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DEVELOPMENT AND EVALUATION OF MOLECULAR MARKERS LINKED TO DISEASE RESISTANCE GENES FOR TOMATO DUS TESTING (OPTION 1A)

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DEVELOPMENT AND EVALUATION OF MOLECULAR MARKERS LINKED TO DISEASE RESISTANCE GENES FOR TOMATO DUS TESTING (OPTION 1A)

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INTRODUCTION

1. UPOV has considered proposals under 3 options for the possible use of molecular markers in DUS testing (see documents TC/38/14 – CAJ/45/5 and TC/38/14 Add. - CAJ/45/5 Add.). Option 1 considers the use of molecular characteristics as a predictor of traditional characteristics. Option 1 covers two possibilities: Option 1(a), the use of molecular characteristics which are directly linked to traditional characteristics (gene specific markers) and Option 1(b), the use of molecular characteristics which can be used reliably to estimate traditional characteristics; e.g. quantitative trait loci (QTLs).

2. It has been noted that molecular markers which are directly linked to traditional characteristics might be particularly useful for the examination of those traditional characteristics that cannot be easily or consistently observed in the field, or that require additional special arrangements (e.g. disease resistance characteristics). The *Ad hoc* Subgroup of Technical and Legal Experts of Biochemical and Molecular Techniques (BMT Review Group) concluded that "Proposal 1 (Option 1(a) for a gene specific marker of a phenotypic characteristic) was, on the basis of the assumptions in the proposal, acceptable within the terms of the UPOV Convention and would not undermine the effectiveness of protection offered under the UPOV system." (see document TC/38/14 Add. - CAJ/45/5 Add.). The Technical Committee (TC) and the Administrative and Legal Committee (CAJ) agreed with that conclusion and that Proposal 1 could be pursued on the basis of the assumptions in the proposal (see documents TC/38/16, paragraph 189 and CAJ/45/8, paragraph 19).

3. For the past 20-30 years, breeding in tomato has been focused on breeding for disease resistance. Resistance genes have been identified for several diseases. A large number (more than 40) of these genes, single (dominant) genes as well as QTLs, have been mapped and molecular markers linked to them identified. Also, a number of genes conferring resistance to diseases in tomato have been cloned and fully sequenced. Based on these sequences marker assays can be developed which directly tag the resistance gene. In this way, the reliability of the test is ascertained.

4. A consequence of the fact that all resistance genes have been introgressed from wild relatives into cultivated tomato is that disease resistance has become an important discriminating characteristic in DUS testing for tomato. Some resistance assays are used for new tomato varieties. Unfortunately, these resistance assays are not always straightforward for various reasons. Therefore gene specific markers for disease resistance might be used to predict disease resistance more efficiently.

5. Before implementing molecular marker assays, several requirements need to be fulfilled, as was also set out by the BMT Review Group in documents TC/38/14 – CAJ/45/5 and TC/38/14 Add. - CAJ/45/5 Add. Amongst others, these include:

(1) Reliability of the molecular marker: it is assumed that the molecular marker is a reliable predictor of the disease resistance characteristic. This will depend on factors such as: how close is the marker linked to the resistance gene, how reliable is the marker assay.

(2) Which resistance genes and influencing factors are involved. It is assumed that: different markers in the same gene would be treated as different methods for examining the same characteristic; and different markers linked to the same gene would all be treated as different methods for examining the same characteristic. Indeed, different genes might result in the same phenotypic expression of a given trait (resistance to TYLCV for example) and influencing factors (like variety genetic background or specific elements in the genome) can modify the phenotype despite the presence of the resistance gene(s) (this, for instance, is the case with some resistances to nematodes).

OBJECTIVES OF THE PROJECT

6. The objective of the project was to develop and evaluate an Option 1(a) approach for the asterisked (obligatory) disease resistance characteristics in the applicable CPVO tomato DUS protocol TP/44/2, which is itself based on UPOV Test Guidelines TG/44/10. For the development, existing mapping data and available sequence information were used. Marker assays were evaluated for robustness and reproducibility. Results from marker analysis were compared to phenotypic characterizations using varieties that were in ongoing DUS trials. Conclusions were drawn and recommendations made on the feasibility of Option 1(a).

