UPOV

International Union for the Protection of New Varieties of Plants

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WHAT INFORMATION IS ESSENTIAL FOR 'CHARACTER-SPECIFIC MOLECULAR MARKERS' IN TEST GUIDELINES

Document prepared by experts from the Netherlands

The annex to this document contains a copy of a presentation on "What information is essential for 'character-specific molecular markers' in Test Guidelines", to be made at the eighteenth session of the BMT.

[Annex follows]

What information is essential for ‘character-specific molecular markers’ in Test Guidelines

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Aim of this presentation

Guidance proposal and discussion:

- NOT On how to develop ‘character-specific molecular markers’
- NOT on how to validate the ‘character-specific molecular markers’ and to establish a reliable correlation between marker and DUS charateristic
- NOT On how to routinely test for ‘character-specific molecular markers’
- NOT On the application of ‘character-specific molecular markers’ in DUS procedure
- **On what information is essential to submit in UPOV test guidelines to ensure harmonized use of the character-specific molecular marker**

TWV/51/10

- In TWV/51/10 (2017) (partial revision of TG for tomato) three molecular markers were proposed as alternative method for the bioassay to examine resistance characteristics (model 1)

- Ad. 48: Resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Fol)
- Ad. 51: Resistance to tomato mosaic virus (ToMV)
- Ad. 58: Resistance to tomato spotted wilt virus (TSWV)

Ad. 58: TSWV

TWV/51/10 (2017)

The marker format follows the format of the bioassay protocol in the TG

Ad. 58: Resistance to Tomato spotted wilt virus (TSWV)

(ii) DNA marker test

Resistance to TSWV strain 0 is often based on resistance gene Sw-5. The presence of the resistant allele and/or susceptible allele(s) can be detected by the co-dominant markers as described in Dianese, E.C. *et al* (2010). Specific aspects:

1. Pathogen	Tomato spotted wilt virus	
2. Functional gene	Sw-5b	
4. Format of the test		
4.1 Number of plants per genotype	at least 20 plants	
4.2 Control varieties	homozygous susceptible allele 1 present: Moneymaker	
	homozygous susceptible allele 2 present: Mountain Magic	
	homozygous resistant allele present: Montealto	
8. Interpretation of test results		
absent	[1]	susceptible allele(s) present and resistant allele absent
present	[9]	resistant allele present (homozygous or heterozygous)
	In case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the resistance is absent or present for the variety (on another mechanism).	

Little details in TG

Literature reference does not give ´ready-to-use´ primers but supply target sequences for resistance and susceptibility alleles

Flexibility in primer and assay design

TWV/51/10 (2017)

Ad. 51: ToMV

The marker format follows the format of the bioassay protocol in the TG

Ad. 51: Resistance to Tomato mosaic virus (ToMV)

(ii) DNA marker test

Resistance to ToMV is often based on resistance gene Tm2 (allele Tm2 or $Tm2^2$). The presence of the resistant alleles Tm2 and $Tm2^2$ and/or susceptible allele tm2 can be detected by the co-dominant markers as described in Arens, P. *et al* (2010). Specific aspects:

1. Pathogen ..... Tomato mosaic virus
2. Functional gene ..... $Tm2/2^2$
4. Format of the test
4.1 Number of plants per genotype ..... at least 20 plants
4.2 Control varieties ..... homozygous susceptible allele tm2 present: Moneymaker
resistant allele Tm2 present: Moperou
resistant allele $Tm2^2$ present: Momor, Persica, Campeon
8. Interpretation of test results ..... the presence of the alleles tm2, Tm2, $Tm2^2$ lead to different interpretation for characteristics 51.1, 51.2 and 51.3, see table. In case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the resistance is absent or present for the variety (on another mechanism, e.g. gene Tm1).

