UPOV

International Union for the Protection of New Varieties of Plants

E

Technical Working Party for Vegetables

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

TWV/54/7 Add.

Original: English
Date: April 29, 2020

ADDENDUM TO 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 addendum contains a proposal by the experts from the Netherlands for the revision of document TGP/12 “Guidance on Certain Physiological Characteristics”, for consideration by the Technical Working Party for Vegetables (TWV), at its fifty-second session.

The proposal by the experts from the Netherlands should be considered in conjunction with the presentation provided in document TWV/54/7 “Use of molecular techniques in DUS examination”.

[Annex follows]

ANNEX

PROPOSED AMENDMENTS TO DOCUMENT TGP/12
Prepared by the experts from the Netherlands

5. Characteristic-specific molecular markers

5.1 Introduction

As characteristics based on a response to an external factor are challenging, a characteristic-specific molecular marker test can be used to examine the particular characteristic of a variety under certain conditions. In documents INF/18 '*Possible Use of Molecular Markers in the Examination of Distinctness, Uniformity and Stability (DUS)*' and TGP/15 *'Guidance on the use of biochemical and molecular markers in the examination of Distinctness, Uniformity and Stability (DUS)'*, the use of characteristic-specific molecular markers is described as an accepted application model. Molecular markers can be applied when accepted by the relevant TWP and included in the Test Guidelines for a certain crop. It often concerns molecular markers for disease resistance characteristics, but also for others.

5.2 Criteria for the use of Characteristic-Specific Molecular Markers (see TGP/15 Annex I)

5.2.1 Molecular markers can be used as a method of examining DUS characteristics that satisfy the criteria for characteristics set out in the General Introduction, Chapter 4, section 4.2, on the following basis:

(a) the test for the marker is conducted on the same number of individual plants, with the same criteria for distinctness, uniformity and stability as for the examination of the characteristic by a bioassay;

(b) there is verification of the reliability of the link between the marker and the characteristic;

(c) different markers for the same characteristic are different methods for examining the same characteristic;

(d) markers linked to different genes conferring expression of the same characteristic are different methods for examining the same characteristic; and

(e) markers linked to different regulatory elements for the same gene conferring expression of the same characteristic are different methods for examining the same characteristic.

5.2.2 It is a matter for the relevant authority to consider if the assumptions are met when applying the model.

5.2.3. In order to include a method based on this model in Test Guidelines the relevant Technical Working Party and the TC would need to agree that the requirement for reliability of the link between the gene and the expression of the characteristic was satisfied.

5.3 Developing characteristic-specific molecular markers

5.3.1 Publicly available information on the functional gene(s) and the DNA sequence(s) that is(are) predictive for the particular state(s) of expression of the characteristic should be provided. This information can be referred to as:
a) file(s) containing the DNA sequence information (order of nucleotides)
b) reference to DNA information in public databases (like GeneBank)
c) reference to (scientific) publications in which the DNA sequence information of the states of expression of the characteristic is revealed.
d) reference to a particular position on the published reference genome version.

5.3.2 For a qualitative characteristic with two states of expression; absent (1) and present (9) information of either allelic DNA sequence responsible for 'absent' (for example susceptibility in case of disease resistance characteristics) and the allelic DNA sequence responsible for 'present' (resistance) should be provided.

5.3.3 For a quantitative characteristic all DNA sequences (alleles) and/or proportion of expression levels that are predictive for all particular states of expression of the characteristic should be provided.

5.3.4 The method should be validated by monitoring performance characteristics (such as repeatability).

5.3.5 Reliability of the link between the molecular marker and the characteristic should be verified on a large set of varieties of a broad genetic background. A test with participants from several members and companies is advisable. The technical working party decides whether the link is acceptable.

5.3.6 Interpretation of the test result needs extra attention. The characteristic is describing the morphology or the physiology, not the absence or presence of a molecular marker. When there is a discrepancy between the claim of the breeder in the Technical Questionnaire (TQ) and the molecular marker test, a morphological / physiological test is decisive.

5.3. 7 When only 'part of the puzzle' can be predicted by using molecular markers because only part of the functional genes are known and published, their application is still valuable.

Example:

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). It is known that resistance to ToMV strain 0 and 2 can also be obtained by another mechanism (like gene Tm1). However, the DNA sequence of Tm1 is not known and therefore no molecular marker is available for Tm1. Specific aspects:

1.	Pathogen	Tomato mosaic virus
2.	Functional gene	$Tm2/2^2$ (with two alleles for resistance Tm2 and $Tm2^2$ and one allele for susceptibility tm2)

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 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 $Tm2^2/Tm2^2$ or $Tm2^2$/Tm2	Reliable marker for Tm1 not yet developed
51.1 Strain 0	[1] absent	[9] resistant	[9] resistant	resistant
51.2 Strain 1	[1] absent	[9] resistant	[9] resistant	absent
51.3 Strain 2	[1] absent	[1] absent	[9] resistant	resistant

5.4 Explanation of characteristic-specific molecular markers in Test Guidelines

5.4.1. To ensure harmonized use of the characteristic-specific molecular marker it is important to standardize the conditions and methodology used. However, a balance between standardization and room/flexibility for improvement of the method in the light of technological developments should be aimed for. What is essential for harmonization and should be described in a technical guideline (protocol) and what is non-essential information but valuable information and can be provided in an annex or example is described in Table 2.

