

Appendix 1: Minutes of first project meeting

Development and evaluation of molecular markers linked to disease resistance genes for tomato DUS testing (option 1a) (Jan 1st, 2006 – Jan 1st 2008)

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| Minutes of the first meeting March 27, 2006 - Wageningen (NL) |
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Participants

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|-------------------|-------------------------------------|
| David Calvache | INIA (Spain) |
| Carmen Mansilla | INIA (Spain) |
| Rene Mathis | GEVES (France) |
| Cecile Collonnier | GEVES (France) |
| Carole Caranta | INRA (France) |
| Sergio Semon | CPVO |
| Diederik Smilde | Naktuinbouw (The Netherlands) |
| Paul Arens | PRI (The Netherlands) |
| Ben Vosman | PRI (The Netherlands) – Coordinator |

Opening

Ben Vosman opened the meeting and welcomed all participants.
The agenda was accepted
Participants introduced themselves

Brief introduction to project

Ben highlighted the main objectives and deliverables of the project.

Asterisked (obligatory) disease resistance characteristics

Current testing procedures

A number of problems within the currently performed disease testing have been notified.

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 harmonisation between the different countries. Inoculums are exchanged and tests carried out. 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 ringtest. Differences found in definition of susceptibility cut-off values in harmonisation 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 inoculum by dilution series on tobacco plants first.

It is impossible to obtain a strain for ToMV:1.2. Also a problem is that there might be other genes conferring resistance around.

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.

Resistance genes

What is known, markers available, cloned genes

Meloidogyne incognita Mi-1 gene mapped on # 6. Mi1-2 confers resistance, in same contig gene Mi1-1 and a pseudogene has been found.

Linked marker: Mi-1 CAPS marker REX-1 reported by Williamson et al. (1994) is known to give false positives in some backgrounds.

Gene cloned Milligan (1998) resistance only effective at temperatures below 32°C, references found up to now that describe a molecular test for Mi resistance;

- Mehrach et al. (from the Int. Plant Virology Laboratory website) closely linked marker (1000bp 3' of Mi) two step PCR protocol with possibility to discriminate homo/heterozygous locus.
- Goggin et al. (2004) specific detection of Mi1-2
- Devran & Elekcioglu (2004) Mi specific primers from Milligan (1998)
- Bendezu (2004) discriminating primers for Mi/mi

Other resistance genes identified Mi-3 and Mi-9. Mi-3 effective above 30°C, gives resistance to virulent strains of *M. incognita* (who 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 so far.

Verticillium dahliae Ve1 and Ve2 genes mapped on #9. Both genes confer resistance to Verticillium.

Linked marker: Kawchuk et al (1998) co-dominant SCAR marker as well as allele specific SCAR markers available. Markers are based on same locus (± 0.67 cM from Mi).

Genes cloned: two closely linked inverted genes (Kawchuk et al. 2001) both conferring resistance, in literature only sequences from resistant line described. For the design of a molecular assay sequence information for susceptible plants has to be obtained.

Tomato Mosaic Virus Tm-1, Tm-2 and Tm-2² genes mapped on #2 and #9 respectively (Tm-2 and Tm-2² are alleles).

Tm-1 confers resistance to strains ToMV:0 and 2.

Linked markers: Ohmori et al. (1996) 6 closely linked SCAR markers of which 2 co-dominant. Only linked markers available.

Tm-2 confers resistance to strains ToMV:0 and 1. Tm-2² confers resistance to strains ToMV:0, 1, 2, 1.2.

Genes are allelic.

Linked markers: Dax et al. (1998) co-dominant SCAR marker to Tm2², closely linked RFLP markers Young et al. (1988, 1989), Sobir et al (2000) CAPS marker to Tm-2.

Gene cloned: Lanfermeijer (2003) Tm-2², Lanfermeijer (2005) Tm-2,

Molecular test described; CAPS marker from Lanfermeijer (2005) two digestions for distinction between tm-2, Tm-2 and Tm-2²

Sequences available from resistant alleles Tm-2, Tm-2² and susceptible alleles tm-2 and lptm-2. These will be used to design an assay that is less labour intensive as the described CAPS marker.

Fusarium oxysporum f. sp. Lycopersici genes I and I-2 mapped on short and long arm of #11 respectively.

I locus confers resistance to race Fol:0 (ex1).

Linked markers: Eshed unpublished data in Ori et al. (1997) 5cM interval between 2 RFLP markers (TG523 (sequence available) and CP58A). Other linked RFLP markers are described by Sela-Buurlage et al.(2001).

I-2 confers resistance to race Fol:1 (ex2), I2C-1 confers partial resistance.

Linked markers: Simons et al. (1998) TG 105 (sequence available) closely linked to I2 (0.4 cM)

Gene cloned: Simons et al. (1998) I-2, Ori et al (1997) I2C-1

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 at # 8 and 9. Work in potato indicated that additional members of this family can be expected at other chromosomes as well. Due to this high number of genes and homologs present development of an assay will be a challenging task.

Based on the present knowledge of the different resistance genes and the expected workload to develop molecular assays for these genes the development of the different assays has been distributed among the three partners for assay development (GEVES, INRA and PRI):

GEVES Mi resistance gene assay and resistance gene I assays

INRA Fusarium resistance gene I2 resistance gene assays

PRI Verticillium resistance gene Ve and Tomato Mosaic Virus resistance gene Tm-1, Tm-2 and Tm-2² assays

Inventory of equipment

Inventory of the equipment present among the partners showed that basically all PCR based assays can be performed. Therefore it is concluded that for this project any assay can be developed. To complement tests for resistance genes it is desirable to include a control for PCR amplification. This can be based on the locus being investigated but also based on a general control marker for the PCR reaction. For this INRA will send around details.

It is important to note that no marker platform will be excluded beforehand, provided that it will be cost effective

Evaluation of markers

For the development of the molecular assays at least 5 resistant and 5 susceptible varieties that have been evaluated in DUS testing and were granted Plant Breeders' rights will be used. At least 1 of the standards for testing resistant and susceptible cultivars mentioned in the CPVO should be included in this set of 5.

GEVES will supply INRA with the varieties needed and Naktuinbouw will supply PRI, but both will ask consent from the breeders first.

Further appointments regarding the number of varieties to be tested in the second year and choices regarding the test set will be made later.

Contract & finance details

- All contract, contract between CPVO and PRI as well as the subcontracts, have been sent to the participants last week.
- Diederik asks a question regarding ownership of the results. This is covered by the contract. Ownership of results is with the beneficiaries.
- There is general agreement that publication of results in a joint publication is the preferred way to disseminate results.

Next meeting and other business

- Next meeting will be organised by GEVES and will be held on December 1th 2006 in Paris.
- The first report is due at the end of this year four weeks after this meeting.
- There will be a notification of the start of the project in the next BMT meeting.

Closing of meeting

Ben thanks all participants for their contributions and the constructive discussions.

Listed collection of decisions made and actions to be taken:

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.

- Division of the work for the development of molecular assays
 - GEVES: Mi resistance gene and resistance gene I assays
 - INRA: Fusarium resistance gene I-2 assays
 - PRI: Verticillium resistance gene Ve and Tomato mosaic virus resistance gene Tm-1, Tm-2 and Tm-2² assays
- All PCR based assays for resistance gene testing can be designed
- Carole will send around details for a positive control marker for PCR result
- Plant material for the development of assays (5 resistant and 5 susceptible cultivars) will be supplied by GEVES and Naktuinbouw to INRA and PRI
- Next meeting will be organised by GEVES on December 1th 2006 in Paris

Appendix 2: Minutes of second project meeting

Project for the Community Plant Variety Office (CPVO)
Research and Development Section

Development and evaluation of molecular markers linked to disease resistance genes for tomato DUS testing (option 1a) (Jan 1st, 2006 – Jan 1st 2008)

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| Draft Minutes of the second meeting November 13, 2006 - Paris (F) |
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Participants

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|--------------------|-------------------------------------|
| Mar Liñán | INIA (Spain) |
| Carmen Mansilla | INIA (Spain) |
| René Mathis | GEVES (France) |
| Chrystelle Jouy | GEVES (France) |
| Laetitia Cavellini | GEVES (France) |
| Cecile Collonnier | GEVES (France) |
| Carole Caranta | INRA (France) |
| Sophie Rolland | INRA (France) |
| Sergio Semon | CPVO |
| Diederik Smilde | Naktuinbouw (The Netherlands) |
| Paul Arens | PRI (The Netherlands) |
| Ben Vosman | PRI (The Netherlands) – Coordinator |

Opening

Ben Vosman opened the meeting and welcomed all participants. The agenda was adopted and the minutes of the previous meeting were accepted with the addition that on page 2 in the remark (± 0.67 cM from Mi) Mi has to be changed into Ve.

Progress report on molecular assays and conclusions regarding assay development

Presentations by Paul Arens (PRI) concerning progress in Verticillium and Tomato Mosaic virus, by René Mathis (GEVES) concerning Meloidogyne incognita and the Fusarium I locus, and by Carole Caranta (INRA) concerning the Fusarium I2 locus. Cecile will send the three presentations as pdf-files to all participants.

Conclusions from the molecular assay developments for the different disease resistance genes are:

Verticillium genes Ve1 and Ve2; tetra primers ARMS tests were developed which allow a control on amplification and co-dominant scoring of phenotype. Assays ready for further testing.

Tomato Mosaic Virus Tm1 (linked marker); only dominant SCAR markers from literature work but often a weak amplification can be observed in susceptible varieties. More susceptible varieties will be analysed to assess whether this may result in problems of false positives. Meanwhile this has been done in a set of 13 susceptible cultivars none of which gave a false positive result. Assay ready for further testing. Because of dominant nature a positive control for amplification has to be included (Rubisco or Lat gene primers).

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. Assays ready for further testing.

Meloidogyne incognita Mi1-2; Co-dominant SCAR marker available from Mehrach et al (2005). Assay ready for further testing.

Fusarium I locus (linked marker); Attempts to develop a molecular test for this locus have been unsuccessful up to now. A number of linked RFLP markers (TG523 and TG7) that have been described in literature will be sequenced and/or sequence data of linked markers will be used in an attempt to obtain sequence information from resistant and susceptible varieties at these marker loci.

Fusarium I2 locus; Dominant PCR marker available for I2 locus (and specific to the *L. pimpinellifolium* resistance allele) that can be combined with a Rubisco positive control on amplification. Possibility to develop co-dominant marker is under assessment. Dominant marker assay ready for further testing.

Type of assays developed can be used by GEVES, INIA and Naktuinbouw without further modification regarding methods used.

Detailed reports on marker assay development and protocols for marker assays will be made by the PRI, GEVES and INRA. These reports and protocols will be included in the interim report and will be used for the robustness tests (see evaluation of marker assays).

Evaluation of marker assays

Robustness test

Before the task “Evaluation of marker assays” starts, a test for reproducibility/robustness will be performed for all available tests by all partners using the detailed protocols (included in the interim report). DNA from varieties used for the development of the tests (at least one resistant and one susceptible variety per test) will be sent around. Besides these DNA samples, each of the five partners will isolate DNA from the varieties Marmande and Moneymaker representing susceptible varieties and from two resistant cultivars for each resistance gene to be suggested by Diederik and Chrystelle. Reports on the robustness tests have to be sent to all project partners. Each group will identify a person to receive the data. Reports will include photos of the gels and conclusions. Robustness tests should be ready before March 1, 2007 so that evaluation of marker assays can start early enough in the year.

Evaluation of markers

Evaluation of marker assays will be carried out on 20 varieties (two plants for each variety) for each of the DUS stations (GEVES, INIA and Naktuinbouw). A list of material will be compiled by NAK, GEVES and INIA together with the CPVO. Varieties will be selected from common catalogue and national lists of the last two years. Provided that robustness test are successful, the evaluation of markers should be finished before the end of May.

To get some indication on possible future problems with the assays developed, we will evaluate some wild tomato material. A number of wild accessions known to be used in breeding schemes will be selected by INRA and PRI and these will be tested on the different assays (PRI and INRA to prepare a list and perform the assays). To be finished before the end of May.

Finally, testing stations will identify possibly suspected material in the tomato DUS tests to be carried out in 2007 and/or a few varieties for which DUS disease tests gave rise to questions in the previous years. These varieties may be whole candidate varieties not behaving as expected in the resistance tests or varieties appearing as non-uniform. They will be tested using the marker assays.

Contract & finance details

- The interim report is due at the end of this year (before January 1st, 2007). This report will mainly consist of a report on the marker development work. Ben will send around a format for a report on each of the resistance genes before November 17th. Reports on assays should be sent to Ben and Paul before December 8th 2006. Based on the reports and the minutes of the two meetings a draft interim report will be made and send around for comments before December 15th 2006, comments and remarks have to be sent back ASAP but at least before December 21. The agreed interim report will be send to the CPVO as a PDF file before Christmas.
- The format for the financial report will be send around before November 17th by Ben. The financial report will cover the period Jan 1 – Dec 31 2006. Reports from the partners should reach PRI before January 15 2007. The financial report will be send, as a PDF file, to the CPVO before the end of January 2007.

Next meeting and other business

- Next meeting will be organised by INIA and will be held on June 25th 2007 in Madrid.
- There will be a notification of the start of the project in the BMT meeting in Seoul November 2006.

Closing of meeting

Ben thanks all participants for their contributions and the constructive discussions. Cecile was thanked for organizing the meeting and the lunch.

List of decisions made and actions to be taken:

- Pdf files of presentations will be sent to everyone by Cecile

- PRI will perform some additional test for ToMV: strains 0 and 2 susceptible varieties for the Tm1 assay (this has been done) and will test marker in conjunction with a positive control
- GEVES will obtain sequence information for markers linked to Fusarium resistance gene I and try to obtain sequence information at the loci of these linked markers in both resistant and susceptible varieties. If successful, a marker assay should be developed
- INRA will perform last tests to see if co-dominant assay for the Fusarium I2 locus can be developed
- PRI, GEVES and INRA will write a detailed report on the marker assay development (containing standardised descriptions of the protocols) based on a format that will be provided by Ben (format before November 17, report before December 8)
- Based on the report on marker assay development, a robustness test for all suitable disease tests will be performed by all partners. Robustness test to be finalised before March 2007
- Format for financial report will be send to all partners by Ben (before November 17). Partners should send their information before January 15
- Draft interim report will be send to all partners by Ben (before December 15)
- Comments on draft report will be made by all partners before December 21 after which the final interim report will be send to CPVO before December 23
- Evaluation of marker assays on 20 varieties (two plants for each variety) for each of the DUS stations (GEVES, INIA and Naktuinbouw). To be finished before the end of May
- Testing stations will identify possibly suspected material in the tomato DUS tests to be carried out in 2007. Applicants, for which DUS disease tests give rise to questions (e.g. segregation of resistance), will be tested using the marker assays and larger numbers of plants
- A number of wild accessions known to be used in breeding schemes will be selected by INRA and PRI and these will be tested on the different assays (PRI and INRA to be settled). Proposal: joint set will be selected and tested on all suitable assays. PRI will do the tests for Verticillium and TMV and INRA will do the tests for Fusarium and Meloidogyne incognita
- Next meeting will be organized by INIA on June 25th 2007 in Madrid

Appendix 3: Minutes of the third project meeting

Project for the Community Plant Variety Office (CPVO)
Research and Development Section

Development and evaluation of molecular markers linked to disease resistance genes for tomato DUS testing (option 1a) (Jan 1st, 2006 – Jan 1st 2008)

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| Draft Minutes of the third meeting June 25, 2007 - Madrid (S) |
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Participants

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|-----------------|-------------------------------------|
| Mar Liñán | INIA (Spain) |
| Carmen Mansilla | INIA (Spain) |
| Fernando Ponz | INIA (Spain) |
| David Calvache | INIA (Spain) |
| René Mathis | GEVES (France) |
| Carole Caranta | INRA (France) |
| Sergio Semon | CPVO |
| Diederik Smilde | Naktuinbouw (The Netherlands) |
| Paul Arens | PRI (The Netherlands) |
| Ben Vosman | PRI (The Netherlands) – Coordinator |

Welcome

All participants were welcomed by the director of INIA

Opening

Ben Vosman opened the meeting and welcomed all participants. The agenda was adopted and the minutes of the previous meeting were accepted with the addition that where it reads NAK, it should read Naktuinbouw. Items on the Action list were reviewed. There were no points arising from that, all items were carried out or on the agenda of this meeting.

Progress report on robustness test of molecular assays and conclusions regarding future use

Paul Arens presented the overview on the basis of the reports that were sent in by all participants. Partners added to this when additional work was carried out. Powerpoints presented will be sent around as pdf to all participants by Carmen.

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. Ve2 markers also work in almost all labs. Improvement was obtained for some by using a touch down PCR protocol. At INRA Ve1 and Ve2 still need some optimisation although expected pattern is visible. Perhaps use a different Taq- polymerase.
- **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 work 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):** PCR assay developed by Geves works well in most labs. At INRA no amplification. Geves to send an aliquot of their primers to INRA. At PRI, INIA and Naktuinbouw there was a problem with Marmande. This cultivar should be susceptible, but is according to the PCR test resistant. Naktuinbouw will do a disease test.
- **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. However, there are some minor lab specific issues to be solved.

Evaluation of marker assays

Diederik already carried out some further evaluation of the markers. He discussed two examples one on the segregation of Verticillium resistance and the other on ToMV resistance (see powerpoint). Major conclusion: We need better background knowledge on the genetics of resistance. Also the genetics of the resistance in the reference varieties is not always clear. Also these varieties might not behave as expected. Focus on the problems, expect surprises. His suggestion is to make knowledge factsheets for each resistance.

Further evaluation of marker assays will be carried out on 20 varieties (two plants for each variety) for each of the DUS stations (GEVES, INIA and Naktuinbouw). A list of material will be compiled by NAK, GEVES and INIA together with the CPVO. Varieties will be selected from common catalogue and national lists of the last two years. The list of materials that will be evaluated by each partner will be send around before July 15.

To get some indication on possible future problems with the assays developed, we will evaluate some wild tomato material. A number of wild accessions known to be used in breeding schemes will be selected by INRA and PRI and these will be tested on the different assays (PRI and INRA to prepare a list and perform the assays). Paul and Carole will come with a final list of material and a division of work before July 15.

Finally, when testing stations identify possibly suspected material in the tomato DUS tests of 2007, these materials will be characterized with markers as well. These varieties may be whole candidate varieties not behaving as expected in the resistance tests, check boundary cases that occur in the pathogenesis and varieties appearing as non-uniform.

All practical (lab) work should be finished before November 1.

Implementation of marker assays in daily DUS testing

This item resulted in a lively discussion in which several different views were expressed. It was decided that we will make a separate discussion on this issue by email. The end result of this should be a chapter that we can include in the final report. In this chapter the different views should be visible. Ben will start the discussion.

Issues raised during the discussion:

If proven reliable implementation should follow in dialog with the breeders. Others questioned the need for dialog. The question was raised whether the tests were reliable enough. However, this should be looked at in perspective (same criteria as for pathogenesis test). The pathogenesis test should always be the ultimate test, as new genes might be used that are not detectable by the present tests. If a DNA replaces the pathogenesis test it is not an additional characteristic but a normal characteristic evaluated by a molecular test. Implementation has to be viewed on the technical merits together with the acceptance of biomolecular techniques within the DUS test by regulatory/granting authorities. .

Contract & finance details

- The interim report has been approved by the CPVO and PRI received the CPVO contribution earlier this month. Partners that have not yet done so are requested to send their banking details ASAP to PRI to facilitate the transfer of money to them.
- We aim at submitting the final report to the CPVO before Christmas on the marker development work. Reports on evaluation of assays should be sent to Ben and Paul before November 8th 2007. Based on the reports and the minutes of our meetings a draft final report will be made and send around for comments before November 20th 2007. Comments and remarks have to be sent back ASAP but at least before December 21. The final report may include recommendations for future research. The agreed final report will be send to the CPVO as a PDF file before Christmas. Hardcopies will be send as soon as the draft is agreed by the CPVO.
- The format for the financial report will be send around before November 17th by Ben. The financial report will cover the period Jan 1 2006 – Dec 31 2007. Reports

from the partners should reach PRI before January 15 2008. The financial report will be send, as a PDF file, to the CPVO before the end of January 2008.

Next meeting and other business

- It is most likely that the project results and conclusions will first be presented to the CPVO's annual vegetable DUS experts' meeting in the second half of January 2008 in Angers. After presenting the project results and conclusions to the UPOV BMT in May 2008 and the UPOV TWV in June 2008, it could be envisaged that a meeting is organised between the CPVO, the project partners, and representatives from tomato breeding companies to analyse the possible implementation of the recommendations emanating from the project.
- Publication of results of the project was discussed. PRI will make a proposal for this. Development of the assays may be a separate publication in TAG. Issues related to implementation and also the validation may be published at a later stage (after the final report is written and discussed).

Closing of meeting

Ben thanks all participants for their contributions and the constructive discussions. Carmen was thanked for organizing the meeting and the lunch.

List of decisions to be taken:

- Pdf files of presentations will be sent to everyone by Carmen.
- The list of materials that will be evaluated by each partner using the assays developed will be send around before July 15.
- Paul and Carole will produce a final list of wild tomato material that will be evaluated using the assays developed, and a division of work before July 15.
- Possibly suspected material in the tomato DUS tests of 2007, these materials will be characterized with markers as well (Naktuinbouw, Geves and INIA).
- All practical (lab) work should be finished before November 1.
- Reports on evaluation of assays should be sent to Ben and Paul before November 8th 2007.
- Ben will start a discussion on the implementation of the assays in DUS testing
- A draft final report will be made by PRI and send around for comments before November 20th 2007, comments and remarks have to be sent back ASAP but at least before December 21 (All).
- The agreed final report will be send to the CPVO as a PDF file before Christmas (Ben)

Appendix 4: Assay for Verticillium resistance genes Ve1 and Ve2

Development of markers for Verticillium resistance gene Ve-1 and Ve-2

Authors

Paul Arens, Hanneke van der Schoot and Ben Vosman
Plant Research International, Wageningen UR

1. Introduction/Background

The CPVO-TP/44/2 protocol for distinctness, uniformity and stability tests mentions resistance to *Verticillium dahliae* race 0 as an obligatory test. Heterozygote varieties can show symptoms of a slightly lower level of expression.

Resistance against *Verticillium dahliae* was introgressed from line Peru Wild (Schaible et al 1951). The Verticillium resistance gene was fine mapped to chromosome 9 using SCAR markers (~0.67cM) developed by Kawchuk et al. (1998). From this locus primers were developed that can be used as a co-dominant SCAR as well as an allele specific SCAR (Ve or ve specific).

Characterisation of the Ve locus showed two closely linked inverted genes that both conferred resistance to *Verticillium albo-atrum* in susceptible potato plants (Kawchuk et al. 2001). The two resistance genes cloned were named Ve-1 (AF272367 genomic DNA; AF272366 cDNA) and Ve-2 (AF365929 genomic DNA; AF365930 cDNA). Genes have an amino acid identity of 84% and no introns were detected. To develop molecular assays for these two genes, sequence information of susceptible varieties has to be obtained as well as additional sequences from different resistant varieties to look for possible allelic variation in these two genes.

2. Materials and Methods

From the 2005 tomato tests of Naktuinbouw 6 resistant and 6 susceptible cultivars have been selected for the assessment of allelic diversity at the two loci.

From the alignment of the sequences deposited by Kawchuk et al. (2001) sequence specific primers have been developed to amplify either Ve1 or Ve2 specific fragments (note, primers in paper of Kawchuk are not labeled correctly and for Ve1 mentioned primers in paper are not specific for the gene furthermore genomic and cDNA sequences are not in full agreement).

The developed gene specific primers have been used to amplify fragments from both resistant and susceptible varieties. These fragments have been sequenced and sequences have been aligned to detect possible variation between resistant and susceptible varieties. Based on resistance specific SNPs, tetra primer ARMS PCR assays have been designed (Ye et al 2001). These assays have been verified and optimized using the available 12 cultivars.

3. Results

For Ve 2 one primer pair was used that amplified a 721 bp fragment of the gene (gene 3419 bp in total, primer locations Ve2_2315F and Ve2_3036R). Fragments from both resistant and susceptible varieties could be obtained. These were subsequently sequenced. Sequences showed two SNPs that were distinguishing resistant from susceptible varieties. Susceptible varieties always showed two distinct bases at these positions whereas resistant varieties showed either another base or a mix of the two bases found at this position indicating heterozygous varieties at this locus. For Ve1 the homologous fragment showed no sequence variation between resistant and susceptible varieties. Therefore, a larger part of this gene has been sequenced (1400 bp of 5'UTR and ~3000 bp of Ve1 gene, total size is 3162 bp). Five SNPs could be detected in the coding part and an additional three SNPs were found in the 5'UTR. Results were in agreement with results from the linked SCAR markers (Kawchuk et al. 1998) with one exception. One cultivar (681 (13)) that was not claimed as being resistant to *Verticillium* shows the resistant phenotype in the linked markers as well as in the gene markers. For Ve1 four SNPs have been tested in a tetra primer ARMS PCR assay and for Ve2 two SNPs have been tested in a tetra primer ARMS PCR assay. Outer primers were chosen such that fragments amplified were specifically Ve1 or Ve2 derived. Out of the six assays five showed the expected pattern whereas for one (assay 2.2) the primer for the SNP base of the resistant genotype was not specific. Two assays were chosen for further testing (assays 1.2 and 2.1). Ve1 assay 1.2 and Ve2 assay 2.1 both depend on a nonsynonymous SNP.

Table 1: Variety characteristics and summary of resistance assays (R= resistant, S= susceptible)

| Cultivar (NAK number) | Resistance according to breeders application ¹ | Resistance Linked marker | Resistance Ve1 SNP locus ² | Resistance Ve2 SNP locus ² |
|-----------------------|---|--------------------------|---------------------------------------|---------------------------------------|
| 1113 (29) | R | R/S | R/S | R/S |
| 1116 (30) | R | R/S | R/S | R/S |
| 1123 (32) | R | R | R | R |
| 1151 (34) | R | R/S | R/S | R/S |
| 1161 (35) | R | R | R | R/S |
| 473 (5) | S | (R) ³ /S | R/S | S |
| 605 (10) | S | S | S | S |
| 681 (13) | S | R | R | R |
| 706 (15) | S | S | S | S |
| 741 (19) | S | S | S | S |
| 743 (20) | S | S | S | S |

¹ Susceptible cultivars: resistance is not claimed by breeder in application

² Resistance SNP bases deduced from haplotype of (homozygous) susceptible and resistant varieties

³ Weak band visible with resistant specific SCAR marker.

4. Discussion

The data of the linked markers are in agreement with the data from the SNP assays cultivars zygosity states agree except for the finding of a heterozygous state in locus Ve2 (cultivar 1161) whereas the linked marker and the Ve1 locus are homozygous. As the different markers are linked such discrepancies between markers are always possible due to cross-over's. Furthermore there are two possible discrepancies with data on resistance coming from breeders. For cultivar 473 no resistance has been reported whereas markers indicate that the resistant allele for Ve1 may be present. Further inquiry on this cultivar learned that this has a weak resistance phenotype and seems to segregate in its resistance level. This corresponds well with the marker data because in the initial sampling leaf material may have been obtained from different plants for one DNA extraction. For cultivar 681 no resistance has been claimed by the breeder whereas all markers indicate this variety is homozygous resistant and SNP assay indicate the homozygous presence of both resistance genes. For this cultivar further research is warranted to confirm data from both the breeder as well as the genetic analysis to exclude possible mistakes being made.

5. Primer list and PCR protocol

Young leaf material has been collected which has been freeze dried for storage until DNA extraction. All DNA extractions have been performed using the Qiagen plant DNA extraction kit following the instructions of the manufacturer.

DNA samples have been tested with two different tetra primer ARMS PCR tests one for the Ve1 gene and one for the Ve2 gene (see Table 2 for primer sequences).

