an extracellular LRR region, a transmembrane domain with a short cytoplasmic tail with no homology to known signalling domains (Joosten & De Wit, 1999).

Avr2

The Cf-2 gene of tomato confers resistance to strains of the biotrophic pathogenic fungus *C. fulvum* carrying avirulence gene *Avr2*. Avr2 encodes for a 78aa cystein-rich protein containing a signal peptide of 20aa. The protein is secreted during the infection in the apoplastic fluid of tomato leaves (Luderer *et al.*, 2002). The Cf2 locus contain 2 genes Cf2-1 and Cf2-2 only differing in three aa (Dixon *et al.*, 1996) and both confer recognition of the same

avirulence protein Avr2. Furthermore, the RCR3 represents an additional plant protein that is specifically required for Cf2- mediated resistance (Luderer *et al.*, 2002). It has been shown that *Avr2* binds and inhibits the secreted tomato cysteine protease RCR3 thereby the formed RCR3-Avr2 complex enables the Cf2 protein to activate an HR. The role of Rcr-3 in the perception of *Avr2* by Cf2 is consistent with the guard hypothesis (Rooney *et al.*, 2005).
Analysis of strains that are virulent on Cf2 tomato lines revealed various independent frameshift mutations in the *Avr2* open reading frame and a point mutation resulting in a premature stop codon. All modifications result in the production of truncated Avr2 proteins. Interestingly, an additional modification involves the insertion of a LINE-like element identified in *C. fulvum* and provides the first example of loss of avirulence of a plant pathogen caused by the insertion of a retrotransposable element in an *Avr* gene (Luderer *et al.*, 2002).

Avr4

The avirulence gene *Avr4* conditions avirulence on tomato genotypes carrying resistance gene Cf4. The mature protein is about 86aa. The expression of Avr4 is very high during infection and is secreted in the apoplastic fluid. The gene is present in one copy in all *C. fulvum* strains either virulent or avirulent. The *Avr4* effector contains a chitin binding domain and is thought to shield the fungal cell wall from plant chitinases (Van den Burg *et al.*, 2003). Instead of deletion of the gene, circumvention of Cf4-mediated resistance was found to reside in single base pair changes in the ORF of the *Avr4* gene. However the gene is still expressed but its encoded protein is unstable and not recognized anymore by the host plant carrying Cf4 gene (Joosten *et al.*, 1997).

Avr9

The *Avr9* gene encodes a secreted peptide, the Avr9 elicitor that is extremely protease resistant and consists in its mature form of 28aa. The Cf9 gene confers resistance to strains that carry the *Avr9* gene. Strains of *C.fulvum* that are virulent on tomato containing the Cf9 resistance gene completely lack the *Avr*9 gene (Van Kan *et al.*, 1991). This deletion has revealed no pathogenicity role (Marmeisse *et al.*, 1993). However, the expression of Avr9 is induced during infection but also during nitrogen limitation condition (Van den Ackerveken *et al.*, 1994). Cf9 encodes for a protein with an extracellular LRR domain and no direct interaction between Avr9 and Cf9 has been demonstrated.

Ecp2

Inoculation of an Ecp2-deficient replacement mutant on susceptible tomato plants showed a reduced virulence and induced accumulation of pathogenesis-related (PR) proteins (Laugé *et al.,* 1997). Consequently, Ecp2 was proposed to play a role in virulence of *C. fulvum* by suppression of host defence responses and it has been shown that certain cultivars carrying the Cf-Ecp2 resistance gene are involved in the recognition of Ecp2. Hence, Ecp2 gene is simultaneously an avirulence factor and a pathogenicity factor (Laugé & De Wit, 1998).

- **Interaction *Melampsora lini* and Flax**

Several *Avr* genes such as *AvrM*, *AvrP4*, *AvrP123,* have been isolated from *M. lini*, which are recognized by the L5, L6, and L7 resistance genes. A cDNA marker cosegregating with the avirulence phenotype in an F2 rust family was used to identify a genomic DNA region from the avirulence locus.
The *M. lini* avirulence genes are expressed in haustoria and encode small secreted proteins. Recognition of these proteins occurs when they are expressed inside the plant cell, suggesting that they are delivered into host cells during rust infection (reviewed by Chrisholm *et al.*, 2006)

- **Interaction *Leptosphaeria maculans* and oilseed**

Leptosphaeria maculans is a dothideomycete that causes the stem canker on oilseed rape. To date, nine resistance genes have been identified and their corresponding nine avirulence genes (AvrLm1-9) in *L.maculans* have been mapped at four independent loci. Recently, through the map-based cloning strategy, *AvrLm1* was cloned. The gene is present as a single copy gene within a 269 kb non-coding region. The predicted AvrLm1 protein is composed of 205aa, of which only one is a cysteine residue and it contains a signal sequence suggesting extracellular localization (Gout *et al.*, 2006).

