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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS
Geneva

**WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES
AND DNA-PROFILING IN PARTICULAR**

Fourteenth Session
Seoul, Republic of Korea, November 10 to 13, 2014

MOLECULAR MARKERS AS PREDICTORS FOR 'TRADITIONAL' CHARACTERISTICS

Document prepared by an expert from the Netherlands

Disclaimer: this document does not represent UPOV policies or guidance

The Annex to this document contains a copy of a presentation “Molecular markers as predictors for ‘traditional’ characteristics” made at the fourteenth session of the Working Group on Biochemical and Molecular Techniques and DNA-Profiling in particular (BMT).

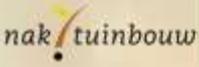
Hedwich Teunissen, Naktuinbouw R&D
The Netherlands

[Annex follows]



Molecular markers as predictors for 'traditional' characteristics

Hedwich Teunissen, Naktuinbouw R&D



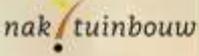
UPOV/INF/18/1



Model 1: Molecular Characteristics as Predictors of 'Traditional' Characteristics



- Gene-specific markers for predicting individual phenotypic characteristics. (Reliable linkage between the marker and the expression of the characteristic required)
 - e.g. *disease resistance*
- Use of a set of molecular characteristics which can be used to reliably estimate traditional characteristics; e.g. *quantitative trait loci (QTL)*



Current situation



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DUS testing tomato (only morph)

- Resistance / Susceptibility for the obligatory diseases is used to select relevant reference varieties (grouping characteristics)
- With the information of the candidate on the Technical Questionnaire (TQ) references in the same group are selected.
- Information on TQ for a candidate variety must be confirmed
- Confirmation is done by bioassay
- PCR test is only performed when problems in bioassay as extra confirmation
- **What should be done if a bioassay is not available, not possible or when a bioassay is difficult?**

Proposed strategy



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DUS testing tomato (both morph and DNA)

- First PCR test performed on seedlings:
 - for resistant candidate varieties - min. 20 individual plants (also check on uniformity).
 - for susceptible candidate varieties – 2 individual.
- When resistance gene present and TQ resistant; enough proof – no bioassay needed.
- When resistance gene absent; bioassay will be performed (min. 20 plants, also check on uniformity).
- When PCR result and TQ do not match; bioassay (if possible).
- When there is any (other) doubt; bioassay (if possible).

Benefits of PCR tests



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1. PCR results are complementary to bioassay results. Increased reliability
2. PCR tests are faster and cheaper than bioassays.
3. Good alternative when bioassay is:
 1. Not available
 2. Not possible (because of e.g. Quarantine status)
 3. Difficult to perform and/or to reproduce (false positives and false negatives)

Overview of possible tests I



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Disease resistance in tomato:

- *Meloidogyne incognita*
 - MI1.2 (traditional PCR)
- *Tomato Mosaic Virus (ToMV)*
 - Tm1 (traditional PCR)
 - Tm2 and Tm2² (tetra ARMS)
- *Verticillium dahliae*
 - Ve1 and Ve2 (same locus) (tetra ARMS)
- *Fusarium oxysporum f. sp. lycopersici*
 - I-2 gene (traditional PCR)

Development and evaluation of robust molecular markers linked to disease resistance in tomato for distinctness, uniformity and stability testing

Paul Aron - Corine Theille - David Béranger - Lucille Couderc - André Mounier - Eugénie Rabreau - Bénédicte van der Velden - Ghislain Colquhoun - Dominique Pilet - Christophe Bousquet - Marie-Hélène - Stéphane Nédélec - Christophe Lecomte - Benoit Vigneron

Why these tests I



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- *Meloidogyne incognita*
 - M1.2 (traditional PCR)
- *Tomato Mosaic Virus (ToMV)*
 - Tm1 (traditional PCR)
 - Tm2 and Tm2² (tetra ARMS)
- *Verticillium dahliae*
 - Ve1 and Ve2 (same locus) (tetra ARMS)
- *Fusarium oxysporum f. sp. lycopersici*
 - I-2 gene (traditional PCR)