Assay 1.2 Ve1 SNP706 (T/A > T = Resistant genotype)

Outer primers

Ve1_2072F

Ve1_2651R

SNP specific primers

Ve1_SNP706Ft

Ve1_SNP706Ra

Expected fragments:

Outer fragment 580 bp (control)

R-fragment 476 bp (resistant allele).

S-fragment 158 bp (susceptible allele)

Assay 2.1 Ve2 SNP 2827 (C/G > C = Resistant genotype)

Outer primers

Ve2_2720F

Ve2_3040R

SNP specific primers

Ve2_SNP2827F

Ve2_SNP2827R

Expected fragments:

Outer fragment 2720 - 3040 = 321 bp (PCR-control)

2720 - 2850 = 131 bp (G-allele, S-band)

2799 - 3040 = 242 bp (C-allele, R-band)

For both assays PCR reaction components are:

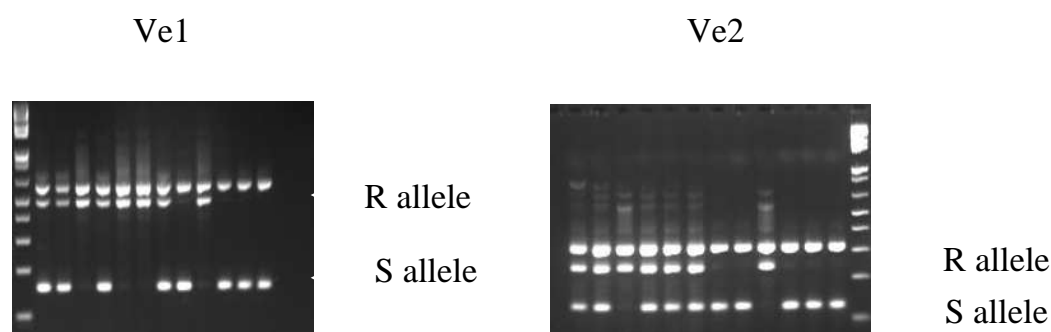
| | | |
|---------------------------|---|------|
| 10x PCR-buffer | | 2 |
| MgCl ₂ (25mM) | | 1.2 |
| dNTP's (5mM) | | 0.4 |
| SNP primers (2 pmol/μl) | F | 2 |
| | R | 2 |
| Outer primers (2 pmol/μl) | F | 0.5 |
| | R | 0.5 |
| Goldstar (5 U/μl) | | 0.04 |
| MQ | | 3.36 |
| DNA (2 ng/μl) | | 8 |
| Total | | 20 |

Goldstar Taq polymerase and PCR-buffer are from Eurogentec.

PCR cycling parameters: 3 min.94°C 1 cycle
 30sec.94°C }
 1 min.55°C } 35 cycli
 2 min.72°C }
 10 min.72°C

PCR reactions were performed on an MJ PTC-200 thermal cycler with maximal ramping. Amplification products (10 ul) were resolved in a 2% (w/v) agarose gel and length estimates were deduced from the 1 kb Plus DNA Ladder (Invitrogen)

Tetra primer ARMS assay for Ve 1 and 2



Order of samples from left to right and similar to Table 1.

Table 2: Primer names and sequences for assays used.

| Primer name | Primer sequence |
|--------------|---------------------------------|
| assay1.2 | |
| Ve1_2072F | CCTTGATGGGGTTGATCTTTCGT |
| Ve1_2651R | GTAGGTGAGTTTCTTGGACAGTCGA |
| Ve1_SNP706Ft | CAGGCCCTTTGGATGAATCACATT |
| Ve1_SNP706Ra | GTTGGACAAAAGAGAGAAAGTGAAGCTAAGT |
| assay2.1 | |
| Ve2_2720F | GGATCTTAGCTCACTTTATGTTTTGAAC |
| Ve2_3040R | GGTGCTGGTTTCAACTCTGAAGT |
| Ve2_SNP2827F | CAAATGCTTGAATCACTAGACCTGTGAAC |
| Ve2_SNP2827R | GGATCTCCCCGGACAGGTGGATTC |

For the robustness test DNA of samples 1113 and 605 will be send to each of the partners.

6. References

Kawchuk LM, Hachey J, Lynch DR (1998) Development of sequence charaterized DNA markers linked to a dominant Verticillium wilt resistance gene in tomato. Genome 41:91-95.

Kawchuk LM, Hachey J, Lynch DR, et al. (2001) Tomato Ve disease resistance genes encode cell surface-like receptors. PNAS 98, 6511-6515.

Schaible L, Cannon OS, Waddoups V (1951) Inheritance of resistance to Verticillium wilt in a tomato cross. Phytopathology 41: 986-990.

Ye S, Dhillon S, Ke X, Collins AR, Day IN (2001) An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Research 29: e88-8.

Principle of SNP detection using tetra primer ARMS PCR.

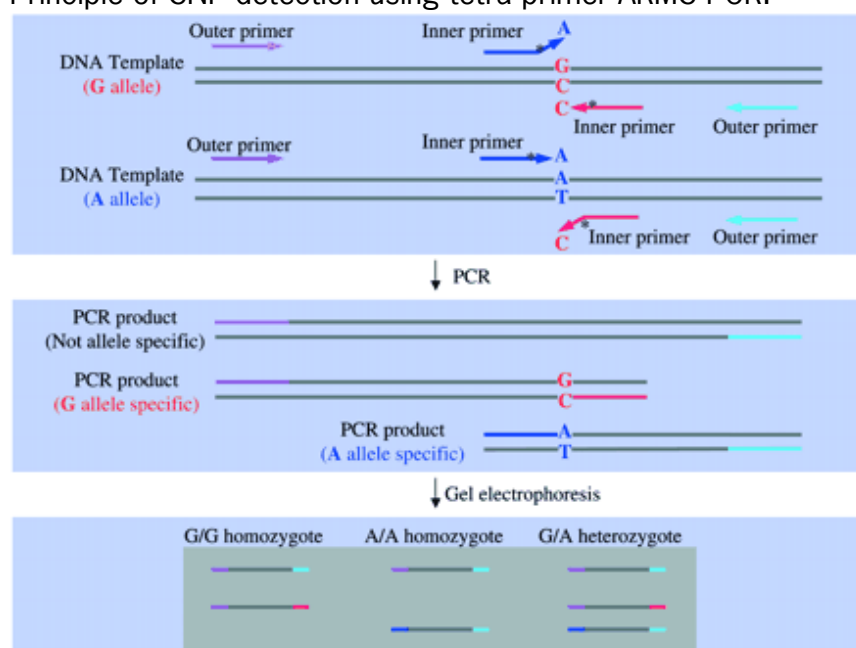


Figure from Ye et al. (2001). A deliberate mismatch has been included at the 3rd base from the 3' end of the primer to improve specificity.

Appendix 5: Assay for Tomato Mosaic Virus resistance Tm1 gene

Development of markers for ToMV resistance gene Tm1

Authors

Paul Arens, Hanneke van der Schoot and Ben Vosman
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1. Introduction/Background

CPVO-TP/44/2 protocol for distinctness, uniformity and stability tests mentions resistance to Tomato Mosaic Virus strains 0, 1, 2 and 1-2 as an obligatory test. ToMV 1-2 is not further considered for the project because up to now it has been impossible to obtain this strain and in practice there is no testing for this strain. Resistance gene Tm1 confers resistance to ToMV strains 0 and 2. For Tm-1 resistance currently no resistance gene has been cloned, the gene has been mapped to the short arms of chromosome 2 and the resistance gene originated from *L. hirsutum*. Ohmori et al. (1996) describe six closely linked SCAR markers. Two SCAR markers are co-dominant after digestion (SCB10 and SCL10), data on exact map position in relation to Tm-1 is lacking but a tight linkage is suggested. The set of six markers developed by Ohmori et al. (1996) will be tested for their applicability on a selection of tomato varieties.

2. Materials and Methods

Varieties with known Tm-1 derived resistance are scarce. Two Tm-1 genotypes were available among which Mobaci the standard variety mentioned in the CPVO Guidelines. Also a set of 16 susceptible cultivars was used as well as a variety that showed resistance to strain 2 but not to strain 0 (which within our current understanding is impossible). All plant material was obtained from Naktuinbouw.

SCAR markers identified by Ohmori et al (1996) were all tested on this set of material either using the conditions described in this paper or by less stringent conditions (i.e. lower annealing temperatures of 55 and 60 °C). Amplification products (10 ul) were separated on 1% agarose gels. Fragments amplified using marker SCG12 in resistant and susceptible varieties were sequenced directly from the obtained fragments. Rubisco and Lat52 primers were tested on compatibility with the dominant SCAR markers in order to have a control on PCR amplification of reactions.

3. Results

Using the conditions described by Ohmori et al. (1996) none of the samples showed any amplification product. Lowering annealing temperature to 55 °C resulted in strong amplification product in the dominant SCAR markers but not in the two co-dominant SCAR markers. In our hands it was impossible to obtain a single product of the expected length from the two described co-dominant SCAR markers. Therefore effort was concentrated

on the four dominant SCAR markers for Tm-1 resistance. With markers SCA15, SCG12 and SCN20 single fragments of the expected length could be amplified although reduced amounts of amplification products were also visible in susceptible varieties although in later tests using a wider set of susceptible varieties newly included varieties remained without amplification. For marker SCG12 for which amplification in the initially used susceptible varieties was strongest both fragments from resistant and susceptible varieties were sequenced in order to try to find sequence variation. Sequences between both resistant and susceptible varieties were identical.

Finally, the two best performing dominant SCAR markers SCA15 and SCN20 were used for testing in conjunction with the Rubisco marker used by INRA or the LAT52 markers used by GEVES in order to complement the test with a positive control for amplification. No amplification could be detected from the SCAR markers after addition of the Rubisco primers and the length of the fragment amplified with these primers is very similar to that of the SCAR markers. Therefore further effort was concentrated on the Lat52 primers. SCAR markers were tested with primer pairs Lat1F/Lat2R, Lat 3F/Lat2R and Lat1F/4R. Combinations of SCA15 and SCN20 with primers Lat1F/Lat2R resulted in the clearest pattern.

4. Discussion

Because only linked markers are available there is a possibility of finding cross-over events that may lead to wrong interpretation of results from the molecular tests. No information is known about the position of the SCAR loci with respect to each other and to the Tm-1 locus. False positives may be a prominent problem in larger scale testing of these loci. Because no additional information on relative map position exist and both SCAR markers are equal in their handling costs or ease of use an arbitrary choice for marker SCN20 has been made for the robustness test and evaluation of markers.

5. Primer list and PCR protocol

Young leaf material has been collected which has been freeze dried for storage until DNA extraction. All DNA extractions have been performed using the Qiagen plant DNA extraction kit following the instructions of the manufacturer.

For PCR reaction components are:

Either SCA15 or SCN20 can be amplified simultaneously with primers Lat1F/Lat2R

| | | |
|--------------------------|---|------|
| 10x PCR-buffer | | 2 |
| MgCl ₂ (25mM) | | 1.2 |
| dNTP's (5mM) | | 0.4 |
| SCAR primers (2 pmol/μl) | F | 2 |
| | R | 2 |
| Lat primers (2 pmol/μl) | F | 0.5 |
| | R | 0.5 |
| Goldstar (5 U/μl) | | 0.04 |
| MQ | | 3.36 |
| DNA (2 ng/μl) | | 8 |

Total

20

Goldstar Taq polymerase and PCR-buffer are from Eurogentec.

PCR cycling parameters:

| | | |
|-----------|------|-------------|
| 2.30 min. | 94°C | 1 cycle |
| 1 min. | 94°C | } 35 cycles |
| 1 min. | 60°C | |
| 2 min. | 72°C | |
| 10 min. | 72°C | |

PCR reactions were performed on an MJ PTC-200 thermal cycler. Amplification products (10 µl) were resolved in a 1% (w/v) agarose gel and length estimates were deduced from the 1 kb Plus DNA Ladder (Invitrogen).

Product sizes:

| | |
|-------------|--------|
| SCA15 F/R | 1000bp |
| SCN20 F/R | 1400bp |
| Lat1F/Lat2R | 92bp |

Figure 1: SCA15 with Lat52

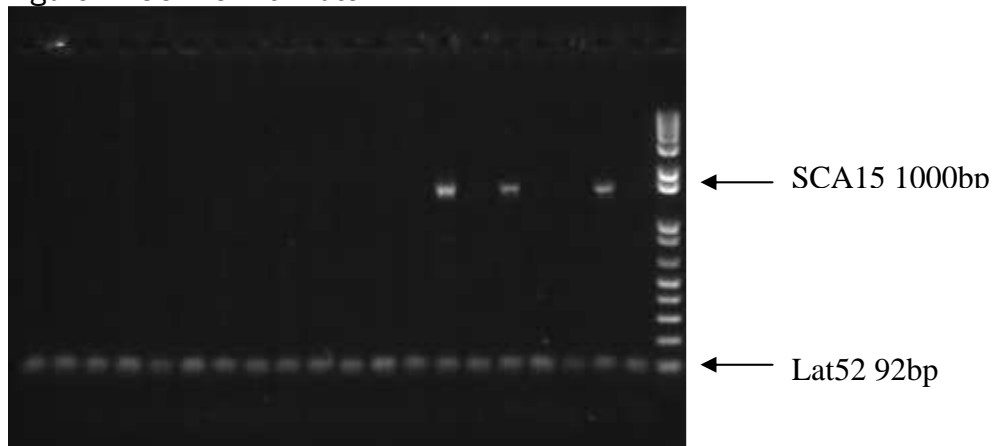


Figure 1: SCN20 with Lat52

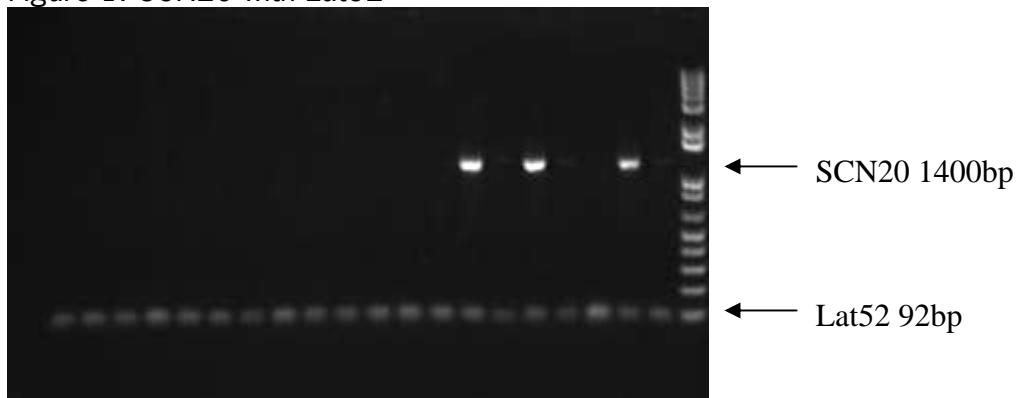


Table 1: Primer names and sequences for assays used.

| | |
|---------------------|----------------------------------|
| Tm-1 SCA15 F | CCGAACCCCTTAAAAATAGTTTCA |
| Tm-1 SCA15 R | CCGAACCCAATCAGGAGGCTCATA |
| Tm-1 SCN20 F | GGTGCTCCGTCGATGCAAAGTGCA |
| Tm-1 SCN20 R | GGTGCTCCGTAGACATAAAATCTA |
| Lat1-F | AGACCACGAGAACGATATTTGC |
| Lat2-R | TTCTTGCCTTTTTCATATCCAGACA |
| Lat3-F | AAAACCTCAGCGAGAACCT |
| Lat4-R | GGAACCTTATCATCCCATA |
| RubisCo F | ATGTCACCACAAACAGAGAC |
| RubisCo R | CTCACAAGCAGCAGCTAGT |

For the robustness test DNA of samples 81 and 83 will be send to each of the partners (nrs 3 and 5 from right in both figures) for testing with SCAR SCN20.

6. References

Ohmori T, Murata M, Motoyoshi F (1996) Molecular characterization of RAPD and SCAR markers linked to the Tm-1 locus in tomato. Theoretical and Applied Genetics 92: 151-156.

Appendix 6: Assay for Tomato Mosaic Virus resistance Tm2 and Tm2² genes

Development of markers for ToMV resistance genes Tm2 and Tm2²

Authors

Paul Arens, Hanneke van der Schoot and Ben Vosman
Plant Research International, Wageningen UR

1. Introduction/Background

CPVO-TP/44/2 protocol for distinctness, uniformity and stability tests mentions resistance to Tomato Mosaic Virus strains 0, 1, 2 and 1-2 as an obligatory test. ToMV 1-2 is not further considered for the project because up to now it has been impossible to obtain this strain and in practice there is no testing for this strain nor a specific resistance gene identified. Resistance gene Tm2 confers resistance to ToMV strains 0 and 1. Resistance gene Tm2² confers resistance to ToMV strains 0, 1 and 2. Both the Tm2 and the Tm2² resistances were introgressed from *L. peruvianum* and are allelic, the resistance gene is localized close to the centromere of chromosome 9. The Tm2² gene was cloned by Lanfermeijer et al (2003) and belongs to the CC-NBS-LRR class of resistance proteins. Both sequences from resistant as well as susceptible varieties have been obtained and show a 2.3% difference at the nucleotide level (AF536201, AF536199). Both among resistant cultivars (ATV847, Craigella GCR267 and MoneyMaker-vir) as well as susceptible cultivars (ATV840, Craigella GCR26 and Stevens) identical sequences have been found Lanfermeijer et al (2003). Lanfermeijer et al (2005) isolated the Tm-2 gene (AF536200). Between the sequences of Tm2 and Tm2² 7 SNPs can be detected, 5 of these SNPs result in four different amino acids. Based on the differences between the three alleles at this locus Lanfermeijer et al. (2005) designed a two CAPS markers with which the genotypes can be discriminated co-dominantly. These two CAPS markers reside in close proximity from each other and can be assessed using the same PCR fragment but using different restriction enzymes. These CAPS markers will be tested for their applicability on a selection of tomato varieties. Furthermore, a co-dominant assay based on PCR will be developed that may be used as an alternative to these mentioned CAPS markers.

2. Materials and Methods

All plant material was obtained from Naktuinbouw. Varieties included the cultivars Monalbo, Moperou and Monalbo x Momor that are mentioned as standard varieties in the CPVO guidelines.

Literature described an assay based on CAPS markers on a fragment of 1085 bp of part of the gene for Tm2⁽²⁾ resistance by Lanfermeijer et al. (2005) which can distinguish between the known alleles tm2, Tm2 and Tm2². This assay has been tested for its applicability on less closely related cultivars. Furthermore two tetra primer ARMS PCR assays have been developed (Ye et al 2001) based on SNPs that are found between

database sequences of these three different alleles. One of these two assays can distinguish between the two resistance alleles Tm2 and Tm2² and is able to identify susceptible cultivars but cannot distinguish heterozygous Tm2/tm2 and Tm2²/tm2 cultivars. The second assay can resolve this but with respect to the question in the DUS testing whether or not a cultivar is resistant this is possibly not essential knowledge. For the testing of the CAPS markers from Lanfermeijer et al. (2005) DNA from susceptible, Tm2 resistant and Tm2² varieties were amplified with the primers Tm-2 PrRuG151 F and Tm-2 PrRuG086 R. An aliquot of the PCR reaction product (10ul) was applied to a 0.8% agarose gel for product conformation and estimation of amount of product. Subsequently two aliquots of 8ul each were digested with either HpaI (GTT[↓]AAC) or BfrI (C[↓]TTAAG) and separated on a 2% agarose gel. For our experiments we have been using BspTI (Fermentas) the isoschizomer of BfrI. For details on protocols see section 5 Primer list and PCR protocol. Based on the sequences from the tm2, Tm2 and Tm2² alleles a number of SNPs have been identified. On SNPs 901 and 2493/2494 two tetra primer ARMS PCR assays have been developed. With assay 1 (based on SNPs 2493/2494) the presence of the alleles Tm2 and Tm2² can be assessed. With assay 2 the presence of the allele tm2 can be assessed as well as the presence of either Tm2 or Tm2². For details on protocols see section 5 Primer list and PCR protocol.

3. Results

CAPS marker assay

Amplification that was performed with primers Tm-2 PrRuG151 F and Tm-2 PrRuG086 R to obtain the fragment for the CAPS analyses needed little optimization. A large amount of PCR product is formed which is more than enough for further processing, resulting restriction digests were very clear and easy interpretable. All 13 tested cultivars (6 susceptible, 6 Tm2² resistant and 1 Tm2 resistant) showed the expected patterns. See section 5 for details on results.

Tetra primer ARMS PCR assays

Alignments have been made from sequences of the tm2, Tm2 and Tm2² and lptm2 alleles (AF536199, AF536200, AF536201, AY765395). Between these sequences a number of SNPs have been identified that were subsequently used to design SNP specific primer for the tetra primer ARMS PCR assays. SNPs 2493/2494 are two adjacent SNPs that have been the target of assay1. From both sides a primer was designed. The primer in the forward direction (SNP2494F Tm2(2)) was based on the sequence of the Tm2² allele, the primer in the reverse direction (SNP2493R Tm2) was based on the sequence of the Tm2 allele. Primers are used in combination to outer primers that allow amplification in any of the alleles. Using this assay1 the presence but not the copy number of the alleles Tm2 and Tm2² can be assessed as well as susceptible cultivars can be distinguished. In order to distinguish resistant cultivars in either heterozygous resistant or homozygous resistant a second assay has been developed. Therefore, two SNPs (positions 848 and 901) between either the susceptible allele or the resistant allele (Tm2 and Tm2²) have been the target of a SNP detection using tetra primers ARMS PCR. Using SNP 901 a suitable pattern could be produced. By combining both assays 1 and 2 the allelic constitution of the cultivars can be assessed. Results are in agreement with the

resistance data and the results from the CAPS markers. In the assays a primer ratio between SNP and outer primers of 4:1 is preferred.

4. Discussion

Both types of assays are relatively easy in use. In the tetra primer ARMS PCR tests a susceptible sample was found that behaved differently from expectations in one of the assays. For cultivar Monalbo which is susceptible a weak product can be seen at the position for the resistant allele in assay 2 whereas assay 1 does not show any amplification indication that neither of the resistance alleles Tm2 or Tm2² is present.

5. Primer list and PCR protocol

Young leaf material has been collected which has been freeze dried for storage until DNA extraction. All DNA extractions have been performed using the Qiagen plant DNA extraction kit following the instructions of the manufacturer.

CAPS marker assay Lanfermeijer et al. (2005)

First step in this assay is the amplification of a 1085bp fragment with the CAPS primers Tm-2 PrRuG151 F and Tm-2 PrRuG086 R.

| | | |
|----------------------|---|------|
| 10x PCR-buffer | | 5 |
| dNTP's (5mM) | | 1 |
| primers (10 pmol/μl) | F | 5 |
| | R | 5 |
| Goldstar polymerase | | 0.1 |
| MQ | | 29.9 |
| DNA (10 ng/μl) | | 4 |
| Total | | 50 |

Goldstar Taq polymerase and PCR-buffer are from Eurogentec.

PCR cycling parameters:

| | |
|-------------|------------|
| 3 min.94°C | 1 cycle |
| 1 min.94°C | } |
| 1 min.55°C | } 35 cycli |
| 2 min.72°C | } |
| 10 min.72°C | |

PCR reactions were performed on an MJ PTC-200 thermal cycler. Amplification products (10 ul) were resolved in a 0.8% (w/v) agarose gel for product confirmation, length estimates were deduced from the 1 kb Plus DNA Ladder (Invitrogen)

Digestion of 8ul PCR-product with 1ul restriction buffer and 1ul restriction enzyme (either HpaI or BrlI) incubation 2 hours at 37°C. Restriction fragments were resolved in a 2%

(w/v) agarose gel and length estimates were deduced from the 1 kb Plus DNA Ladder (Invitrogen).

Fragment sizes with HpaI: Total size of amplified fragment is 1085bp. Susceptible allele (tm-2) no restriction site present, length of fragment 1085bp. Resistant alleles (Tm2 and Tm2²) one restriction site, two fragments of 660bp and 425bp respectively.

Fragment sizes with BfrI (iso BspT I): Total size of amplified fragment is 1085bp. In the fragment there is one restriction site that is present in all three alleles (tm2, Tm2 and Tm2²) resulting in a fragment of 328bp present in all alleles. No other restriction sites are present in alleles tm2 and Tm2 resulting in a second fragment of 757bp. Only allele Tm2² has an additional second restriction site which is situated in the 757bp fragment resulting into a fragment of 559bp and a fragment of 198bp.

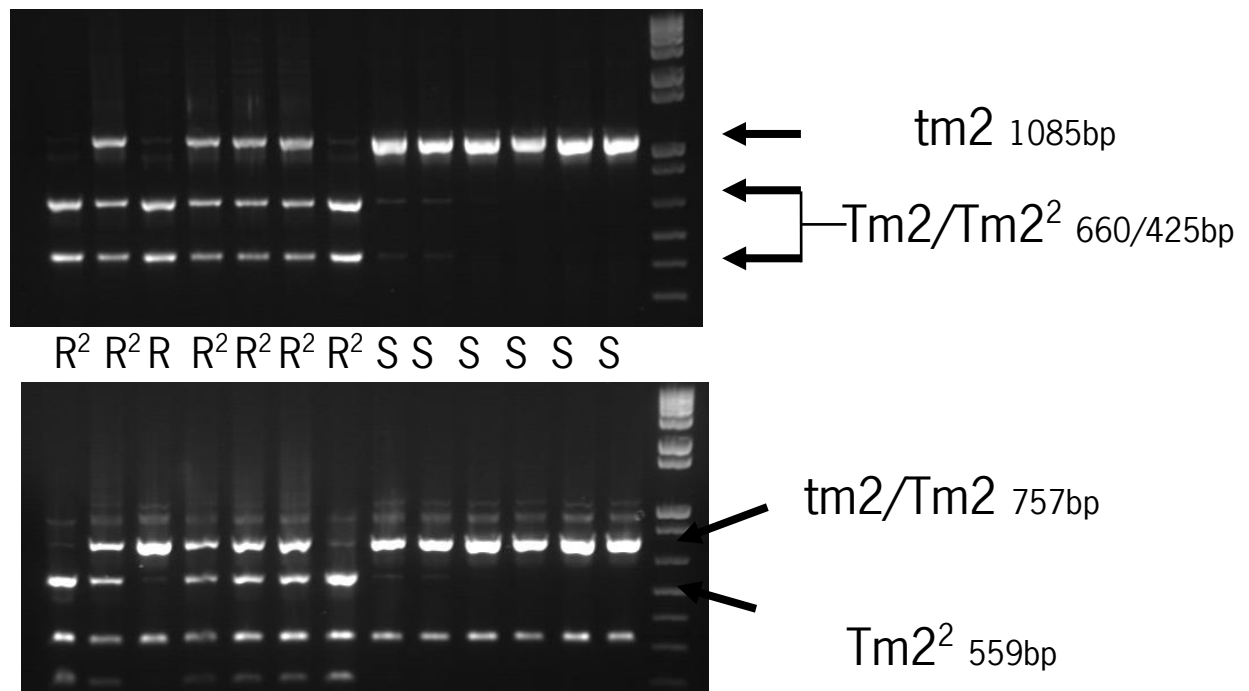


Figure 1. CAPS markers for the discrimination between tm2, Tm2 and Tm2². Upper part HpaI dependent marker, lower part BspTI dependent marker.