- **Interaction *Magnaporthe grisea*-Rice**

In *M. grisea,* more than 30 avirulence genes have been identified; among which nine have been mapped (Dioh *et al.*, 2000). Four of these genes have been isolated by positional cloning (*Avr-Pita*, *Avr-CO39, PWL2* and *Ace1*) (Sweigard *et al.*, 1995; Dioh *et al.*, 2000; Jia *et al.*, 2000 and Farman *et al.*, 2002).

Avr-Pita

The avirulence gene *Avr-Pita* is located in chromosome 1 at the tip of the teleomere. This gene encodes for a metalloprotease with an N-terminal secretory signal. The Pita gene located on chromosome 12 in rice confers resistance to strains that carry the *Avr-Pita* (Jia *et al.*, 2000). The direct interaction between Avr-Pita and Pita has been demonstrated by a two-yeast hybrid system. It has been proposed that Avr-Pita is secreted into the host cells during penetration and will penetrate the cytoplasm in which there is a direct interaction with Pita. *Avr-Pita* is expressed only at later stages of plant infection, suggesting a possible function of the protease after the pathogen is inside the plant (reviewed by Leach *et al.*, 2001).Complete deletion of the *Avr* gene in the virulent strain has been demonstrated and this phenomenon is thought to be frequent as a consequence of its sub-telomeric location (Orbach *et al.*, 2000).

ACE1

The avirulence gene ACE1 that interacts with the resistance gene Pi33 was isolated by positional cloning (Dioh *et al.*, 2000). It encodes a large multifunctional enzyme corresponding to a polyketide synthase fused to a signal peptide synthetase module (Böhnert *et al.*, 2004). The corresponding resistance gene, Pi33, was mapped on rice chromosome 8 (Berruyer *et al.*, 2003). It is frequently detected in semi-dwarf indica rice cultivars, which are extensively grown in Asia and South-America.

The molecular understanding of the function of the *Avr* genes in the disease process, apart from eliciting a defense response, is still unclear although mounting evidence attributes virulence and fitness functions to these genes (reviewed by Leach *et al.*, 2001). Hints of the potential function of some avirulence genes have recently emerged (Van't Slot & Knogge *et al.*, 2002) and a few of the examples, such as the Ecp2 (Laugé *et al.*, 1997), appear to act as virulence factors that enable the fungus to kill host cells and obtain nutrients or the NIP1(for necrosis-inducing protein) identified in the barley scald pathogen *Rhynchosporium secalis* that cause necrosis in a nonspecific manner and thus functions as toxin (Rohe *et al.*, 1995).

Lack of DNA homology but consensus in effector model

Currently 19 fungal/oomycetous *Avr* genes have been cloned from 7 pathosystems (Table 2) in additional a large number on *Avr* genes from bacteria have been cloned. As well as a set of effector proteins that could potentially be a avirulence gene but for which the matching R gene is lacking. The homology on the DNA level of these *Avr* genes even within a single species is low. In some species the *Avr* genes share some common features such as a signal peptide or a particular motif such as the RxXL motif that is shared by oomycete avr genes. The location of the protein can be the apoplast or the cytoplasm. In all studied cases however the AVR proteins seem to promote virulence and seem to interfere with the host physiology.

This lead to the hypothesis that plants exhibit a basic level of resistance against potential pathogens and that pathogens have evolved mechanisms to break down this resistance by specific effectors. Plants have countered this by development of proteins that survey (the guard model). In general the individual contribution of the effectors is low which indicates that they can be lost or mutated without strong effects on the virulence. However if the effector proteins are detected by the host a strong defense cascade is activated that will render the pathogen avirulent.