No significant problem with bioassays

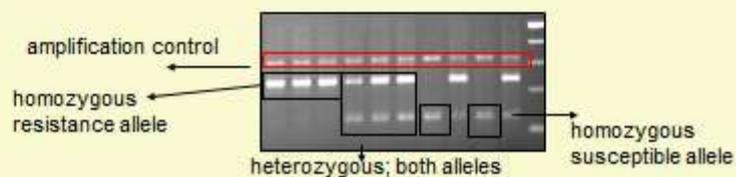
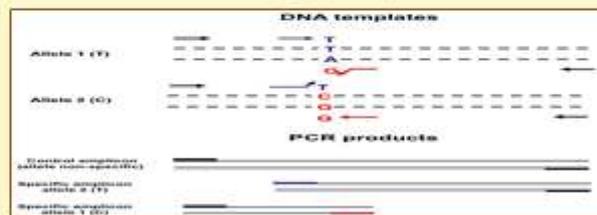
1. Complementary results – more reliability
2. Faster and cheaper – cost efficiency
3. Management of reference collections:
 - Gain new/additional data for old varieties
 - To screen (old) reference varieties

ARMS: example



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Example: Amplification Refractory Mutation System (ARMS) for the detection of Ve-1/Ve-2 and Tm2/Tm2²



Correlation



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Verticillium dahliae Ve1 and Ve2 genes

Total # varieties	Correlation PCR vs TQ and Bioassay
94	98%
2	Ve1-ve2 new haplotype = intermediate resistance

Discovery of combination of new alleles (=haplotype) that might explain newly observed intermediate resistance levels for *Verticillium*.

Meloidogyne incognita MI1.2 gene

Total # varieties	Correlation PCR vs Bioassay
130	99%
1 resistant fragment	Susceptible in bioassay

This candidate variety also had intermediate resistance levels for *Ve*.
This application was not registered. Not DUS.

Correlation



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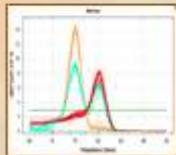
Tomato Mosaic Virus (ToMV) Tm2 and Tm2²

Total # varieties	Correlation PCR vs TQ and Bioassay
100	100%

Fusarium oxysporum f. sp. lycopersici I-2 gene

Total # varieties	Correlation PCR vs TQ and Bioassay
100	100%

Overview of possible tests II



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Disease resistance in tomato:

- **Tomato Spotted Wilt Virus (TSWV)**
 - Sw-5 (TaqMan PCR)
- **Tomato Yellow Leaf Curl Virus (TyLCV)**
 - Ty-1 / Ty-3 (Melt Curve analysis)

TSWV reference:

Dianese E.C., Fonseca M.E.N., Goldbach R., Kormelink R., Inoue-Nagata A.K., Resende R.O., Boiteux L.S. (2009) Development of a locus-specific, co-dominant SCAR marker for assisted-selection of the SW-5 (Tospovirus resistance) gene cluster in a wide range of tomato accessions. Mol Breeding (2010) 25:133-142.

TyLCV reference:

Verisani M.G.: The Tomato Yellow Leaf Curl Virus Resistance Gene Ty-1 and TY-3 are allelic and Code for DFGD-Class RNA Dependent RNA Polymerases. PLOS Genetics March 2013 Volume 9 Issue 3.

Patent: <http://www.google.com/patents/WO2012125025A1?open>

Why these tests II

Disease resistance in tomato:

- **Tomato Spotted Wilt Virus (TSWV)**
- **Tomato Yellow Leaf Curl Virus (TyLCV)**

Problems:

TSWV:

- Quarantain pathogens in EU
- difficult bioassay in a tent
- Trips
- Very instable virus
- Many false negatives sometimes false positives

TyLCV:

- Quarantain pathogens in EU
- No bioassay
- White Fly
- Bio Assay based on Agrobacterium inoculation with transgen



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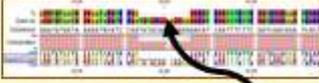
TyLCV: melt curve assay



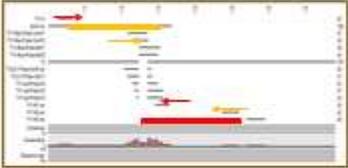


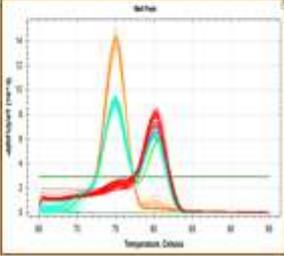
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Example: melt curve analysis for TyLCV