Tetra primers ARMS PCR assays 1 and 2

For both tetra primers ARMS PCR assays PCR reaction components are:

| | | |
|---------------------------|---|------|
| 10x PCR-buffer | | 2 |
| MgCl ₂ (25mM) | | 1.2 |
| dNTP's (5mM) | | 0.4 |
| SNP primers (2 pmol/μl) | F | 2 |
| | R | 2 |
| Outer primers (2 pmol/μl) | F | 0.5 |
| | R | 0.5 |
| Goldstar (5 U/μl) | | 0.04 |
| MQ | | 9.36 |
| DNA (10 ng/μl) | | 2 |
| Total | | 20 |

Goldstar Taq polymerase and PCR-buffer are from Eurogentec.

PCR cycling parameters: 3 min.94°C 1 cycle
 1 min.94°C }
 1 min.55°C } 35 cycli
 2 min.72°C }
 10 min.72°C

PCR reactions were performed on an MJ PTC-200 thermal cycler with maximal ramping. Amplification products (10 ul) were resolved in a 2% (w/v) agarose gel and length estimates were deduced from the 1 kb Plus DNA Ladder (Invitrogen)

Assay1 SNP2493/2494

Outer primers

TMV-2286F.

TMV-2658R

SNP specific primers

SNP2494 F Tm22

SNP2493 R Tm2

Expected fragments

Outer fragment 416 bp (control)

Tm2-fragment 254 bp fragment

Tm2²-fragment 214bp fragment

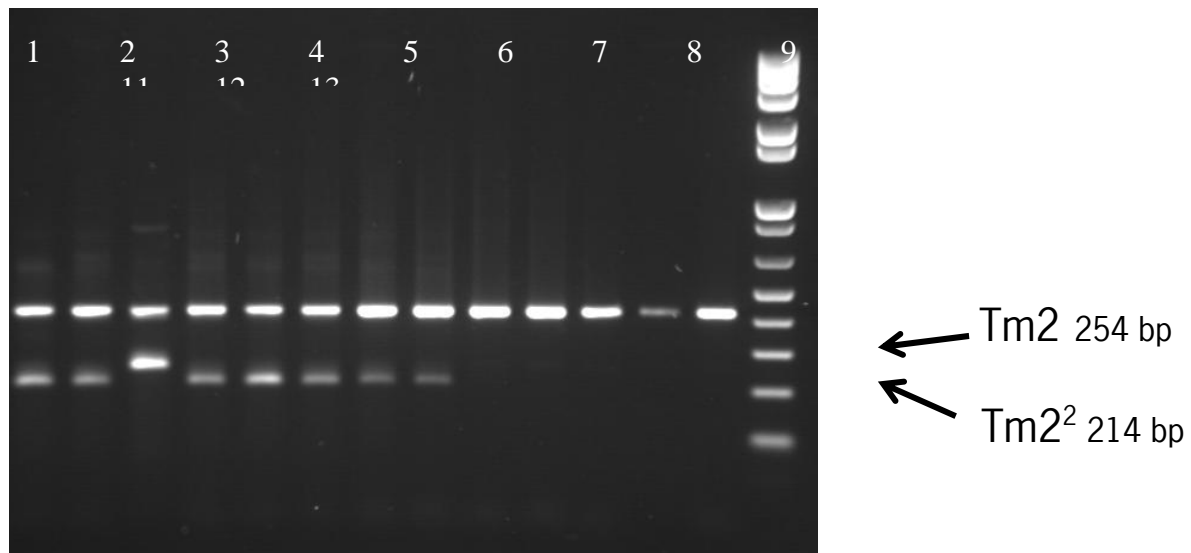


Figure 2: Tetra primer ARMS assay1 (Tm2² nrs1,2,4,5,6,7,8, Tm2 nr. 3, tm2 nrs 9,10,11,12,13)

Assay 2 SNP901

Outer primers

TMV-747F

TMV-1256R

SNP specific primers

TMV-R SNP901misR

TMV-S SNP901misF

Expected fragments:

Outer primers 509 bp

Specific fragments

R-allele 185 bp fragment (Tm2 or Tm2²)

S-allele 381 bp fragment (tm2)

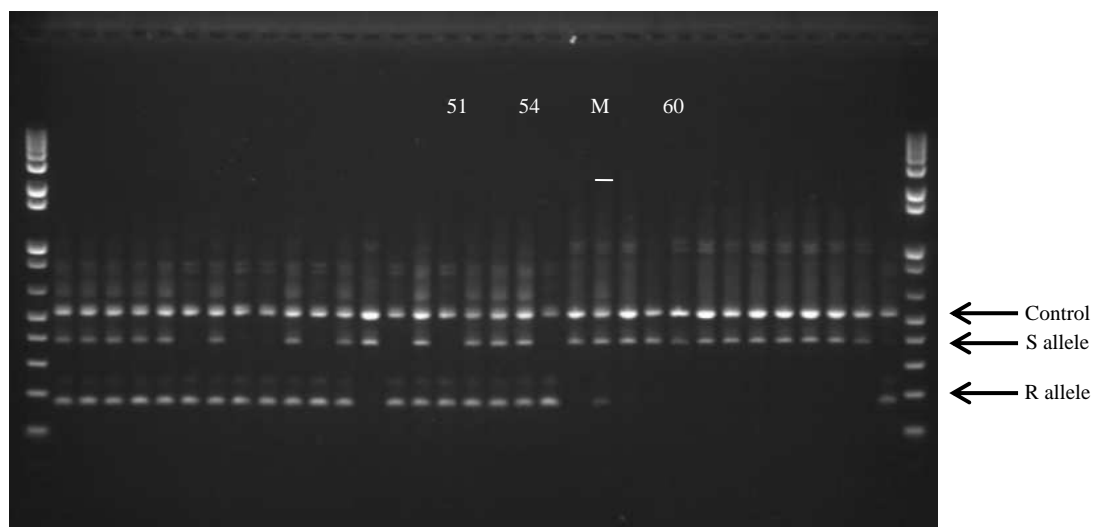


Figure 3: Tetra primer ARMS assay2 (M = Monalbo)

List of primers used

CAPS primers

| | |
|-----------------|-----------------------------------|
| Tm-2 PrRuG151 F | GAGTTCTTCCGTTCAAATCCTAAGCTTGAGAAG |
| Tm-2 PrRuG086 R | CTACTACACTCACGTTGCTGTGATGCAC |

Tetra primer ARMS PCR assay 1 (Tm2/Tm2²)

| | |
|-----------------|--------------------------------|
| TMV-2286F | GGGTATACTGGGAGTGTCCAATTC |
| TMV-2658R | CCGTGCACGTTACTTCAGACAA |
| SNP2493R TM2 | CTGCCAGTATATAACGGTCTACCG |
| SNP2494F TM2(2) | CTCATCAAGCTTACTCTAGCCTACTTTAGT |

Tetra primer ARMS PCR assay 2 (tm/Tm2 and or Tm2²)

| | |
|------------------|-----------------------------|
| TMV-747F | CGGTCTGGGGAAAACAACCTCT |
| TMV-1256R | CTAGCGGTATACCTCCACATCTCC |
| TMV-R SNP901misR | GCAGGTTGTCCTCCAAATTTCCATC |
| TMV-S SNP901misF | CAAATTGGACTGACGGAACAGAAAGTT |

For the robustness test DNA of samples 51 (Moperou – Tm2 sample 3 in figures 1 and 2), 54 (Monalbo x Momor Tm2² sample 6 in figures 1 and 2) and 60 (Marmande tm2 sample 12 in figures 1 and 2) will be send to each of the partners.

6. References

- Lanfermeijer FC, Dijkhuis J, Sturre MJG, de Haan P, Hille J (2003) Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-2² from *Lycopersicon esculentum*. Plant Molecular Biology 52: 1037-1049.
- Lanfermeijer FC, Warmink J, Hille J (2005) The products of the broken Tm-2 and the durable Tm-2² resistance genes from tomato differ in four amino acids. Journal of Experimental Botany 56: 2925-2933.
- Ye S, Dhillon S, Ke X, Collins AR, Day IN (2001) An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Research 29: e88-8.

Appendix 7: Assay for *Meloidogyne incognita* resistance Mi1-2 gene

Development of markers for Mi1-2 resistance gene

Authors

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GEVES (France)

1. Introduction/Background

Mi region (1Mb) is localised on the short arm of chromosome 6 in the wild type resistant strain *Lycopersicon peruvianum*. This locus contains 3 ORF: 2 are intact genes (Mi1-1 and Mi1) and 1 is a pseudogene. Mi1 is a multigenic family (Seah et al 2004) with 7 homologues grouped into 2 clusters on chromosome 6 (separated by 300kb): cluster 1p (Mi1-1, Mi1-2, Mi1-3) and cluster 2p (Mi1-4, Mi1-5, Mi1-6, Mi1-7), and 2 homologues on chromosome 5. Mi1-1 and Mi1-2 share 95% of homology and code for proteins of 1255 and 1257 amino acids respectively. These proteins are members of the leucine zipper, nucleotide binding leucine-rich repeat family of plant genes (NB-LRR proteins). Mi1-2 (but not Mi1-1) is sufficient to confer the resistance to susceptible tomato varieties (Milligan et al 1998).

Mi1-2 resistance gene confers resistance to different species of root-knot nematodes: *Meloidogyne incognita*, *M. javanica* and *M. arenaria*, as well as resistance to potato aphids (Vos et al 1998), and could lead to *Bemisia tabaci* resistance (Nombela et al 2003). Resistance is thermosensitive (effective only if $T^{\circ} < 30^{\circ}\text{C}$) and is HR type. Some strains, known to overcome Mi1-2, are called Mi virulent strains. Mi 1-2 is a cloned and sequenced gene (Milligan et al 1998). Its expression can vary according to the genetic background, as for example in hybrids containing other resistance genes in the same chromosomal region.

Available markers for Mi1-2 resistance gene:

A linked marker, Mi-1 CAPS marker REX-1, was first developed in 1994 by Williamson et al. It can give false positive results in some specific backgrounds.

In 2005, Mehrach et al proposed new PCR markers:

- PMi12 F1 / PMi12 R2 for the specific detection of Mi1-2
- PMi F3 / PMi R3 for the specific detection of Le, Lp and Lh Mi1
- PM3 F/ PM3 R for the specific detection of the 3'-coding region-of Mi1-2
- PM3Fb / PM3Rb for the specific detection of Lp Mi1 (from *L. peruvianum*)

Other PCR markers were recently published for the specific detection of Mi1-2, such as: C2 D1 / C2S4 (Goggin et al 2004) (in the coding region-3') ; C1/2 / C2S4 (Devran & Elekcioğlu 2004) (dominant marker: 1.6kb/Abs) and IMOF1 / IMOR1 (Bendezu 2004).

Tomato reference genes were searched in the literature to be used as an endogenous DNA control for PCR. Two sets of PCR marker hybridizing in LAT52 gene, Lat1F-Lat2R, Lat3F-Lat4R (Yang L. *et al*/2005) were chosen. They can be used as amplification controls in conjunction with dominant markers of Mi gene.

2. Materials and Methods

Plant Material

Tested cultivars were provided by Cavaillon and/or Brion GEVES stations. Five resistant varieties and five susceptible varieties have been tested (see table below).

| | R/S | CPVO Std | Diff Host | Resistance Type |
|-----------------------|-----|----------|-----------|-----------------|
| Anahu | R | X | X | 1 |
| Anahu X Casaque Rouge | R | | X | 2a |
| Anahu X Monalbo | R | X | X | 2a |
| Thomas | R | | | 2b |
| Campeon | R | | | 3 |
| Casaque Rouge | S | X | X | - |
| Rio Grande | S | | | - |
| Montfavit H63.5 | S | | | - |
| Daniela | S | | | - |
| Earlymech | S | | | - |

CPVO Std: standards for testing resistant and susceptible cultivars mentioned in CPVO protocols

Diff Host : Differential host of the French network resistance test.

Resistance type: levels of tolerance defined through an inter-laboratory work on *Meloidogyne incognita* (CTPS, 2002) resistance test: type 1 designates varieties showing a strong resistance, type 3 a very weak resistance, Type 2 is intermediate.

Plants were grown in a growth chamber (16h/8h, light/dark, 20°C/30°C respectively). Seeds are deposited on top of two flat plus one folded blotting paper (5 seeds per fold) with 40ml water. Seedlings are harvested for DNA preparation after 5 to 6 days of growth.

-DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 100mg of plant tissue (5 day-old seedlings) according to procedure (except for tissue crushed directly in lysis buffer).

-Quality DNA control:

-OD measurement

-Amplification of a tomato specific gene: LAT52

-Sequencing: representative PCR products were purified from agarose gel (Wizard SV Gel PCR clean-Up System from Promega) and sequenced by GATC biotech.

-Thermocycler: All experiments were performed on a PTC200 from BIORAD.

PCR marker tested with PCR conditions:

a. LAT1F-LAT2R

| | |
|--------|---------------------------------|
| Lat1-F | AGA CCA CGA GAA CGA TAT TTG C |
| Lat2-R | TTC TTG CCT TTT CAT ATC CAG ACA |

| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| Lat1F | 0,5 μ M |
| Lat2R | 0,5 μ M |
| RedTaq Sigma | 1U / PCR |

| | | |
|-------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 54 °C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

b. LAT3F-LAT4R

| | |
|--------|-------------------------|
| Lat3-F | AAA ACT CAG CGA GAA CCT |
| Lat4-R | GGA ACT TAT CAT CCC ATA |

| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| Lat3F | 0,5 μ M |
| Lat4R | 0,5 μ M |
| RedTaq Sigma | 1U / PCR |

| | | |
|-------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 50 °C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

c. PMi12F1-R2

| | |
|---------|--|
| PMi12F1 | GCA ATT CTA GAT CTA GCT ATT TGT TGT TC |
| PMi12R2 | CCT GCT CGT TTA CCA TTA CTT TTC CAA CC |

| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| PMi12F1 | 0,5 μ M |
| PMi12R2 | 0,5 μ M |
| RedTaq Sigma | 1U / PCR |

| | | |
|------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 50°C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

d. PMiF3-R3

| | |
|-------|--|
| PMiF3 | GGT ATG AGC ATG CTT AAT CAG AGC TCT C |
| PMiR3 | CCT ACA AGA AAT TAT TGT GCG TGT GAA TG |

| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| PMiF3 | 0,5 μ M |
| PMiR3 | 0,5 μ M |
| RedTaq Sigma | 1U / PCR |

| | | |
|-------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 50 °C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

e. PM3F-R

| | |
|------|-----------------------------------|
| PM3F | CCT GTG ATG AGA TTC CTC TTA G |
| PM3R | ACC CTT TGT TGA GCG ACT TTG CAG C |

| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| PM3F | 0,5 μ M |
| PM3R | 0,5 μ M |
| RedTaq Sigma | 1U / PCR |

| | | |
|-------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 50 °C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

f. PM3Fb-Rb

| | |
|-------|-------------------------------------|
| PM3Fb | CAC ACA TGA GGT ATG TTC GTA TTA TGG |
| PM3Rb | TCA CAG CCT AGC TTT TGA ATC AGT ACC |

| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| PM3Fb | 0,5 μ M |
| PM3Rb | 0,5 μ M |
| RedTaq Sigma | 1U / PCR |

| | | |
|-------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 50 °C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

g. C2D1-C2S4

| | |
|------|--|
| C2D1 | CTA GAA AGT CTG TTT GTG TCT AAC AAA GG |
| C2S4 | CTA AGAGGAATCTCATCACAGG |

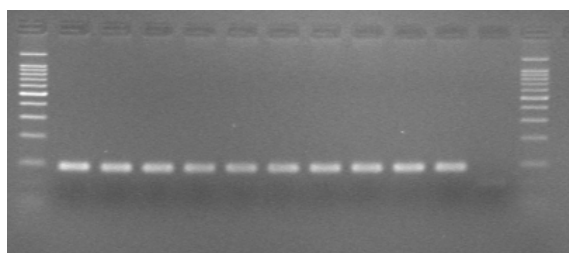
| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| C2D1 | 0,5 μ M |
| C2S4 | 0,5 μ M |
| RedTaq Sigma | 1U / PCR |

| | | |
|-------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 54 °C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

3. Results

a. LAT1F-LAT2R (tomato specific gene marker)

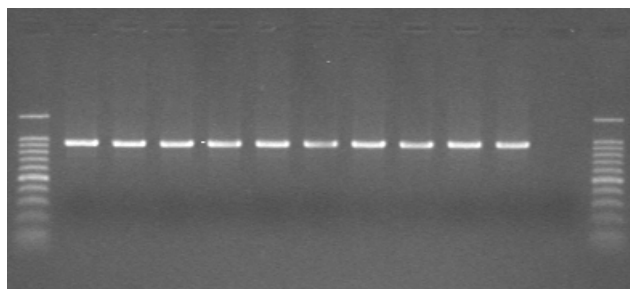
| Cultivar | R / S | Result LAT1-2 |
|-------------------|-------|------------------|
| Anahu | R:1 | 92pb |
| Anahu x Casaque R | R:2a | 92pb |
| Anahu x Monalbo | R:2a | 92pb |
| Thomas | R:2b | 92pb |
| Campeon | R:3 | 92pb |
| Casaque Rouge | S | 92pb |
| Rio Grande | S | 92pb |
| Montfavet H63.5 | S | 92pb |
| Daniela | S | 92pb |
| Earlymech | S | 92pb |
| Negative control | - | - |



- Conclusion
 - PCR product according to published data (92bp)

b. LAT3F-LAT4R (tomato specific gene marker)

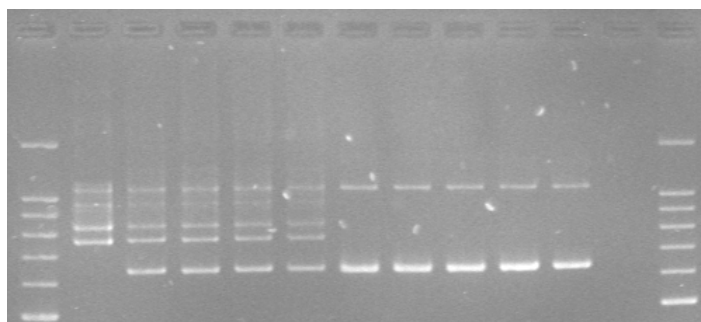
| Cultivar | R / S | Result |
|-------------------|-------|--------|
| Anahu | R:1 | 946pb |
| Anahu x Casaque R | R:2a | 946pb |
| Anahu x Monalbo | R:2a | 946pb |
| Thomas | R:2b | 946pb |
| Campeon | R:3 | 946pb |
| Casaque Rouge | S | 946pb |
| Rio Grande | S | 946pb |
| Montfavet H63.5 | S | 946pb |
| Daniela | S | 946pb |
| Earlymech | S | 946pb |
| Negative control | | - |



- Conclusion
 - PCR product according to published data (946bp).

c. PMi12F1-R2

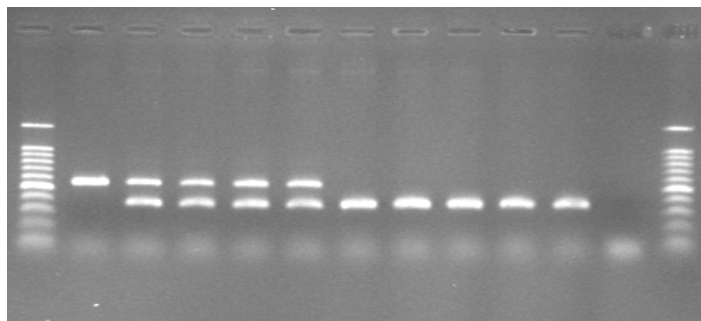
| Cultivar | R / S | Result |
|-------------------|-------|--------------------------|
| Anahu | R:1 | 720, 800 and 1000pb |
| Anahu x Casaque R | R:2a | 620, 720, 800 and 1000pb |
| Anahu x Monalbo | R:2a | 620, 720, 800 and 1000pb |
| Thomas | R:2b | 620, 720, 800 and 1000pb |
| Campeon | R:3 | 620, 720, 800 and 1000pb |
| Casaque Rouge | S | 620 and 1000pb |
| Rio Grande | S | 620 and 1000pb |
| Montfavet H63.5 | S | 620 and 1000pb |
| Daniela | S | 620 and 1000pb |
| Earlymech | S | 620 and 1000pb |
| Negative control | | - |



- Conclusions
 - PCR products of 720, 800 and 1000bp for homozygote resistant cultivar Anahu but published results show a unique band of 720bp for resistant homozygote cultivar Motelle
 - amplicons of 800 and 1000bp are also present for resistant heterozygotes (Anahu x Casaque Rouge and Anahu x Monalbo)
 - Amplification profile is difficult to read in case of routine application (3% agarose gel)

d. PMiF3-R3

| Cultivar | R / S | Result |
|-------------------|-------|---------------|
| Anahu | R:1 | 550pb |
| Anahu x Casaque R | R:2a | 350 and 550pb |
| Anahu x Monalbo | R:2a | 350 and 550pb |
| Thomas | R:2b | 350 and 550pb |
| Campeon | R:3 | 350 and 550pb |
| Casaque Rouge | S | 350pb |
| Rio Grande | S | 350pb |
| Montfavet H63.5 | S | 350pb |
| Daniela | S | 350pb |
| Earlymech | S | 350pb |
| Negative control | | - |

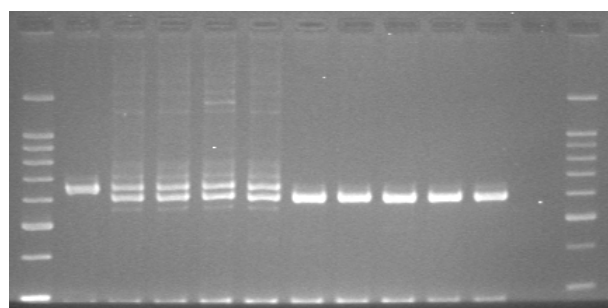


PCR product of 550bp obtained from resistant cultivar Anahu was checked by sequencing as well as PCR product of 350bp obtained from susceptible cultivar Casaque Rouge.

- Conclusions
 - Amplification of 350 and 550bp products corresponding published data.
 - With this single PCR, each cultivar gives DNA product, and a unique profile is obtained for any susceptible (5 tested), any heterozygous resistant (4 tested) and any homozygous resistant (1 tested).
 - Sequence alignment problem for cultivar Motelle : 2 sequences of 495 and 676bp does not correspond to 550 bp PCR product and to database sequence of 552bp of cultivar Anahu. Motelle is an "in silico" example of resistant cultivar that would give a result different to resistant tested here.

e. PM3F-R

| Cultivar | R / S | Result |
|-------------------|------------------|------------------------|
| Anahu | R:1 | 650pb and 300pb |
| Anahu x Casaque R | R:2 ^a | 650pb, 600pb and 300pb |
| Anahu x Monalbo | R:2 ^a | 650pb, 600pb and 300pb |
| Thomas | R:2b | 650pb, 600pb and 300pb |
| Campeon | R:3 | 650pb, 600pb and 300pb |
| Casaque Rouge | S | 600pb and 300pb |
| Rio Grande | S | 600pb and 300pb |
| Montfavet H63.5 | S | 600pb and 300pb |
| Daniela | S | 600pb and 300pb |
| Earlymech | S | 600pb and 300pb |
| Negative control | | - |

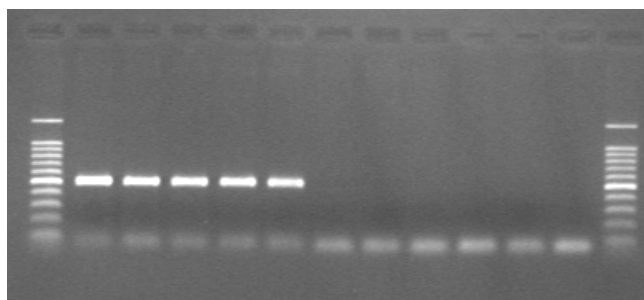


PCR product of 650bp obtained from resistant cultivar Anahu was checked by sequencing as well as PCR product of 600bp obtained from susceptible cultivar Casaque Rouge.

- Conclusions
 - Amplification of 600 and 650bp products for susceptible cultivars Casaque Rouge and resistant Anahu
 - Unclear results from the related publication: « these primers gave a 750-bp fragment for RKN-susceptible breeding line Gh13 « ... » This PCR fragment was sequenced (GenBank 657582) and 593 bp compared with the similar region for the Mi-1.2 gene from the genomic sequence (U81378) »
 - Amplification profile would be difficult to read in routine application (3% agarose gel)

f. PM3Fb-Rb

| Cultivar | R / S | Result |
|-------------------|-------|--------|
| Anahu | R:1 | 500pb |
| Anahu x Casaque R | R:2a | 500pb |
| Anahu x Monalbo | R:2a | 500pb |
| Thomas | R:2b | 500pb |
| Campeon | R:3 | 500pb |
| Casaque Rouge | S | X |
| Rio Grande | S | X |
| Montfavet H63.5 | S | X |
| Daniela | S | X |
| Earlymech | S | X |
| Negative control | | - |

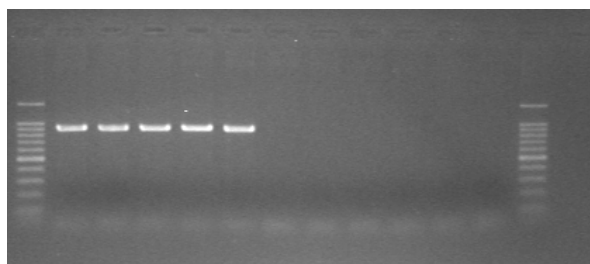


PCR product of 500bp obtained from resistant cultivar Anahu was checked by sequencing.

- Conclusion
 - Amplification of 500bp product (as expected)

g. C2D1-C2S4

| Cultivar | R / S | Resultts C2D1-C2S4 |
|-------------------|-------|-----------------------|
| Anahu | R:1 | 915pb |
| Anahu x Casaque R | R:2a | 915pb |
| Anahu x Monalbo | R:2a | 915pb |
| Thomas | R:2b | 915pb |
| Campeon | R:3 | 915pb |
| Casaque Rouge | S | X |
| Rio Grande | S | X |
| Montfavet H63.5 | S | X |
| Daniela | S | X |
| Earlymech | S | X |
| Negative control | | - |



PCR product of 915bp obtained from resistant cultivar Anahu was checked by sequencing.

- Conclusion
 - Amplification of a 915bp product (as expected).

4. Discussion

| Primers | | Cultivars | | |
|------------|-----------------|--------------------|--------------------------|---------------|
| | | Résistant type 1 | Résistant type 2a, 2b, 3 | Susceptible |
| Lat1-2 | tomato specific | 92pb | 92pb | 92pb |
| Lat3-4 | tomato specific | 946pb | 946pb | 946pb |
| PMi12F1-R2 | Codominant | 720, 800 et 1000pb | 620, 720, 800 et 1000pb | 620 et 1000pb |
| PMiF3-R3 | Codominant | 550pb | 350 et 550pb | 350pb |
| PM3F-3R | Codominant | 650pb | 600 et 650pb | 600pb |
| PM3Fb-Rb | Dominant | 500pb | 500pb | X |
| C2D1-C2S4 | Dominant | 915pb | 915pb | X |

X : no PCR product

All the PCR markers described as Mi1-2 specific were able to discriminate resistant and susceptible cultivars as published.

Two markers are dominant (PM3Fb-Rb and C2D1-S4). They showed very clear results and may be used with one of the two LAT52 markers as control which would require adjusting PCR conditions.

The three other markers are codominant (PMi12F1-R2, PMiF3-R3, PM3F-3R) but only one of them (PMiF3-R3) fits to the requirement of a routine application (i.e. no additional band, clear separation of both R and S specific bands on agarose gel). This Mi1-2 resistance gene specific marker appears to be the best marker to be used in a routine assay on the base of this first round of tests. It should be confirmed by others tests using a larger set of cultivars.