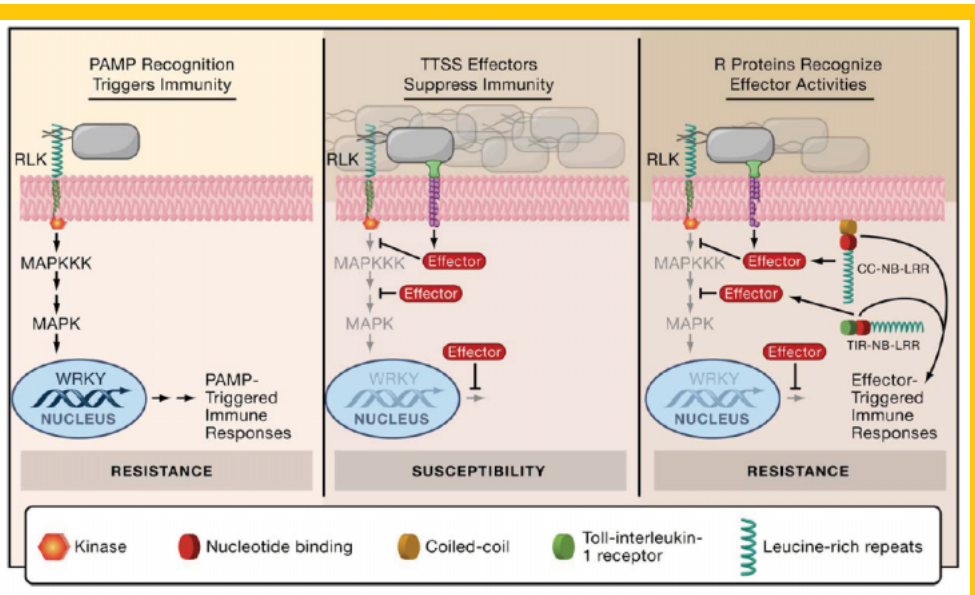

Figure 1. Model for the Evolution of Bacterial Resistance in Plants
Left to right, recognition of pathogen-associated molecular patterns (such as bacterial flagellin) by extracellular receptor-like kinases (RLKs) promptly triggers basal immunity, which requires signaling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors. Pathogenic bacteria use the type III secretion system to deliver effector proteins that target multiple host proteins to suppress basal immune responses, allowing significant accumulation of bacteria in the plant apoplast. Plant resistance proteins (represented by CC-NB-LRR and TIR-NB-LRR; see text) recognize effector activity and restore resistance through effector-triggered immune responses. Limited accumulation of bacteria occurs prior to effective initiation of effector-triggered immune responses.

Loss of avirulence/Functional polymorphism

With the cloning of the avirulence genes the allelic variation at these loci can now be studied. Although for many *Avr* genes no extensive population studies were performed (Gout, 2007). Many of the avirulence genes seem to be in telomeric regions associated with transposable elements. When the natural variation for these genes and the function was studied it appeared that in most cases the avirulence genes were lost. In these cases the gene was completely absent in the virulent isolates or large deletion were present that delete part of the gene. In few cases single nucleotide polymorphisms were found often leading to stop codons. In addition in some cases frameshifts were found. In oomycetes two *Avr* genes have the same coding sequence in avirulent and virulent isolates but the transcript level is much lower in the virulent isolate (AVr1b and Avr2). In other cases it was shown by antisense RNA that lower expression could result in virulent isolates. This means that all possible combinations were found but that deletion of the gene seems to be the dominant mechanism. Although the situation for *S. endobioticum* and *P. horiana* is yet unknown we can expect similar mechanism given the large evolutionary distribution of the studied pathogenes (ascomycetes, basidiomycetes, oomycetes).

