Rapid identification of *Trialeurodes vaporariorum*, *Bemisia tabaci* (MEAM1 and MED) and tomato-infecting criniviruses in whiteflies and in tomato leaves by real-time reverse transcription-PCR assay

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**Abstract**

The whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* (Hemiptera Aleyrodidae) are harmful pests of vegetable and ornamental crops in many countries. Also, they are vectors of emergent viruses on tomato including the criniviruses (*Closteroviridae* genus *Crinivirus*) Tomato chlorosis virus (ToCV) and Tomato infectious chlorosis virus (TICV). Since different vectors are involved in the transmission of both viruses (ToCV is transmitted by *B. tabaci*, *Trialeurodes abutiloneus* and *T. vaporariorum* while TICV is transmitted only by *T. vaporariorum*), and they induce similar symptoms on tomato plants, a sensitive and specific diagnosis method is desirable. In addition, a rapid discriminating method of the vectors is essential for monitoring and control activities and epidemiological studies. For these reasons, a combined protocol based on one-step multiplex real-time reverse transcription (RT)-PCR has been developed for the identification of *T. vaporariorum*, two invasive species of the complex *B. tabaci* (MEAM1 and MED) and for the specific detection of ToCV and TICV in whiteflies and plants.

**Key words:** Aleyrodidae, virus vector, tomato yellowing, molecular diagnosis.

**Introduction**

During the last decades, whitefly populations (Hemiptera Aleyrodidae) have rapidly increased throughout the world as well as the associated virus diseases (Wisler et al., 1998b; Brown, 2007; Wintermantel, 2010; Navas-Castillo et al., 2011). Although a high number of whitefly species have been described, only a few are virus vectors and these include *Bemisia tabaci* (Gennadius) and *Trialeurodes vaporariorum* (Westwood). *B. tabaci* is considered the most important pest due to its wide distribution, host range and capacity to transmit most of the whitefly transmitted-viruses. The viruses transmitted by *B. tabaci* include members of the genera Begomovirus (Geminiviridae), Crinivirus (Closteroviridae), Carlavirus (Betaflexiviridae), Ipomovirus (Potyviridae) and Torravovirus (Secoviridae) (Jones, 2003; Navas-Castillo et al., 2011; EFSA, 2013).

*B. tabaci* includes a complex mix of genetically but not morphologically distinguishable populations, which have been referred as biotypes. Recently, it has been proposed that *B. tabaci* is a complex of different species (Dinsdale et al., 2010; De Barro et al., 2011). Different molecular tools have been developed in the last decade to study this genetic diversity and to identify the different biotypes/species (Guirao et al., 1997; Frohlich et al., 1999; Cervera et al., 2000; De Barro et al., 2000). Middle East-Asia Minor 1 (MEAM1, formerly biotype B) (Demichelis et al., 2000) and Mediterranean (MED, formerly biotype Q) are the most common and polyphagous species of the *B. tabaci* complex found in Italy (Demichelis et al., 2000; Bosco et al., 2001); they both are responsible for the transmission and emergence of begomoviruses and some criniviruses worldwide. *T. vaporariorum* is also a polyphagous pest but transmits a limited number of viruses, all within the genera *Crinivirus* and *Torravovirus* (Wisler et al., 1998a; Jones, 2003; Brown et al., 2007; Navas-Castillo et al., 2011). Compared to *B. tabaci*, fewer studies were carried out with *T. vaporariorum*. Recent studies analyzed the genetic variation of this whitefly using COI gene and the internal transcribe spacer (ITS) sequence of ribosomal DNA, finding a very low genetic diversity in different populations of *T. vaporariorum* (Roopa et al., 2012; Prijović et al., 2014). Other authors, using biochemical and allozime analysis, found two distinct populations of *T. vaporariorum* separated by geographical barriers (Shin et al., 2013).

The genus *Crinivirus* includes a number of species emerged in the past two decades (Wintermantel and Hladky, 2010). The criniviruses *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV), firstly identified on tomato (*Solanum lycopersicum* L.) in USA and characterized in the mid of 1990 (Duffus et al., 1996; Wisler et al., 1998b), are now emergent viruses worldwide, becoming a serious threat in many countries including those in the Mediterranean Basin (Tzanetakis et al., 2013). In Italy, both criniviruses have been found in several regions on protected tomato (Acotto et al., 2001; Vaira et al., 2002; Davino et al., 2007) and other crops (Parrella, 2008).