5. Primer list and PCR protocol

-Plants: 5/6 days old plants (see chapter 2)

-DNA preparation: DNeasy Plant Mini Kit (Qiagen)

-Primers: PMiF3/PMiR3 (Mehrch, 2005)

| | |
|-------|--|
| PMiF3 | GGT ATG AGC ATG CTT AAT CAG AGC TCT C |
| PMiR3 | CCT ACA AGA AAT TAT TGT GCG TGT GAA TG |

PCR conditions:

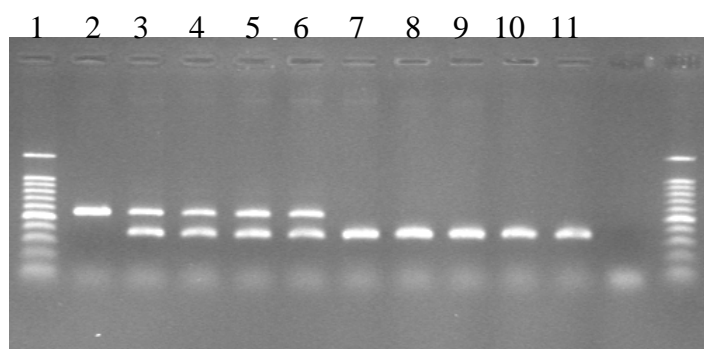
| | |
|-------------------|-------------|
| MgCl ₂ | 2 mM |
| dNTP | 0,2 mM |
| PMiF3 | 0,5 μ M |
| PMiR3 | 0,5 μ M |
| Red Taq Sigma | 1U / PCR |

| | | |
|-------|--------|-------------|
| 94°C | 10 min | } 35 cycles |
| 94°C | 30 sec | |
| 50 °C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

PCR product visualization: electrophoresis on a 1.5% agarose gel followed by BET staining.

-Representative result:

| | Cultivar | R / S | Result |
|----|-------------------|-------|---------------|
| 1 | Anahu | R:1 | 550pb |
| 2 | Anahu x Casaque R | R:2a | 350 and 550pb |
| 3 | Anahu x Monalbo | R:2a | 350 and 550pb |
| 4 | Thomas | R:2b | 350 and 550pb |
| 5 | Campeon | R:3 | 350 and 550pb |
| 6 | Casaque Rouge | S | 350pb |
| 7 | Rio Grande | S | 350pb |
| 8 | Montfaret H63.5 | S | 350pb |
| 9 | Daniela | S | 350pb |
| 10 | Earlymech | S | 350pb |
| 11 | Negative control | - | - |



The DNA of varieties Anahu and Casaque Rouge as resistant and susceptible variety respectively will be sent around for testing the Mi marker.

6. References

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Appendix 8: Assay for *Fusarium* I resistance locus

Development of a molecular marker for I (f.o.l. 0) resistance locus

Authors:

René Mathis, Laetitia Cavellini, Cécile Collonnier

GEVES (France)

1. Introduction/Background

I locus (introgression from *L. pimpinellifolium*) confers resistance to *Fusarium oxysporum* f. sp. *Lycopersici* race 0 (f.o.l. 0) (ex race 1) but not to race 1 (ex2) (Diener and Ausubel; 2005). This region is mapped on the short arm of chromosome 11.

The gene is not cloned but some linked markers have been published

- Ori et al. (1997) mentioned unpublished data obtained by Eshed on 2 RFLP markers: TG523 (sequence available) and CP58A. The estimated interval between TG523 and CP58A is 7.5 cM interval (sol genomics network, Cornell University).

- Sela-Buurlage et al (2001) proved that some clones with NBS domain mapped to the I locus (no linked sequence available)

- Scott et al (2004) found that the I locus was positioned between TG523 and TG7 markers (approximate interval of 3.8 cM).

In conclusion, no marker is available for *i* gene detection and there are not enough sequence data to investigate for a marker positioned inside the *i* resistance gene. However available sequence of markers linked to the I locus can be analysed to look for any polymorphism possibly associated with R/S phenotypes. The discussion that took place during the 2nd meeting of the program in Paris concluded that such related polymorphism could be useful for detecting resistance to fol race 0. This analysis was started immediately after our project second meeting (Paris 13/11/2006) the work done until 19/01/2007 is presented in this document.

2. Materials and Methods

a) Available data on DNA fragment related to *i*/locus (see Figure 1)

- Sequenced DNA from RFLP fragments:

- TG523-F: two sequences available: DQ097537 cv. Heinz (R) (339bp sequence); and SGN-M121 (445bp sequence) (Sol Genomics Network)

- TG523-R: two sequences available: DQ097530 cv. Heinz (R) (455bp sequence); and SGN-M121 (475bp sequence) (Sol Genomics Network)

- TG7: 548 bp sequence (SGN-M263; Sol Genomics Network)

▪ Testing intronic universal primers for Asterid species positioned near the TG723 markers

-At5-F/At5-R: targeting COSII Marker C2_At5g16710 (SGN-M4725; Sol Genomics Network) for a PCR product of about 1400bp

| | |
|-------|----------------------------|
| At5-F | ACTTGATGAGCTGACAGCTTTCAATG |
| At5-R | AGCTTTGGTCCAAGCGACAAATC |

-At2-F/At2-R: targeting COSII Marker C2_At2g22570 (SGN-M4717; Sol Genomics Network) for a PCR product of about 1200bp.

| | |
|-------|----------------------------|
| At2-F | ACTGAAGAGTGAGATTCCGGTGGAG |
| At2-R | TCTGTTCCAGTGATACAATGAGGAGG |

(All experiments were performed on a PTC200 thermocycler from BIORAD.)

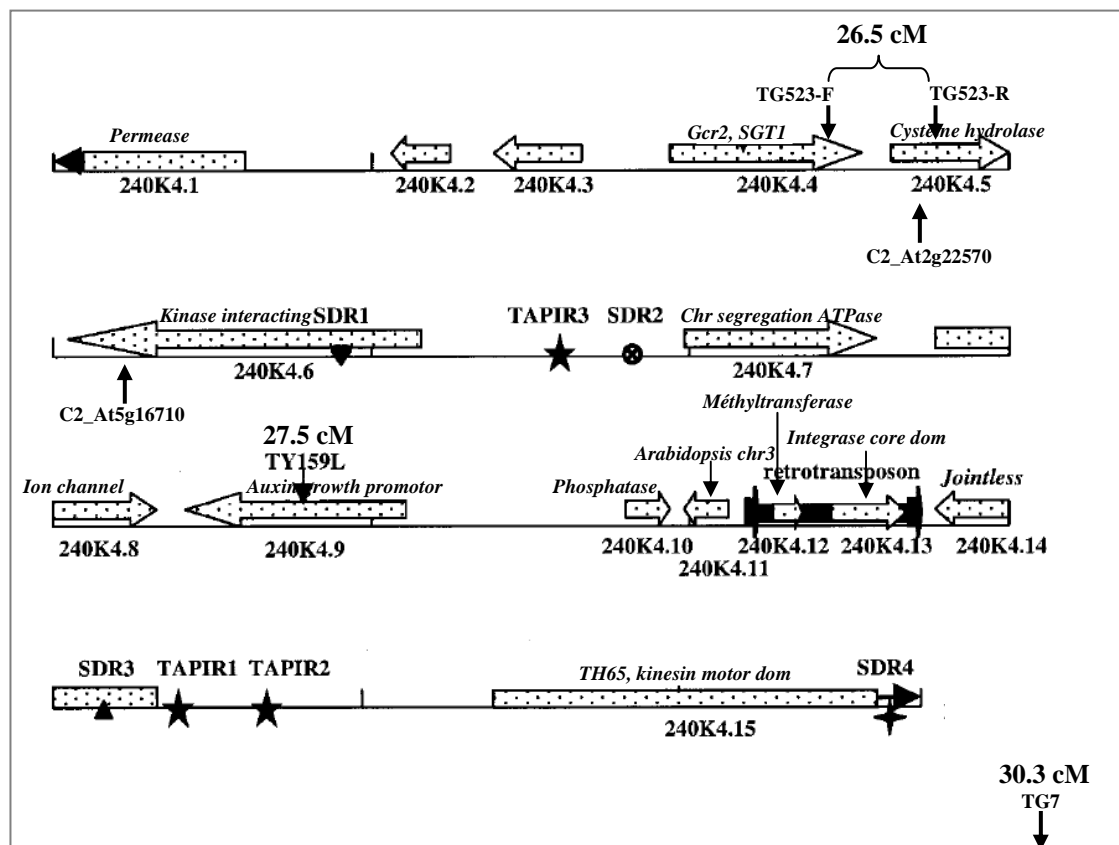


Figure 1: Map of I region (Modified Figure I from Mao *et al*, 2001, A graphical display of the predicted ORFs and the DNA elements on BAC 240K04 (GenBank accession no. AF275345). (Arrows for each ORF indicate the coding orientation.),

b) Tested cultivars

Tested cultivars were provided by Cavaillon GEVES station and SNES Pathology laboratory. Five resistant varieties and three susceptible varieties have been sequenced and tested in PCR

| Plant Material | R/S |
|---------------------------|-----|
| Marporum | R |
| Nemo-Netta | R |
| Campeon | R |
| Thomas | R |
| Marporum x Marmande Verte | R |
| Montfavet H63.5 | S |
| Marmande Verte | S |
| Marmande | S |

c) DNA preparation

-DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 100mg of plant tissue according to procedure (except for tissue crushed directly in lysis buffer).

-Quality DNA control (OD measurement, amplification of a tomato specific gene: LAT52)

d) Sequencing

PCR products were purified from agarose gel (Wizard SV Gel PCR clean-Up System from Promega) and sequenced by GATC biotech.

3. Results

1/ Design of primers

To increase the number of available sequences linked to I locus, we designed 12 new primers using DQ097537, DQ097530 (NCBI) and SGN-M263, SGN-M4725, SGN-M4717 (Sol Genomics Network) Together with 4 primers from Sol Genomics Network, they were used to amplify TG523-F, TG253-R, TG7, C2_At5g16710 and C2_At2g22570 fragments, as well as a DNA fragment between TG523-F and TG523-R (see figure 1 for markers positions).

| Primer name | Sequence 5'-3' | Origin | Amplicon length | Target | Utilization |
|-------------|----------------------------|------------|-----------------|------------------------------|--------------------|
| TG523-F1 | ATGGACTTCCAGGACCTGCT | this study | 303bp | TG523-F | PCR and Sequencing |
| TG523-F2 | TTCCTGGTCATGATAAATTCG | “ | | | |
| TG523-R1 | CCAGTAAGGAGCTTCATTCAAT | “ | 455bp | TG253-R | |
| TG523-R2 | GACCACATTCACAAAACACT | “ | | | |
| TG523(F)-F3 | CGAAATTTATCATGACCAGGAA | “ | Unknown | Junction TG523-F and TG523-R | |
| TG523(R)-R3 | AACAAATGGCACAAAATCACA | “ | | | |
| TG7-F | GCAAGTTTAGTGATCAGGGATG | “ | 499bp | TG7 | |
| TG7-R | CCAAGAAAGTAATACAAGGACCAA | “ | | | |
| At5-F | ACTTGATGAGCTGACAGCTTTCAATG | SGN | 1400bp | C2_At5g16710 | |
| At5-R | AGCTTTGGTCCAAGCGACAAATC | “ | | | |
| At2-F | ACTGAAGAGTGAGATTCCGGTGGAG | “ | 1200bp | C2_At2g22570 | |
| At2-R | TCTGTTCAGTGATACAATGAGGAGG | “ | | | |
| At2-F1 | GGAGCTGGCAATTTGGTAAA | this study | 400bp | | |
| At2-R1 | TTCCAAATACACCATTTTCACTTT | “ | | | |
| At2-F3 | CGAATCTGTATATTACATCCGTCGT | “ | 130bp | C2_At2g22570 | PCR |
| At2-R3 | GGTGAATACCGATCATAGTCGAG | “ | | | |

Table 1: sequences of primers used for the first round of sequencing
SGN : Sol Genomics Network

2/ Sequencing and sequences analysis

The search for sequence polymorphism between resistant and susceptible cultivars based on our PCR products is reported in table 2.

| Cultivar | PCR Primers | Sequencing Primers | PCR product | Sequence available | Observation |
|----------------|-------------|--------------------|-------------|--------------------|--|
| Marmande Verte | TG7-F/R | TG7-F | X | X | 100% sequence homology between S (Marmande Verte) and R (Marporum) |
| Marporum | | | X | X | |
| Marmande Verte | TG523-F1/F2 | TG523-F1 | X | X | |
| Marporum | | | X | X | |
| Marmande Verte | TG523-R1/R2 | TG523-R1 | X | X | |
| Marporum | | | X | X | |
| Marmande Verte | TG523-F3/R3 | TG523-F3 | X | no | No sequence produced (presence of secondary structures) |
| Marporum | | | X | no | |
| Marmande Verte | | TG523-R3 | X | no | |
| Marporum | | | X | no | |
| Marmande Verte | At2-F/R | At2-F | X | X | One gap difference between resistant (Marporum) and susceptible (Marmande Verte) cultivars |
| Marporum | | | X | X | |
| Marmande Verte | | At2-R | X | X | Sequence homology between S (Marmande Verte) and R (Marporum) |
| Marporum | | | X | X | |
| Marmande Verte | At5-F/R | At5-F | X | X | Sequence homology between S (Marmande Verte) and R (Marporum) |
| Marporum | | | X | X | |
| Marmande Verte | | At5-R | X | X | |
| Marporum | | | X | X | |

Table 2: Primers used for sequence analysis and deduced information.

Conclusion:

Out of 18 DNA fragments we could have sequence information from only 14 of them.

One amplicon could not be used to compare the resistant and susceptible cultivars.

The 5 possible sequence comparisons between one resistant and one susceptible cultivar showed :

- 4 cases of 100% nucleotide identity with TG7-F/R, TG523-F1/F2, TG523-R1/R2, At5-F/R

- and one case with a clear difference: a gap of seven nucleotides, with At2-F. (see Figure 2)

This last case is the unique indication towards a sequence difference between resistant and susceptible cultivars (out of 2310 nt).

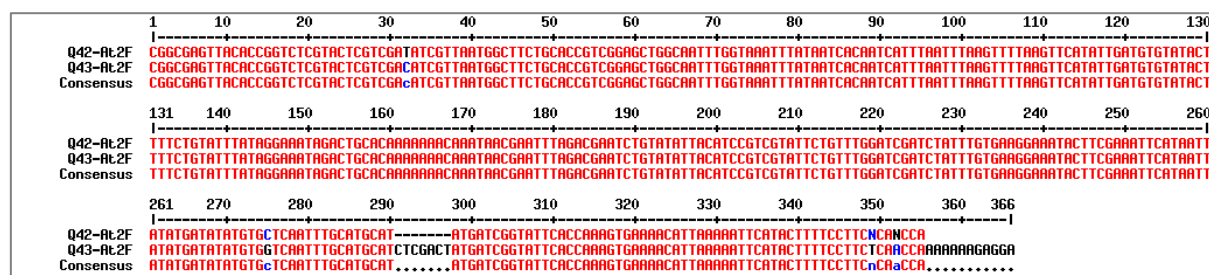


Figure 2: First sequence difference between a resistant cultivar (Q43 or Marporum) and a susceptible one (Q42 or Marmande Verte).

```

gi|83629189|gb|AF275345.3| Lycopersicon esculentum putative permease gene,
partial cds; Length=118761
/organism="Solanum lycopersicum"
/mol_type="genomic DNA"
/cultivar="Heinz 1706"
/chromosome="11" /map="TG523-TY159L"

Query : Q42-At2F
Length=348

Score = 565 bits (285), Expect = 4e-158
Identities = 335/346 (96%), Gaps = 7/346 (2%)

Query_10 ACACCGGTCTCGTACTCGTCGATATCGTTAATGGCTTCTGCACCGTCGGAGCTGGCAATT 69
|||||
Sbjct_27486 ACACCGGTCTCGTACTCGTCGACATCGTTAATGGCTTCTGCACCGTCGGAGCTGGCAATT 27545

Query_70 TGGTAAATTTATAATCACAAATCATTAAATTTAAGTTTAAAGTTCATATTGATGTGTATAC 129
|||||
Sbjct_27546 TGGTAAATTTATAATCACAAATCATTAAATTTAAGTTTAAAGTTCATATTGATGTGTATAC 27605

Query_130 TTTTCTGTATTTATAGGAAATAGACTGCACaaaaaaCAAATAACGAATTTAGACGAATC 189
|||||
Sbjct_27606 TTTTCTGTATTTATAGGAAATAGACTGCACAAAAAACAAATAACGAATTTAGACGAATC 27665

Query_190 TGTATATTACATCCGTCGTATTCTGTTTGGATCGATCTATTTGTGAAGGAAATACTTCGA 249
|||||
Sbjct_27666 TGTATATTACATCCGTCGTATTCTGTTTGGATCGATCTATTTGTGAAGGAAATACTTCGA 27725

Query_250 AATTCATAATTATATGATATATGTGCTCAATTTGCATGCA-----TATGATCGGTATT 302
|||||
Sbjct_27726 AATTCATAATTATATGATATATGTGCTCAATTTGCATGCACTCTGACTATGATCGGTATT 27785

Query_303 CACCAAAGTGAAAACATTAATAATTCATACTTTTCCTTCNCANCCA 348
|||||
Sbjct_27786 CACCAAAGTGAAAACATTAATAATTCATACTTTTCCTTCNCAACA 27831

Query : Q43-At2F
Length=366

Score = 642 bits (324), Expect = 0.0
Identities = 345/345 (100%), Gaps = 0/345 (0%)

Query_10 ACACCGGTCTCGTACTCGTCGACATCGTTAATGGCTTCTGCACCGTCGGAGCTGGCAATT 69
|||||
Sbjct_27486 ACACCGGTCTCGTACTCGTCGACATCGTTAATGGCTTCTGCACCGTCGGAGCTGGCAATT 27545

Query_70 TGGTAAATTTATAATCACAAATCATTAAATTTAAGTTTAAAGTTCATATTGATGTGTATAC 129
|||||
Sbjct_27546 TGGTAAATTTATAATCACAAATCATTAAATTTAAGTTTAAAGTTCATATTGATGTGTATAC 27605

Query_130 TTTTCTGTATTTATAGGAAATAGACTGCACaaaaaaCAAATAACGAATTTAGACGAATC 189
|||||
Sbjct_27606 TTTTCTGTATTTATAGGAAATAGACTGCACAAAAAACAAATAACGAATTTAGACGAATC 27665

Query_190 TGTATATTACATCCGTCGTATTCTGTTTGGATCGATCTATTTGTGAAGGAAATACTTCGA 249
|||||
Sbjct_27666 TGTATATTACATCCGTCGTATTCTGTTTGGATCGATCTATTTGTGAAGGAAATACTTCGA 27725

Query_250 AATTCATAATTATATGATATATGTGCTCAATTTGCATGCACTCTGACTATGATCGGTATT 309
|||||
Sbjct_27726 AATTCATAATTATATGATATATGTGCTCAATTTGCATGCACTCTGACTATGATCGGTATT 27785

Query_310 CACCAAAGTGAAAACATTAATAATTCATACTTTTCCTTCNCAACC 354
|||||
Sbjct_27786 CACCAAAGTGAAAACATTAATAATTCATACTTTTCCTTCNCAACC 27830

```

Figure 3: Alignment of resistant cultivar (Q43 or Marporum) and a susceptible cultivar (Q42 or Marmande Verte) with AF275347 showing the 7-nt-gap restricted to the susceptible cultivars

3/ Confirmation of one I-linked sequence difference between resistant and susceptible cultivars

Further sequencing work was performed in order to confirm the first observation of sequence difference between resistant and susceptible tomato cultivars.

This difference was confirmed by sequencing a new PCR product from the same cultivars (Marporum and Marmande Verte). Same sequencing using At2-F/At2-R primers

for PCR was also intended on other resistant and susceptible cultivars but failed. Therefore, new primers (At2-F1/R1) were designed to border the gap region and facilitate the sequencing of this region. The PCR product from five additional cultivars were then sequenced and clearly showed again the presence of a 7 nucleotides gap in susceptible cultivars. The result of this work is reported in table 3 and figure 4.

| Cultivar | PCR Primers | Sequencing Primers | PCR product | Sequence available | Observation |
|-----------------|-------------|--------------------|-------------|--------------------|--|
| Marporum | At2-F/R | At2-F | X | X | Repetition of n°10-At2-F, perfect homology |
| Campeon | | | X | no | No clear sequence data in the gap area, see below for repetition |
| Montfayet H63.5 | | | X | no | |
| Marmande Verte | | | X | X | Repetition of n°9-At2-F, perfect homology |
| Marmande | | | X | no | No clear sequence data in the gap area, see below for repetition |
| Nemo-Netta | At2-F1/R1 | At2-F1 | X | X | One gap difference between resistant (Nemo-Netta, Campeon, Thomas) and susceptible (Montfayet H63.5, Marmande) cultivars |
| Campeon | | | X | X | |
| Thomas | | | X | X | |
| Montfayet H63.5 | | | X | X | |
| Marmande | | | X | X | |

Table 3: Sequence analysis and deduced information using new sets of primers.

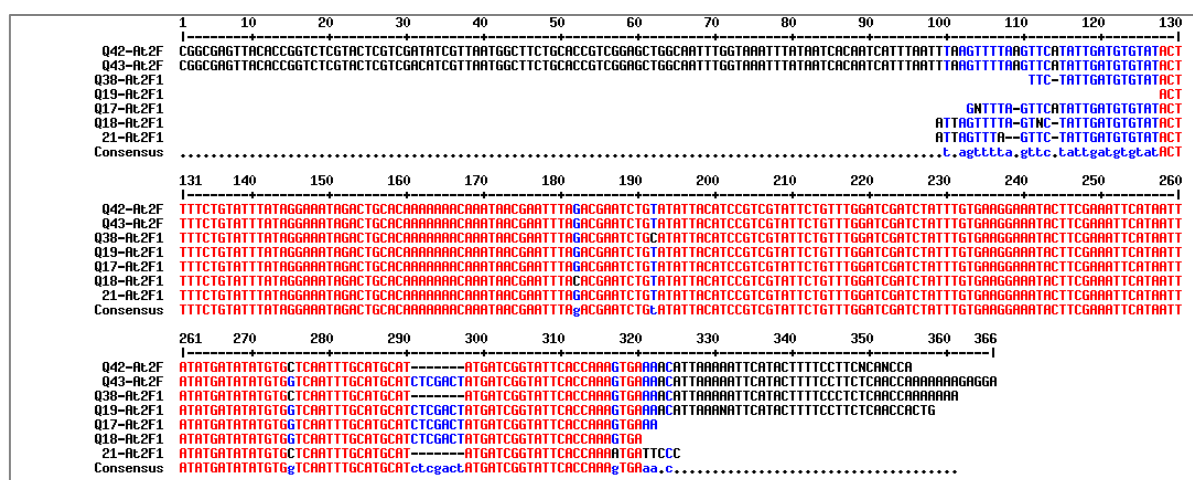


Figure 4: Alignment showing the 7-nt-gap restricted to the susceptible cultivars (Q42 = Marmande Verte, Q43 = Marporum, Q38 = Montfayet H63.5, Q19 = Campeon, Q17 = Thomas, Q18 = Nemo-Netta, 21 = Marmande)

4/ A first PCR marker for I linked resistance

Since the occurrence of a gap in susceptible cultivars was confirmed, we undertake the design of a PCR based marker. One discriminating primer was designed to match the sequence present only in resistant cultivars (reverse primer), the forward primer was designed in a sequence region common to resistant and susceptible cultivar (see figure 5).

These two new primers to be used for a PCR based marker were named At2-F3 / At2-R3 :

| | |
|--------|---------------------------|
| At2-F3 | CGAATCTGTATATTACATCCGTCGT |
| At2-R3 | GGTGAATACCGATCATAGTCGAG |

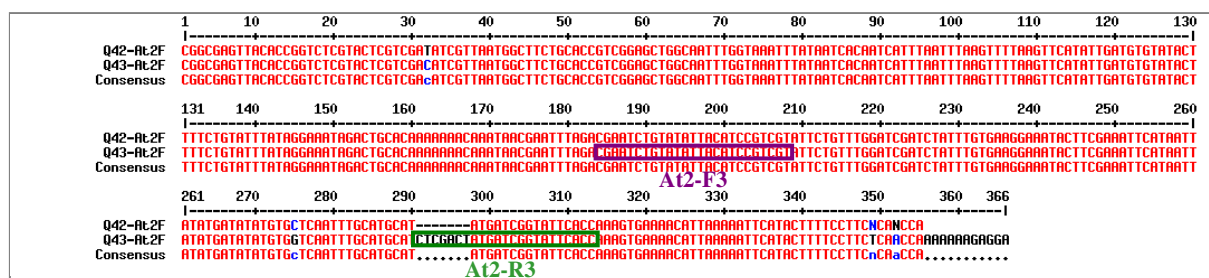


Figure 5: PCR marker tested and their position on sequence C2_At2g22570 (Q42 = susceptible cultivar Marmande Verte and Q43 = resistant cultivar Marporum)

This PCR specific test for i-linked resistance (using the couple of primers At2-F3/R3) was tested on 8 cultivars available (five resistant varieties and three susceptible varieties).

- PCR conditions

| | |
|--------------|-------------|
| MgCl2 | 1,5 mM |
| dNTP | 0,2 mM |
| At2-F3 | 0,5 μ M |
| At2-R3 | 0,5 μ M |
| RedTaq Sigma | 1U par PCR |

| | |
|-------|--------|
| 94°C | 5 min |
| 94°C | 30 sec |
| 66 °C | 30 sec |
| 72°C | 30 sec |
| 72°C | 5 min |

} 35 cycles

- Results

| Plant Material | R/S | Result At2-F3/R3 |
|---------------------------|-----|------------------|
| Marporum | R | 130bp |
| Nemo-Netta | R | 130bp |
| Campeon | R | 130bp |
| Thomas | R | 130bp |
| Marporum x Marmande Verte | R | X |
| Montfavet H63.5 | S | X |
| Marmande Verte | S | X |
| Marmande | S | X |

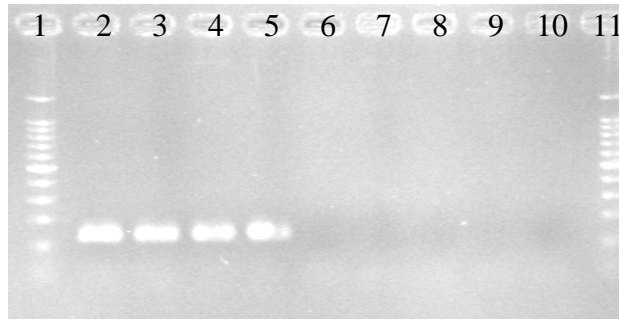


Figure 6: Gel picture showing the result of a PCR test specific for i-linked resistance (primers At2-F3/ At2-R3). Lane 1 and 11, 100pb marker; lane 2, Marporum; lane 3, Nemo-Netta; lane 4, Campeon, lane 5, Thomas; lane 6, Marporum x Marmande Verte; lane7, Montfavet H63.5, lane8, Marmande Verte; lane 9, Marmande; lane10, empty.

- Conclusion
 - Amplification of 130bp product (as expected)
 - Every resistant lines gives DNA product but not heterozygous resistant hybrids (Marporum x Marmande Verte)

4. Discussion

The PCR marker described as I specific was able to discriminate resistant and susceptible cultivars. However, no PCR product was amplified by this marker for the heterozygous resistant cultivar (Marporum x Marmande Verte). No explanation is available yet. Because of the dominant character of this marker, a duplex with LAT52 or Rubisco can be tested in PCR in order to complement the test with a positive control for amplification.

The results obtained during this specific work enable us to propose a first molecular marker “At2” for i-linked resistance (fol O). This PCR marker could be included in the next steps of our schedule within the framework of this project. (For the robustness test, DNA of samples Marporum and Marmande Verte should be used.)