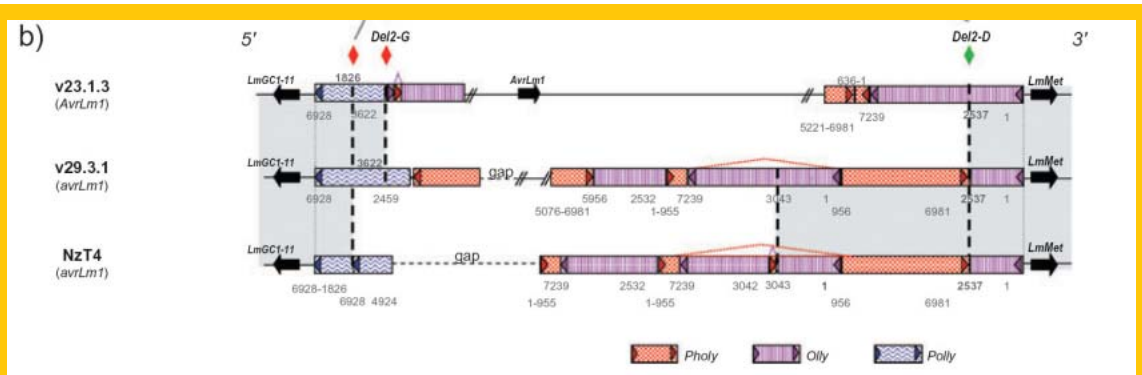

Fig. 2. Genomic organization of the *AvrLm1* region of *Leptosphaeria maculans* in one avirulent isolate (*AvrLm1* – v23.1.3) and two virulent isolates (*avrLm1* – v29.3.1 and NzT4).
A. Schematic representation of the structural organization of the whole *AvrLm1* genomic region. Black and white striped boxes represent gene-rich and repeat-rich chromosomal segments respectively. Regions of sequence identity between the three isolates are connected using dotted lines and shown as grey areas. Positions of some markers are indicated.
B. Organization of the REP2 fragment deletion borders. The plain black arrows indicate predicted coding regions and the *AvrLm1* gene. Boxes represent different LTR retrotransposons flanked or not by LTRs represented by small internal triangles. For each box, base pair numbers below the box indicate where truncation occurred as compared with the deposited sequence of the complete element. The plain red and green diamonds indicate the 5′ and 3′ deletion breakpoints, respectively, identified in the REP2 chromosomal segment following alignment with the sequences of REP2b and REP2c. The corresponding deletion markers *Del2-G* and *Del2-D* are also indicated. Grey areas connecting different contigs represent regions of sequence identity.

Table 2. Avirulence genes identified and the cause of its loss of avirulence function in virulent isolates.

Pathogen	Gene	Loss of avirulence fuction due to
C. fulvum	*Avr2*	SNP and line element insertion
C. fulvum	*Avr4*	various point mutations
C. fulvum	*Avr4e*	Deletion
C. fulvum	*Avr9*	Deletion
P. infestans	*Avr1*	ND
P. infestans	*Avr2*	reduced expression
P. infestans	*Avr3a*	SNP
P. infestans	*Avr4*	frameshift indel
F. oxysporum	*Six1*	G490A
L. maculans	*AvrLm1*	Deletion (260 kb) in >90%
L. maculans	*AvrLm6*	ND
L. maculans	*AvrLm4-7*	ND
P. sojae	*Avr1b-1*	numerous SNP's and reduced expression
R. secalis	*nip-1*	Deletion (>90%) SNP's 9 %
B. graminis	*Avr10a*	ND
B. graminis	*Avrk1*	ND
M. oryzae	*Avr-pita*	Deletion
M. oryzae	*Avr1-CO39*	Deletion
M. lini	*AvrM*	Deletion (230bp)
M. lini	*Avrp4*	SNP's

Association genetics: Tracing identity by descent

In many cases the number of virulent alleles is limited and seem to be associated with a single event. Occasionally this is followed by additional mutations. Such a single event means that the occurrence of virulence can be traced genetically by genealogy (Fig. 3). For instance if the virulence is found in a single clonal line any marker that distinguishes this clone from the other genotypes can serve as a marker for virulence. In this case linkage is independent from the relative position of the marker with respect to the avirulence gene. However, when

recombination occurs, for instance by genome instability or the occurrence of a sexual cycle, the marker should be close to the *Avr* gene preferable within the gene. The ease in which markers can be obtained depends on the genetic variation between the isolates and the marker system used. Novel molecular techniques allow sequencing of fragments thereby generating the highest differentiation possible. In principle sequencing allows the identification of any single point mutation within the genome thereby offering the highest resolution possible. As mentioned not much is known on the genetic variability for the isolates. When more information is available the matching technology can be selected.
Factors that affect the possibility to detect the differences are the genome flexibility, the population size and genetic diversity of the population. Selection pressure, Genetic drift, the gene flow and the frequency of the sexual cycle.