Test result DNA marker test	tm2/tm2	Tm2/tm2 or Tm2/Tm2	$Tm2^2$/tm2 or $Tm2^2$/Tm2 or $Tm2^2$/Tm2
		(occurs incidentally)	
51.1 Strain 0	[1] absent	[9] resistant	[9] resistant
51.2 Strain 1	[1] absent	[9] resistant	[9] resistant
51.3 Strain 2	[1] absent	[1] absent	[9] resistant

Little details in TG

Literature reference does give ´ready-to-use´ primers and also supply references to target sequences for resistance and susceptibility alleles (Genebank)

Useful for immediate use but also provide flexibility in primer and assay design

TWV/51/10 (2017)

Ad. 48: Fol

The marker format follows the format of the bioassay protocol in the TG

Ad. 48: Resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Fol)

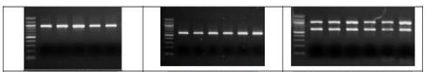
(ii) DNA marker test

Resistance to both race 0 (ex 1) and race 1 (ex 2) is often based on resistance gene I2. The presence of the resistant and/or susceptible allele of gene I2 can be detected by the co-dominant marker as described in this method

1. Pathogen ..... *Fusarium oxysporum* f. sp. *lycopersici*
2. Functional gene I2
3. Primers
3.1 Susceptible allele ..... Z1063-I2-F 5'-GTT TGA CAG CTT GGT TTT GT-3'
Z1063-I2-R 5'-CTC AAA CTC ACC ATC ATT GA-3'
3.2 Resistant allele ..... TFusF1 5'-CTG AAA CTC TCC GTA TTT C-3'
TFusRR1 5'-CGA AGA GTG ATT GGA GAT-3'
4. Format of the test
4.1 Number of plants per genotype ..... at least 20 plants
4.2 Control varieties ..... homozygous susceptible allele present: Moneymaker
homozygous resistant allele present: Tradiro
5. Preparation
5.1 Preparation DNA ..... harvest per individual plant a part of a young leaf. Isolate total DNA with a standard DNA isolation protocol (CTAB/SDS based). Re-suspend in 100 µl $T_{10}E_{0.1}$. Dilute total DNA to 1/10 (H_2O) to obtain a DNA concentration between 1-10 ng/µl.
5.1 Preparation PCR ..... use 3 µl of each diluted DNA sample into individuals PCR reactions. Prepare the PCR master mix, 20µl reaction volume:
- 3 µl of 10x diluted DNA
- 2,5 µl of 10x reaction buffer
- 2 mM $MgCl_2$
- 0,1 µM of resistance primers each
- 0,2 µM of susceptible primers each
- 200 µM of each of the four dNTPs
- 1 unit of Taq DNA polymerase

6. PCR conditions ..... 1. initial denaturation step at 94°C for 3 minutes
2. 35 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes
3. final extension step of 72°C for 10 minutes

7. Observations
7.1 Method ..... visual
7.2 Observation scale .....



amplicon of 940bp only homozygous susceptible allele present | amplicon of 600bp only homozygous resistant allele present | amplicons of 940bp and 600bp susceptible and resistant allele present: heterozygous resistant

7.3 Validation of test ..... control varieties should give the expected band(s).
8. Interpretation of test results
48.1 Race 0 (ex 1)
present ..... [9] homozygous or heterozygous resistant in DNA marker test in case homozygous susceptible allele present a bio-assay on race 0 (ex 1) should be performed.
in case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the resistance is absent or present for the variety (on another mechanism, e.g. gene I2 without I).

48.2 Race 1 (ex 2)
absent ..... [1] homozygous susceptible in DNA merker test
present ..... [9] homozygous or heterozygous resistant in DNA merker test in case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the resistance is absent or present for the variety (on another mechanism, e.g. gene I3).

More details in method description in TG

No reference to literature as primers are only partly described in scientific papers and partly developed by Naktuinbouw

NO Flexibility in primer and assay design

TWV/53/7

Addaped proposal for Fol marker

5. Preparation	
5.1 Preparation DNA	harvest per individual plant a part of a young leaf. Isolate total DNA with a standard DNA isolation protocol (CTAB/SDS based). Re-suspend in 100 µl $T_{10}E_{0.1}$, Dilute total DNA to 1/10 (H_2O) to obtain a DNA concentration between 1-10 ng/µl.
5.1 Preparation PCR	use 3 µl of each diluted DNA sample into individuals PCR reactions. Prepare the PCR master mix, 20µl reaction volume: • 3 µl of 10x diluted DNA • 2,5 µl of 10x reaction buffer • 2 mM $MgCl_2$ • 0,1 µM of resistance primers each • 0,2 µM of susceptible primers each • 200 µM of each of the four dNTPs • 1 unit of Taq DNA polymerase
6. PCR conditions	1. initial denaturation step at 94°C for 3 minutes 2. 35 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes 3. final extension step of 72°C for 10 minutes