5. Primer list and PCR protocol

-Plants: 5/6 days old plants (see chapter 2)

- DNA preparation : DNeasy Plant Mini Kit (Qiagen)

-Primers: At2-F3/R3

| | |
|--------|---------------------------|
| At2-F3 | CGAATCTGTATATTACATCCGTCGT |
| At2-R3 | GGTGAATACCGATCATAGTCGAG |

- PCR conditions:

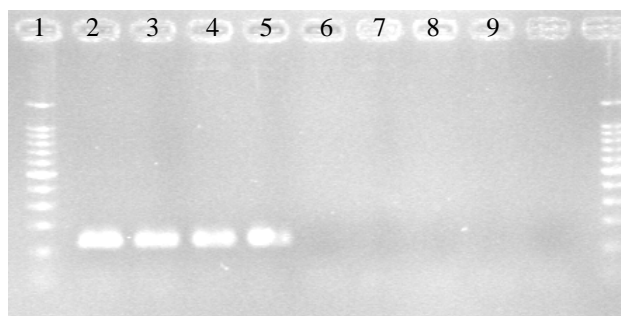
| | |
|-------------------|-------------|
| MgCl ₂ | 1,5 mM |
| dNTP | 0,2 mM |
| At2-F3 | 0,5 μ M |
| At2-R3 | 0,5 μ M |
| RedTaq Sigma | 1U par PCR |

| | | |
|-------|--------|-------------|
| 94°C | 5 min | } 35 cycles |
| 94°C | 30 sec | |
| 66 °C | 30 sec | |
| 72°C | 30 sec | |
| 72°C | 5 min | |

-PCR product visualization: electrophoresis on a 3% agarose gel followed by BET staining.

-Representative result:

| Lanes | Cultivar | R/S | Result At2-F3/R3 |
|-------|---------------------------|-----|------------------|
| 2 | Marporum | R | 130bp |
| 3 | Nemo-Netta | R | 130bp |
| 4 | Campeon | R | 130bp |
| 5 | Thomas | R | 130bp |
| 6 | Marporum x Marmande Verte | R | X |
| 7 | Montfavet H63.5 | S | X |
| 8 | Marmande Verte | S | X |
| 9 | Marmande | S | X |



6. References

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Mao L, Begum D, Goff SA, Wing RA. Sequence and analysis of the tomato JOINTLESS locus. *Plant Physiol*. 2001 Jul;126(3):1331-40.

Ori N, Eshed Y, Paran I, Presting G, Aviv D, Tanksley S, Zamir D, Fluhr R. The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. *Plant Cell*. 1997; 9(4):521-32.

Scott, J. W., H. A. Agrama, and J. P. Jones. RFLP-based analysis of recombination among resistance genes to fusarium wilt races 1, 2 and 3 in tomato. *J. Amer. Soc. Hort. Sci*. 2004 ; 129(3):394-400.

Sela-Buurlage MB, Budai-Hadrian O, Pan Q, Carmel-Goren L, Vunsch R, Zamir D, Fluhr R. Genome-wide dissection of Fusarium resistance in tomato reveals multiple complex loci. *Mol Genet Genomics*. 2001; 265(6):1104-11.

Sol Genomics Network, <http://www.sgn.cornell.edu/index.pl>

Markers for Tomato Chromosomes:

<http://www.plantpath.wisc.edu/GeminivirusResistantTomatoes/Markers/chr11.htm>

Appendix 9: Assay for Fusarium I2 resistance locus

Development of markers for the I2 resistance gene

Authors

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1. Introduction/Background

The *I2* locus was introduced into tomato from *Lycopersicon pimpinellifolium* and confers resistance to race 2 of the soil-borne fungus *Fusarium oxysporum* f sp *lycopersici-foI* (Sela-Buurlage *et al.*, 2001). *I2* was mapped on the long arm of chromosome 11 and cloned (Ori *et al.*, 1997). It encodes for a protein of 1266 amino acids and belongs to the nucleotide binding sites (NBS), leucine rich repeat (LRR) superfamily of plant resistance genes. *I2* is a member of a complex resistance locus with 6-7 members (*I2*, *I2C1*, *I2C2*, *I2C3*, *I2C4* and *I2C5*) within 90 kb named the I2C gene family (Ori *et al.*, 1997; Simon *et al.*, 1998). Some of these members share strong sequence similarities with *I2* but can not confer complete resistance to *foI*. Moreover, alignments between the various members of the I2C gene family showed that they can be distinguished by the number of repeats of a 23-amino acid sequence in the LRR region (Simon *et al.*, 1998)(**Table 1**). A probable *I2* ortholog controlling a partial resistance to race 2 of *foI* was identified in *L. pennellii* LA716 (Sela-Buurlage *et al.*, 2001) and other members of the I2C gene family were also mapped on tomato chromosome 8 and 9 (Ori *et al.*, 1997).

In this project, three types of experiments were conducted to develop *I2* specific markers: (I) development of specific PCR markers using the *I2* sequence, (II) characterization of the LRR region of each member of the I2C complex in order to identify SNPs specific for *I2* *vs.* *I2⁺* and *I2* *vs.* other members of the complex, and (III) transformation of RFLP (restriction fragment length polymorphism) markers linked to *I2* into specific PCR markers. This report will focus on experiments I and II. Indeed, we were unsuccessful in the transformation of linked-RFLPs into PCR markers.

2. Materials and Methods

The *I2*-genotypes *L. esculentum* Mogéor, Mobox and Ideucenzi and the *I2⁺*-genotypes *L. esculentum* Marmande verte and Mossol were used. The F1 hybrid between Marmande verte (*I2⁺*) and Motelle (*I2*) and *L. pennellii* LA716 were also included in the assays.

For the development of specific PCR markers based on the *I2* sequence the following primers pairs were tested: Z1063F&R, Z1064F&R and Z1065F&R (see **Table 2**). Multiplex PCR for amplification of both *Rubisco* (used as positive control) and *I2* were performed with primers Z1063 and *Rubisco* described in **Table 2**.

The analysis of LRR sequences of the I2C members was performed using primers Z1062F&R (**Table 2**). PCR products from Ideucenzi (*I2*) and Marmande verte (*I2^v*) obtained during three independent experiments were cloned. Sixty clones per genotypes were selected according to their size and 17 and 18 clones for Ideucenzi and Marmande verte respectively were sequenced.

All PCR conditions are described in section 5.

3. Results

Part I

The position of primers Z1063, Z1064 and Z1065 on the *I2* and *I2C1* to *5* sequences for the development of *I2* specific markers is indicated in **Figure 1**. Among these primers, Z1063F&R permitted the amplification of a single fragment only for genotypes with the *I2* gene from *L. pimpinellifolium* (*i.e.*, no amplification of the *L. pennellii* LA716 ortholog). PCR products were sequenced and confirmed that the amplified fragment correspond to *I2*.

Because of the dominant nature of this marker, a control for amplification (*Rubisco*) was included in the PCR reaction (=> see section 5 multiplex PCR for *I2* and *Rubisco*). Optimization of multiplex PCR conditions gave specific and reproducible results by using three times more *I2* primers in comparison with *Rubisco* primers, and an annealing temperature comprised between 54 and 56°C (**Figure 2**).

Part II

PCR were performed with Z1062 primers flanking the LRR repeats (Simon *et al.*, 1998). **Figure 3** provides a photo of the PCR amplification obtained together with hypothesis about the nature of each fragment according to its size. The cloning and sequencing of 17 fragments of different size from Ideucenzi allowed the characterization of the LRR domains of *I2*, *I2C1*, *I2C2*, *I2C3*, *I3C5* (with 99 to 100% identity with AF118127, AF004878, AF004879, AF004880 and AF408704, respectively). For Marmande verte, the cloning and sequencing of 18 PCR fragments allowed the characterization of *I2^v* (with 74-77% identity with AF118127), *I2C1^v* and *I2C2^v* (with 83-86% identity with AF004878 and AF004879), *I2C3^v* (99% identity with AF004880) and *I2C5^v* (90% identity with AF408704). Surprisingly, *I2C4* was never cloned. These experiments permitted to obtain part of the sequence of the *I2^v* susceptibility allele and to identify polymorphism between *I2* and *I2^v*. During next months (December 06 & January 07), tests for obtaining a codominant marker specific for *I2* will be performed.

4. Discussion

A specific and reproducible dominant PCR marker that can be combined with a *Rubisco* positive control is available for the *I2* resistance gene originating from *L. pimpinellifolium*.

The sequencing of the LRR region of members of the I2C complex in both resistant and susceptible genotypes allowed the identification of polymorphism between *I2* and *I2^v*. Assays to try to develop a codominant marker based on this polymorphism are underway.

5. Primer list and PCR protocol

Total DNAs were extracted from 2-3 young leaves per genotype according to the protocol described by Fulton *et al.* (1995).

PCR amplifications were routinely performed using 2 µl of DNA (20 ng/µl) in 25µl-reactions containing 5 µl of 5X buffer (Promega), 1.5 mM MgCl₂ (1,5 µl at 25 mM, Promega), the 4 dNTPs at 0.16 mM each (1 µl at 4mM @), primers at 0.2 mM each (0.5 µl @ at 10 mM) and GoTaq DNA polymerase from Promega at 0.04 U/µl (0.2 µl at 5U/µl).

Tests for optimization of the multiplex PCR for amplification of both *Rubisco* and *I2* (with Z1063F&R primers) lead us to use *Rubisco* primers at 0.12 mM (0.3 µl @ at 10mM) and *I2* primers at 0.4 mM (1 µl @ at 10mM) and an annealing temperature comprised between 54 and 56 °C. A photo of the dominant PCR marker available for *I2* combined with the *Rubisco* positive control is provided at **Figure 2**.

For these reactions, 35 cycles (30 s of denaturation at 94°C, 45 s of annealing at different temperatures according to the primer used, and 1 min of elongation at 72°C) were performed in a Eppendorf (Mastercycler ep gradient) thermal cycler after an initial denaturation at 94°C for 2 min.

Amplification products were resolved by electrophoresis in a 1% (w/v) agarose gel except for products obtained with Z1062F&R where a 3% agarose gel was used.

List of primer names, sequences, annealing temperatures and size of the expected fragment are indicated in **Table 2**.

For the robustness test DNA of Ideucenzi (I2) and Motelle (I2+) will be sent to each of the partners.

6. References

- Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., Zamir, D. and Fluhr, R. (1997) The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine rich repeat superfamily of plant resistance genes. *Plant Cell*, 9: 521-532.
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7. Tables

| Gene | Accession n° | Number of 23-amino acid repeats in the LRR |
|-------|--------------|--|
| I2 | AF118127 | 3 |
| I2C-1 | AF004878 | 2 |
| I2C-2 | AF004879 | 2 |
| I2C-3 | AF004880 | 5 |
| I2C-4 | AF004881 | 6 |
| I2C-5 | AF408704 | 4 |

Table 1. Characteristics of members of the I2C gene family

| Primer name | Forward sequence (5'-3') | Reverse sequence (5'-3') | Annealing temperature | Size of expected fragment |
|-------------|--------------------------|--------------------------|-----------------------|---------------------------|
| Z1063 | ATTGAAAGCGTGGTATTGC | CTTAACTCACCATTAAATC | 54-56 | 940 bp |
| Rubisco | ATGTCACCACAAACAGAGAC | CTCACAAGCAGCAGCTAGT | 54-56 | 1380 bp |
| Z1062 | CCTCCTTTTCTCACCTCACTTCGC | ATTTGTGGCCAGTATTCCCC | 58 | several |
| Z1064 | TTCTCCTGTCATCTTGTGCTG | ATGTTTTAAGATTCTTTATGG | 54 | 1400 bp |
| Z1065 | TGAAATATGGGAGCTGCCAC | AACAACCTGGACAATCACT | 50-53 | 1500 bp |

Table 2. Characteristics of primers used in this study.

Primer pairs for the *I2* specific assay are indicated in black whereas other primers used in this study are indicated in grey.

8. Figures

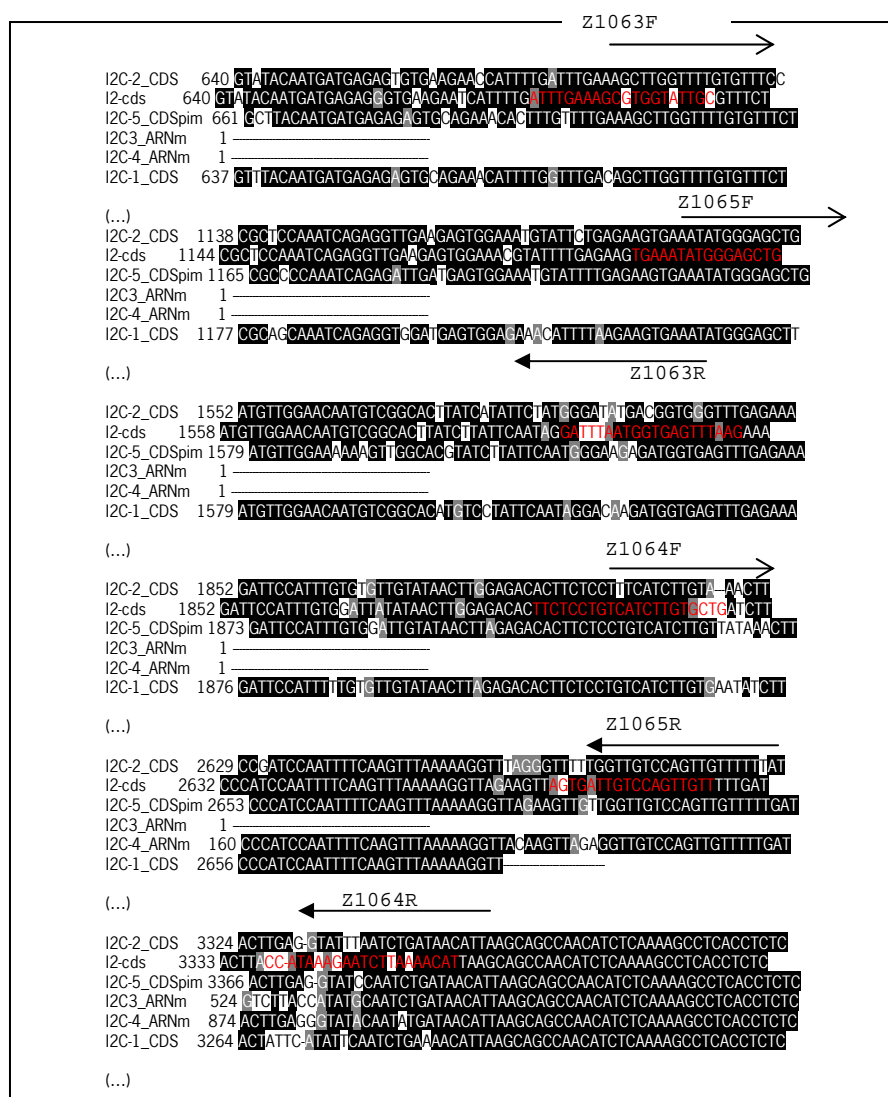


Figure 1. Position of primers Z1063 F and R, Z1064 F and R and Z1065 F and R on the *I2*, *I2C1*, *I2C2*, *I2C3*, *I2C4* and *I2C5* sequences

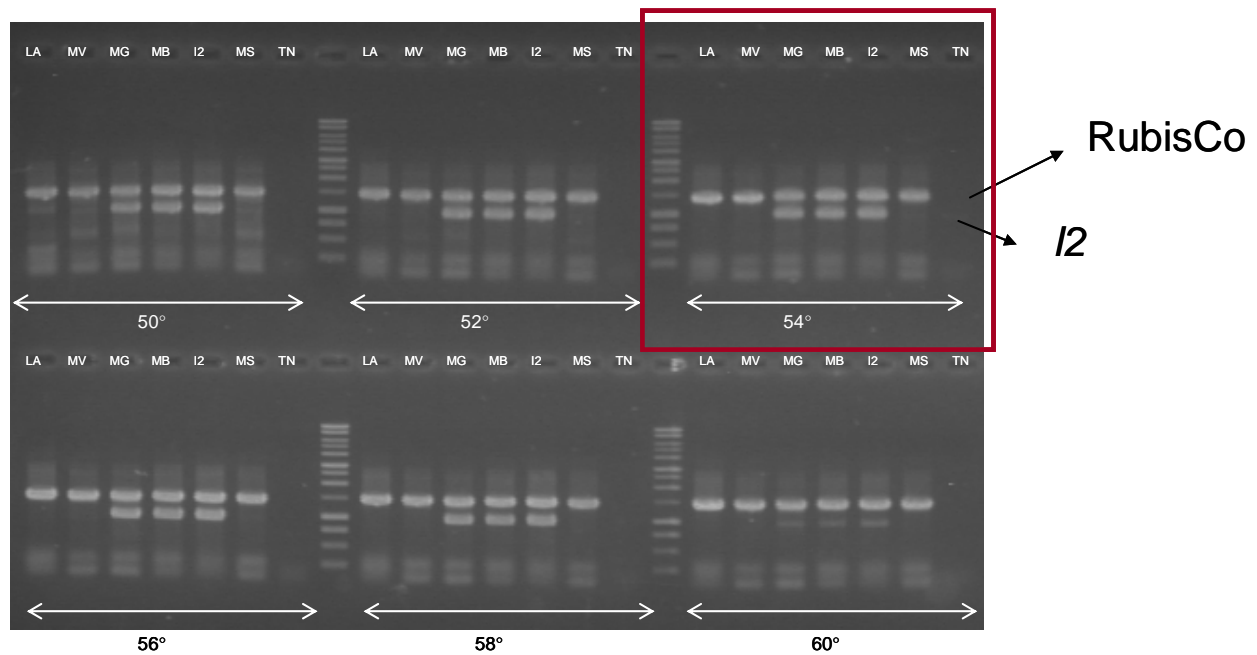


Figure 2. PCR amplification with primers Z1063 F and R (specific for I2) and Rubisco (positive control) at different annealing temperatures.
 LA=LA716; MV=Marmande verte; MG=Mogéor; MB=Mobox; I2=Ideucenzi; MS=Mossol

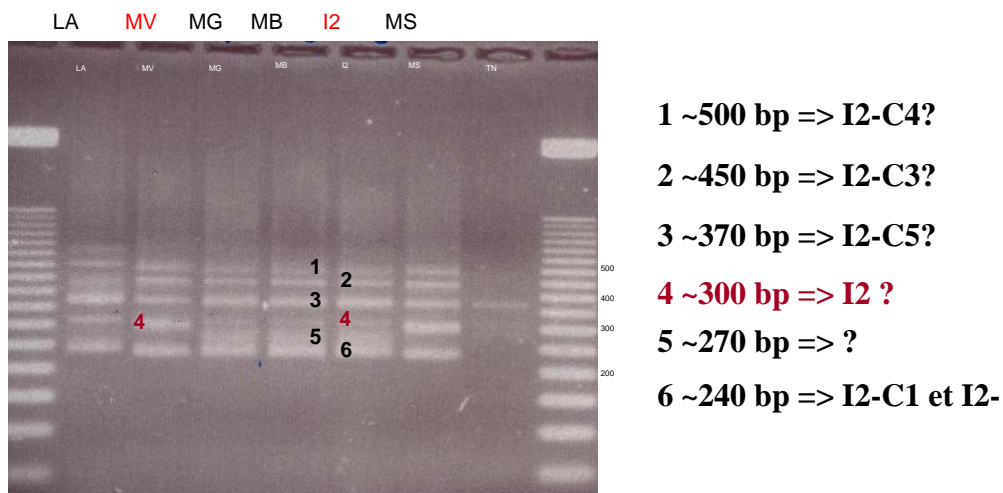


Figure 3. PCR amplification obtained with primers Z1062 F and R together with hypothesis about the nature of each fragment according to its size.
 LA=LA716; MV=Marmande verte; MG=Mogéor; MB=Mobox; I2=Ideucenzi; MS=Mossol

Appendix 10: Results robustness test

Compiled results of robustness test

General conclusions

Transfer of molecular markers from one lab to the next often requires optimisation because between most labs differences exist in the equipment (notably different PCR machines) and reaction components used (mainly Taq polymerases). For each assay results could be reproduced without prior optimisation in at least two other labs. Lab individual optimisation was needed to get all assays at the required level of scorability. Despite the problems in a number of tests that needed attention even these 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 and interpretation of included photos. First a general overview is given followed by result for each assay separately. Note that results given here are the results obtained by the individual labs before a round of optimisation of the conditions was performed, final results after individual optimisation can be found in the main body of this report.

Specific conclusions per assay and optimisations needed

For each of the tests an overview of the test results/actions needed is given in the table below. For each of the partners some issues still need to be addressed to improve results.

- **Ve1** - marker assay shows a number of problems; at NAKtuinbouw amount of product may need improvement. At GEVES and INRA optimisation of PCR conditions needed. PRI will try a set of slightly adapted primers (deliberate mismatch at base directly next to SNP at 3'prime end) to test whether these perform better.
- **Ve2** - marker assay shows a number of problems. At GEVES and INRA optimisation of PCR conditions needed. PRI will try a set of slightly adapted primers.
- **Tm1** – Problem with test for the LAT product used to check PCR fidelity. Optimisation of primer ratio needed at CBGP-INIA and GEVES.
- **Tm2 – ARMS** problems with amount of product and specificity. At INIA and (may be) NAKtuinbouw amount of product in assay 2 needs improvement. Optimisation of specificity of assays at INRA.
- **Mi 1-2** - marker assay is without problems in all labs – ready for testing in DUS samples
- **Fusarium I** – cultivar specific problem (Campeon) at INIA that should be repeated together with independent sample from other partner. Unexpected amplification with Marmande further discussion needed on next step. Unexpected amplification with Marmande verte at PRI will need further checking and possibly optimisation of PCR conditions.
- **Fusarium I2** – Problem with co-amplification of rubisco primers. Optimisation of primer ratio needed at CBGP-INIA and NAKtuinbouw.

Overview of test results

| Test | CBGP-INIA | Naktuinbouw | GEVES | INRA | PRI |
|------------|---------------------|---------------------|-------|------|-----|
| Ve1 | OK | OK/OPA? | OPT | OPT | OK |
| Ve2 | OK | OK | OPT | OPT | OK |
| Tm1 | OPT | OK | OPT | OK | OK |
| Tm2 – CAPS | OK | na | OK | na | OK |
| Tm2 - ARMS | OK/OPA? (assay2) | OK/OPA? (assay2) | OK | OPT | OK |
| Mi1-2 | OK | OK | OK | OK | OK |
| I | Rep | OK | OK | na | Rep |
| I2 | OPT | OPT | OK | OK | OK |

Note: For all tests, GEVES and INRA tested Marmande Verte whereas CBGP-INIA, NAKtuinbouw and PRI have tested Marmande.

OK = no further actions needed, result clear enough for proceeding to further testing.

OPT = optimisation of conditions to achieve clearly scorable pattern.

OPA = increasing amount of PCR product by adding a few cycles may be needed to increase scorability (to be assessed by individual partners).

Rep = Repeated testing of specific samples together with new sample from different origin

na = not analysed or unable to amplify fragments

Specific results per test

Verticillium resistance genes Ve1 & Ve2

Ve1

| Test | Campeon | Marmande | Moneymaker | Persica | 10 (605) | 29 (1113) |
|---|---------|----------|------------|---------|----------|-----------|
| CBGP-INIA ¹ | R | S | S | R/S | S | R/S |
| Naktuinbouw ² | R | S | S | R/S | S | R/S |
| GEVES (2 ^e PCR) ^{1,3} | - (R) | - (S) | - (S) | - (R/S) | - (S) | - (R/S) |
| INRA ^{4,5} | R | S | S | R/S | S | R/S |
| PRI | R | S | S | R/S | S | R/S |
| | | | | | | |

¹Control band is weak or missing

²Faint S-band?

³Differences between PCRs in intensity of bands

⁴Additional bands amplified

⁵Run through of SNP specific primer resulting in weak bands in samples that lack specific SNP scoring based on intensity difference

Ve2

| Test | Campeon | Marmande | Moneymaker | Persica | 10 (605) | 29 (1113) |
|---------------------|---------|----------|------------|---------|----------|-----------|
| CBGP-INIA | R | S | S | R/S | S | R/S |
| Naktuinbouw | R | S | S | R/S | S | R/S |
| GEVES | - | - | - | - | - | - |
| INRA ^{3,4} | R | S | S | R/S | S | R/S |
| PRI | R | S | S | R/S | S | R/S |
| | | | | | | |

³Additional bands amplified

⁴Run through of SNP specific primer resulting in weak bands in samples that lack specific SNP scoring based on intensity difference

Remarks: Being co-dominant assays amplification of outer fragment (control band) is not required.

Actions required: For NAKtuinbouw enhancement of amount of PCR products by adding a few cycles may be needed (to own judgement). For GEVES and INRA the assay needs optimisation to the lab conditions. This may need changing primer ratios and testing of specific PCR conditions (annealing temperature, MgCl concentration). Current SNP specific primers contain a deliberate mismatch at the third base from the 3' end to increase specificity, PRI will order and test primers where this mismatch is at the second base (directly next to SNP) to see if this gives stronger results with less side product.

Tm1

| Test | Campeon | Marmande | Moneymaker | Persica | 81 | 83 |
|------------------------------|---------|----------|------------|---------|----|----|
| CBGP-INIA ¹ SCA15 | 0 | 0 | 0 | 0 | R | 0 |
| SCN20 | 0 | 0 | 0 | 0 | R | 0 |
| Naktuinbouw SCN20 | 0 | 0 | 0 | 0 | R | 0 |
| GEVES ¹ SCA15 | 0 | 0 | 0 | 0 | R | 0 |
| SCN20 | 0 | 0 | 0 | 0 | R | 0 |
| INRA SCN20 | 0 | 0 | 0 | 0 | R | 0 |
| PRI SCA15 | 0 | 0 | 0 | 0 | R | 0 |
| SCN20 | 0 | 0 | 0 | 0 | R | 0 |

¹INIA and GEVES Lat primers no band amplified

Remarks: In those labs where both SCAR markers have been tested the SCA15 (1200bp) marker seems to perform best. Given that most cultivars will not have the Tm-1 gene this assay in general will render no amplification product therefore simultaneous amplification with the LAT primers would be preferred to check amplification conditions.

Actions required: Optimisation of PCR conditions at INIA and GEVES eg by adjusting relative primer concentrations in order to co-amplify LAT product.

Tm2/Tm2²

Tm2/Tm2² CAPS assay Bfr I (or isoschizomer)

| Test | Campeon | Marmande | Moneymaker | Persica | 51 | 54 | 225 |
|-------------|------------------------|----------|------------|------------------------|---------------------|------------------------|-----|
| CBGP-INIA | R or R ² /S | S | S | R or R ² /S | R or R ² | R or R ² /S | S |
| Naktuinbouw | nt | nt | nt | nt | nt | nt | nt |
| GEVES | R or R ² /S | S | S | R or R ² /S | R or R ² | R or R ² /S | S |
| INRA | nt | nt | nt | nt | nt | nt | nt |
| PRI | R or R ² /S | S | S | R or R ² /S | R or R ² | R or R ² /S | S |
| | | | | | | | |

Tm2/Tm2² CAPS assay Hpa I

| Test | Campeon | Marmande | Moneymaker | Persica | 51 | 54 | 225 |
|-------------|------------------------|----------|------------|------------------------|--------|------------------------|--------|
| CBGP-INIA | R ² /S or R | S or R | S or R | R ² /S or R | S or R | R ² /S or R | S or R |
| Naktuinbouw | nt | nt | nt | nt | nt | nt | nt |
| GEVES | R ² /S or R | S or R | S or R | R ² /S or R | S or R | R ² /S or R | S or R |
| INRA | nt | nt | nt | nt | nt | nt | nt |
| PRI | R ² /S or R | S or R | S or R | R ² /S or R | S or R | R ² /S or R | S or R |
| | | | | | | | |

Tm2/Tm2² CAPS assay conclusion

| Test | Campeon | Marmande | Moneymaker | Persica | 51 | 54 | 225 |
|-------------|-----------------------|----------|------------|-----------------------|---------|-----------------------|---------|
| CBGP-INIA | Tm2 ² /tm2 | tm2/tm2 | tm2/tm2 | Tm2 ² /tm2 | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| Naktuinbouw | nt | nt | nt | nt | nt | nt | nt |
| GEVES | Tm2 ² /tm2 | tm2/tm2 | tm2/tm2 | Tm2 ² /tm2 | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| INRA | nt | nt | nt | nt | nt | nt | nt |
| PRI | Tm2 ² /tm2 | tm2/tm2 | tm2/tm2 | Tm2 ² /tm2 | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| | | | | | | | |

Remarks: Tested by three groups with good results. Slight amount of undigested PCR amount if any is clearly weaker than other bands and poses no problems in interpretation. PCR assay is preferred compared to the enzyme digestion made in this assay
 Actions required: None.