TICV is transmitted exclusively by *T. vaporariorum* (Duffus et al., 1996), while ToCV is transmitted by *Trialeurodes abutiloneus* (Haldeman), *T. vaporariorum* and the New World 1 (NW1, formerly biotype A), MEAM1 and MED species of the *B. tabaci* complex (Wisler et al., 1998a). Studies have shown differences in the efficiency to transmit ToCV among species, showing that *T. abuti-
lonicus and T. tabaci MEAM1 and MED are more efficient than T. tabaci NW1 and T. vaporariorum (Wintermantel and Wisler, 2006). Both viruses are transmitted in a semi-persistent manner to plant hosts. In addition to tomato, the viruses infect several cultivated plants, ornamental and weeds that represent a potential virus reservoir and a source for whitefly feeding and colonization resulting in movement of the virus into the near fields (Wintermantel, 2010).

A number of studies on epidemiology, virus-vector species specificity and diagnosis were made during the last years. Sensitive molecular techniques were developed to identify TICV and ToCV in plants and insects, the most recent by real-time RT-PCR (Morris et al., 2006; Papayiannis et al., 2011; Cavalieri et al., 2011; Tiberini et al., 2011). Concerning vectors, real-time PCR was also developed for T. tabaci identification (Zhang et al., 2007) and to differentiate the MEAM1 and MED species (Jones et al., 2008; Papayiannis et al., 2009). Since a different host preference is usually shown by different parasitoids, the correct identification of these whitefly species is fundamental for applying an effective biological control strategy of the vectors which has a basic role in reducing their attacks to crops, within an integrated pest management strategy of primary importance especially in lack of commercial tomato varieties resistant to the two viruses.

The present paper describes combined protocols based on one-step real time RT-PCR (Heid et al., 1996; Mackay et al., 2002; Bustin et al., 2005) that were specially performed to deliver a complete and rapid method to investigate the distribution and prevalence of T. vaporariorum and T. tabaci in the two main Italian islands, Sicily and Sardinia. Also, the real-time RT-PCR assay was developed for rapid identification of vector species and simultaneously detection of tomato criniviruses into their bodies. Because vector species identification is not an easy task based on whitefly morphology, specific primers and TaqMan probes were designed to allow identification in a single reaction of MEAM1 and MED species of T. tabaci as well as of T. vaporariorum.

Materials and methods

Whitefly and plant sampling in tomato greenhouses

Whitefly adults were collected during 2011 in four Sicilian and one Sardinian tomato greenhouses (table 1) by means of a manual aspirator and maintained in 70% ethanol at 4 °C until use. At the same time, symptomatic leaves colonized by the captured whiteflies were also collected and analyzed to confirm the presence of TICV or ToCV infection. T. vaporariorum, T. tabaci MEAM1 and T. tabaci MED populations were also identified from different localities to be used as controls in real-time TaqMan assays. TICV and ToCV positive controls from CRA-PAV collection, validated in a test performance study (ARNADIA Italian project) (Mangli et al., 2013), were used in all assays.

Identification of whitefly species

Each pooled sample of whitefly individuals were discriminated exclusively on a morphological basis (Martin et al., 2000; EPPO, 2004), through both initial field observations and subsequent lab analysis by means of a stereomicroscope.

For T. tabaci species discrimination, a molecular technique was applied based on microsatellite marker BEM 23 described by De Barro et al. (2003) and used in surveys in Serbia (Zanić et al., 2005), Sardinia (Ortu et al., 2007), Sicily (Cavalieri and Rapisarda, 2008), and Tunisia (Bel-Kadhi et al., 2008). Therefore, five specimens from each T. tabaci population (samples and controls) were individually subjected to DNA extraction according to the protocol described by Walsh et al. (1991) and modified by De Barro and Driver (1997). The PCR reaction was conducted using the primers forward BEM 23 (5'-CGAGCTTGCCTGCTGTCTGC-3') and reverse BEM 23 (5'-CCTTTATCATGCTCCTT-3') (De Barro et al., 2003). All PCR reactions were performed in 20 µl volumes with 1X PCR buffer, 3.5 mM of MgCl₂, 125 µM of dNTPs, 7 pmol of each primer, 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2 µl of DNA template. The cycling conditions were: 94 °C for 5 min, then 40 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, followed by final cycle at 72 °C for 7 min. Reactions and cycling conditions were conducted in an automated thermal cycler [GeneAmp® PCR System 2700 (Applied Biosystems)]. PCR products were run in 1.6% agarose gel and visualized with SYBR® Safe DNA gel stain (Invitrogen). MEAM1 shows a characteristic band of about 200 bp, while MED shows a band of about 400 bp.