5.	Preparation	
5.1	Preparation DNA	harvest per individual plant a part of a young leaf. Isolate total DNA of each individual plant separately with a standard DNA isolation protocol (CTAB/SDS based). Re-suspend in 100 µl $T_{10}E_{0.1}$ or another suitable buffer. Dilute total DNA to 1/10 (H_2O) to obtain a DNA concentration between 1-10 ng/µl.
5.2	Preparation PCR	for example, use 3 µl of each diluted DNA sample into individuals PCR reactions. prepare the PCR master mix, 20µl reaction volume, for example: • 3 µl of 10x diluted DNA • 2,5 µl of 10x reaction buffer • 2 mM $MgCl_2$ • 0,1 µM of resistance primers each • 0,2 µM of susceptible primers each • 200 µM of each of the four dNTPs • 1 unit of Taq DNA polymerase
6.	PCR conditions	for example: 1. initial denaturation step at 94°C for 3 minutes 2. 35 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes 3. final extension step of 72°C for 10 minutes

Bringing back the flexibility in primer and assay design?

In the report of the TWV/51 meeting

TWV/51/16

Ad. 51 (ii)	to add chapter 3 and 6 of Char. 48
Ad. 58 (ii)	to add chapter 3 and 6 of Char. 48

3. Is primers
6. PCR conditions

Request to add the information of the primer sequences and the PCR conditions for both ToMV and TWSV as was done for Fol

Request for more detailed information

TC-EDC/mar18/8

Current status of the TG tomato

Ad. 58: TSWV

old

(ii) DNA marker test

Resistance to TSWV strain 0 is often based on resistance gene Sw-5. The presence of the resistant allele and/or susceptible allele(s) can be detected by the co-dominant markers as described in Dianese, E.C. *et al* (2010). Specific aspects:

1. Pathogen ..... Tomato spotted wilt virus
2. Functional gene Sw-5b
4. Format of the test
4.1 Number of plants per genotype ..... at least 20 plants
4.2 Control varieties ..... homozygous susceptible allele 1 present: Moneymaker
homozygous susceptible allele 2 present: Mountain Magic
homozygous resistant allele present: Montealto
8. Interpretation of test results .....
absent ..... [1] susceptible allele(s) present and resistant allele absent
present ..... [9] resistant allele present (homozygous or heterozygous)
In case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the resistance is absent or present for the variety (on another mechanism).

new

(ii) DNA marker test

Dominant resistance gene Sw-5 is always associated with resistance to TSWV strain 0. The presence or absence of the resistance allele can be detected by the co dominant marker as described in Dianese, E.C. et al (2010). Specific aspects:

1.	Pathogen	Tomato spotted wilt virus
2.	Functional gene	Sw-5b
3.	Primers	
3.1	Allele for susceptibility	Sw5-Vat1-F: 5'-ACAACATCAAACAATGTTAGCC-3' Sw5-Vat2-F: 5'-CATCAAACAATGCAGTTAGCC-3'
3.2	Allele for resistance	Sw5-Res-F: 5'-ATCAACCAATACAGCCTAACC-3
3.3	Universal reverse	Sw5-universal-R: 5'-TTTCTCCCTGCAAGTTCACC-3'
3.4	Allele specific probes	Sw5-Sus1: 5'-VIC-TACATTATGAAGGGTTAACAAG-MGB-NFQ-3' Sw5-Sus2: 5'-6FAM-ACAACAGAGGGTTAACAAGTTTAGG-BHQ1-3' Sw5-Res: 5'-TEXAS RED-TGGGCGAAAATCCCAACAAG-BHQ2-3'
4.	Format of the test	
4.1	Number of plants per genotype	at least 20 plants
4.2	Control varieties	homozygous allele 1 for susceptibility present: Moneymaker homozygous allele 2 for susceptibility present: Mountain Magic homozygous allele for resistance present: Montealto heterozygous (allele for resistance and allele 1 for susceptibility present): Bodar
6.	PCR conditions	1. Initial denaturation step 10 min 95 °C 2. 40 cycles 15 sec 95 °C and 1 min 60°C. Every cycle ends with a plate reading.
8.	Interpretation of test results	
	absent	[1] susceptible allele(s) present and resistant allele absent
	present	[9] resistant allele present (homozygous or heterozygous) In case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the variety is resistant due to another mechanism