5.4.2 Standard elements in a characteristic-specific molecular marker test protocol are shown in Table 2. Indicated which information is essential for harmonization of characteristic-specific molecular markers in the Test Guidelines irrespective of the technology that is used.

Table 2:

	Elements in a Standard characteristic-specific molecular marker protocol	Example	Essential information for harmonization	Remark
1	characteristic	Resistance to Tomato mosaic virus (ToMV) *See TG/44/11/rev3 – Ad 51: ii DNA marker test*	YES	
2	Genes and alleles	*See TG/44/11/rev3 – Ad 51: ii DNA marker test add 2*		
2.1	Functional gene(s)	Resistance Gene Tm2 Arens, P. et al (2010)	YES	a) file(s) containing the DNA sequence information (order of nucleotides) b) reference to DNA information in public databases (like GeneBank) c) reference to (scientific) publications in which the DNA sequence information of the states of expression of the characteristic is revealed. d) reference to a particular position on the published reference genome version.
2.2	Allele corresponding to expression state 9 (present)	Tm2 and $Tm2^2$ Arens, P. et al (2010)	YES	a) file(s) containing the DNA sequence information (order of nucleotides) b) reference to DNA information in public databases (like GeneBank) c) reference to (scientific) publications in which the DNA sequence information of the states of expression of the characteristic is revealed. d) reference to a particular position on the published reference genome version in combination with the SNP or INDEL that is responsible for the state of expression.
2.3	Allele corresponding to expression state 1 (absent)	tm2 Arens, P. et al (2010)	YES	a) file(s) containing the DNA sequence information (order of nucleotides) b) reference to DNA information in public databases (like GeneBank) c) reference to (scientific) publications in which the DNA sequence information of the states of expression of the characteristic is revealed. d) reference to a particular position on the published reference genome version in combination with the SNP or INDEL that is responsible for the state of expression.
3	Primers (and probes)	*See TG/44/11/rev3 – Ad 51: ii DNA marker test add 3, 3.1 and 3.2*	NO	Primer and probe sequences, reference to accessions and sequences in public databases (Genebank numbers), literature
3.1	Primers to detect allele ‘9’		NO	Primer Sequences corresponding to allele(s) for expression ‘9’ (resistance)
3.2	Primers to detect allele ‘1’		NO	Primer Sequences corresponding to allele(s) for expression ‘1’ (susceptibility)

3.3	Primers to detect allele 'x'		NO	Primer Sequences corresponding to allele(s) for expression 'x'
4	Format of the test			
4.1	Number of plants	>#20	YES	A minimal number of individual plants required . (see 5.2.1a)
4.2	Control varieties	*See TG/44/11/rev3 – Ad 51: ii DNA marker test add 4.2*	YES	Control varieties (same as in bioassay) as standards representing all relevant combination of alleles. For example homozygous for Allele corresponding to expression state 9 (present), homozygous for allele corresponding to expression state 1 (susceptible) and heterozygous (both alleles are present in a diploid) corresponding to either resistant, susceptible or intermediate state of expression (depending on gene function; dominant - recessive).
5	Preparations		NO	e.g. Sampling of seedings 4 days old followed by DNA extraction using CTAB method Depending on the method used. Not in the Test Guideline. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker
6	Performance or protocol of the method	e.g. conventional PCR, TETRA-ARMS, qPCR, KASP, amplicon sequencing *See TG/44/11/rev3 – Ad 51: ii DNA marker test add 6*	NO	Depending on the method used. Not in the Test Guideline. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker
6.1	Particular conditions	e.g. PCR protocol describing primer, enzyme, dNTP concentrations, PCR cycle scheme	NO	Depending on the method used. Not in the Test Guideline. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker
6.2	Particular hardware or infrastructure	e.g. machines, commercial kits, manufactures of components, lot numbers of chemicals	NO	Depending on the method used. Not in the Test Guideline. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker
7	Observations	e.g. Bands on agarose gel (conventional PCR), Ct values (qPCR)Variant call based on sequencing reads	NO	Depending on the method used. Not in the Test Guideline. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker
8	Interpretation of the test results	*See TG/44/11/rev3 – Ad 51: ii DNA marker test add 8*	YES	Relation between alleles and expressions (with its notes)

[End of Annex and of document]