Tm2/Tm2² Tetra primer ARMS assay 1 Tm2 or Tm2²

| Test | Campeon | Marmande | Moneymaker | Persica | 51 | 54 | 225 |
|--------------------------------|----------------|----------|------------|----------------|----|----------------|-----|
| CBGP-INIA ¹ | R ² | S | S | R ² | R | R ² | S |
| Naktuinbouw | R ² | S | S | R ² | R | R ² | S |
| GEVES | R ² | S | S | R ² | R | R ² | S |
| INRA (2 ^e PCR) 3 | R ² | S | S | R ² | R | R ² | S |
| PRI | R ² | S | S | R ² | R | R ² | S |
| | | | | | | | |

¹In two samples control band is lacking

³Additional band amplified together with specific band

Tm2/Tm2² Tetra primer ARMS assay 2 Tm2/Tm2² or S

| Test | Campeon | Marmande | Moneymaker | Persica | 51 | 54 | 225 |
|------------------------|-----------|----------|------------|---------|----|-----|-----|
| CBGP-INIA ¹ | - no ampl | S | S | R/- | R | R/S | S |
| Naktuinbouw | R/S | S | S | R/S | R | R/S | S |
| GEVES | R/S | S | S | R/S | R | R/S | S |
| INRA ^{3,4} | R/S | S | S | R/S | R | R/S | S |
| PRI | R/S | S | S | R/S | R | R/S | S |
| | | | | | | | |

¹Low amplification S allele

³Additional band amplified together with specific band

⁴Run through of S-SNP specific primer resulting in weak band in sample 51 (Moperou) that lacks susceptible allele, scoring based on intensity difference.

Tm2/Tm2² Tetra primer ARMS assay conclusion

| Test | Campeon | Marmande | Moneymaker | Persica | 51 | 54 | 225 |
|-------------|-----------------------|----------|------------|-----------------------|---------|-----------------------|---------|
| CBGP-INIA | Tm2 ² /? | tm2/tm2 | tm2/tm2 | Tm2 ² /? | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| Naktuinbouw | Tm2 ² /tm2 | tm2/tm2 | tm2/tm2 | Tm2 ² /tm2 | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| GEVES | Tm2 ² /tm2 | tm2/tm2 | tm2/tm2 | Tm2 ² /tm2 | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| INRA | Tm2 ² /tm2 | tm2/tm2 | tm2/tm2 | Tm2 ² /tm2 | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| PRI | Tm2 ² /tm2 | tm2/tm2 | tm2/tm2 | Tm2 ² /tm2 | Tm2/Tm2 | Tm2 ² /tm2 | tm2/tm2 |
| | | | | | | | |

Remarks: Some problems especially in assay 2.

Actions required: For INIA and NAKtuinbouw enhancement of amount of PCR products in assay 2 by adding a few cycles may be needed. At INRA optimisation of PCR conditions to avoid additional bands needed.

Mi1-2

| Test | Campeon | Marmande | Moneymaker | Persica | Anahu | Casaque Rouge |
|-------------|---------|----------|------------|---------|-------|---------------|
| CBGP-INIA | R/S | S | S | R/S | R | S |
| Naktuinbouw | R/S | S | S | R/S | R | S |
| GEVES | R/S | S | S | R/S | R | S |
| INRA | R/S | S | S | R/S | R | S |
| PRI | R/S | S | S | R/S | R | S |
| | | | | | | |

Remarks: No problems encountered.

Actions required: None, ready for further testing.

Fusarium I-locus

| Test | Campeon | Marmande ² | Moneymaker | Persica | Marmande verte | Marporum |
|-------------|----------------|-----------------------|------------|---------|-----------------|----------|
| CBGP-INIA | S ¹ | R | S | R | S | R |
| Naktuinbouw | R | R | S | R | S | R |
| GEVES | R | not tested | S | R | S | R |
| INRA | na | na | na | na | na | na |
| PRI | R | R | S | R | R? ³ | R |
| | | | | | | |

¹No band amplified in resistant cultivar

²Band amplified in Marmande is unexpected

³ Clear band visible but of lower intensity

na = no amplification testing in progress

Remarks: Marmande was incorporated as susceptible cultivar in the test but seems resistant, Campeon did not amplify a product at INIA possible due to DNA quality of specific sample, and amplified band in Marmande verte at PRI is strange.

Actions required: Testing of another Marmande DNA sample or testing with Fol:0, to be discussed. Repeated testing at INIA of the Campeon sample next to another Campeon sample send by PRI, and repeated testing at PRI of Marmande verte and another Marmande verte sample send by another partner.

Fusarium I2-locus

| Test | Campeon | Marmande | Moneymaker | Persica | Ideucenzi | Motelle |
|--------------------------|---------|----------|------------|---------|-----------|---------|
| CBGP-INIA ¹ | S | S | S | R | R | R |
| Naktuinbouw ² | R | S | S | R | R | R |
| GEVES | R | S | S | R | R | R |
| INRA | R | S | S | R | R | R |
| PRI | R | S | S | R | R | R |
| | | | | | | |

¹ I2 specific Z1063 primers product very faint compared to rubisco band.

² Combination with Rubisco primers failed to produce I2 specific Z1063 primer product

Remarks: Combination of I2 specific Z1063 primers with rubisco primers gives problems for the I2 specific Z1063 primers at INIA and NAKtuinbouw. Combination with positive control for amplification is preferable, further reduction of rubisco primer concentration may resolve the problem, possibly similar problem of DNA of Campeon at INIA.

Actions required: Testing with a further reduced amount of rubisco primers (INIA and NAKtuinbouw), optimisation of relative primer amounts.

Appendix 11: Validation results INIA



The results were resumed in tables at the end of this document.

Comments:

- **Verticillium:** the results of the biological assay (BA) were confirmed by molecular markers (MM) in susceptible and resistant varieties, except in Agraz5.

Varieties with non-conclusive results in BA:

- Yedi variety: homogeneous MM resistant result. In ten different BA, with a total number of 115 plants, only 13% showed weak infection symptoms and none of them died.
- Santanyi variety: heterogeneous MM result, corresponding with resistant plants (*Ve1Ve1 Ve2Ve2*, 2:3). The BA assay result was very similar to Yedi, 17% plants with symptoms of 161 plants assayed.
- Conty variety: heterogeneous MM result, corresponding with resistant plants (*ve1ve1 Ve2ve2*:*Ve1ve1 Ve2ve2*, 4:1). The BA assay result showed 80% of plants (5 different assays, 66 plants) with symptoms; a 4:1 symptoms:no-symptoms segregation.
- Clarion: heterogeneous MM result, corresponding with susceptible and resistant mixed plants (*ve1ve1 ve2ve2*:*Ve1ve1 ve2ve2*, 4:1). The BA assay result showed susceptible plants with fugues.

- **ToMV:** in all the assayed plants the resistance was conferred by *Tm2*. The results of BA were confirmed by MM in all susceptible and resistant varieties.

Varieties with non-conclusive results in BA:

- Vanity variety: heterogeneous MM result, corresponding with susceptible and resistant mixed plants. The same segregation was observed in the BA, with 46% of resistant plants (7 different assays, 84 plants).
- Monalbo x Momor: homogeneous MM resistant result. Without susceptibility symptoms, marked with "X" because presented necrosis.

- **Meloidogyne:** results of BA were confirmed by MM in all susceptible and resistant varieties.

Varieties with non-conclusive results in BA: all of them present R/S homogeneous MM result. Mariana, Aroa and Denis showed an heterogeneous result, with more susceptible plants than resistant plants. Madita is considered as intermediate resistance control.

- **Fusarium race 0:** the results of BA were confirmed by MM in the susceptible and resistant varieties, except in Cascada.

Varieties with non-conclusive results in BA: in all these varieties the BA result was heterogeneous, with susceptible and resistant plants.

- Susceptible MM result: Kalanda, First Love
- Resistant MM result: Yanelli, Ranco, Marporum x Marmante verte, Larisa, Marporum

- **Fusarium race 1:** results of BA were confirmed by MM in all susceptible and resistant varieties.

Varieties with non-conclusive results in BA: all of them present susceptible homogeneous MM result.

| VARIETY | Verticillium | | Result | | ToMV | | Result | | Meloidog. | Result | | Fo 0 | Result | | Fo 1 | Result | |
|---------------------------|--------------|--------|---------|----|--------|-----------------------------------|--------|----|------------|--------|----|--------|--------|----|----------|--------|----|
| | Ve1 | Ve2 | MM | BA | Tm1 | Tm2 | MM | BA | Mi | MM | BA | Ful | MM | BA | Ful2 | MM | BA |
| | 4:1 | | | | | | | | | | | | | | | | |
| KALANDA | | | | | | | | | | | | fulful | S | X | | | |
| AROA SEM | Ve1ve1 | Ve2ve2 | R/S R/S | R | tm1tm1 | Tm2 ² Tm2 ² | S R | R | Mi1-2mi1-2 | R/S | X | Ful | R | R | Ful2 | R | R |
| DENIS SEM | | | | | | | | | Mi1-2mi1-2 | R/S | X | | | | | | |
| GORRION | ve1ve1 | Ve2ve2 | S R/S | R | tm1tm1 | tm2 ² tm2 ² | S S | S | | | | Ful | R | R | Ful2 | R | R |
| RIEL | | | | | tm1tm1 | tm2 ² tm2 ² | S S | S | | | | Ful | R | R | ful2ful2 | S | S |
| FIMANDE | ve1ve1 | ve2ve2 | S S | S | tm1tm1 | tm2 ² tm2 ² | S S | S | mi1-2mi1-2 | S | S | fulful | S | S | ful2ful2 | S | S |
| FIRST LOVE | | | | | | | | | | | | fulful | S | X | | | |
| JOSEFINA | ve1ve1 | ve2ve2 | S S | S | | | | | mi1-2mi1-2 | S | S | | | | | | |
| GUADALHORC E | Ve1Ve1 | Ve2Ve2 | R R | R | | | | | Mi1-2mi1-2 | R/S | R | | | | | | |
| MAGNOLIA | | | | | | | | | mi1-2mi1-2 | S | S | fulful | S | S | ful2ful2 | S | S |
| BOND | | | | | | | | | Mi1-2mi1-2 | R/S | R | | | | | | |
| CASCADE | | | | | | | | | mi1-2mi1-2 | S | S | Ful | R | S | ful2ful2 | S | S |
| RANCO | | | | | | | | | | | | Ful | R | X | ful2ful2 | S | X |
| MARPORUM x MARM. VERTE | | | | | | | | | | | | Ful | R | X | | | |
| LARISA | | | | | | | | | | | | Ful | R | X | | | |
| MARPORUM | | | | | | | | | | | | Ful | R | X | ful2ful2 | S | X |
| ROMA | | | | | | | | | | | | | | | ful2ful2 | S | X |
| CHERRY BELLE | | | | | | | | | | | | | | | ful2ful2 | S | X |
| CLARION | ve1ve1 | ve2ve2 | S S | X | | | | | | | | | | | | | |
| | Ve1ve1 | ve2ve2 | R/S S | X | | | | | | | | | | | | | |
| | 4:1 | | | | | | | | | | | | | | | | |
| MONALBO MOMOR x | | | | | tm1tm1 | Tm2 ² Tm2 ² | S R | X | | | | | | | | | |
| MADITA | | | | | | | | | Mi1-2mi1-2 | R/S | X | | | | | | |

MM: molecular marker assay result, BA: Biological assay result; S: susceptible, R: resistant, X: non-conclusive result; light grey: non assayed.

Appendix 12: Validation results GEVES

Authors :

René Mathis, Laetitia Cavellini, Céline Andro, Cécile Collonnier

GEVES (France)

Molecular assay developed for the different disease resistance genes

Verticillium genes Ve1 and Ve2: tetra primers ARMS tests were developed which allow a control on amplification and co-dominant scoring of phenotype. (chap II)

Tomato Mosaic Virus Tm1 (linked marker): only dominant SCAR markers from literature work but often a weak amplification can be observed in susceptible varieties. More susceptible varieties will be analysed to assess whether this may result in problems of false positives. Meanwhile this has been done in a set of 13 susceptible cultivars none of which gave a false positive result. Because of dominant nature a positive control for amplification has to be included (Lat gene primers). (chap III)

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. (chap IV)

Meloidogyne incognita Mi1-2: Co-dominant SCAR marker available from Mehrach et al (2005). (chap V)

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

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

Verticillium genes Ve1 and Ve2

Conditions

Plants were grown in a growth chamber (16h/8h, light/dark, 20°C/30°C respectively).

DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 2 plants

Quality DNA control:

-OD measurement

-Amplification of a tomato specific gene: LAT52

DNA samples have been tested with two different tetra primer ARMS PCR tests: one for the Ve1 gene and one for the Ve2 gene. Ve1 SNP primers were modified for optimisation during “robustness test” step.

| Primer name | Primer sequence | Expected fragments |
|----------------------|---------------------------------|--|
| Assay 1 / Ve1 | | Control fragment 580 bp |
| Ve1_2072F | CCTTGATGGGGTTGATCTTTCGT | R-fragment 476 bp S-fragment 158 bp |
| Ve1_2651R | GTAGGTGAGTTTCTTGGACAGTCGA | |
| Ve1_SNP706Ft | CAGGCCCTTTGGATGAATCACATT | |
| Ve1_SNP706Ra | GTTGGACAAAAGAGAGAAAGTGAAGCTAAGT | |
| Ve1_SNP706misF1t | CAGGCCCTTTGGATGAATCACIAT | |
| Ve1_SNP706misR1a | GTTGGACAAAAGAGAGAAAGTGAAGCTIACT | |
| Assay 2 / Ve2 | | Control fragment 321 bp |
| Ve2_2720F | GGATCTTAGCTCACTTTATGTTTGAAC | R-fragment 242 bp S-fragment 131 bp |
| Ve2_3040R | GGTGCTGGTTTCAACTCTGAAGT | |
| Ve2_SNP2827F | CAAATGCTTGAATCACTAGACCTGTGAAC | |
| Ve2_SNP2827R | GGATCTCCCCGGACAGGTGGATTC | |

PCR conditions for Ve1 assay

| | Units | Initial concentration | Final concentration | 1 PCR |
|-------------------------|-------|-----------------------|---------------------|-------|
| H ₂ O | | | | 3.36 |
| 10X PCR Buffer | mM | 10 | 1 | 2 |
| MgCl ₂ | mM | 25 | 1.5 | 1.2 |
| dNTP | μM | 5 | 0.1 | 0.4 |
| SNP Primers | μM | 2 | 0.2 | 2 |
| Outer primers | μM | 2 | 0.05 | 0.5 |
| Goldstar Polymerase Taq | U/μl | 5 | 1 | 0.04 |
| DNA | | 2 ng/μl | 16ng | 8 |
| Total volume | μl | | | 20 |

PCR program for Ve1 assay

| MJ PTC-200 thermal cycler | | |
|---------------------------|--------|-----------|
| 94°C | 3 min | 35 cycles |
| 94°C | 30 sec | |
| 55°C | 1 min | |
| 72°C | 2 min | |
| 72°C | 10 min | |
| 10°C | - | |

PCR program for Ve2 assay (modified for optimisation during “robustness test” step)

| MJ PTC-200 thermal cycler | | |
|---------------------------|--------|-----------|
| 94°C | 3 min | |
| 94°C | 30 sec | |
| 60°C (-1°C/cycle) | 1 min | 10 cycles |
| 72°C | 2 min | |
| 94°C | 30 sec | 30 cycles |
| 50°C | 1 min | |
| 72°C | 2 min | |
| 72°C | 10 min | |
| 10°C | - | |

Amplification products (10µl) were resolved in a 2% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 100bp (Promega)

Evaluation of the assays

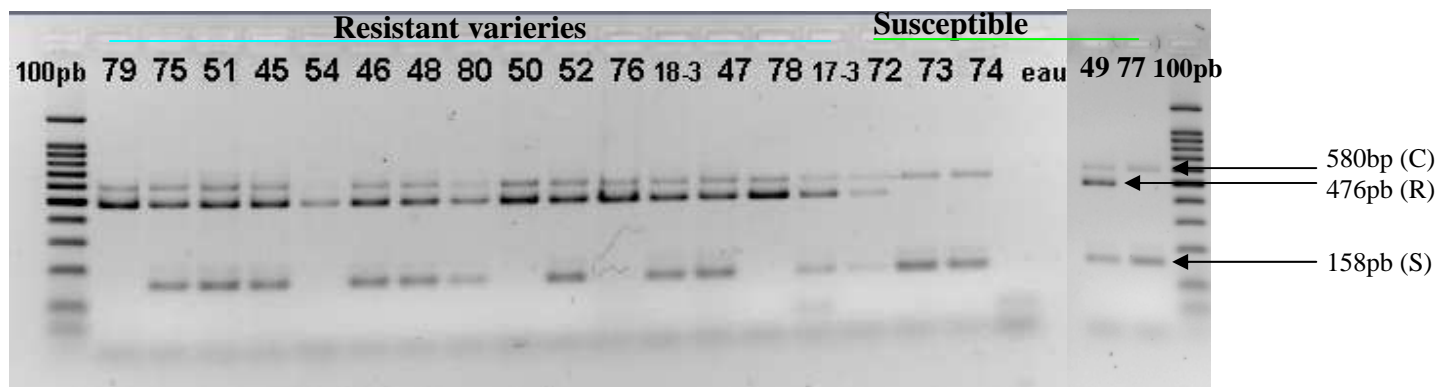
Plant material and results

| Code | Cultivar | Code | Cultivar |
|------|-----------------|-------|------------|
| Q68 | Momor | Q52 | Gaheris |
| Q69 | Momor x Monalbo | Q18-3 | Nemo-Netta |
| Q70 | Moperou 161 | Q47 | Petula |
| Q67 | Mobaci | Q17-3 | Thomas |
| Q51 | Abellus | Q49 | Tracie |
| Q45 | Abigail | Q71 | Corazon |
| Q46 | Cencara | Q66 | Grandimat |
| Q48 | Cibellia | Q64 | Model |
| Q80 | Clermon | Q65 | Osnat |
| Q50 | Facility | Q79 | Monalbo |

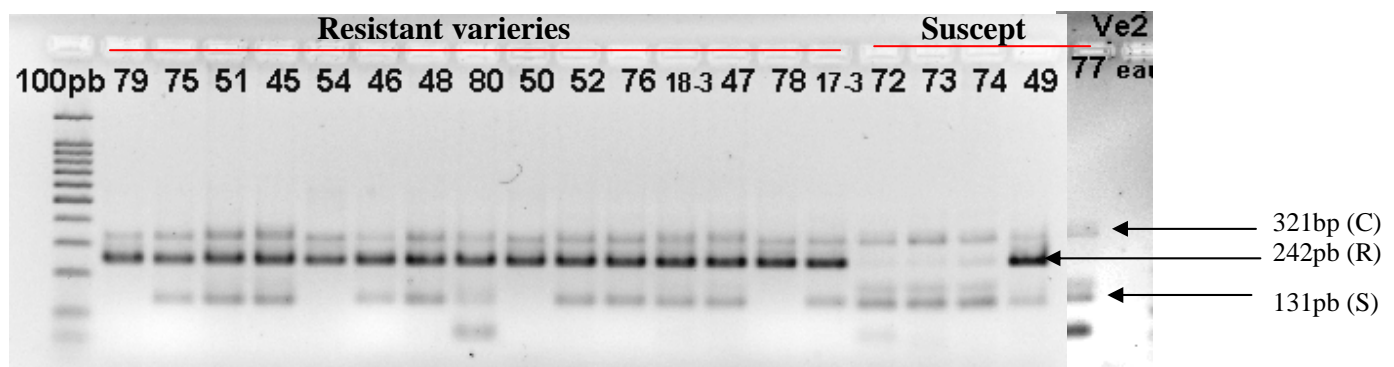
Verticillium dahliae

| Code | Cultivar | Type resistance |
|-------|--------------------------|-----------------|
| Q79 | Monalbo | R |
| Q75 | Monalbo x Marmande verte | R |
| Q51 | Abellus | R |
| Q45 | Abigail | R |
| Q54 | Allflesh 1200 | R |
| Q46 | Cencara | R |
| Q48 | Cibellia | R |
| Q80 | Clermon | R |
| Q50 | Facility | R |
| Q52 | Gaheris | R |
| Q76 | Hipop | R |
| Q18-3 | Nemo-Netta | R |
| Q47 | Petula | R |
| Q78 | Relaxx | R |
| Q17-3 | Thomas | R |
| Q72 | Harmony | S |
| Q73 | Ogusto | S |
| Q74 | Shiren | S |
| Q49 | Tracie | S |
| Q77 | Marmande Verte | S |
| T- | water | |

Assay 1 (Ve1)



Assay 2 (Ve2)



Conclusion

Expected results except for two varieties: Q49 (Ve1 and Ve2 R band) and Q72 (Ve1 R band).

Tomato Mosaic Virus gene Tm1

Conditions

Plants were grown in a growth chamber (16h/8h, light/dark, 20°C/30°C respectively).

DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 2 plants

Quality DNA control:

-OD measurement

-Amplification of a tomato specific gene: LAT52

Either SCA15 or SCN20 can be amplified simultaneously with primers Lat1F/Lat2R

| Primer name | Primer sequence | Expected fragments |
|--------------|--------------------------|--------------------|
| Tm-1 SCA15 F | CCGAACCCCTTAAAAATAGTTTCA | 1000bp |
| Tm-1 SCA15 R | CCGAACCCAATCAGGAGGCTCATA | |
| Tm-1 SCN20 F | GGTGCTCCGTCGATGCAAAGTGCA | 1400bp |
| Tm-1 SCN20 R | GGTGCTCCGTAGACATAAAATCTA | |
| LAT1-F | AGACCACGAGAACGATATTTGC | 92bp |
| LAT2-R | TTCTTGCCTTTTCATATCCAGACA | |

PCR conditions (modified for optimisation during “robustness test” step : increase in LAT primer concentration)

| | Units | Initial concentration | Final concentration | 1 PCR |
|-------------------------|-------|-----------------------|---------------------|-------|
| H ₂ O | | | | 2.86 |
| 10X PCR Buffer | mM | 10 | 1 | 2 |
| MgCl ₂ | mM | 25 | 1.5 | 1.2 |
| dNTP | μM | 5 | 0.1 | 0.4 |
| SC Primers | μM | 2 | 0.2 | 2 |
| LAT primers | μM | 2 | 0.1 | 1 |
| Goldstar Polymerase Taq | U/μl | 5 | 1 | 0.04 |
| DNA | | 2 ng/μl | 16ng | 8 |
| Total volume | μl | | | 20 |

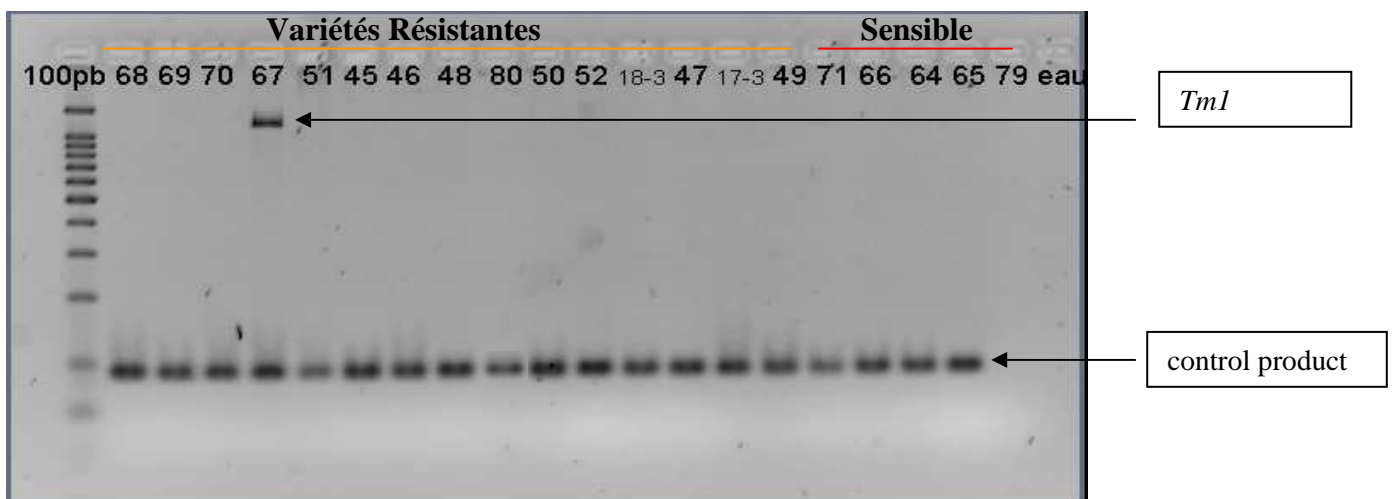
| MJ PTC-200 thermal cycler | | |
|---------------------------|--------------|-----------|
| 94°C | 2 min 30 sec | |
| 94°C | 1 min | 35 cycles |
| 60°C | 1 min | |
| 72°C | 2 min | |
| 72°C | 10 min | |
| 10°C | - | |

Amplification products (10 µl) were resolved in a 2% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 1kb, 100bp and 50bp (Promega)

Evaluation of the assays

Plant material and results

| Code | Cultivar | Code | Cultivar |
|------|-----------------|-------|------------|
| Q68 | Momor | Q52 | Gaheris |
| Q69 | Momor x Monalbo | Q18-3 | Nemo-Netta |
| Q70 | Moperou 161 | Q47 | Petula |
| Q67 | Mobaci | Q17-3 | Thomas |
| Q51 | Abellus | Q49 | Tracie |
| Q45 | Abigail | Q71 | Corazon |
| Q46 | Cencara | Q66 | Grandimat |
| Q48 | Cibellia | Q64 | Model |
| Q80 | Clermon | Q65 | Osnat |
| Q50 | Facility | Q79 | Monalbo |



Conclusion

Expected results, since resistance is due to *Tm2* gene in the majority of tested varieties.

Tomato Mosaic Virus Tm2 and Tm2²

Conditions

Plants were grown in a growth chamber (16h/8h, light/dark, 20°C/30°C respectively).

DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 2 plants

Quality DNA control:

-OD measurement

-Amplification of a tomato specific gene: LAT52

CAPS Markers

| Primer name | Primer sequence | Expected fragments |
|-----------------|-----------------------------------|--------------------|
| Tm-2 PrRuG151 F | GAGTTCTTCCGTTCAAATCCTAAGCTTGAGAAG | 1085bp |
| Tm-2 PrRuG086 R | CTACTACACTCACGTTGCTGTGATGCAC | |

PCR conditions

| | Units | Initial concentration | Final concentration | 1 PCR |
|-------------------------|-------|-----------------------|---------------------|-------|
| H ₂ O | | | | 26.9 |
| 10X PCR Buffer | mM | 10 | 1 | 5 |
| MgCl ₂ | mM | 25 | 1.5 | 3 |
| dNTP | μM | 5 | 0.1 | 1 |
| CAPS Primers | μM | 10 | 0.2 | 5 |
| Goldstar Polymerase Taq | U/μl | 5 | 1 | 0.1 |
| DNA | | 10 ng/μl | 40ng | 4 |
| Total volume | μl | | | 50 |

| MJ PTC-200 thermal cycler | | |
|---------------------------|--------|-----------|
| 94°C | 3 min | 35 cycles |
| 94°C | 1 min | |
| 55°C | 1 min | |
| 72°C | 2 min | |
| 72°C | 10 min | |
| 10°C | - | |

Amplification products (10 μl) were resolved in a 1% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 1kb (Promega)

Digestion of 7μl PCR-product with 1μl restriction buffer, 1μl BSA buffer and 1μl restriction enzyme (either HpaI or BrlI) incubation 2 hours at 37°C. Restriction fragments were resolved in a 2% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 1kb (Promega)

Fragment sizes with HpaI (GTT↓AAC): Total size of amplified fragment is 1085bp. Susceptible allele (tm-2) no restriction site present, length of fragment 1085bp. Resistant alleles (Tm2 and Tm2²) one restriction site, two fragments of 660bp and 425bp respectively.

Fragment sizes with BfrI or MspC I (C↓TTAAG): Total size of amplified fragment is 1085bp. In the fragment there is one restriction site that is present in all three alleles (tm2, Tm2 and Tm2²) resulting in a fragment of 328bp present in all alleles. No other restriction sites are present in alleles tm2 and Tm2 resulting in a second fragment of 757bp. Only allele Tm2² has an additional second restriction site which is situated in the 757bp fragment resulting into a fragment of 559bp and a fragment of 198bp.

Tetra ARMS PCR

| Primer name | Primer sequence | Expected fragments |
|------------------|--------------------------------|--|
| Assay 1 | | Control fragment 416 bp Tm2-fragment 254 bp Tm2 ² -fragment 214 bp |
| TMV-2286F | GGGTATACTGGGAGTGTCCAATTC | |
| TMV-2658R | CCGTGCACGTTACTTCAGACAA | |
| SNP2493R TM2 | CTGCCAGTATATAACGGTCTACCG | |
| SNP2494F TM2(2) | CTCATCAAGCTTACTCTAGCCTACTTTAGT | |
| Assay 2 | | Control fragment 509 bp R-allele fragment (Tm2 or Tm2 ²) 185 bp S-allele fragment (tm2) 381 bp |
| TMV-747F | CGGTCTGGGGAAAACAACCTCT | |
| TMV-1256R | CTAGCGGTATACCTCCACATCTCC | |
| TMV-R SNP901misR | GCAGGTTGTCCTCCAAATTTTCCATC | |
| TMV-S SNP901misF | CAAATTGGACTGACGGAACAGAAAGTT | |

PCR conditions

| | Units | Initial concentration | Final concentration | 1 PCR |
|-------------------------|-------|-----------------------|---------------------|-------|
| H ₂ O | | | | 9.36 |
| 10X PCR Buffer | mM | 10 | 1 | 2 |
| MgCl ₂ | mM | 25 | 1.5 | 1.2 |
| dNTP | μM | 5 | 0.1 | 0.4 |
| SNP Primers | μM | 2 | 0.2 | 2 |
| Outer primers | μM | 2 | 0.05 | 0.5 |
| Goldstar Polymerase Taq | U/μl | 5 | 1 | 0.04 |
| DNA | | 10 ng/μl | 20ng | 2 |
| Total volume | μl | | | 20 |

| MJ PTC-200 thermal cycler | | |
|---------------------------|--------|-----------|
| 94°C | 3 min | 35 cycles |
| 94°C | 1 min | |
| 55°C | 1 min | |
| 72°C | 2 min | |
| 72°C | 10 min | |
| 10°C | - | |

Amplification products (10 μl) were resolved in a 2% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 1kb (Promega)

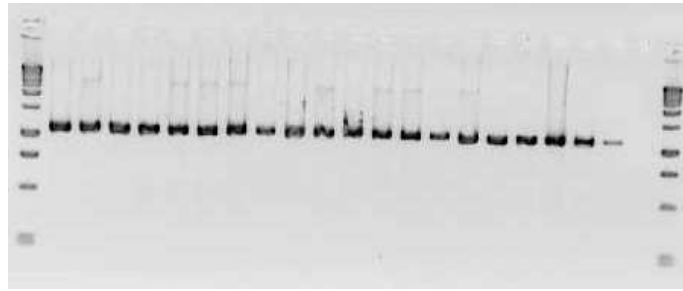
Evaluation of the assays

CAPS Markers

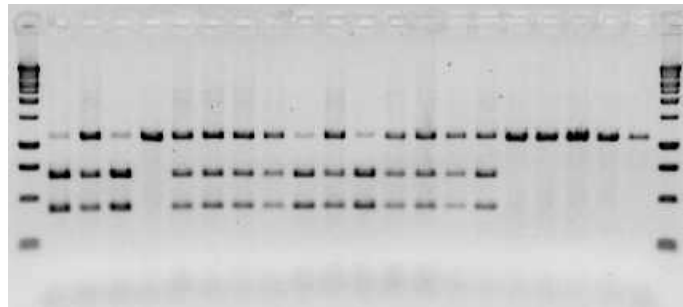
Plant material and results

| Lane | Cultivar | R/S | Results (bp) HpaI Digestion | Results (bp) BfrI Digestion |
|------|-----------------|-----|--------------------------------|--------------------------------|
| 2 | Momor | R | 425 and 660 | 198, 328 and 559 |
| 3 | Momor x Monalbo | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 4 | Moperou 161 | R | 425 and 660 | 328 and 757 |
| 5 | Mobaci | R | 1085 | 328 and 757 |
| 6 | Abellus | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 7 | Abigail | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 8 | Cencara | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 9 | Cibellia | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 10 | Clermon | R | 425 and 660 | 198, 328 and 559 |
| 11 | Facility | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 12 | Gaheris | R | 425 and 660 | 198, 328 and 559 |
| 13 | Nemo-Netta | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 14 | Petula | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 15 | Thomas | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 16 | Tracie | R | 425, 660 and 1085 | 198, 328, 559 and 757 |
| 17 | Corazon | S | 1085 | 328 and 757 |
| 18 | Grandimat | S | 1085 | 328 and 757 |
| 19 | Model | S | 1085 | 328 and 757 |
| 20 | Osnat | S | 1085 | 328 and 757 |
| 21 | Monalbo | S | 1085 | 328 and 757 |

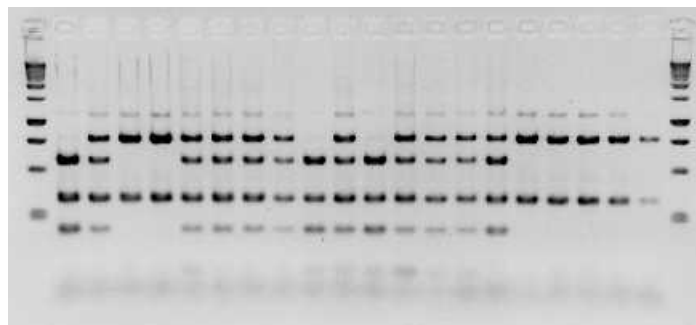
Before
Digestion



After
Digestion
HpaI



After
Digestion
BfrI



Conclusion for CAPS

Only expected results

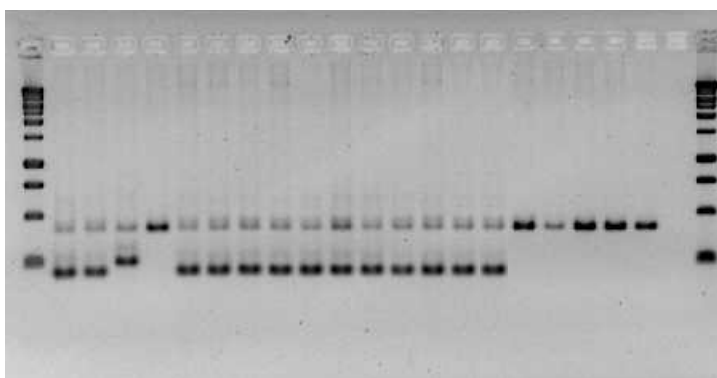
(HpaI digestion distinguishes well R and S cultivars. Partially undigested DNA, if any, is weaker than non digested. BfrI digestion distinguishes well R Tm2 and R Tm2(2))

Tetra ARMS PCR

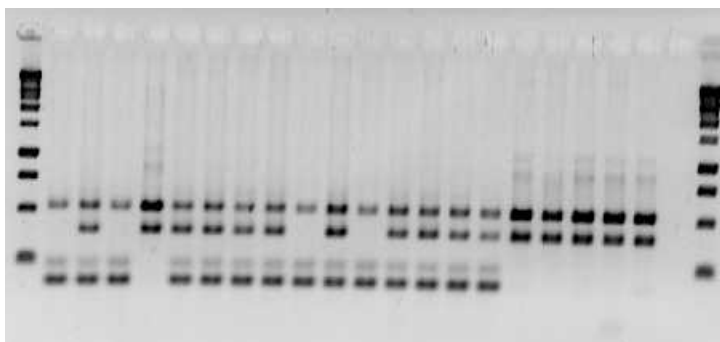
Plant material and results

| Cultivar | R/S | Results (bp) Assay 1 | Results (bp) Assay 2 |
|-----------------|-----|-------------------------|-------------------------|
| Momor | R | 214 and 416 | 185 and 509 |
| Momor x Monalbo | R | 214 and 416 | 185, 381 and 509 |
| Moperou 161 | R | 254 and 416 | 185 and 509 |
| Mobaci | R | 416 | 381 and 509 |
| Abellus | R | 214 and 416 | 185, 381 and 509 |
| Abigail | R | 214 and 416 | 185, 381 and 509 |
| Cencara | R | 214 and 416 | 185, 381 and 509 |
| Cibellia | R | 214 and 416 | 185, 381 and 509 |
| Clermon | R | 214 and 416 | 185 and 509 |
| Facility | R | 214 and 416 | 185, 381 and 509 |
| Gaheris | R | 214 and 416 | 185 and 509 |
| Nemo-Netta | R | 214 and 416 | 185, 381 and 509 |
| Petula | R | 214 and 416 | 185, 381 and 509 |
| Thomas | R | 214 and 416 | 185, 381 and 509 |
| Tracie | R | 214 and 416 | 185, 381 and 509 |
| Corazon | S | 416 | 381 and 509 |
| Grandimat | S | 416 | 381 and 509 |
| Model | S | 416 | 381 and 509 |
| Osnat | S | 416 | 381 and 509 |
| Monalbo | S | 416 | 381 and 509 |

| |
|---------------------------|
| Assay 1 |
| TMV-2286F |
| TMV-2658R |
| SNP2493R TM2 |
| SNP2494F TM2 ² |



| |
|------------------|
| Assay 2 |
| TMV-747F |
| TMV-1256R |
| TMV-R SNP901misR |
| TMV-S SNP901misF |



Conclusion for SCARs

Only expected results

(Assay 1 distinguishes R Tm2, R Tm2(2) and susceptible. Assay 2 distinguishes R heterozygotes, R homozygotes and S)

Conclusion of Robustness tests

| R/S | Cultivar | HpaI Digestion | BfrI Digestion | Assay 1 | Assay 2 | Genotype |
|-----|-----------------|--------------------------------------|-------------------------------|------------------|--------------------------------------|-------------------------------|
| R | Momor | Tm2 or Tm2 ² Homozygous | Tm2 ² Homozygous | Tm2 ² | Tm2 or Tm2 ² Homozygous | Tm2 ² Homozygous |
| R | Momor x Monalbo | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Moperou 161 | Tm2 or Tm2 ² Homozygous | Tm2 or tm2 | Tm2 | Tm2 or Tm2 ² Homozygous | Tm2 Homozygous |
| R | Mobaci | tm2 | Tm2 or tm2 | tm2 | tm2 | tm2 |
| R | Abellus | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Abigail | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Cencara | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Cibellia | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Clermon | Tm2 or Tm2 ² Homozygous | Tm2 ² Homozygous | Tm2 ² | Tm2 or Tm2 ² Homozygous | Tm2 ² Homozygous |
| R | Facility | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Gaheris | Tm2 or Tm2 ² Homozygous | Tm2 ² Homozygous | Tm2 ² | Tm2 or Tm2 ² Homozygous | Tm2 ² Homozygous |
| R | Nemo-Netta | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Petula | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Thomas | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| R | Tracie | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous | Tm2 ² | Tm2 or Tm2 ² Heterozygous | Tm2 ² Heterozygous |
| S | Corazon | tm2 | Tm2 or tm2 | tm2 | tm2 | tm2 |
| S | Grandimat | tm2 | Tm2 or tm2 | tm2 | tm2 | tm2 |
| S | Model | tm2 | Tm2 or tm2 | tm2 | tm2 | tm2 |
| S | Osnat | tm2 | Tm2 or tm2 | tm2 | tm2 | tm2 |
| S | Monalbo | tm2 | Tm2 or tm2 | tm2 | tm2 | tm2 |

Meloidogyne incognita Mi1-2

Conditions

Plants were grown in a growth chamber (16h/8h, light/dark, 20°C/30°C respectively).

DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 2 plants

Quality DNA control:

-OD measurement

-Amplification of a tomato specific gene: LAT52

| Primer name | Primer sequence | Expected fragments |
|-------------|--|--------------------|
| PMiF3 | GGT ATG AGC ATG CTT AAT CAG AGC TCT C | 350 and 550 bp |
| PMiR3 | CCT ACA AGA AAT TAT TGT GCG TGT GAA TG | |

PCR conditions

| | Units | Initial concentration | Final concentration | 1 PCR (μl) |
|-------------------|-------|-----------------------|---------------------|------------|
| H ₂ O | | | | 8.6 |
| 10X PCR Buffer | mM | 10 | 1 | 2 |
| MgCl ₂ | mM | 25 | 2 | 1.6 |
| dNTP | μM | 2 | 0.2 | 2 |
| PMi Primers | μM | 20 | 0.5 | 0.5 |
| Sigma Red Taq | U/μl | 1 | 0.04 | 0.8 |
| DNA | | 10 ng/μl | 40ng | 4 |
| Total volume | μl | | | 20 |

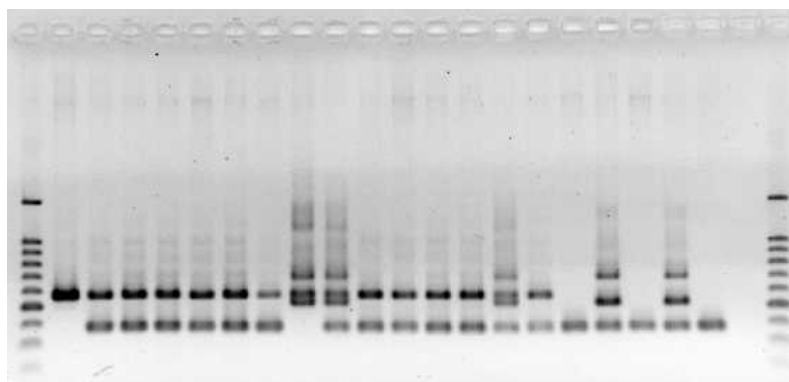
| MJ PTC-200 thermal cycler | | |
|------------------------------|--------|-----------|
| 94°C | 10 min | 35 cycles |
| 94°C | 30 sec | |
| 50°C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |
| 10°C | - | |

Amplification products (10 μl) were resolved in a 1.5% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 100bp (Promega).

Evaluation of the assay

Plant material and results

| Lane | Cultivar | R/S | Results (bp) |
|------|-----------------------|-------|-----------------------|
| 2 | Anahu | R(1) | 550 |
| 3 | Anahu x Casaque Rouge | R(2a) | 350 and 550 |
| 4 | Thomas | R(2b) | 350 and 550 |
| 5 | Campeon | R(3) | 350 and 550 |
| 6 | Abellus | R(1) | 350 and 550 |
| 7 | Allflesh 1120 | R(1) | 350 and 550 |
| 8 | Allflesh 900 | R(3) | 350 and 550 |
| 9 | Anastasia | R(1) | 500, 550 and 700 |
| 10 | Brentylia | R(2) | 350, 500, 550 and 700 |
| 11 | Cencara | R(1) | 350 and 550 |
| 12 | Cibellia | R(1) | 350 and 550 |
| 13 | Gaheris | R(1) | 350 and 550 |
| 14 | Nemo-Netta | R(2b) | 350 and 550 |
| 15 | Pristylia | R(3) | 350, 500, 550 and 700 |
| 16 | Tangra | R(1) | 350 and 550 |
| 17 | Abigail | S | 350 |
| 18 | Facility | S | 350, 500 and 700 |
| 19 | Petula | S | 350 |
| 20 | Tracie | S | 350, 500 and 700 |
| 21 | Casaque Rouge | S | 350 |



Conclusion

A duplex of additional bands (700pb + 500pb) appears in 5 cases giving rise to unexpected profiles. Informative bands are always present (350bp for susceptibility, 550bp for resistance and 350, 550bp for heterozygous) so that conclusion about resistance or susceptibility can still be made in any case.

Fusarium I locus

Conditions

Plants were grown in a growth chamber (16h/8h, light/dark, 20°C/30°C respectively).

DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 2 plants

Quality DNA control:

-OD measurement

-Amplification of a tomato specific gene: LAT52

| Primer name | Primer sequence | Expected fragments |
|-------------|---------------------------|--------------------|
| At2-F3 | CGAATCTGTATATTACATCCGTCGT | 130 bp |
| At2-R3 | GGTGAATACCGATCATAGTCGAG | |

PCR conditions

| | Units | Initial concentration | Final concentration | 1 PCR |
|-------------------|-------|-----------------------|---------------------|-------|
| H ₂ O | | | | 9 |
| 10X PCR Buffer | mM | 10 | 1 | 2 |
| MgCl ₂ | mM | 25 | 1.5 | 1.2 |
| dNTP | μM | 2 | 0.2 | 2 |
| At2 Primers | μM | 20 | 0.5 | 0.5 |
| Sigma Red Taq | U/μl | 1 | 0.04 | 0.8 |
| DNA | | 10 ng/μl | 40ng | 4 |
| Total volume | μl | | | 20 |

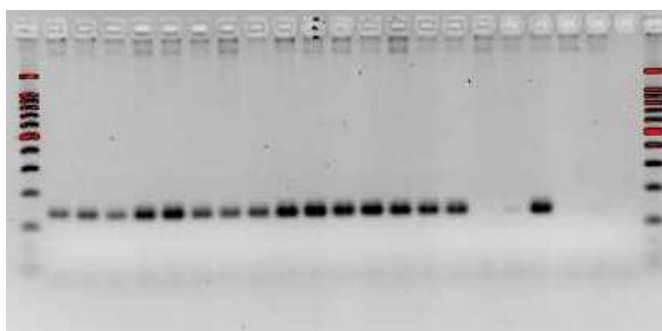
| MJ PTC-200 thermal cycler | | |
|------------------------------|-----------|--------------|
| 94°C | 5 min | |
| 94°C | 30 sec | 35 cycles |
| 66°C | 30 sec | |
| 72°C | 30 sec | |
| 72°C | 5 min | |
| 10°C | - | |

Amplification products (10 μl) were resolved in a 3% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 100bp (Promega).

Evaluation of the assay

Plant material and results

| Lane | Cultivar | R/S | Results (bp) |
|------|---------------------------|-----|--------------|
| 2 | Marporum | R | 130 |
| 3 | Marporum x Marmande Verte | R | 130 |
| 4 | Motelle | R | 130 |
| 5 | Abellus | R | 130 |
| 6 | Abigail | R | 130 |
| 7 | Cencara | R | 130 |
| 8 | Cibellia | R | 130 |
| 9 | Clermon | R | 130 |
| 10 | Facility | R | 130 |
| 11 | Gaheris | R | 130 |
| 12 | Hipop | R | 130 |
| 13 | Nemo-Netta | R | 130 |
| 14 | Petula | R | 130 |
| 15 | Thomas | R | 130 |
| 16 | Tracie | R | 130 |
| 17 | Corazon | S | - |
| 18 | Costabel | S | - |
| 19 | Piccolo | S | 130 |
| 20 | Vemone | S | - |
| 21 | Marmande Verte | S | - |



Conclusion

Expected results, distinguishes R and S. Piccolo is not susceptible but resistant from our result.

Resistant cultivars show different intensities of bands, not due to the quantity of DNA in PCR

Fusarium I2 locus

Conditions

Plants were grown in a growth chamber (16h/8h, light/dark, 20°C/30°C respectively).

DNA extraction procedure: DNeasy Plant Mini Kit (Qiagen) from 2 plants

Quality DNA control:

-OD measurement

-Amplification of a tomato specific gene: LAT52

| Primer name | Primer sequence | Expected fragments |
|-------------|----------------------|--------------------|
| Z1063F | ATTTGAAAGCGTGGTATTGC | 940 bp |
| Z1063R | CTTAAACTCACCATTAAATC | |
| RubiscoF | ATGTCACCACAAACAGAGAC | 1380 bp |
| RubiscoR | CTCACAAGCAGCAGCTAGT | |

PCR conditions

| | Units | Initial concentration | Final concentration | 1 PCR |
|-------------------|-------|-----------------------|---------------------|-------|
| H ₂ O | | | | 12.7 |
| 10X PCR Buffer | mM | 5 | 1 | 5 |
| MgCl ₂ | mM | 25 | 1.5 | 1.5 |
| dNTP | μM | 4 | 0.16 | 1 |
| Z1063 Primers | μM | 10 | 0.4 | 1 |
| Rubisco Primers | μM | 10 | 0.12 | 0.3 |
| Promega Go Taq | U/μl | 5 | 1 | 0.2 |
| DNA | | 20 ng/μl | 40ng | 2 |
| Total volume | μl | | | 25 |

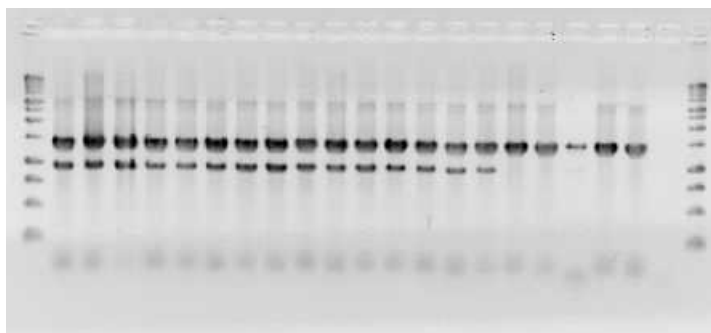
| MJ PTC-200 thermal cyclor | | |
|---------------------------|--------|-----------|
| 94°C | 2 min | 35 cycles |
| 94°C | 30 sec | |
| 55°C | 45 sec | |
| 72°C | 1 min | |
| 72°C | 10 min | |
| 10°C | - | |

Amplification products (10 μl) were resolved in a 1.5% (w/v) agarose gel and length estimates were deduced from the Bench Top Ladder 1kb (Promega).

Evaluation of the assay

Plant material and results

| Cultivar | R/S | Results (bp) |
|--------------------------|-----|--------------|
| Motelle | R | 940 and 1380 |
| Motelle x Marmande verte | R | 940 and 1380 |
| Abellus | R | 940 and 1380 |
| Abigail | R | 940 and 1380 |
| Cibellia | R | 940 and 1380 |
| Clermon | R | 940 and 1380 |
| Facility | R | 940 and 1380 |
| Gaheris | R | 940 and 1380 |
| Hipop | R | 940 and 1380 |
| Nemo-Netta | R | 940 and 1380 |
| Petula | R | 940 and 1380 |
| Relaxx | R | 940 and 1380 |
| Thomas | R | 940 and 1380 |
| Tracie | R | 940 and 1380 |
| Allflesh 900 | R | 940 and 1380 |
| Bellezza | S | 1380 |
| Cencara | S | 1380 |
| Lancelot | S | 1380 |
| Marporum | S | 1380 |
| Marmande Verte | S | 1380 |



Conclusion:

Expected results

Essays applied on ambiguous varieties

| Maladie | Gène de résistance | Variété | Résultat phénotypique | Résultat moléculaire |
|---|---|---------|-----------------------|---------------------------------------|
| Maladies dues aux nématodes (<i>Meloidogyne incognita</i>) | <i>Mi-1.2</i> | 2612 | 5 S + 29 R (R2) | Résistant hétérozygote |
| | | 2640 | 28 S + 2 R (sensible) | Sensible |
| | | 2528 | 11 S + 19 R (R3) | Résistant hétérozygote |
| | | 2529 | 6 S + 27 R (R2) | Résistant hétérozygote |
| Fusariose due à <i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> race 2 (<i>fo</i> (1)) | <i>I2</i> | 2330 | 9 S + 21 R | Résistant |
| | | 2331 | 21 S + 2 R | Sensible |
| | | 2632 | 12 S + 18 R | Résistant |
| Fusariose due à <i>fo</i> (0) | <i>i</i> | 2504 | 20 S + 2 R | Résistant |
| | | 2546 | 22 S + 2 R | Résistant |
| Verticilliose | <i>Ve1</i> et <i>Ve2</i> | 2325 | 4 S + 26 R | Résistant (<i>Ve1</i> , <i>Ve2</i>) |
| | | 2605 | 24 S + 4R | Sensible |
| | | 2555 | 20 S + 10 R | Sensible |
| Mosaïque de la tomate | <i>Tm1</i> , <i>Tm2</i> et <i>Tm2²</i> | 2328 | 22 S + 10 R | Sensible |

Appendix 13. Results Validation Naktuinbouw

1. Mi1-2 marker
2. I1 linked marker
3. I2 marker
4. Tm1 linked marker
5. Tm2 marker assay 1 (+/-)
6. Tm2 marker assay 2 (het / hom)
7. Ve1 marker
8. Ve2 marker

We have tested two plants of 30 varieties with markers for resistance to nematodes, Fusarium, Tomato mosaic virus and Verticillium. The scores are presented in the table at the end of this appendix. The gel pictures are discussed below. Some of the gel pictures show more lanes than scored in the table, because we analyzed some other material. On the gel pictures we have written plate codes A1-E10 for the 58 plants representing 30 varieties selected for marker validation. In every experiment we also included the DNA of control varieties that were used earlier for robustness-control. The names of these varieties (Persica, Moneymaker, Marmande and Campeon) are sometimes written on the pictures that were scanned from the 2007 labjournal of Daniel Deinum at Naktuinbouw.

We have used a molecular size standard with bands at 100 b distance and a stronger band at 800 b. We followed the prescribed protocols on our PTC200 PCR machine from MJC Research (now also BioRad). In most cases we used Taq polymerase from Invitrogen but for Ve1 we had to use Super Taq from HT Biotechnology.

In this report we present thirty varieties without mentioning their name, to avoid dissemination of sensitive data. However, we have contacted the companies that own these varieties and found them willing to release these data after giving them sufficient opportunity to study the present report.

