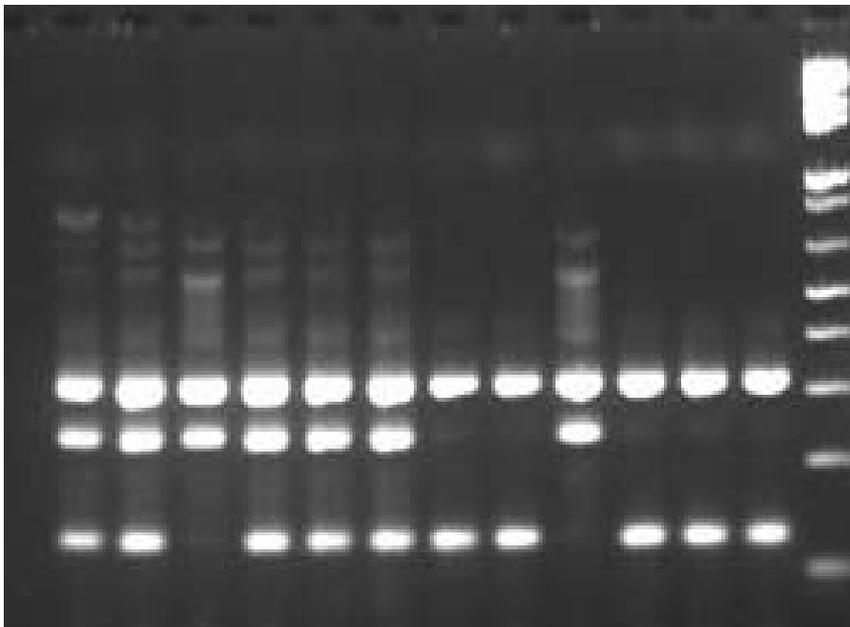


Final report

Development and evaluation of molecular markers linked to disease resistance genes for tomato DUS testing (option 1a)



December 2007

Final report

Development and evaluation of molecular markers linked to disease resistance genes for tomato DUS testing (option 1a)

Paul Arens, Laetitia Cavellini, Sophie Rolland, André Moretti, Hanneke van der Schoot, Carmen Mansilla, Cécile Collonnier, Diederik Smilde, Daniël Deinum René Mathis, Carole Caranta, Ben Vosman

This project is co-funded by the Community Plant Variety Office, the ministry of agriculture, Nature and Food quality of the Netherlands, GEVES, INRA, the French ministry of Agriculture and the Spanish ministry of agriculture.

Institution responsible of the project: Plant Research International
P.O.Box 16
6700 AA Wageningen
The Netherlands

Other institutes involved:

France:

GEVES (Groupe d'Etude et de contrôle des Varietes Et des Semences), contact
Dr. Cecile Collonnier
La Miniere 78 285 Guyancourt cedex, FR
cecile.collonnier@geves.fr

INRA Avignon, FR, contact Dr. Carole Caranta
Unité de recherche Génétique et amélioration des fruits et légumes
INRA Domaine Saint-Maurice - BP 94
84143 MONTFAVET cedex, FR
carole.caranta@avignon.inra.fr

Netherlands:

Naktuinbouw, contact Dr. W.D. Smilde
Postbus 40, Sotaweg 22, 2370 AA Roelofarendsveen, NL
d.smilde@naktuinbouw.nl

Spain:

Jefe de Área del Registro de Variedades, Oficina Española de Variedades
Vegetales (OEVV), Ministerio de Agricultura, Pesca y Alimentación (MAPA),
contact Luis Salaices
Avenida de Ciudad de Barcelona No 6, 28007 Madrid, ES
lsalaice@mapya.es

Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), SP,
contact Mr. David Calvache
Centro de ensayos de Valencia, Calle Joaquin Ballester, 39, ESPAÑA 46009
Valencia.
oevvval@teleline.es

Co-ordinator and responsible scientist: Dr. Ben Vosman (PRI)
ben.vosman@wur.nl

Table of contents

Executive summary

Introduction

Objectives addressed

Resistance genes evaluated in the study

Background on the Resistance genes

Assay development

Robustness test

Validation

Discussion and Conclusions

Literature

Appendices

1. Minutes of first project meeting
2. Minutes of second project meeting
3. Minutes of third project meeting
4. Assay for *Verticillium* resistance genes Ve1 and Ve2
5. Assay for Tomato Mosaic Virus resistance Tm1 gene
6. Assay for Tomato Mosaic Virus resistance Tm2 and Tm22 genes
7. Assay for *Meloidogyne incognita* resistance Mi1-2 gene
8. Assay for *Fusarium* I resistance locus
9. Assay for *Fusarium* I2 resistance locus
10. Results Robustness test
11. Results validation INIA
12. Results validation Geves
13. Results validation Naktuinbouw
14. Evaluation of wild accessions

Executive summary

The UPOV BMT group has proposed three options for implementing molecular markers in DUS testing. Option 1 deals with the use of molecular characteristics as a predictor of traditional characteristics. It is split up into two sub options, 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. In this project we have developed and evaluated an option 1(a) approach for the asterisked (obligatory) disease resistance characteristics in the applicable CPVO tomato DUS protocol TP/44/2. Molecular marker assays were developed for the Verticillium genes Ve1 and Ve2, the Tomato Mosaic Virus Tm1 (linked marker), the Tomato Mosaic Virus Tm2 and Tm2² genes, the Meloidogyne incognita Mi1-2 gene, the Fusarium I locus (linked marker) and the Fusarium I2 locus. The markers were tested for their robustness and subsequently validated in a set of tomato varieties. 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 ToMV resistance genes. For the fungal resistance genes for Verticillium and Fusarium minor deviations between the pathogenesis assay and marker assay were observed. 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. These are more difficult to standardize and are more subjectively interpreted than the assays for virus and nematode resistance. Marker assays have the advantage that the results are clearer and homozygote/heterozygote presence of the resistance gene can be detected. Marker assays are also good at spotting heterogeneity. Consequences of implementation of the marker assay in DUS testing are discussed.

