UPOV

International Union for the Protection of New Varieties of Plants

E

**Technical Working Party for Vegetables**

**TWV/54/7**

**Fifty-Fourth Session**
**Brasilia, Brazil, May 11 to 15, 2020**

**Original:** English
**Date:** April 23, 2020

# USE OF MOLECULAR TECHNIQUES IN DUS EXAMINATION

*Document prepared by the Office of the Union*

*Disclaimer: this document does not represent UPOV policies or guidance*

The annex to this document contains a copy of a presentation “Information on molecular markers in Test Guidelines explanations”, prepared by an expert from the Netherlands, to be considered by the fifty-fourth session of the Technical Working Party for Vegetables (TWV).

[Annex follows]

# Information on molecular markers in TG explanations

Naktuinbouw
Hedwich Teunissen, Amanda van Dijk

## Aim of this presentation

Guidance proposal and discussion:

**Which information is essential to include in the explanation of UPOV test guidelines to ensure harmonized use of the characteristic-specific molecular marker**

- Very detailed: same lab equipment, same supplier of primers, no flexibility fitting the lab
- Very detailed: difficult to adapt to newer technologies, and technologies develop fast (example: conventional PCR method - qPCR method - sequencing)

So, can we do with less details?

# Example: TSWV tomato

## Original proposal

(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

Flexibility in primer and assay design

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

## Current version

(ii) DNA marker test

Dominant resistance gene Sw-5 is always associated with resistance to TSWV strain 0. The presence or absence of the allele for resistance 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'<br>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:<br>5'-VIC-TACATTATGAAGGGTTAACAAG-MGB-NFQ-3'<br>Sw5-Sus2:<br>5'-6FAM-ACAACAGAGGGTTAACAAGTTTAGG-BHQ1-3'<br>Sw5-Res:<br>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<br>homozygous allele 2 for susceptibility present: Mountain Magic<br>homozygous allele for resistance present: Montealto<br>heterozygous (allele for resistance and allele 1 for susceptibility present): Bodar |
| 6. | PCR conditions | 1. Initial denaturation step 10 min 95 °C<br>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] allele(s) for susceptibility present and allele for resistance absent |
| | present | [9] allele for resistance present (homozygous or heterozygous)<br>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. |

Added: primers and PCR conditions

# Example: ToMV tomato

## Original proposal

(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 (occurs incidentally) | $Tm2^2$/tm2 or $Tm2^2$/Tm2 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 |

Little details in TG

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

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

## Current version

(ii) DNA marker test

Resistance gene Tm2 gives resistance to ToMV. Gene Tm2 has two dominant allele for resistance: allele Tm2 is always associated with resistance to strain 0 and 1, allele $Tm2^2$ is always associated with resistance to strain 0, 1 and 2. The presence or absence of both allele for resistance 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 alleles for resistance Tm2 and $Tm2^2$ and |
| 3. | Primers | |
| 3.1 | Assay 1 to check resistance allele Tm2 or $Tm2^2$ | Outer primer TMV-2286F:<br>5'GGGTATACTGGGAGTGTCCAATTC3'<br>Outer primer TMV-2658R:<br>5'CCGTGCACGTTACTTCAGACAA3'<br>$Tm2^2$-SNP2494F:<br>5'CTCATCAAGCTTACTCTAGCCTACTTTAGT3'<br>$Tm2^2$-SNP2493R:<br>5'CTGCCAGTATATAACGGTCTACCG3' |
| 3.2 | Assay 2 to check allele for susceptibility or resistance | Outer primer TM2-748F:<br>5'CGGTCTGGGGAAAACAACTCT3'<br>Outer primer TM2-1256R:<br>5'CTAGCGGTATACCTCCACATCTCC3'<br>TM2-SNP901miniF:<br>5'GCAGGTTGTCCTCCAAATTTTCCATC3'<br>TM2-SNP901miniF:<br>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: Mobaco, Monalbo, Moneymaker<br>homozygous allele for resistance Tm2 present: Moperou<br>homozygous allele for resistance $Tm2^2$ present: Mocimor, Momor |
| 6. | PCR conditions | 1. Initial denaturation step at 94°C for 3 minutes<br>2. 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes<br>3. Final extension step of 72°C for 10 minutes |
| 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.<br>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 like 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 |
|---|---|---|---|
| 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 |