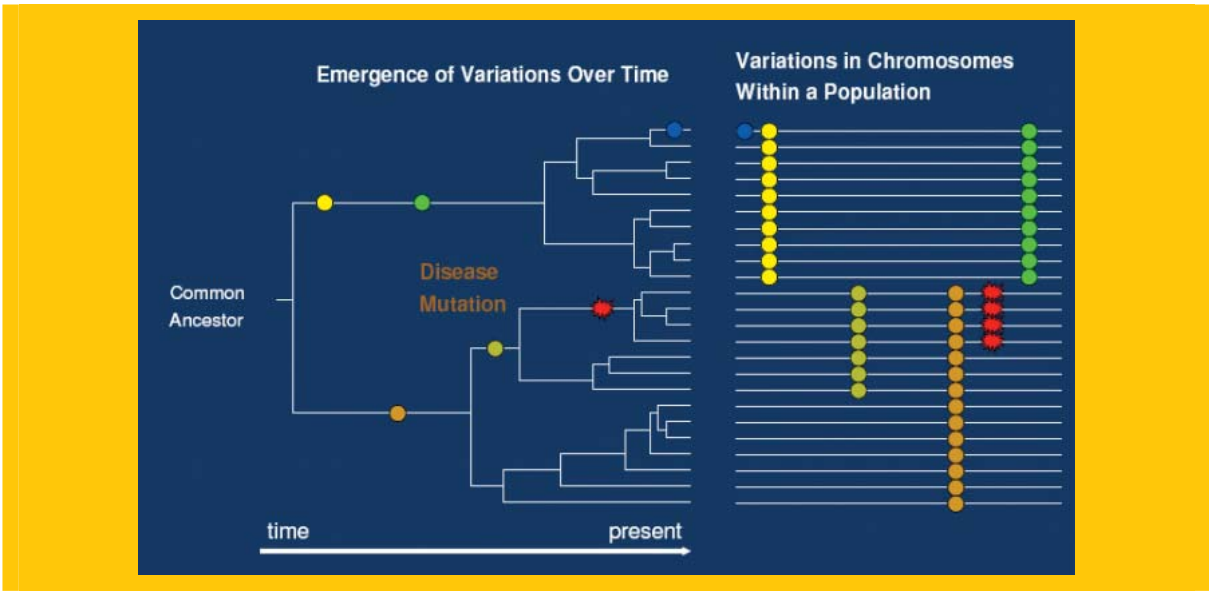

Figure 3. Identifications of genetic markers linked to a trait using genealogy.

Novel Molecular techniques

Roche 454 sequencing

454 Sequencing is a massively-parallel Pyrosequencing system capable of sequencing roughly 100 megabases of raw DNA sequence per 7-hour run of their current sequencing machine, the GSFLX. The system relies on fixing nebulized and adapter-ligated DNA fragments to small DNA-capture beads in a water-in-oil emulsion. The DNA fixed to these beads is then amplified by PCR. Finally, each DNA-bound bead is placed into a ~44 µm well on a PicoTiterPlate, a fiber optic chip. A mix of enzymes such as polymerase, ATP sulfurylase, and luciferase are also packed into the well. The PicoTiterPlate is then placed into the GS20 for sequencing.
At this stage, the four nucleotides (TAGC) are washed in series over the PicoTiterPlate. During the nucleotide flow, each of the hundreds of thousands of beads with millions of copies of DNA is sequenced in parallel. If a nucleotide complementary to the template strand is flowed into a well, the polymerase extends the existing DNA strand by adding nucleotide(s). Addition of one (or more) nucleotide(s) results in a reaction that generates a light signal that is recorded by the CCD camera in the instrument. This technique is based on sequencing-by-synthesis and is called, Pyrosequencing (Ronaghi *et al.*, 1996 and 1998). The signal strength is proportional to the number of nucleotides, for example, homopolymer stretches, incorporated in a single nucleotide flow. However, the signal strength for homopolymer stretches is linear up to eight consecutive nucleotides; signal fall-off is rapid after a stretch of more than eight identical nucleotides. In the procedure genomic DNA is fractionated into smaller fragments (300-500 base pairs) that are subsequently polished (blunted). Short adaptors are then ligated onto the ends of the fragments. These adaptors provide priming sequences for both amplification and sequencing of the sample-library fragments.

Adaptor B contains a 5'-biotin tag that enables immobilization of the library onto streptavidin coated beads. After nick repair, the non-biotinylated strand is released and used as a single-stranded template DNA (sstDNA) library. The sstDNA library is assessed for its quality and the optimal amount (DNA copies per bead) needed for emPCR™ is determined by titration. The sstDNA library is immobilized onto beads. The beads containing a library fragment carry a single sstDNA molecule. The bead-bound library is emulsified with the amplification reagents in a water-in-oil mixture. Each bead is captured within its own microreactor where PCR amplification occurs. This results in bead-immobilized, clonally amplified DNA fragments. The sstDNA library beads are added to the DNA Bead Incubation Mix (containing DNA polymerase) and are layered with Enzyme Beads (containing sulfurylase and luciferase) onto the PicoTiterPlate™ device. The device is centrifuged to deposit the beads into the wells. The layer of Enzyme Beads ensures that the DNA beads remain positioned in the wells during the sequencing reaction. The bead-deposition process maximizes the number of wells that contain a single amplified library bead (avoiding more than one sstDNA library bead per well).
The loaded PicoTiterPlate device is placed into the GS20 Instrument. The fluidics sub-system flows sequencing reagents (containing buffers and nucleotides) across the wells of the plate. Nucleotides are flowed sequentially in a fixed order across the PicoTiterPlate device during a sequencing run. During the nucleotide flow, each of the hundreds of thousands of beads with millions of copies of DNA is sequenced in parallel. If a nucleotide complementary to the template strand is flowed into a well, the polymerase extends the existing DNA strand by adding nucleotide(s). Addition of one (or more) nucleotide(s) results in a reaction that generates a light signal that is recorded by the CCD camera in the Instrument. The signal strength is proportional to the number of nucleotides, for example, homopolymer stretches, incorporated in a single nucleotide flow. The procedure is highly cost efficient and 40 Mb of sequence information can be generated in a single run for approximately 15.000 Euro. At this rate genome sequencing is much more affordable.