Introduction

The UPOV BMT group has developed 3 options for implementing molecular markers in DUS testing. The first, option 1, deals with the use of molecular characteristics as a predictor of traditional characteristics. It was split up into two sub options, 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).

Within the crop subgroups, it was 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). Following the discussion in the BMT review group it was concluded that " Option 1(a) 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 (TC/38/14 Add. – CAJ/45/5 Add.)".

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. These include genes conferring resistance to all major classes of plant pathogens. Amongst others these include *Verticillium* (*Ve*), *Fusarium oxysporum* f.sp. *lycopersici* (races 0, 1 and 2), *Fusarium oxysporum radialis lycopersici* (*Forl*), *Cladosporium* (*Cfs* alleles), *Stemphylium* (*Sm*), *Oidium* (two species), *Phytophthora infestans*, *Pyrenochaeta* (*pyl*), root knot nematodes (*Mi*), Tomato Mosaic Virus (*ToMV*), Tomato Spotted Wilt Virus (*TSWV*), Tomato Yellow Leaf Curl Virus (*TYLCV*), *Pseudomonas* tomato (*Ps*), *Ralstonia solanacearum*, *Clavibacter michiganense*, for reviews see Barone (2002) and Berloo & Lindhout (2001). Also, a number of genes conferring resistance to diseases in tomato have been cloned and fully sequenced. Amongst the sequenced genes are the *Cladosporium* resistance genes *Cf-2*, *Cf-4*, *Cf-5*, *Cf-9*, the *Pseudomonas syringae* resistance gene *Pto* and the nematode resistance gene *Mi*. Sequence data can be found in databases (<http://www.sgn.cornell.edu/> or <http://www.ncbi.nlm.nih.gov/>). 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.

A consequence from the fact that all resistance genes have been introgressed from wild relatives into cultivated tomato is that disease resistance has become an important discriminating character in DUS testing in tomato. Some resistance assays are compulsory for new tomato varieties. Unfortunately, these resistance assays are not always straightforward for various reasons:

(1) Several factors might influence the expression of the characteristic (environmental conditions, level of heterozygosity for monogenic dominant genes). Some diseases like *Fusarium* and *ToMV* can cause major testing problems. Factors like loss of pathogenicity of the isolates, subjectivity of scoring, differences between isolates at testing stations also contribute to this. Molecular markers could help solving these problems.

(2) Pathogenesis tests are often costly (eg. *Pyrenochaeta*, nematodes, *Clavibacter michiganense*, *Ralstonia solanacearum* and *Phytophthora infestans* tests). If such tests could be replaced by molecular marker assays, this project could result in more cost effective disease resistance testing.

(3) Biotests can be submitted to legal rules when dealing with quarantine pests (such as TYLCV). Therefore replacing pathogenesis tests by molecular marker assays can result in a more efficient use of resources and a system which is more robust, and as it depends less on individual expertise, less open to criticism on the basis of perceived subjectivity. It will not change the way the DUS testing is carried out, but it will ensure it is done in the most reliable, technically credible, way. It will also offer breeders better ways to enforce the rights granted to them.

Before implementation molecular marker assays, several requirements need to be fulfilled, as was also set out by the experts of France in document TC/38/14 – CAJ/45/5. Amongst others these include:

- (1) Reliability of the molecular test. 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. 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

The objective of the project is 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. For the development, existing mapping and sequence information will be used. Marker assays will be evaluated for robustness and reproducibility. Results from marker analysis will be compared to phenotypic characterizations using varieties that are in ongoing DUS trials. Conclusions will be drawn and recommendations made on the feasibility of option 1(a).

Resistance genes evaluated in the study

In our research project, we have evaluated the asterisked (obligatory) disease resistance characteristics as mentioned in the applicable CPVO tomato protocol TP/44/2:

- *Meloidogyne incognita*, Mi1-2 gene
- *Verticillium dahliae*, Ve1 and Ve2 gene
- *Fusarium oxysporum* f.sp. lycopersici Race 0, 1 locus
- *Fusarium oxysporum* f.sp. lycopersici Race 1, 12 locus
- Tomato Mosaic Virus – Strain 0
- Tomato Mosaic Virus – Strain 1
- Tomato Mosaic Virus – Strain 2
- Tomato Mosaic Virus – Strain 1-2

During the first meeting of the partners (March 26, 2006, Wageningen, NL), current testing procedures and the problems encountered with the asterisked disease resistance characteristics have been discussed, resulting in the following conclusions:

Meloidogyne incognita (Mi) evaluation; main issues are the production of a good inoculum (multiplication and stability), the identity of the nematodes, the differences in resistance levels depending on the zygotic state of the resistance gene and may be on the background within which the resistance gene is introgressed. The trial is lengthy and takes at least 40 days after inoculation. The test is reliable but expensive. The resistance is not effective for temperatures above 28-30°C.