<u>Resistance genes evaluated and assays developed</u>

7. In this project molecular assays for the asterisked (obligatory) disease resistance characteristics, as mentioned in the applicable CPVO tomato protocol TP/44/2, were developed and evaluated. It is to be noted that it is impossible to obtain a strain for ToMV:1.2., but since the resistance gene controlling it is usually Tm-2^2, this asterisked disease resistance was not further considered for the project. Molecular marker assays were developed and/or tested for the Verticillium Ve1 and Ve2, the Tomato Mosaic Virus Tm1 (linked marker), the Tomato Mosaic Virus Tm2 and $Tm2^2$, the *Meloidogyne incognita* Mi1-2, the Fusarium I locus (linked marker) and the Fusarium I2. Depending on the already available data (markers, sequences of the resistance/susceptibility alleles) for each resistance gene, the starting situation for the development of an easy and robust molecular assay was different. For instance for *Meloidogyne incognita*, a number of different assays were already described in literature from which the best assay with respect to clearness and robustness was chosen. For some other diseases, sequence information from the linked markers or the

susceptible allele was missing and had to be obtained prior to assay development. Type of marker assay developed is briefly described below:

Verticillium genes Ve1 and Ve2: Based on identified SNPs between resistant and susceptible cultivars, two tetra primers ARMS tests were developed which allow co-dominant scoring of phenotype for both genes.

Tomato Mosaic Virus Tm1 (linked marker): Only two dominant SCAR markers from literature (Ohmori et al. 1996) could be selected. Because of dominant nature, a positive control (Lat gene primers) for amplification has been included.

Tomato Mosaic Virus Tm2 and Tm2^2: These resistance genes are allelic. Combination of two CAPS markers available from Lanfermeijer et al. (2005) and also a developed combination of two tetra primer ARMS PCR tests allow co-dominant scoring of phenotypes.

Meloidogyne incognita Mi1-2: Co-dominant SCAR marker (based on promoter region) available from Mehrach et al (2005) was selected.

Fusarium I locus (linked marker): Based on identified indel (derived from linked marker C2_At2g22570), a dominant linked marker is available. Because of dominant nature, a positive control (Lat gene primers) for amplification has been included.

Fusarium I2 locus: Dominant PCR marker developed for I2 locus (and highly specific to the *S. pimpinellifolium* resistance allele) that can be combined with a Rubisco positive control on amplification.

Robustness test

8. The aim of this part of the study was to assess how robust the developed marker assays are. Based on the reports of the marker development (which contained detailed instructions on the test and the conditions to be used in the assays), all partners performed the developed assays in their own laboratory on an agreed set of varieties. For the robustness test, DNA from varieties used for the development of the tests (at least one resistant and one susceptible variety per test) were distributed. Besides these DNA samples, each of the five partners isolated DNA from the varieties ‘Marmande’ and ‘Moneymaker’, representing susceptible varieties and from ‘Campeon’ (from Clause Tezier) and ‘Persica’ (from Gautier) representing resistant varieties. These varieties were used in the different tests by all partners.

9. Transfer of molecular markers from one laboratory to the next often requires optimization because, between most laboratories, there are differences in the equipment (notably different PCR machines) and in the reaction components used (mainly Taq polymerases). Nevertheless, for each assay, results could be reproduced without prior optimization in at least two other laboratories (five labs in total). Individual laboratory optimization was needed to achieve assays at the required level of scorability. Despite some initial problems in a number of tests that needed attention, even these first results often indicated the expected patterns and the necessary conditions for unambiguous scoring seemed close. After optimization, all assays worked well.