TC-EDC/mar18/8

Current status of the TG tomato

Ad. 51: ToMV

old

(ii) DNA marker test

Resistance to ToMV is often based on resistance gene Tm2 (allele Tm2 or Tm2^2). The presence of the resistant alleles Tm2 and Tm2^2 and/or susceptible allele tm2 can be detected by the co-dominant markers as described in Arens, P. *et al* (2010). Specific aspects:

1. Pathogen ..... Tomato mosaic virus
2. Functional gene ..... Tm2/2^2
4. Format of the test
4.1 Number of plants per genotype ..... at least 20 plants
4.2 Control varieties ..... homozygous susceptible allele tm2 present: Moneymaker
resistant allele Tm2 present: Moperou
resistant allele Tm2^2 present: Momor, Persica, Campeon
8. Interpretation of test results ..... the presence of the alleles tm2, Tm2, Tm2^2 lead to different interpretation for characteristics 51.1, 51.2 and 51.3, see table. In case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the resistance is absent or present for the variety (on another mechanism, e.g. gene Tm1).

Test result DNA marker test	tm2/tm2	Tm2/tm2 or Tm2/Tm2	Tm2^2/tm2 or Tm2^2/Tm2^2 or Tm2^2/Tm2
		(occurs incidentally)	
51.1 Strain 0	[1] absent	[9] resistant	[9] resistant
51.2 Strain 1	[1] absent	[9] resistant	[9] resistant
51.3 Strain 2	[1] absent	[1] absent	[9] resistant

new

(ii) DNA marker test

Resistance gene Tm2 gives resistance to ToMV. Gene Tm2 has two dominant resistance alleles: resistance allele Tm2 is always associated with resistance to strain 0 and 1, resistance allele Tm2^2 is always associated with resistance to strain 0, 1 and 2. The presence or absence of both resistance alleles can be detected by the co-dominant markers as described in Arens, P. et al (2010). Specific aspects:

1.	Pathogen	Tomato mosaic virus
2.	Functional gene	Tm2/2^2 (with two resistance alleles Tm2 and Tm22 and one susceptibility allele tm2)
3.	Primers	
3.1	Assay 1 to check resistance allele Tm2 or Tm2^2	Outer primer TMV-2286F: 5'GGGTATACTGGGAGTGTCCAATTC3' Outer primer TMV-2658R: 5'CCGTGCACGTTACTTCAGACAA3' Tm2^2 SNP2494F: 5'CTCATCAAGCTTACTCTAGCCTACTTTAGT3' Tm2 SNP2493R: 5'CTGCCAGTATATAACGGTCTACCG3'
3.2	Assay 2 to check allele for susceptibility or resistance	Outer primer TM2-748F: 5'CGGTCTGGGGAAAACAACTCT3' Outer primer TM2-1256R: 5'CTAGCGGTATACCTCCACATCTCC3' TM2-SNP901misR: 5'GCAGGTTGTCCTCCAAATTTTCCATC3' TM2-SNP901misF: 5'CAAATTGGACTGACGGAACAGAAAGTT3'
4.	Format of the test	
4.1	Number of plants per genotype	at least 20 plants
4.2	Control varieties	homozygous allele for susceptibility tm2 present: Mobaci, Monalbo, Moneymaker homozygous allele for resistance Tm2 present: Moperou homozygous allele for resistance Tm2^2 present: Mocimor, Momor
6.	PCR conditions	1. Initial denaturation step at 94°C for 3 minutes 2. 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes 3. Final extension step of 72°C for 10 minutes
8.	Interpretation of test results	In case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the variety is resistant due to another mechanism gene Tm1.