Verticillium dahliae (Vd) evaluation; main issues are the stability of the inoculums; there is loss of aggressiveness of strains after multiplication and differences in intensity of symptoms. There is no standard inoculum. The reliability of the test is not always the same, depending on the year. Different strains of *Verticillium* exist; there has been breakthrough of resistance reported for some strains. It is a test with problems. Disease is part of an ongoing ring test. This test aims at harmonization between the different countries. Inoculums are exchanged and tests carried out between INIA, GEVES and Naktuinbouw. The ring test is in its second year.

Fusarium oxysporum f. sp. *lycopersici* (Fol) evaluation; for both race Fol:0 (ex 1) and Fol:1 (ex 2) differences in inoculums exist and loss of aggressiveness of strains occurs. Disease is also part of the ongoing ring test. Differences found in definition of susceptibility cut-off values in harmonization test.

Tomato Mosaic Virus (ToMV) evaluation; good standards for definition of virus strains do not exist and standards are difficult to obtain. Gene Tm-2² is mainly used and confers resistance to all strains (ToMV: 0,1,2). Therefore, in Spain, when the applicant declares resistance to ToMV to be absent or present to the four strains (corresponding to the Tm-2² allele), the test is routinely carried out with race ToMV:0. Other races are used in a randomized way, or in concrete cases. There are difficulties noted in accessing the levels of necrosis, levels depend on temperature, light and lifecycle stage of plants. It is best to test inoculums by dilution series on tobacco plants first.

It is impossible to obtain a strain for ToMV: 1.2.

ToMV: 1.2 is not further considered for the project. Because the resistance gene controlling it is usually Tm-2² this has no further implications for work load or shifts therein.

Background on the Resistance genes

Details on each of the genes can be found in the description made for each of these genes, see appendices 4-10.

Meloidogyne incognita Mi-1. The gene maps on chromosome # 6, Mi1-2 confers resistance to nematodes and is in a contig with gene Mi1-1 and a pseudogene. Linked CAPS markers have been described. The Mi-1 CAPS marker REX-1 reported by Williamson et al. (1994) is known to give false positives in some backgrounds. The Mi1-2 gene is cloned (Milligan 1998). Other Mi resistance genes are identified (Mi-3 and Mi-9). Mi-3 effective above 30°C, gives resistance to virulent strains of *M. incognita* that break Mi1-2 resistance. Mi-9 confers resistance to nematodes also at higher temperatures, is localized at the short arm of # 6 within the same genetic interval that spans the Mi-1 region. No existing commercial varieties with these new genes are available so far.

Verticillium dahliae Ve1 and Ve2. Both genes map on chromosome #9 and confer resistance to Verticillium. Linked markers are available (Kawchuk et al 1998), this includes a co-dominant SCAR marker as well as an allele specific SCAR marker. The Ve1 and Ve2 Gene are cloned (Kawchuk et al. 2001) both conferring resistance. In literature only the sequences from resistant lines are described. For the design of a molecular assay sequence information from susceptible plants has to be obtained.

Tomato Mosaic Virus Tm-1, Tm-2 and Tm-2² The genes map on chromosomes #2 and #9 respectively (Tm-2 and Tm-2² are alleles). Tm-1 confers resistance to strains ToMV:0 and 2. Linked markers for Tm-1 are available. Ohmori et al. (1996) described 6 closely linked SCAR markers of which 2 are co-dominant. The gene is not cloned yet. The Tm-2 gene confers resistance to strains ToMV: 0 and 1. Tm-2² confers resistance to strains ToMV: 0, 1, 2, 1.2. Both genes are allelic. Linked markers are available Dax et al. (1998) report a co-dominant SCAR marker to Tm2², closely linked RFLP markers are reported by Young et al. (1988, 1989), Sobir et al (2000) report a CAPS marker to Tm-2. The Tm-2² and Tm-2 gene are cloned: Lanfermeijer (2003, 2005). Molecular test was described to distinguish the alleles; CAPS marker from Lanfermeijer (2005). Sequences are available from resistant alleles Tm-2, Tm-2² and susceptible alleles tm-2 and lptm-2.

Fusarium oxysporum f. sp. Lycopersici Genes I and I-2 map on short and long arm of chromosome #11 respectively. The I locus confers resistance to race Fol:0 (ex1). Linked markers are available (Eshed unpublished data in Ori et al. ,1997); 5cM interval between 2 RFLP markers (TG523 and CP58A). Other linked RFLP markers are described by Sela-Buurlage et al.(2001). The I-2 gene confers resistance to race Fol:1 (ex2), I2C-1 confers partial resistance. Linked markers are described by Simons et al. (1998); TG 105 is closely linked to I2 (0.4 cM). The genes are cloned, I-2 by Simons et al. (1998) and I2C-1 by Ori et al (1997)

Other sources of resistance have been mapped by Sela-Buurlage (2001) and the I2C-1 gene is member of a large family of which I2C-2 is also cloned and other members have been mapped on chromosomes # 8 and 9. Work in potato indicates that additional members of this family can be expected at other chromosomes as well.