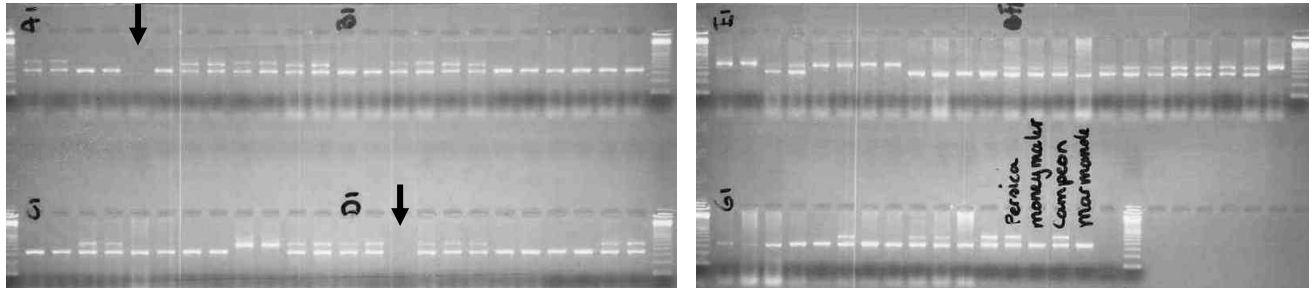
1. Mi marker for nematode resistance

550 bp resistant band for Mi1-2
350 bp susceptible band for Mi1-2

Susceptible controls Moneymaker and Marmande show only the susceptible band. Resistant controls Persica and Campeon show both bands. These varieties are heterozygous for the Mi1-2 gene. Thirteen susceptible varieties showed only the expected band. Twelve resistant varieties showed both bands and four resistant varieties showed only the resistant band. One variety which is used as an intermediate resistant control in our bioassay (Variety 6, lane A11 and A12) showed both bands. This probably indicates that the Mi1-2 gene is incompletely dominant in some genetic backgrounds.

Two plants (arrow) produced no product. This is not due to the DNA quality because we used the same DNA for the other markers. The most likely explanation is PCR failure due to technical problems.

For one variety (no. 27) we encountered the problem that the breeder claimed susceptibility while we detected resistance in our bioassay. The marker assay supported our finding because we detected the resistant band. The variety is heterozygous for Mi1-2 and is marketed in a country with Mediterranean climate. We assume that the dispute arises from the fact that the Mi gene is not effective at temperatures over 28°C. This weakness is probably more pronounced in heterozygous plants.

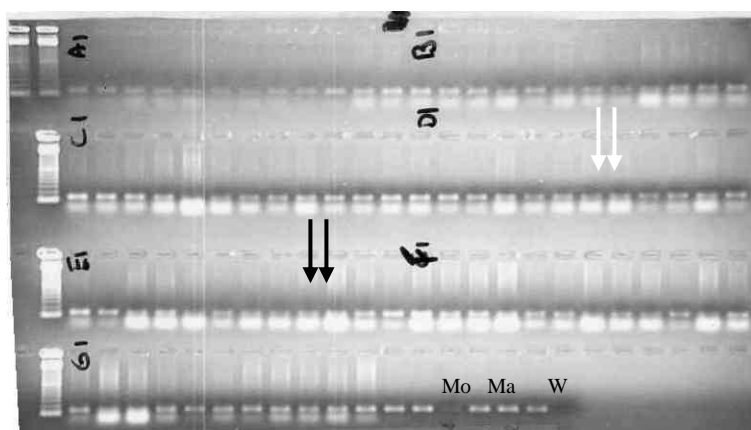


2. I1 linked marker C2_At22570 for Fol:1 resistance

130 bp resistant band

Susceptible control variety Marmande (indicated with Ma) shows an strong false positive band, while susceptible control Moneymaker (indicated with Mo) shows a very weak false positive band. The white arrows indicate a susceptible variety with rather weak false positive bands. All other varieties are resistant to Fol:0 and Fol:1. One variety (indicated by a black arrow) is resistant to Fol:2 and is reputed to carry I3. This variety also shows a band. We did not expect this, but it is not clear whether this is a false positive or whether I1 is present in the background.

The water control indicated with W shows that the false positives are not due to contamination. Nevertheless, the picture has poor quality and may easily be over interpreted. Our feeling is that the PCR should be repeated before drawing final conclusions about the practical value of this marker.



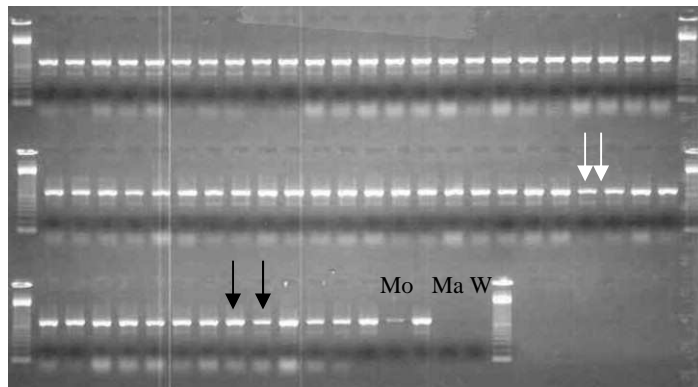
3. I2 marker for Fol:2 resistance

940 bp resistant band of I2

We were not able to use Rubisco as an internal control. After adding Rubisco primers, no product for the I2 marker was obtained, most likely because the I2 amplification competes poorly with the Rubisco amplification. We Rubisco:I2 primers in 1:4 ratio in 20 and 1:3 in 25 μ L.

The I2 marker was absent in the susceptible control Marmande (Ma), weakly present in the susceptible Moneymaker (Mo) and absent in the water control (W). The I2 marker was present in most varieties, including the I3 variety (black arrows) and one susceptible variety (white arrows).

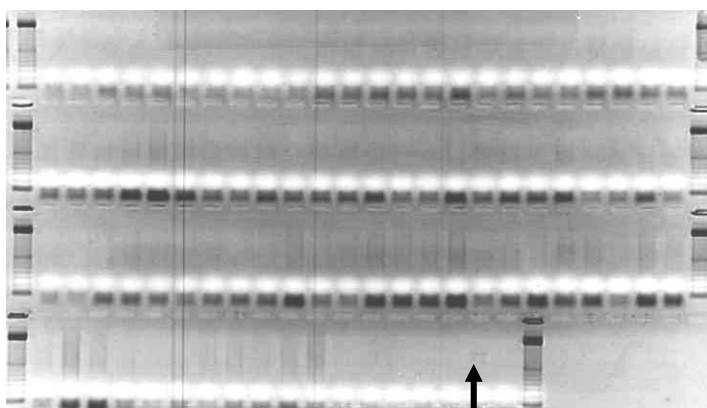
Further confirmation of the false positives (arrows) is needed.



4. Tm1 linked marker SCN20 for ToMV:0 and ToMV:2 resistance

1400 bp SCN1 resistant band linked to Tm1
92 bp Lat1 internal control band

The Tm1 linked marker produced a weak band in one control variety (arrow). This variety is known to have Tm1. The conclusion that none of the other varieties has Tm1 seems valid, although we feel that the 1400 bp is too weak for reliable routine testing. The Tm1 gene has not been popular among breeders because it allows latent infection with the virus. This may generate trouble whenever Tm1 varieties are grown near susceptible varieties.



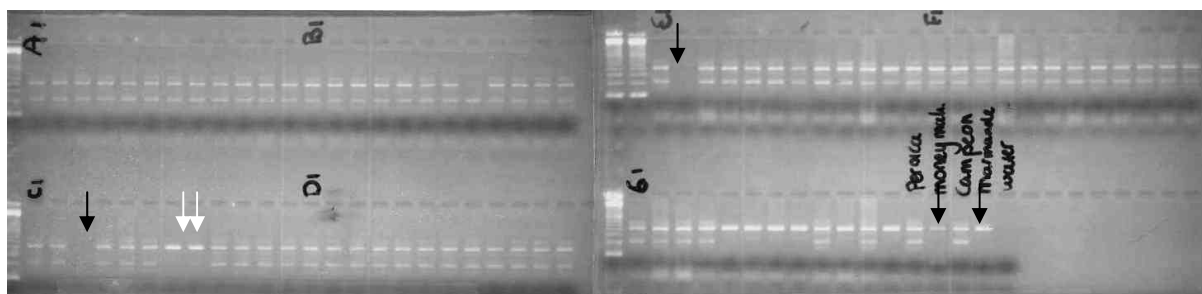
5. Tm2 marker (assay 1) for ToMV:0 and ToMV:2 resistance

509 bp tetra-arms control
254 bp Tm2 resistant allele
214 bp Tm22 resistant allele
No band for susceptible allele

Susceptible control varieties Moneymaker and Marmande show only the control band. The two resistant alleles are absent, as expected.

Among the thirty varieties used for validation only one variety was susceptible (white arrows). We noticed in the biotest that this variety has reduced susceptibility. We could not evaluate this variety within the normal test period, but after extension of the period for symptom development we decided to agree with the claimed susceptibility. The marker results confirm that this procedure is justifiable.

Black arrows point out two failing PCR reactions in lane C3 and E2. The control band is missing in lane B8.



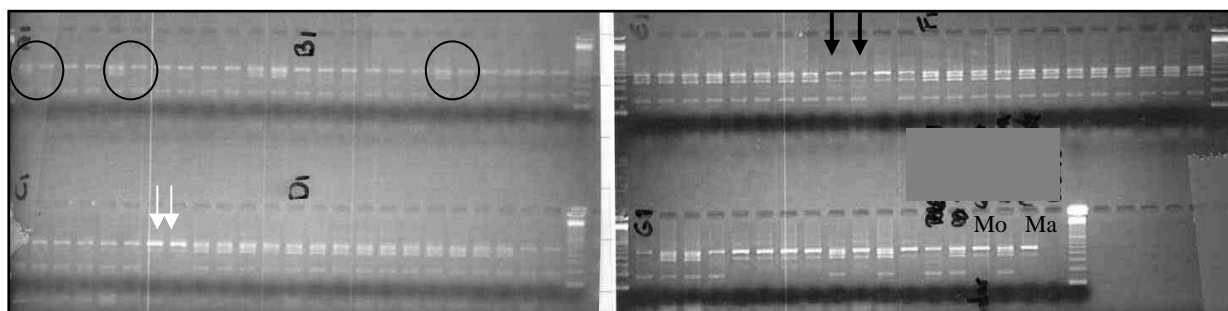
6. Tm2 marker (assay 2) for ToMV:0 and ToMV:2 resistance

509 bp tetra-arms control band
 381 bp susceptible band
 185 bp resistant band Tm2 or Tm22

In this assay the heterozygote can be distinguished from the homozygote, but no distinction of Tm2 and Tm22 is possible.

The susceptible band is present in Marmande and Moneymaker, as expected. Persica and Campeon are heterozygous. This agrees with our earlier result for these resistant control varieties. One variety in the panel was susceptible (white arrows). This variety showed reduced susceptibility (see above). Assay 2 again confirmed the absence of resistance based on Tm2 or Tm22.

Some of the varieties in the validation panel were homozygous resistant and some were heterozygous resistant. In three cases (encircled) we encountered one homozygous and one heterozygous plant from the same variety. This is in agreement with information provided informally by breeders, although we are not able to confirm this heterogeneity in our bioassays. Heterozygous plants are more likely to have necrotic symptoms than homozygous plants, but the number of necrotic plants also depends on light, temperature and plant stage at inoculation. Both the bioassay and the marker test show a form of (apparent) heterogeneity that is not relevant to DUS evaluation, but they do not show this in exactly the same way.



7. Ve1 marker for Verticillium resistance

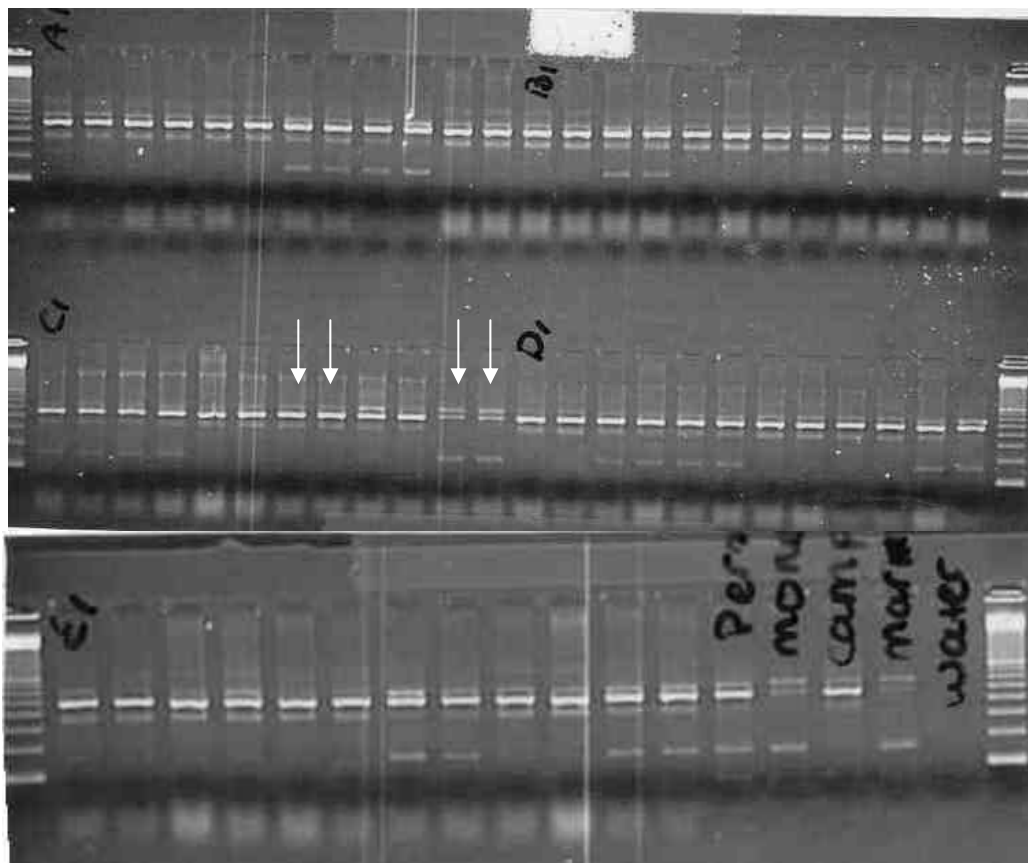
580 bp tetra-arms control band

476 bp resistant Ve1 band

158 bp susceptible Ve1 band

We could not immediately reproduce our earlier success with the Ve1 marker. The 580 bp control band did not appear at all with normal Taq polymerase, and, critically, the susceptible band was often not present in samples that should have it. Only the resistant band was present where expected and absent in the susceptible controls. After we changed over to SuperTaq polymerase, the result became acceptable (see picture below), although this change-over resulted in an awkward extra band in Marmande and Moneymaker just above the 476 bp band and an extra band in most other samples around 370 bp.

Two susceptible varieties are indicated by white arrows. Only one of these has the susceptible band. We have difficulty in deciding whether the resistant band is present or absent in this variety.



8. Ve2 marker for Verticillium resistance

321 bp tetra-arms control
242 bp resistant Ve2 band
131 bp susceptible Ve2 band

The susceptible standard varieties Moneymaker and Marmande produced strong a control band and a rather weak susceptible band. On the picture the susceptible band is somewhat obscured by co-migrating loading dye. The resistant band was sometimes accompanied by a band at ca. 180 bp and the heterozygous genotype consistently produced an extra band at ca. 280 bp. This 280 bp band is probably due to heteroduplex formation during PCR amplification.

Two susceptible varieties are indicated with white arrows. Surprisingly, no product can be seen *in both plants* of one of the susceptible varieties. This variety had the resistant allele of Ve1. The other susceptible variety has the weak susceptible band and the strong control band that are also seen in Moneymaker and Marmande.

Comparison of the Ve1 and Ve2 results shows that three varieties are homozygous for Ve1 and heterozygous for Ve2. We did not notice a difference in resistance level. Nevertheless we cannot exclude the presence of a small difference that has escaped our attention in our routine testing.

These results indicate that it is critically important to have better information available about the functionality of both Ve1 and Ve2, two genes that are considered to act independent and usually as a tandem. However, it appears that recombination between these genes has taken place and that a recombined Ve1-Ve2 locus is rather frequently used in modern tomato varieties. This information is new and has a potential impact on breeding strategies and DUS evaluations.

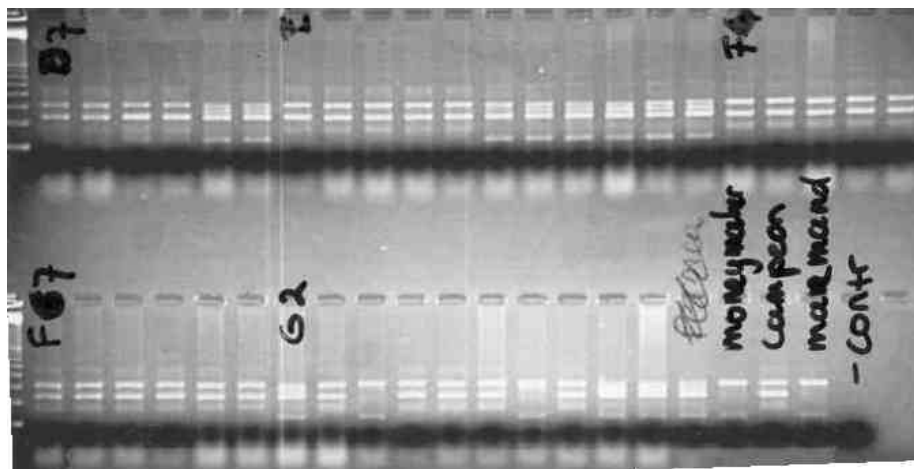
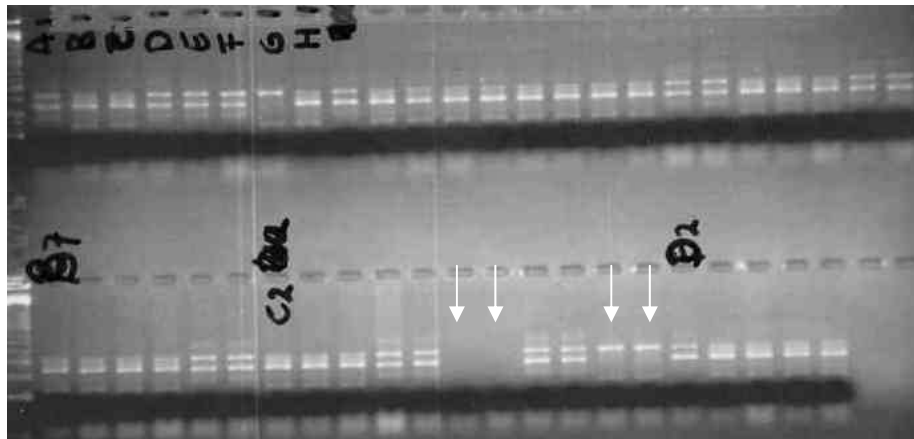


Table1. Validation of molecular markers for resistance tomato DUS testing by Naktuinbouw. December 2007.

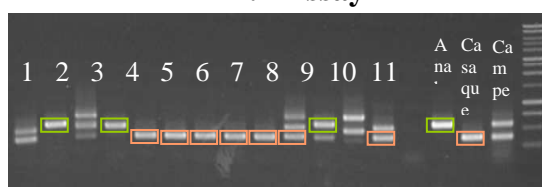
| Tomato variety | Mi | Mi resistant 550bp | Mi 350 bp susceptible | Fol0 | Fol:1 | Fol1 locus 130 bp Resistance | Fol12 940bp resistant | ToMV:0 | ToMV:1 | ToMV:2 | R Tm1 1400 bp | Tm1 92 bp control | Tm assay 1 509 bp control | R Tm2 254 bp | R Tm2(2) Tm22 | Tm assay 2 509 bp control | S Tm2(2) Hetero/homo 381 bp | R Tm2(2) Hetero/homo 185 bp | Ve | VE1 580 bp control | VE1 476 bp resistant | VE1 158 bp susceptible | VE2 321bp control | VE2 242 bp resistant Ve2 | VE2 131 bp susceptible | | | |
|----------------|----|--------------------|-----------------------|-------|-------|------------------------------|-----------------------|--------|--------|--------|---------------|-------------------|---------------------------|--------------|---------------|---------------------------|-----------------------------|-----------------------------|----|--------------------|----------------------|------------------------|-------------------|--------------------------|------------------------|-----|-------------|-----|
| 1 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 2 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 0 | 1 | 1 | 1 | het | rec Ve1-Ve2 | |
| 3 | S | 0 | 0? | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 1 | het | rec Ve1-Ve2 | |
| 4 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 5 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 6 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 7 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 0 | 1 | 1 | 1 | het | rec Ve1-Ve2 | |
| 8 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 0 | 1 | 1 | 1 | | | |
| 9 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 10 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 11 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 1 | het | rec Ve1-Ve2 | |
| 12 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 0 | 1 | 1 | 1 | het | rec Ve1-Ve2 | |
| 13 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 14 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 15 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 0 | 0 | 0? | 1 | 0 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 16 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 17 | S | 0 | 1 | R | R | 1 | 1 | S | S | S | 0 | 1 | 1 | 0 | 0 | ok | 1 | 1 | 0 | S | 1 | 1 | 0 | false+? | 0 | 0 | null | ??? |
| 18 | R | 1 | 0 | hom R | R | R | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 19 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | S | 1 | 0 | 1 | 1 | 0 | 1 | | | |
| 20 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 21 | R | 0 | 0? | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 22 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 23 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 24 | S | 0 | 1 | S | S | 1 | 1* | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 25 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 26 | R | 1 | 0 | hom R | R | R | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 27 | R | 1 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 0? | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 28 | R | 1 | 0 | hom R | R | R | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| 29 | R | 1 | 0 | hom R | R | R | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| 30 | S | 0 | 1 | R | R | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 1 | het | rec Ve1-Ve2 | |
| Persica | R | 1 | 1 | R | | 1 | 1 | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 1 | het | 1 | 1 | het | | |
| Moneymaker | S | 0 | 1 | S | 1 | 1* | false+ | S | S | S | 0 | 1 | 1 | 0 | 0 | ok | 1 | 1 | 0 | S | 1 | 0 | 1 | ok | 1 | 0 | 1 | ok |
| Campeon | R | 1 | 1 | R | 1 | 1 | | R | R | R | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | R | 1 | 1 | 0 | 1 | 1 | 0 | | | |
| Marmande | S | 0 | 1 | S | 1 | 0 | 1 | S | S | S | 0 | 1 | 1 | 0 | 0 | ok | 1 | 1 | 0 | S | 1 | 0 | 1 | ok | 1 | 0 | 1 | ok |
| Master no 2 | | | | | | 1 | | R | S | R | 1 | 1 | ok | | | | 1 | 1 | 0 | | | | | | | | | |
| Moperou | | | | | | | | | | | | | | | | | 1 | 1 | 0 | | | | | | | | | |
| Water Control | | 0 | 0 | | | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | | 0 | 0 | 0 | | 0 | 0 | 0 | |

Appendix 14. Evaluation of wild accessions

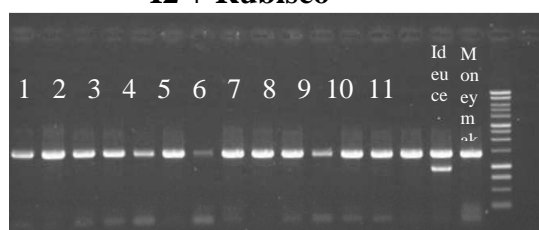
Assays on Wild species – P. Sanchez & C. Caranta – INRA – Nov.

| | | |
|----|----------|-----------------------------------|
| 1 | LA1777 | <i>S. habrochaites</i> |
| 2 | PI247087 | <i>S. habrochaites</i> |
| 3 | LA0385 | <i>S. peruvianum</i> |
| 4 | LA1601 | <i>S. parviflorum</i> |
| 5 | LA1615 | <i>S. cheesmaniae</i> |
| 6 | LA1401 | <i>S. cheesmaniae</i> |
| 7 | LA1554 | <i>S. rickii/pimpinellifolium</i> |
| 8 | LA1589 | <i>S. pimpinellifolium</i> |
| 9 | LA1602 | <i>S. pimpinellifolium</i> |
| 10 | LA2172 | <i>S. peruvianum</i> |
| 11 | LA1556 | <i>S. chilense</i> |
| 12 | LA0716 | <i>S. pennellii</i> |
| 13 | LA2157 | <i>S. peruvianum</i> |
| 14 | LA1840 | <i>S. chmielewskii</i> |

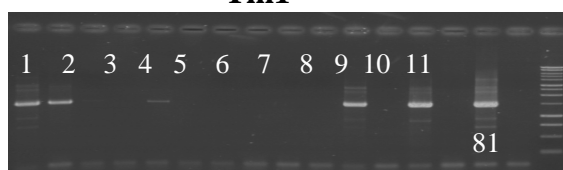
Mi 1.2 Assay



I2 + Rubisco



Tm1



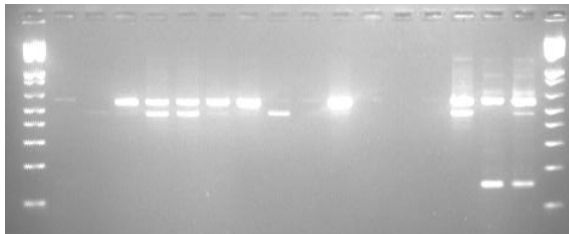
Conclusions :

- Mi 1.2 assay (Mi 1.2 from *S. peruvianum*): Very complex pattern of amplification. In Green, fragment of the same MW as the resistance allele; in orange, fragment of the same MW as the susceptibility allele. New bands can be expected
- I2 assay (I2 from *S. pimpinellifolium*) : PCR fragment specific for the I2 gene never amplified in the set of wild species => Highly specific marker
- Tm1 assay (Tm1 from *S. habrochaites*) : fragment of the same molecular weight amplified in the two *S. habrochaites* accessions (LA1777 and PI247087), in a single accession of *S. cheesmaniae* (among 2), in the *S. chilense* accession and in a single *S. Peruvianum* accession (among 2) => do we need to sequence to check that amplified fragments correspond to the same locus???

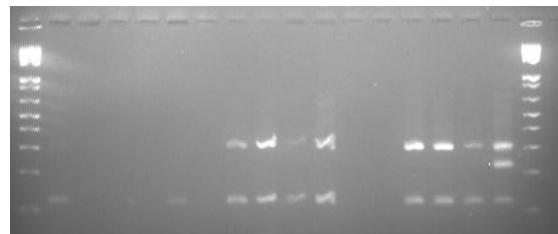
Assays on Wild species – P. Arens – PRI – Nov.

| | | |
|----|----------|-----------------------------------|
| 1 | LA1777 | <i>S. habrochaites</i> |
| 2 | LA0385 | <i>S. peruvianum</i> |
| 3 | LA1601 | <i>S. parviflorum</i> |
| 4 | LA1615 | <i>S. cheesmaniae</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. cheesmaniae</i> |
| 13 | LA1589 | <i>S. pimpinellifolium</i> |
| 14 | LA1602 | <i>S. pimpinellifolium</i> |

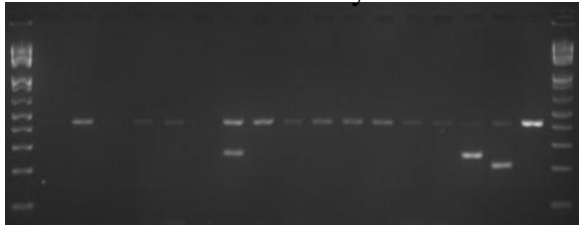
Ve1 assay



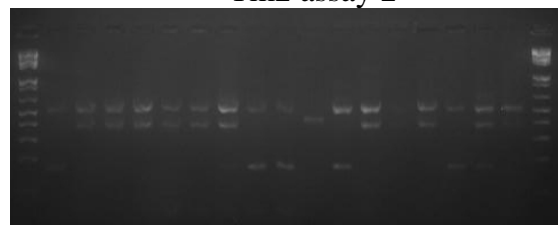
Ve2 assay



Tm2 assay 1



Tm2 assay 2



For the tests also the resistant and susceptible controls used in the development of the marker assays were added

Conclusions :

Ve1/Ve2 assay (Ve from *S. lycopersicon*): Many species show amplification of either the outer fragment or one of SNP specific bands. Orthologs or paralogs may be present.

Tm2 assay (Tm2 from *S. peruvianum*): Many species show amplification of either the outer fragment or one of SNP specific bands. Orthologs or paralogs may be present.