Test result DNA marker test	tm2/tm2	Tm2/tm2 or Tm2/Tm2	Tm2^2/tm2 or Tm2^2/Tm2^2 or Tm2^2/Tm2
51.1 Strain 0	[1] absent	[9] resistant	[9] resistant
51.2 Strain 1	[1] absent	[9] resistant	[9] resistant
51.3 Strain 2	[1] absent	[1] absent	[9] resistant

EU situation vs UPOV

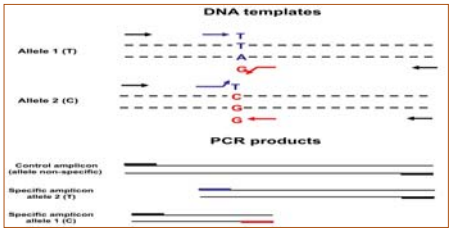
In general
- In EU the UPOV TGs are generally translated into protocols TPs
- These protocols are not guidelines but are mandatory

In the tomato situation
- In parallel with UPOV partial revision of tomato, CPVO revision of technical protocol of tomato.
- Based on the discussions in TWV and VEM the molecular markers for ToMV and TSWV were added to the CPVO TP including all technical details on primers sequences and PCR conditions

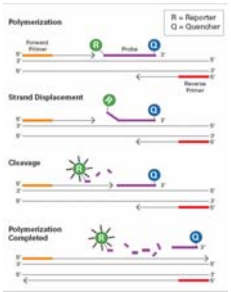
CPVO-TP/044/4 Rev.3 Ad. 55 for TSWV resistance; CPVO-TP/044/4 Rev.3 Ad. 48.1-48.3 for ToMV resistance

Improvements of the method to detect the marker

ToMV



Improved method:
TaqMan SNP detection
Only 1 assay:
Using tm2, Tm2 and Tm2^2 specific probes



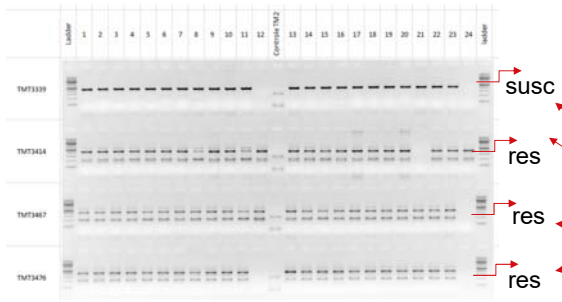
Original method:
ARMS-PCR SNP detection
1st assay: Tm2 vs Tm2^2 (which resistance allele is present)
2nd assay: tm2 vs Tm2/Tm2^2 (susceptible allele vs one of the resistance alleles)

Improvements of the method to detect the marker

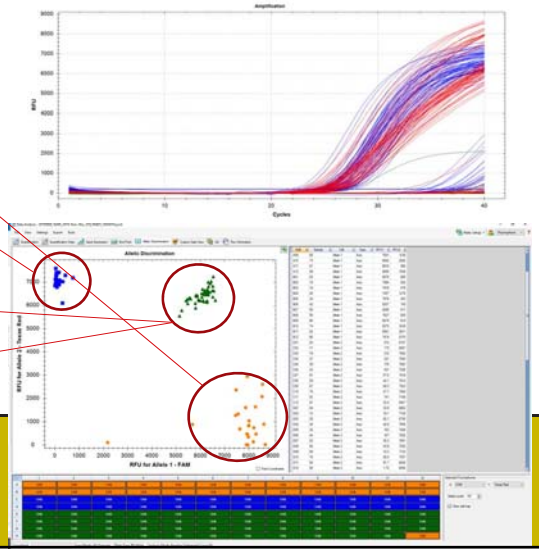
24 plants per variety

Old Assay 1

Tm2 vs $Tm2^2$ (which resistance allele is present)



New TaqMan assay combines old Assay 1 and 2



Old Assay 2:
tm2 vs Tm2/$Tm2^2$ (susceptible allele vs one of the resistance alleles)….. Replaced by TaqMan

What information is essential?

		Essential?	Proposal or remark
1	Pathogen		
2	Functional gene		
3	Primers		
3.1	Primers to detect allele susceptibility		
3.2	Primers to detect allele for resistance		
4	Format of the test		
4.1	Number of plants		
4.2	Control varieties		
5	Preparation		
6	PCR conditions		
7	Observations		
8	Interpretation of the test results		

[End of Annex and of document]