Assay development

Molecular assays were developed for the different disease resistance genes. As described in the previous chapter, for each disease the starting situation was different. Research efforts depended on the already available data and the requirements for developing an easy and robust molecular assay. 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. Results from the marker development are described briefly below and primers for the different assays given in table 1. A full description of the experiments that have been performed to develop the tests including results and experimental conditions can be found in the appendixes 4 through 9.

Verticillium genes Ve1 and Ve2: Sequence information was retrieved on Ve 1 and Ve2 fragments from resistant and susceptible cultivars (gene specific primers were developed from sequences deposited by Kawchuk et al. 2001). These sequences showed a number of specific SNPs on which 2 (Ve1 and Ve2) tetra primers ARMS tests were developed which allow co-dominant scoring of phenotype for both genes (Appendix 4). For the tetra primers ARMS tests also primer sets have been made in which the deliberate mismatch was closer to the 3' SNP end of the primer (Table 1).

Tomato Mosaic Virus Tm1 (linked marker): only dominant SCAR markers from literature available (Ohmori et al. 1996). Because of dominant nature, a positive control (Lat gene primers) for amplification has been included (Appendix 5).

Tomato Mosaic Virus Tm2 and Tm2²: 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 (Appendix 6).

Meloidogyne incognita Mi1-2: Co-dominant SCAR marker available from Mehrach et al (2005) has been selected (Appendix 7).

Fusarium I locus (linked marker): A number of linked RFLP markers (TG523, TG7, C2_At2g22570 and C2_At5g16710) that have been described in literature were sequenced in an attempt to obtain sequence information from resistant and susceptible varieties at these marker loci. Dominant linked marker based on C2_At2g22570 is available (Appendix 8).

Fusarium I2 locus: Dominant PCR marker available for I2 locus (and highly specific to the *S. pimpinellifolium* resistance allele) that can be combined with a Rubisco positive control on amplification. (Appendix 9)

Table 1: Primers used, type of assay and amplification products expected

Disease and gene involved	primer name	Sequence (5' – 3') ²	assay type	Amplification products (bp)			Origin of test
				R-band	S-band	Other	
M. incognita Mi1-2	PMiF3	GGTATGAGCATGCTTAATCAGAGCTCTC	A	550	350		Literature El Mehrach et al. (2005)
	PMiR3	CCTACAAGAAATTATTGTGCGTGTGAATG					
ToMV Tm-1	SCA15F	CCGAACCCCTTAAAAATAGTTTCA	B	1400	-	92 (Lat product)	Literature Ohmori et al. (1996)
	SCA15R	CCGAACCCAATCAGGAGGCTCATA					
	SCN20F	GGTGCTCCGTCGATGCAAAGTGCA	B	1200	-	92 (Lat product)	Literature Ohmori et al. (1996)
	SCN20R	GGTGCTCCGTAGACATAAAATCTA					
Tm-2/Tm2 ² assay 1 SNP2493/2494	Outer primers TMV-2286F.	GGGTATACTGGGAGTGCCAATTC	C			416	Primer design based on Lanfermeijer et al. (2005)
	TMV-2658R	CCGTGCACGTTACTTCAGACAA					
	Tm2 ² SNP 2494 F	CTCATCAAGCTTACTCTAGCCTACTTTAGT		214 (Tm2 ²)			
	Tm2 SNP2493 R	CTGCCAGTATATAACGGTCTACCG		254 (Tm2)			
Tm-2/Tm2 ² assay 2 SNP901	Outer primers TM2-747F	CGGTCTGGGGAAAACTCT	C			509	Primer design based on Lanfermeijer et al. (2005)
	TM2-1256R	CTAGCGGTATACCTCCACATCTCC					
	TM2- SNP901misR	GCAGGTTGTCCTCCAAATTTCCATC		185 (Tm2 or Tm2 ²)			
	TM2- SNP901misF	CAAATTGGACTGACGGAACAGAAAAGTT			381 (tm2)		
V. dahliae Ve1 Assay SNP706	Outer primers Ve1_2072F	CCTTGATGGGGTTGATCTTTCGT	C	476	158	580	Sequence retrieval (based on Kawchuk et al. 2001) and development of assay this study
	Ve1_2651R	GTAGGTGAGTTTCTTGGACAGTCGA					
	Ve1_SNP706Ft	CAGGCCCTTTGGATGAATCACATT					
	Ve1_SNP706Ra	GTTGGACAAAAGAGAGAAAAGTGAAGCTAAGT					
	Ve1_SNP706F1t ¹	CAGGCCCTTTGGATGAATCACTAT					

	Ve1_SNP706R1a ₁	GTTGGACAAAAGAGAGAAAAGTGAAGCTTACT					
Ve2 Assay SNP 2827	Outer primers Ve2_2720F	GGATCTTAGCTCACTTTATGTTTTGAAC	C			321	Sequence retrieval (based on Kawchuk et al. 2001) and development of assay this study
	Ve2_3040R	GGTGCTGGTTTCAACTCTGAAGT					
	Ve2_SNP2827F	CAAATGCTTGAATCACTAGACCTGTGAAC			131		
	Ve2_SNP2827R	GGATCTCCCCGGACAGGTGGATTC		242			
F. oxysporum race 0, Fo 1	At2-F3	CGAATCTGTATATTACATCCGTCGT	D	130			Sequence retrieval of markers (based on data from Sol Genomic Network) and development of assay this study
	At2-R3	GGTGAATACCGATCATAGTCGAG					
F. oxysporum race 1, Fo 2	Z1063F	ATTTGAAAGCGTGGTATTGC	E	940		1380 (Rubisco product)	Primer design based on sequences Simons et al. (1998)
	Z1063R	CTTAAACTCACCATTAAATC					
Controls							
LAT	Lat1-F	AGACCACGAGAACGATATTTGC				92	
	Lat2-R	TTCTTGCCTTTTCATATCCAGACA					
	Rubisco F	ATGTCACCACAAACAGAGAC				1380	
	Rubisco R	CTCACAAGCAGCAGCTAGT					