CRoPS

CRoPS also uses the Roche 454 technology only in this case the DNA is not sheared into small fragments but genomic representations are made by AFLP restriction digests (Fig. 4). Depending on the required complexity reduction the enzymes and selective bases are chosen. The adaptors that are attached to the fragments can contain additional sequence information and can thus label the different samples such as genotypic origin. This technology provides high resolution information on a limited set of fragments

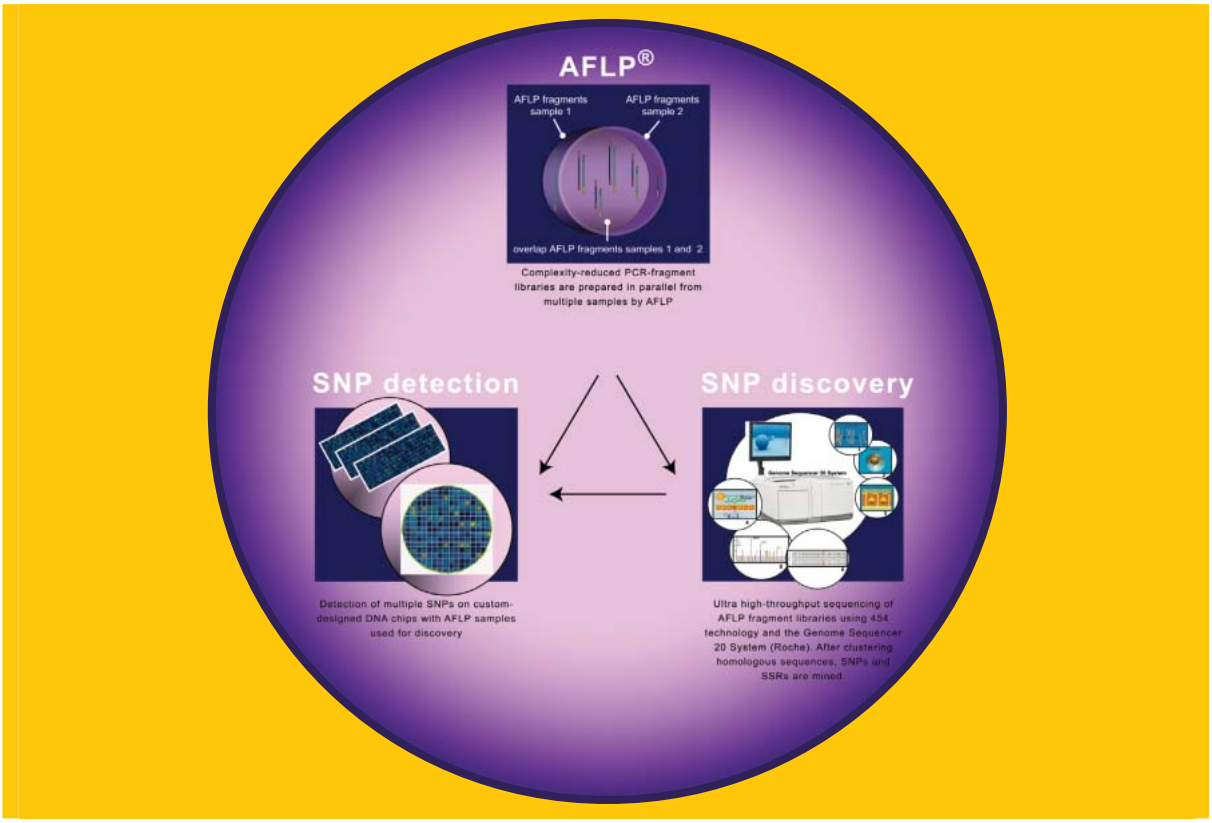

Figure 4. Application of the 454 technology to sequence genome representations generated by AFLP.