Added: Primers and PCR conditions

# EU situation vs UPOV

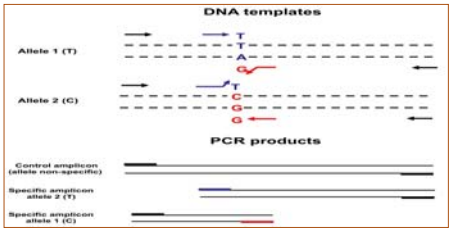
In general
- In EU the UPOV TGs are converted into European Technical Protocols (TPs) wich are mandatory

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

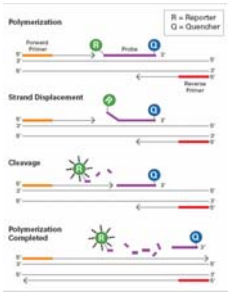
CPVO-TP/044/4 Rev.3 Ad. 55 for TSWV resistance; CPVO-TP/044/4 Rev.3 Ad. 48.1-48.3 for ToMV resistance

# 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



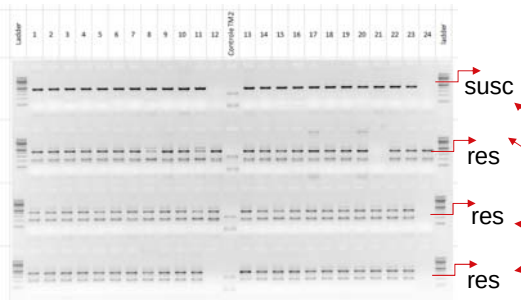

Original method:
ARMS-PCR SNP detection
1st assay: Tm2 vs $Tm2^2$ (which resistance allele is present)
2nd assay: tm2 vs Tm2/$Tm2^2$ (susceptible allele vs one of the resistance alleles)

# Improvements of the method to detect the marker

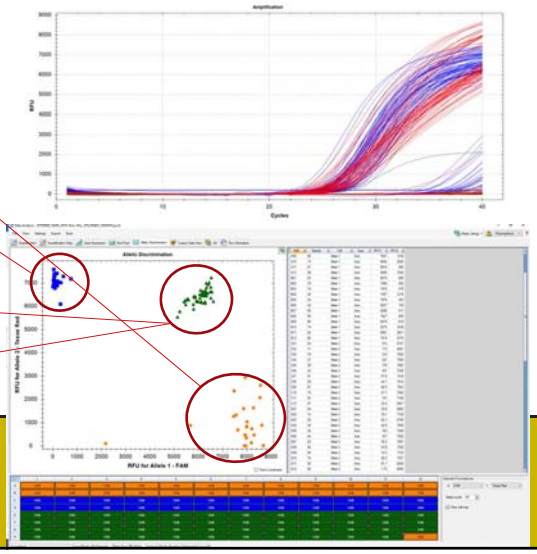
24 plants per variety

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

# Which information is essential?

| | | Essential? | Proposal or remark |
|---|---|---|---|
| 1 | Pathogen | YES | |
| 2.1 | Functional gene | YES | Reference to literature and/or position on ref genome/sequence in public databases |
| 2.2 | Functional allele for resistance | YES | Reference to literature and/or position on ref genome/sequence in public databases |
| 2.3 | Functional allele for susceptibility | YES | Reference to literature and/or position on ref genome/sequence in public databases |
| 3 | Primers | NO | Sequences, reference to accessions and sequences in public databases (Genebank numbers), literature |
| 3.1 | Primers to detect allele susceptibility | NO | Sequences corresponding to allele(s) for expression A (susceptibility) |
| 3.2 | Primers to detect allele for resistance | NO | Sequences corresponding to allele(s) for expression B (resistance) |
| 4 | Format of the test | | |
| 4.1 | Number of plants | YES | A minimal number of plants required |
| 4.2 | Control varieties | YES | Control varieties (same as in bioassay) as standards representing alleles homozygous for susceptibility, homozygous for resistance, heterozygous. |
| 5 | Preparation | NO | Depending on the method used. Not in the TG but detailed protocol(s) as an example in annex or available on request from the institute that developed the marker |
| 6 | PCR conditions | NO | Depending on the method used. Not in the TG but detailed protocol(s) as an example in annex or available on request from the institute that developed the marker |
| 7 | Observations | NO | Depending on the method used. Not in the TG but detailed protocol(s) as an example in annex or available on request from the institute that developed the marker |
| 8 | Interpretation of the test results | YES | Relation between alleles and expressions (with its notes) |
| | | | |


[End of Annex and of document]