Robustness test

Goal 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 each of the developed assays in their own lab 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 sent around. 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 (see Appendix 10). The results of these tests were compiled and are reported here.

Compiled results of robustness test

Transfer of molecular markers from one lab to the next often requires optimization because between most labs differences exist in the equipment (notably different PCR machines) and in the reaction components used (mainly Taq polymerases). For each assay results could be reproduced without prior optimization in at least two other labs. Lab individual optimization was needed to get all assays at the required level of scorability. Despite some initial problems in a number of tests that needed attention even these first results were often indicating the expected patterns and the good conditions for unambiguous scoring seemed close. In the following report the results have been compiled on basis of the individual reports, the interpretation of included photos and the additional information added in the Madrid meeting (June 25, 2007). The compiled results of the robustness tests after the first series of experiments and before the individual optimization are added in the appendix of this report (Appendix 10). The test set of cultivars used in the assays for each of the resistance genes can also be found in this appendix. A summary of the results is presented in table 2.

Conclusions from the robustness tests of the molecular assays for the different disease resistance genes are:

- **Verticillium genes Ve1 and Ve2.** Tetra primers ARMS tests. Ve1 markers were tested and work in almost all labs. Initial problems were solved by raising the annealing temperature to 57°C or by using new primers in which the deliberately introduced mismatch was placed directly next to the SNP (see Table1 marker development). Ve2 markers also work in almost all labs. Improvement was obtained for one by using a touch down PCR protocol; another had to use a different Taq polymerase to reduce additional bands. The assay works well after optimisation.
- **Tomato Mosaic Virus Tm1 (linked marker):** dominant marker + LAT primers. Gene is hardly used in cultivars. Marker is OK, but LAT primers caused some problems. Concentration of LAT primers was doubled, which solved the problem. INIA also did a first 5 cycles at 55 °C before raising the annealing temperature to 60 °C. The assay works well after optimisation.
- **Tomato Mosaic Virus Tm2 and Tm2²:** These resistance genes are allelic. Combination of two CAPS markers available from Lanfermeijer et al. (2005) and combination of two tetra primer ARMS PCR tests allow co-dominant scoring of phenotypes. CAPS markers were tested in 3 labs and worked well. However, tetra primer ARMS PCR tests are preferred. Improvement of these tests was obtained

for some by increasing annealing temperature. The assay works well after optimisation.

- **Meloidogyne incognita Mi1-2:** Co-dominant SCAR marker available from Mehrach et al (2005). Assay works well in all labs.
- **Fusarium I locus (linked marker):** Dominant PCR assay works well in most labs. INRA was never able to obtain any amplification even with an aliquot of primers sent by GEVES. The background of this problem remains unclear and was not further investigated. At PRI, INIA and Naktuinbouw, there was a problem with Marmande (not tested at GEVES). This cultivar is expected to be susceptible, but is according to the PCR test resistant. Naktuinbouw did a disease test with plants from the same seedlot and the biotest indeed indicated that 13 plants were resistant and 10 were susceptible. This means that the plant that was used in the robustness test may have been a resistant plant.
- **Fusarium I2 locus:** Dominant PCR marker available for I2 locus combined with a Rubisco positive control. Marker is OK, Rubisco fragment is too dominant. Reduction of primer concentration needed for some.

In general, all assays work well.

Table 2: Overview of test results

Test	CBGP-INIA	Naktuinbouw	GEVES	INRA	PRI
Ve1	OK	OK ¹	OK ¹	OK ¹	OK
Ve2	OK	OK	OK ¹	OK ¹	OK
Tm1	OK ¹	OK	OK ¹	OK	OK
Tm2 – CAPS	OK	na	OK	na	OK
Tm2 - ARMS	OK	OK	OK	OK ¹	OK
Mi1-2	OK	OK	OK	OK	OK
I	OK	OK	OK	X	OK
I2	OK ¹	OK ¹	OK	OK	OK

¹ Test works well after optimization, na not analysed, X amplification failed.

Validation of marker assays

Because the robustness test showed that, except for some minor problems, all assays could be reproduced, the marker assays were evaluated for their usefulness in predicting disease resistance. For this, evaluation of marker assays was carried out on 20 varieties for each of the DUS stations (GEVES, INIA and Naktuinbouw).

The detailed results on the evaluations (including cultivar list) from each of the DUS stations are given in appendices (11 = results INIA, 12 = results GEVES, 13 results Naktuinbouw). Results from INIA were obtained by analyzing two plants per variety, and in those varieties with heterogeneous or not clear result in the biological assay (X in table of Appendix 10) 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 (except for two varieties). For each of the separate test the overall conclusions are described below and summarized in table 3. 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.