Validation of marker assays

10. The marker assays developed were evaluated for their usefulness in predicting disease resistance. For this, evaluation of marker assays was carried out on 20 varieties per disease test (except Tm1) for each of the DUS stations (GEVES, INIA and Naktuinbouw). The Tm1 gene (conferring resistance to strains 0 and 2) is hardly used in varieties. Most varieties contain $Tm2^2$ based resistance (which includes resistance to strains 0 and 2). In a $Tm2^2$ background it is not possible to assess with a pathogenesis assay whether or not Tm1 is present. Therefore, this resistance assay was not validated.

11. Results from INIA were obtained by analyzing two plants per variety, except for varieties with heterogeneous or unclear results in the biological assay, in which case five plants were used. Results from GEVES were obtained from one sample that contained the pooled DNA of 2 plants. Results from Naktuinbouw were obtained from 2 plants per variety. For each of the separate tests, the overall conclusions are described below. It is important to realize that not all the pathogenesis assays were made on the same plant material that was used for the molecular assay. Also, pathogenesis assays may have been performed in different years, but using the same seed lot.

12. For the ToMV resistance, results from pathogenesis test and molecular marker assay fitted exactly. For the other diseases, in a number of cases, the pathogenesis tests deviated from the molecular marker assays or showed unclear results. In the Ve resistance tests, in 5 out of the 69 cases, varieties or a number of individual plants of a variety were found to be susceptible or weakly affected (none of the plants died) in the pathogenesis assay whereas the molecular marker assay indicated that the plants tested in this assay should be resistant. In one case, a variety was found to be resistant in the pathogenesis assay, but showed a susceptible phenotype in the molecular marker assay. The discrepancies observed may be related to the inoculums used or due to the sensitivity of the pathogenesis test to environmental conditions.

13. In the Mi resistance pathogenesis assay, four varieties (including the intermediate resistant control variety ‘Madyta’) showed heterogeneous results with more susceptible than resistant plants, whereas the molecular marker assay for each of the 2 plants tested suggested a Mi1-2/mi1-2 heterozygous (resistant) phenotype. Recent studies at Naktuinbouw and INIA indicate that these varieties show a band at approximately 500-510 bp (instead of 550 bp) which may be linked to the weak Mi resistance gene Mi-J giving intermediate resistance (D. Smilde and C. Mansilla, pers. comm.). The size difference is due to a small deletion, which might explain (part of) the “intermediate resistance” phenotypes.

14. In the Fusarium I pathogenesis assay, 2 varieties were found to be susceptible, whereas the molecular marker assays suggested that they would be resistant. Seven varieties showed heterogeneous results from the pathogenesis assay (including control ‘Marporum’) whereas the 2 plants tested for each variety showed either resistant or susceptible results. This suggests that the pathogenesis assay may be affected by environmental factors, which do not obscure the molecular marker assay, or that the samples size for the molecular assay was too small to find the heterogeneity in the variety.

15. Finally, the Fusarium I2 pathogenesis assay showed 5 varieties for which the results were inconclusive, all of them were scored as susceptible in the molecular marker assay.

DISCUSSION AND CONCLUSION

Assay development and testing

16. In this project, molecular assays for 6 disease resistance loci in tomato were developed and validated. The assays are based on either tetra ARMS PCR markers, CAPS markers or SCAR markers. When possible, the cloned resistance gene was taken as the starting point for marker development. The assays developed targeted the Verticillium resistance genes Ve1 and Ve2, the Tomato mosaic virus resistance genes Tm1 (linked marker) and Tm2/Tm2^2, the nematode resistance gene Mi1-2, the Fusarium I Iocus (linked marker) and the Fusarium I2 locus. All assays were tested by the partners of the project and proved to be robust. In most cases the assays work well in the different laboratories. In a few cases, optimization of the PCR reaction was needed, but in the end all laboratories succeeded in running the assays.

Assay validation

17. Assay validation was carried out by the three DUS testing stations that participated in the project. Each station had selected around 20 varieties per disease with different resistance profiles, as judged from the pathogenesis assays that had been carried out in the framework of DUS testing. All those varieties were also genotyped using the molecular assays.