Solexa sequencing

Solexa sequencing uses a parallel sequence strategy starting from single molecules fixed on a solid glass slide (Fig. 5). In this procedure the DNA is fragmented in 150 bp fragments to which adaptors or ligated after which they are spotted on glass slides. DNA is amplified on the glass slide and sequenced directly (SOLEXA sequencing) the sequence results in short stretches of DNA (25-30 bp) from two directions (paired) that can be assembled on a template such as a previously assembled genome sequence of the organism or used for SNP detection. 10-20 X coverage. Regions that do not match loop out and can be assembled the novo. This procedure can not assemble repetitive regions, but the single copy, gene rich regions, can be determined at high confidence. Currently many fungi are sequenced by this approach. As the sequencing is performed by service labs such as Service Express no set- up costs are required. However, analysis of the results is complex and calls for specific expertise.
The procedure is highly cost efficient and 40 Mb of sequence information can be generated in a single run for approximately 15.000 Euro. At this rate genome sequencing is much more affordable.

For explanation on the SOLEXA sequencing technology check: http://www.illumina.com/pages.ilmn?ID=203

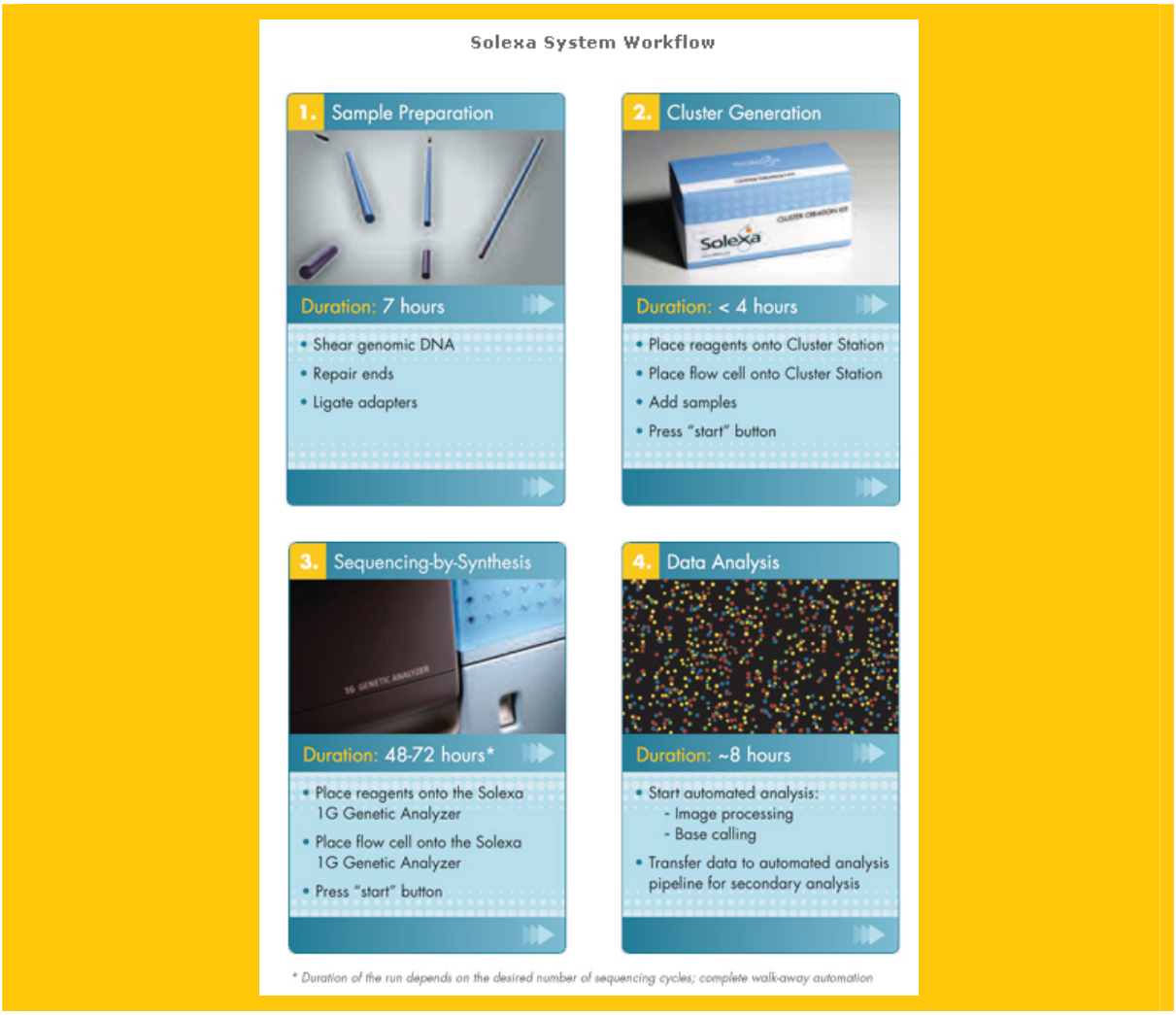

Figure 5. Description of the Solexa solid phase sequencing procedure.