- **Verticillium genes Ve1 and Ve2.** Tetra primers ARMS tests. Both genes are believed to confer resistance.

Results:

At INIA, the results of the pathogenesis assay were confirmed by molecular markers in susceptible and resistant varieties, except in Agraz5. This cultivar is susceptible according to the molecular marker assay whereas it was scored resistant in the pathogenesis assay. Furthermore in the pathogenesis assay inconclusive results were obtained with 4 cultivars due to heterogeneity. In the molecular marker assays 3 of these cultivars showed heterogeneity in the scoring of the 5 different plants whereas for the fourth only two plants have been analyzed that showed the same phenotype in the molecular marker assay.

At GEVES, all results were conform expectations from the pathogenesis assay except for Q49 cv. Tracie which is scored susceptible in the pathogenesis assay whereas the molecular marker assay indicates there is at least one copy of the resistant allele for both Ve1 and Ve2 in the pooled sample. Similarly, Q72 cv. Harmony is scored susceptible whereas the molecular marker assay indicates the presence of at least one copy of the resistant Ve1 allele in the pooled sample.

At Naktuinbouw, two susceptible varieties did not give expected results. One variety had the resistant allele of Ve1. The other variety had no product for Ve2. Other results of the molecular marker assay were in agreement with the pathogenesis assay. All the resistant varieties were either homozygous or heterozygous resistant for Ve1 and Ve2. Six varieties were homozygous resistant for Ve1 and heterozygous resistant for Ve2.

- **Tomato Mosaic Virus Tm1 (linked marker):** dominant marker used in combination with LAT primers. Because the gene is hardly used in cultivars whereas the most cultivars contain Tm² based resistance, it is not possible to assess with a pathogenesis assay whether or not Tm-1 is present.

Results:

INIA in all the assayed plants the resistance was conferred by Tm2². Similarly for GEVES none of the tested cultivars indicated Tm1 derived resistance except for the included standard variety Mobaci. At Naktuinbouw also none of the tested cultivars indicated Tm1 derived resistance except for the one variety that was included as a Tm1 control

- **Tomato Mosaic Virus Tm2 and Tm2²:** These resistance genes are allelic. Combination of two tetra primer ARMS PCR tests allows co-dominant scoring of phenotypes. GEVES also tested CAPS markers.
Results:
At INIA, the results of the pathogenesis assay were confirmed by molecular markers in all susceptible and resistant varieties, including cv. Vanity which is heterogeneous in both the pathogenesis assay and molecular markers.
At GEVES, all results for the CAPS and tetra primer ARMS PCR assays were as expected from the pathogenesis assay.
At Naktuinbouw using assay1; in two plants of different varieties the PCR failed, otherwise all results are in agreement with the pathogenesis assay. Marker results from assay 2 also agreed with the results from the resistance test.

- **Meloidogyne incognita Mi1-2:** Co-dominant SCAR marker (Mehrch et al. 2005).
Results:
At INIA, results of the pathogenesis assay were confirmed by molecular marker assays, 4 cultivars (including intermediate resistant control Madyta) with inconclusive results in the pathogenesis assay (more susceptible than resistant plants) showed a R/S phenotype in the molecular marker assay for the 2 plants tested.
At GEVES, results from molecular marker assays are as expected based on the pathogenesis assay. In 5 cultivars besides the expected R- and S-fragments two other fragments amplify but this doesn't interfere with the scoring.
At Naktuinbouw, in two plants of different varieties the PCR failed, probably due to a minor technical problem. Other marker results are in agreement with the pathogenesis assay.

- **Fusarium I locus (linked marker):** Dominant SCAR marker
Results:
At INIA, the results of the pathogenesis assay were confirmed by molecular markers in the susceptible and resistant varieties, except in Cascada a susceptible cultivar from the pathogenesis assay that was scored resistant in the molecular marker assay. In 7 cultivars the pathogenesis assay results were heterogeneous with resistant and susceptible plants (including standard variety Marporum), the 2 plants analysed in the molecular marker assays showed either the susceptible (2) or resistant (5) phenotype.
At GEVES, molecular marker assay shows expected results except for cv Piccolo (resistant in molecular markers) with was scored susceptible in the pathogenesis assay.
At Naktuinbouw, one susceptible variety showed a positive band in the marker test. Also an I3 variety was positive. A very weak band was present in Moneymaker. It is not clear whether this reaction is always reliable. Further optimisation may be needed. .

- **Fusarium I2 locus:** Dominant SCAR marker used in combination with Rubisco primer.

Results:

At INIA, results of the pathogenesis assay were confirmed by molecular markers in all susceptible and resistant varieties. Inconclusive results in the pathogenesis assay all give susceptible molecular marker results.

At GEVES, all results were as expected.

At Naktuinbouw, one false positive detected. Moneymaker showed a weak band and may or may not be false positive. Also the I3 variety was positive. Only Marmande showed no band.