Susceptible allele = deletion of 12 base pairs



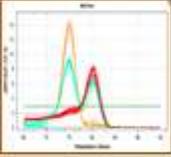


PCR product for resistant allele melts at 75°C (yellow peak)

PCR product for susceptible allele melts at 80°C (red peak)

In a heterozygous variety both peaks are visible (blue)

Correlation


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TSWV Sw-5

Total # varieties	Correlation PCR vs TQ
118	100%
Total # varieties	Correlation PCR vs TQ and Bioassays
37	100%

TyLCV Ty-1/Ty-3

Total # varieties	Correlation PCR vs TQ
15	100%

Bioassay is not (yet) possible.

Overview of tests III



Cytoplasmic Male Sterility (CMS) in *Brassicaceae*

- Broccoli
- Cauliflower
- Red Cabbage
- White Cabbage
- Savoy Cabbage
- Brussels Sprout
- Curly Kale
- Kohlrabi

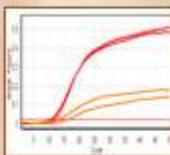
Ogura-type cytoplasmic male-sterility from Japanese radish
(covered by confidentiality clause)

CMS marker is located in *orf138*

Fertile varieties do not have *orf138*

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Procedure CMS-PCR testing



Cytoplasmic Male Sterility (CMS) in *Brassicaceae*

Seeds are sown on wet filter paper. After one week seedlings are harvested.



For fertile varieties:

5 pools of 5 individuals are sampled and analysed by TaqMan PCR.

For sterile varieties:

min. 20 individual plants were sampled and analysed by TaqMan PCR.



Only when problem: uniformity problem or when contradictory to TQ a morphology test will be performed

A				B			
	Cy. var. orf138	Cy. var. orf138 VC	Control		Cy. var. orf138 VC	Cy. var. orf138 VC	Control
0-01	19.24	19.09	Sterile	0-01	N/A	19.8	Fertile
0-02	19.80	19.01	Sterile	0-02	N/A	19.01	Fertile
0-03	19.17	19.06	Sterile	0-03	N/A	19.14	Fertile
0-04	19.28	19.27	Sterile	0-04	N/A	19.10	Fertile
0-05	19.2	19.11	Sterile	0-05	N/A	19.7	Fertile
0-06	19.11	19.09	Sterile	0-06	N/A	19.78	Fertile
0-07	19.16	19.08	Sterile	0-07	N/A	19.07	Fertile
0-08	19.08	19.00	Sterile	0-08	N/A	19.14	Fertile
0-09	19.02	19.13	Sterile	0-09	N/A	19.81	Fertile
0-10	19.00	19.08	Sterile	0-10	N/A	19.78	Fertile

Internal
DNA
control
NAD5

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Correlation CMS

Results 2013:

Accession	Test number	Reference/Accession	OGURA test/TQ	OGURA test/PCR	OGURA test/PCR
OG1	OG1-1	11	OGURA	OGURA	OGURA
OG2	OG2-1	11	OGURA	OGURA	OGURA
OG3	OG3-1	11	OGURA	OGURA	OGURA
OG4	OG4-1	11	OGURA	OGURA	OGURA
OG5	OG5-1	11	OGURA	OGURA	OGURA
OG6	OG6-1	11	OGURA	OGURA	OGURA
OG7	OG7-1	11	OGURA	OGURA	OGURA
OG8	OG8-1	11	OGURA	OGURA	OGURA
OG9	OG9-1	11	OGURA	OGURA	OGURA
OG10	OG10-1	11	OGURA	OGURA	OGURA
OG11	OG11-1	11	OGURA	OGURA	OGURA
OG12	OG12-1	11	OGURA	OGURA	OGURA
OG13	OG13-1	11	OGURA	OGURA	OGURA
OG14	OG14-1	11	OGURA	OGURA	OGURA
OG15	OG15-1	11	OGURA	OGURA	OGURA
OG16	OG16-1	11	OGURA	OGURA	OGURA
OG17	OG17-1	11	OGURA	OGURA	OGURA
OG18	OG18-1	11	OGURA	OGURA	OGURA
OG19	OG19-1	11	OGURA	OGURA	OGURA
OG20	OG20-1	11	OGURA	OGURA	OGURA
OG21	OG21-1	11	OGURA	OGURA	OGURA
OG22	OG22-1	11	OGURA	OGURA	OGURA
OG23	OG23-1	11	OGURA	OGURA	OGURA
OG24	OG24-1	11	OGURA	OGURA	OGURA
OG25	OG25-1	11	OGURA	OGURA	OGURA
OG26	OG26-1	11	OGURA	OGURA	OGURA
OG27	OG27-1	11	OGURA	OGURA	OGURA
OG28	OG28-1	11	OGURA	OGURA	OGURA
OG29	OG29-1	11	OGURA	OGURA	OGURA
OG30	OG30-1	11	OGURA	OGURA	OGURA
OG31	OG31-1	11	OGURA	OGURA	OGURA
OG32	OG32-1	11	OGURA	OGURA	OGURA
OG33	OG33-1	11	OGURA	OGURA	OGURA
OG34	OG34-1	11	OGURA	OGURA	OGURA
OG35	OG35-1	11	OGURA	OGURA	OGURA
OG36	OG36-1	11	OGURA	OGURA	OGURA
OG37	OG37-1	11	OGURA	OGURA	OGURA
OG38	OG38-1	11	OGURA	OGURA	OGURA
OG39	OG39-1	11	OGURA	OGURA	OGURA
OG40	OG40-1	11	OGURA	OGURA	OGURA
OG41	OG41-1	11	OGURA	OGURA	OGURA
OG42	OG42-1	11	OGURA	OGURA	OGURA
OG43	OG43-1	11	OGURA	OGURA	OGURA
OG44	OG44-1	11	OGURA	OGURA	OGURA
OG45	OG45-1	11	OGURA	OGURA	OGURA
OG46	OG46-1	11	OGURA	OGURA	OGURA
OG47	OG47-1	11	OGURA	OGURA	OGURA
OG48	OG48-1	11	OGURA	OGURA	OGURA
OG49	OG49-1	11	OGURA	OGURA	OGURA
OG50	OG50-1	11	OGURA	OGURA	OGURA
OG51	OG51-1	11	OGURA	OGURA	OGURA
OG52	OG52-1	11	OGURA	OGURA	OGURA
OG53	OG53-1	11	OGURA	OGURA	OGURA
OG54	OG54-1	11	OGURA	OGURA	OGURA
OG55	OG55-1	11	OGURA	OGURA	OGURA
OG56	OG56-1	11	OGURA	OGURA	OGURA
OG57	OG57-1	11	OGURA	OGURA	OGURA
OG58	OG58-1	11	OGURA	OGURA	OGURA
OG59	OG59-1	11	OGURA	OGURA	OGURA
OG60	OG60-1	11	OGURA	OGURA	OGURA
OG61	OG61-1	11	OGURA	OGURA	OGURA
OG62	OG62-1	11	OGURA	OGURA	OGURA
OG63	OG63-1	11	OGURA	OGURA	OGURA
OG64	OG64-1	11	OGURA	OGURA	OGURA
OG65	OG65-1	11	OGURA	OGURA	OGURA
OG66	OG66-1	11	OGURA	OGURA	OGURA
OG67	OG67-1	11	OGURA	OGURA	OGURA
OG68	OG68-1	11	OGURA	OGURA	OGURA
OG69	OG69-1	11	OGURA	OGURA	OGURA
OG70	OG70-1	11	OGURA	OGURA	OGURA
OG71	OG71-1	11	OGURA	OGURA	OGURA
OG72	OG72-1	11	OGURA	OGURA	OGURA
OG73	OG73-1	11	OGURA	OGURA	OGURA
OG74	OG74-1	11	OGURA	OGURA	OGURA
OG75	OG75-1	11	OGURA	OGURA	OGURA
OG76	OG76-1	11	OGURA	OGURA	OGURA
OG77	OG77-1	11	OGURA	OGURA	OGURA
OG78	OG78-1	11	OGURA	OGURA	OGURA
OG79	OG79-1	11	OGURA	OGURA	OGURA
OG80	OG80-1	11	OGURA	OGURA	OGURA
OG81	OG81-1	11	OGURA	OGURA	OGURA
OG82	OG82-1	11	OGURA	OGURA	OGURA
OG83	OG83-1	11	OGURA	OGURA	OGURA
OG84	OG84-1	11	OGURA	OGURA	OGURA
OG85	OG85-1	11	OGURA	OGURA	OGURA
OG86	OG86-1	11	OGURA	OGURA	OGURA
OG87	OG87-1	11	OGURA	OGURA	OGURA
OG88	OG88-1	11	OGURA	OGURA	OGURA
OG89	OG89-1	11	OGURA	OGURA	OGURA
OG90	OG90-1	11	OGURA	OGURA	OGURA
OG91	OG91-1	11	OGURA	OGURA	OGURA
OG92	OG92-1	11	OGURA	OGURA	OGURA
OG93	OG93-1	11	OGURA	OGURA	OGURA
OG94	OG94-1	11	OGURA	OGURA	OGURA
OG95	OG95-1	11	OGURA	OGURA	OGURA
OG96	OG96-1	11	OGURA	OGURA	OGURA
OG97	OG97-1	11	OGURA	OGURA	OGURA
OG98	OG98-1	11	OGURA	OGURA	OGURA
OG99	OG99-1	11	OGURA	OGURA	OGURA
OG100	OG100-1	11	OGURA	OGURA	OGURA