18. In 97% of the cases, the molecular marker assays confirmed the results obtained from the pathogenesis assays. Pathogenesis assays and marker assays gave identical results for the nematode resistance gene Mi1-2 and TMV resistance genes. Minor deviations between the pathogenesis assay and marker assay were observed for the fungus resistance genes (Verticillium and Fusarium). The poorest results were obtained for the Verticillium assay. In 8% of the cases, there was a discrepancy between the pathogenesis and the marker assay. Observed discrepancies are most likely due to the pathogenesis assay, which is strongly dependent on the conditions used to carry out the assay and on the inoculums. Those are more difficult to standardize and are more subjectively interpreted than the assays for virus and nematode resistance. Marker assay seem to perform better. Not only because the results are clearer but also because homozygote/heterozygote presence of the resistance gene can be detected. Resistance genes that are present in a heterozygote state are more difficult to score in a pathogenesis test. The results obtained so far may indicate that the sole presence of either the Ve1 or the Ve2 allele in heterozygous state is not sufficient to keep plants from showing symptoms in the pathotests. Markers are also good at spotting heterogeneity, as was shown for a number of cases. Therefore, it is recommended to develop marker assays for other diseases where pathogenesis tests are similarly or even more difficult to perform (e.g. for TSWV it is found feasible to implement such a test). With regard to a possible loss of linkage for the Fusarium I (linked) marker; this issue is difficult to address with the current data, but if present, the frequency does not exceed 4 in 70 (5.7%) based on the number of differing results between the molecular marker and pathogenesis results.

Implementation of marker assays in daily DUS testing.

19. In current DUS testing, the evaluation of disease resistance is carried out by performing a pathogenesis test using an isolate of the pathogen under study. The phenotype of the candidate variety, in terms of the global level of expression of the disease resistance, is described in the conditions of this test. So far, the presence of the genes involved in the observed phenotype is not assessed.

20. In the last decade, several disease resistance genes from tomato have been cloned and sequenced. These sequences have been used to develop molecular markers hybridizing directly in the genes or in their near regions (linked markers). Potentially all these markers can be used to detect whether a particular resistance gene is present in a variety or not.

21. However, they do not always allow a conclusion on the level of the variety resistance, which depends on the nature of the pathogenic strain used, on the genetics of the resistance and on the genetic background of the variety. Therefore, using molecular markers of resistance genes to characterize candidate varieties implies a precise knowledge of the effects of these genes in the resistance phenotype.

22. Option 1(a) applied to disease resistance characteristics is an approach adapted to diseases that; firstly, are controlled by very few major genes; and secondly, that do not evolve too rapidly (to avoid a continuous development of new molecular tests). For those reasons, disease resistance in tomato is a good model. Additionally, during the last decades, breeding in tomato has been focused on resistance breeding using single sources for resistance for the obligatory diseases mentioned in the CPVO guidelines. This has resulted in many varieties containing different combinations of reasonably well known resistance genes. Within the group of partners involved in this project, several molecular tests have been successfully developed and tested that target these genes.

Consequences for the tomato DUS system:

23. The implementation of marker assays in tomato DUS testing triggers some questions that remain to be addressed:

- Should breeders have the obligation to indicate which resistance genes are present in a particular variety?
- When the resistance data provided by the breeder of the candidate variety exactly match with marker data obtained by a DUS testing station, could it be considered as sufficient evidence for the presence or absence of resistance and a reason not to carry out the pathogenesis test again?
- Could an approved biomolecular test be recognized in the Test Guidelines as a predictor of the resistance? Who would approve it? Based on which criteria?
- If more than one gene can confer the same resistance, could different predictors be considered separately in the Test Guidelines?
- In the official variety description, the exact information that the official authority has about the resistance presence in a variety should be indicated. So the description of resistance characteristics would be a little more complex than now.
- Should DUS testing stations carry out the molecular tests or could they also be subcontracted out?

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