Table 3: Identical scores from the pathogenesis assay and molecular markers (including heterogeneous scorings from the pathogenesis assay that were not in disagreement to the molecular marker assay)

Testing Station	Verticillium	Tomato Mosaic Virus	Meloidogyne incognita	Fusarium I	Fusarium I2
INIA	17/19 ^a	20/20	21/21 ^b	20/20 ^c	20/20 ^d
GEVES	18/20	20/20	20/20	19/20	20/20
Naktuinbouw	29/30	30/30	30/30	28/30	29/30
Overall	64/69	70/70	71/71	67/70	69/70

^a pathogenesis assay inconclusive for 2 possibly 4 cultivars

^b pathogenesis assay inconclusive for 4 cultivars including intermediate resistant control variety Madita

^c pathogenesis assay inconclusive for 7 cultivars including resistant standard variety Marporum

^d pathogenesis assay inconclusive for 5 cultivars

Evaluation of wild accessions

To get some indication on possible future problems with the assays developed, some wild tomato material was evaluated. A number of wild accessions, known to be used in breeding schemes, have been selected by INRA and PRI and these were tested on the different assays.

List of wild tomato material been evaluated

1	LA1777	<i>S. habrochaites</i>
2	LA0385	<i>S. peruvianum</i>
3	LA1601	<i>S. parviflorum</i>
4	LA1615	<i>S. cheesmanii</i>
5	LA1554	<i>S. rickii/pimpinellifolium</i>
6	LA2172	<i>S. peruvianum</i>
7	LA1556	<i>S. chilense</i>
8	LA0716	<i>S. pennelli</i>
9	LA2157	<i>S. peruvianum</i>
10	LA1840	<i>S. chmielewskii</i>

11	PI247087	<i>S. habrochaites</i>
12	LA1401	<i>S. cheesmanii</i>
13	LA1589	<i>S. pimpinellifolium</i>
14	LA1602	<i>S. pimpinellifolium</i>

Results of the tests can be found in appendix 14. Amplification of PCR products in wild species was prominent in the tests for which the primers amplified part of a NBS-LLR gene (Mi 1-2, Ve1/2, Tm2). Patterns are complex and it is likely that more than one fragment is amplified. At present it is unclear whether these fragments actually relate to the homologous resistance gene.

Discussion and Conclusion

Assay development and testing

In this project, we have developed and validated molecular assays for 6 disease resistance loci in tomato. 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. Assays developed target the verticillium resistance genes Ve1 and Ve2, the tomato mosaic virus resistance genes Tm1 (linked marker) and Tm2/Tm2², the nematode resistance gene Mi1-2, fusarium I locus (linked marker) and the fusarium I2 locus (Table 1). 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 case optimization of the PCR reaction was needed, but in the end all laboratories succeeded in get all assays up and running.

Assay validation

Assay validation was carried out by the three DUS testing stations that participated in the project. Each station had selected 20-30 varieties with different resistance profiles, as judged from the pathogenesis assays that had been carried out in the framework of DUS testing. All these varieties were also genotyped using the molecular assays. From these evaluations, it could be concluded that in 97% of the cases the molecular marker assays confirmed the pathogenesis assays. For the ToMV resistance, results from pathogenesis test and molecular marker assay fitted exactly. In a number of cases the pathogenesis test deviated from the molecular marker assays or showed no clear results. This was the case for Ve resistance, were in 5 out of the 39 cases (3 INIA, 2 GEVES) varieties or a number of individual plants of a variety were found susceptible or weakly affected (none of the plants died) in the pathogenesis assay whereas the molecular marker assay indicates that the plants tested in this assay should be resistant. In one case a variety was found 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 pathogenesis test results to environmental conditions. 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. In the Fusarium I pathogenesis assay, 2 varieties were found to be susceptible, whereas the molecular marker assays suggested that they would be resistant. At INIA

7 varieties showed heterogeneous results from the pathogenesis assay (including control Marporum) whereas the two 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.

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

For those cultivars with clear discrepancies between pathogenesis assay and molecular marker assays it will be important to find out whether test results for this cultivar are available from different years and different testing stations. Generally speaking, pathogenesis tests are done once. After the result is obtained, it is compared with the claim of the applicant. This is important because it is an independent confirmation of the result. If there is a contradiction, it is discussed with the applicant. The applicant will check if the same seed lot is used etc. If the contradiction persists the test is repeated and the applicant is invited to look at the test. After this the applicant has to agree to the test carried out at the DUS station. Thus results are usually based on two (breeder and DUS station) and sometimes three tests. Breeders also use pedigree information and marker information, but this is not taken into account during the DUS test.

Also the issues concerning the pathogenesis assay tests that were surfacing from the disease testing harmonization project should be considered with respect to these findings. The fungus disease tests show more often cases that are difficult to explain. This might be related to the less clear pathological test results in the pathogenesis assay. The "less clear pathological test result" in the Fusarium assays maybe related to the race 0 isolate used in the biological assays.

In conclusion, pathogenesis 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. These 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 more clear 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. Markers are also good at spotting heterogeneity, as was shown for a number of cases. Therefore it is recommended to develop markerassays 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 the possible loss of linkage for the Fusarium I marker. This issue is difficult to address with the current data, but if present the frequency does not exceed 3 in 70 (4.5%) based on the number of differing results between the molecular marker and pathogenesis results.

Implementation of marker assays in daily DUS testing.

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.

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.

However, they do not always allow concluding 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.

Recently, the UPOV BMT group proposed three options for implementing molecular markers in DUS testing. Option 1 deals with the use of molecular characteristics as a predictor of traditional characteristics. It is split up into two sub options, 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.