From 165 candidate varieties (2011-2014) in all *Brassicaceae* tested only one PCR result not in accordance with TQ/morph test (99,4%).

Different source of sterility (not OGURA-type)



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Future perspective tests I



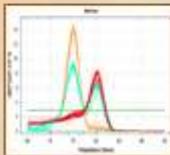
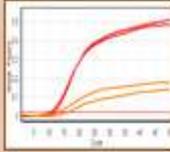
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- *Meloidogyne Incognita*
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 - Tm2 and Tm2^c (tetra ARMS)
- *Verticillium dahliae*
 - Ve1 and Ve2 (same locus) (tetra ARMS)
- *Fusarium oxysporum f. sp. lycopersici*
 - I-2 gene (traditional PCR)

- PCR is complementary to the bioassay.
- Faster and cheaper
- Good help to screen and manage the reference collection

Proposal:
include PCR tests in the UPOV guidelines
on crop-by-crop basis

Future perspective tests II



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- **Tomato Spotted Wilt Virus (TSWV)**
 - Sw-5 (TaqMan PCR)
- **Tomato Yellow Leaf Curl Virus (TYLCV)**
 - Ty-1 / Ty-3 (Melt Curve analysis)

Proposal: include PCR tests in the UPOV guidelines

From CPVO protocol tomato (TP/044/4 Rev):

11. Observations	
11.1 Method	visual
11.2 Observation scale	Symptoms: top mosaic, bronzing, various malformations, necrosis
11.3 Validation of test	evaluation of variety resistance should be calibrated with results of resistant and susceptible controls
12. Interpretation of data in terms of UPOV characteristic states	
absent	[1] symptoms
present	[9] no symptoms
13. Critical control points:	
TSWV has a quarantine status in some countries. TSWV is transmitted by <i>Thrips tabaci</i> and Western flower thrips (<i>Frankliniella occidentalis</i>). Pathotype 0 is defined by its inability to break resistance in tomato varieties carrying the resistance gene Sw-5. TSWV resistance based on Sw-5 may be detected without using the pathogen.	
Note: Option for testing without using the pathogen	
Resistance to TSWV-0 is often based on the resistance gene Sw-5. The presence of the resistance gene Sw-5 can be detected by molecular marker Sw-5o-LRR (Garland et al. 2005) or by a co-dominant SCAR marker (Dianese et al. 2010). This molecular test is validated to be used instead of the pathogen, as foreseen in UPOV document TC/38/17 Add. – CA/45/5 Add. Under Option 1(a). Each molecular marker should be applied to a minimum of twenty plants and validated with proper controls.	
If the biomolecular test is inconclusive, then the pathogen needs to be carried out.	

Future perspective



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Cytoplasmic Male Sterility (CMS) in *Brassicaceae*

Ogura-type cytoplasmic male-sterility from Japanese radish
covered by confidentiality clause

Company is willing to make the marker available for EOs
for DUS research only

Legal discussion within UPOV about how to deal
with confidentiality
to make sure that the marker is used for DUS purposes only



Quality in Horticulture

[End of Annex and of document]