Inventory available material

For *Synchytrium endobioticum* we were able to obtain 18 different isolates from 3 races
PPS and HLB delivered necessary material form different countries (Table 3). Those different isolates are inoculated on one susceptible cultivar (Markies) at HLB. If inoculation is successful DNA will be isolated from warts produced in May-June 2008.
For *Puccinia horiana* ILVO together with some Dutch chrysanthemum growers delivered 12 different isolates and more material will be sampled (Table 4). From research at ILVO it became clear that at the moment at least 3 races can be distinguished:

- 1 main race: race 1 ⇒ three cultivars from the set are susceptible, other cultivars are less susceptible or resistant.
- 2 other races (more aggressive; race 2 and 3). The mixed isolate of PPS belongs to this category. Unfortunately the history of this mixed isolate is unknown.

DNA of all isolates tested will become available in May 2008.

Table 3. Set of isolates available for Synchytrium endobioticum.

Nr	Fysio	location	source	Method
1	6(O1)	Drenthe, NL	PD	Spieckermann
2	6(O1)	Overijssel, NL	PD	Spieckermann
3	6(O1)	Drenthe, NL	PD	Spieckermann
4	6(O1)	Drenthe, NL	PD	Spieckermann
5	6(O1)	Drenthe, NL	PD	Spieckermann
6	18(T1)	Zweden	PD	Spieckermann
7	18(T1)	Drenthe, NL	PD	Spieckermann
8	18(T1)	Weser-Ems, DE	PD	Spieckermann
9	1(D1)	Limburg, NL	PD	Spieckermann
10	1(D1)	Limburg, NL	PD	Spieckermann
11	1(D1)	Drenthe, NL	PD	Spieckermann
12	1(D1)	Limburg, NL	PD	Spieckermann
13	1(D1)	BBA F1 01/05	HLB	Spieckermann
14	1(D1)	HLB P1 (D1) 01-05	HLB	Spieckermann
15	2(G1)	BBA F2 09/04	HLB	Spieckermann
16	2(G1)	BBA F2 01/05	HLB	Spieckermann
17	6(O1)	BBA F6 01/05	HLB	Spieckermann
18	6(O1)	HLB P6 (O1) 01-05	HLB	Spieckermann

Table 4. Set of isolates available for Puccinia horiana.

Nr	Fysio	Location	Naam ILVO	Source
1	1	Belgium	Ph 308	BE 2003 via ILVO
2	2	The Netherlands	Ph NL1	NL via PD (2006)
3	1	Belgium	Ph 301	BE 2003 via ILVO
4	1	Belgium	Ph 307	BE 2003 via ILVO
5	1	Poland	Ph Poland	PO via INSAD
6	1	United Kingdom	Ph Spalding	UK via CSL
7	1	Belgium	Ph 522	BE 2005 via ILVO
8	1	United Kingdom	Ph SGP UK	UK via CSL
9	1	United Kingdom	Ph Red UK	UK via CSL
10	3	Belgium	Ph 707	BE 2007 via ILVO

Concluding remarks

In this literature study race specificity is addressed. It describes the current knowledge on race specificity in several model pathosystems in relation to two specific obligate fungi: *Synchytrium endobioticum* and *Puccinia horiana*. It is generally accepted that in *Synchytrium endobioticum* and *Puccinia horiana* several races exists although the use of a specific set of differentials is internationally very difficult to achieve and therefore scorings of races are difficult to compare. However, we are confident that the race determination for both *Synchytrium endobioticum* and *Puccinia horiana* is uniform because the bioassays were performed either at a single laboratorium or in two laboratoria using the same procedures. These scoring are also relevant for Dutch practices as the methods set the standard in The Netherlands and isolates come from different countries in Europe. Another difficult aspect is to get several isolates of the same race due to their obligate nature. A limited set of isolates belonging to different races are available for DNA genotyping in order to identify race specific markers.

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