The purpose of this project was to develop an Option 1(a) approach for tomato DUS testing with the aim of investigating how molecular markers could be used to optimize the evaluation of disease resistance for the obligatory tomato disease resistance characteristics mentioned in the CPVO guidelines.

Option 1(a) applied to disease resistance characteristics is an approach adapted to diseases that, first, are controlled by very few major genes and, second, that do not evolve too fast (to avoid a continuous development of new molecular tests). For these 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 the program, several molecular tests have been successfully developed and tested. Here are our views concerning their potential implementation in tomato DUS testing.

Nature and conditions of implementation:

1) Marker assays could be used to complement pathogenesis tests.

For that matter, they could be used at two different stages of the procedure:

a) in a first step to select the samples that need to be further analyzed with a pathogenesis test.

For example, varieties containing a major gene involved in the resistance could be first identified with a marker assay, and then be analysed with a pathogenesis test to evaluate their global level of resistance and the part due to other minor genes or to their genetic background.

b) as a second step after the pathogenesis tests to help the decision for some samples presenting ambiguous results.

Marker assays could also replace pathogenesis tests but only in the cases where a complete correlation between the presence of the markers used and the resistance characteristic tested has been demonstrated. For all the host-pathogen couples tested in the project, this assumption has so far proven to be true.

- 2) If a DNA test replaces the pathogenesis test, it should not be considered as an additional characteristic but a normal characteristic evaluated by a molecular test.
- 3) The implementation of any new marker assay should be submitted to a preliminary consultation of all stakeholders including breeders, DUS testing stations, regulatory and granting authorities.
- 4) The reliability of the marker assays should have been demonstrated on a representative panel of varieties. For that matter, the same criteria as for pathogenesis tests should be used. The results should be compared to those obtained with pathogenesis tests, keeping in mind that, as for pathogenesis tests, 100% reliability will never be guaranteed but is anyway not necessary.
- 5) Marker assays should provide a gain in reliability, cost, time and /or be easier to perform than the pathogenesis tests (e.g. when manipulating quarantine pathogens).

Advantages:

- 1) Improved reliability
 - a) In complement of the pathogenesis tests, marker assays increase the reliability of the conclusions by providing additional information to the analysis.
 - b) In substitution to the pathogenesis tests, marker assays provide an answer to the problems related to the sensitivity of the pathogenesis tests to environmental conditions, virulence of strains, and interpretation of the symptoms.
- 2) Gain in cost, time and feasibility

If substituting the pathogenesis tests, marker assays provide gain in terms of:

 - a) Cost: by the possibility of automation of the analyses, and the multiplexing of markers. Cost will most likely be lower than the standard pathogenesis tests.
 - b) Time: because they can be performed at early stages
 - c) Feasibility: because they remove the necessity of the maintenance of a good quality inoculum, and avoid manipulating quarantine pathogens.

Limitations:

- 1) Limited representativeness

Marker assays are developed and tested on a representative but limited panel of varieties, which means that they could easily be ineffective to detect resistance genes coming from other sources of resistance used by the breeders but not

represented in the panel. Moreover, other (new) resistance genes could be used by the breeders in the future and could be not detected by the molecular tests. The solution to these problems could be to develop new specific molecular assays. In addition, breeders could be asked to indicate if other genes than the ones for which tests are available have been used. In such cases the DUS testing station could perform the standard pathogenesis test. A similar system is needed for the pathogenesis testing when the involved new genes give resistance to other strains/fysios. Recombination within the detected sequences, modifying the strength of the resistance, could not be detected depending on the kind of markers used. Only markers positioned inside the resistance genes are considered fully reliable. Markers that are merely linked to a resistance gene are not reliable enough to replace pathotests in variety registration.

2) No evaluation of the global level of resistance

The global level of resistance of a given variety may depend on more than the genes detected by the molecular assays. Other specific genes, often part of complex loci or gene clusters, can interfere. The genetic background of the variety can also have an impact. Pathogenesis tests allow evaluating the result of all these different components. However one has to keep in mind that that also inoculums are not always of the same quality and strength. In addition the resistance measured in a pathogenesis test is affected by environmental conditions so that as such the level of resistance is a difficult to assess 'trait'.

3) Cost of the technology

Small companies may have difficulties to afford the investments needed for the application of molecular markers. For them, the necessity to use molecular markers could mean more difficulties to enter the market with new varieties. However, there are several service companies specialized in performing marker analysis. They can perform the tests for the smaller companies for competitive prizes. However, this also applies to the pathotests.

Consequences for the tomato DUS system:

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

- Should DUS testing stations carry out the molecular tests or can they also be contracted out?
- 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, it could be considered as sufficient evidence for the resistance present or absent and a reason not to carry out the pathogenesis test again. This would save time and money.
- For future use the introduction of the marker assays also offer the possibility to declare a variety distinct when different genes are used. This may be imported as it is not sure that different sources of resistance will behave the same under all circumstances.
- An approved biomolecular tests should be recognized in the guideline as predictor of the resistance.

- If more than one gene can confer the same resistance, different predictors can be considered separately in the guideline.
- Considering the easiness and low cost of molecular compared with pathogenesis test, the pathogenesis should not be compulsory for registration.
- In the official variety description, the exact information that the official authority has about the resistance present in a variety it should be indicated. So the description of resistance characteristics should be a little more complex than now.