Primer and probe design

In order to achieve the combined identification expected through the present work, a region of the cytoch-
Tome oxidase sub unit I gene (COI) was selected for discrimination of *T. vaporariorum* and *B. tabaci* MEAM1 and MED. This marker has been employed successfully in the study of genetic variability of whiteflies and *B. tabaci* in particular (Frohlich *et al.*, 1999; Zhang *et al.*, 2005; Bosco *et al.*, 2006; De la Rúa *et al.*, 2006; Boykin *et al.*, 2007; Dinsdale *et al.*, 2010; Roopa *et al.*, 2012). A number of sequences of COI region of *T. vaporariorum* and both MEAM1 and MED species of *B. tabaci* deposited in GenBank (GenBank accession: gb:[AF110708.2], gb:[JF512474.1], gb:[JF693935.1], emb:[AM179445.1], gb:[JF693931.1], gb:[HQ992961.1], gb:[EU760751.1], gb:[EU760747.1], gb:[EU760746.1], gb:[EU760736.1], gb:[JF754925.1], gb:[HQ992959.1], gb:[FJ188589.1], emb:[FN821808.1], emb:[FN821807.1], gb:[JF754925.1], gi:[308026346]) were aligned with Clustal W2 (Larkin *et al.*, 2007) to search for variable regions of COI gene between whitefly species, in order to design species-specific TaqMan-probes and primers for every whitefly type.

A total of 10 probes and 17 primers were designed for the TaqMan assay using the Primer Express™ version 3.0 software (Applied Biosystems, Life Technologies Corporation, Grand Island, USA) with an amplified fragment size of 67-84 bp. Primers were supplied by Invitrogen Custom DNA Oligos (Life Technologies Corporation) and probes each labeled by differently colored fluorophores were supplied by Sigma-Aldrich (St Louis, USA) and Bio-Fab Research (Roma, Italy). The sequences of probes and primers selected in this work are given in table 2, where internal vector control, designed in the consensus region of rRNA 18s, is also included. Furthermore, primers and probes have been designed in order to use them under the same reaction conditions of TIV and ToCV primers and probes previously designed and used in multiplex TaqMan RT-PCR assay. Three primer and probe sets, one for each whitefly type, were selected for multiplex real time RT-PCR for whitefly identification (table 2). The triplex real-time RT-PCR assay was set-up in 96-well reaction plates using TaqMan® One Step PCR Master Mix Reagents Kit (Applied Biosystems) as following: 12.5 µl 2X Master Mix, 0.625 µl 40X MultiScibe™ and RNase Inhibitor Mix, 0.75 µl of each primer (10 µM), 0.5 µl of each probe (5 µM) and 1 µl of RNA in a final volume of 25 µl. One reaction mix was prepared for discrimination of the three whiteflies; another mix was prepared for detection of the two viruses into the vectors (table 2). Therefore, every sample was analyzed in the same multi-well preloaded with the two reaction mixes each in one half of the plate. The amplification reaction was carried out as follows: 48 ºC for 30 min, 95 ºC for 10 min, followed by 40 cycles of denaturation at 95 ºC for 15 sec and an elongation step at 60 ºC for 1 min. All reactions were performed in duplicate and the results were visualized and analyzed using SDS software. Primers and probes were tested using RNA from known specimens of the investigated whitefly species, and reference virus isolates. These samples were also used as positive controls. RNA from healthy tomato leaves and virus-free whiteflies were used as negative control Assays were performed in ABI PRISM 7500 Fast. Thresholds cycles (Ct) were automatically calculated and data analyzed by the system software in the thermocycler.

**Results**

Three primer and probe sets, one for each whitefly type, were selected for multiplex real time RT-PCR for whitefly identification (table 2). The triplex real-time RT-PCR assays allowed the simultaneous and specific identification of all whitefly species. The results obtained were in accordance with morphological discrimination of species and molecular identification of species through PCR. The TaqMan probes selected were: BEM COI MEAM1-probe, BEM COI MED-probe, TRAIL COI-probe. In Sicilian greenhouses, all whiteflies colonized by whiteflies, 1g of tissue was minced in 5 mL of 0.1M PBS (3.63 g NaHPO4, 1.2 H2O, 0.24 g KH2PO4, 8.0 g NaCl and 0.2 g KCl) and 100 µl of the extract were used for total RNA extraction using the above mentioned Qiagen kit.

<table>
<thead>
<tr>
<th>Name</th>
<th>Probe sequences</th>
<th>Forward primer sequences</th>
<th>Reverse primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEM COI MEAM1</td>
<td>HEX-TATTTTACATATGTTGGTTAACCTGA-CBHQ1</td>
<td>5’-TGGCCCTTTGATTACAGGATTTTT-3’</td>
<td>5’-ACACCATCTACAGAAGAATCCACAAAGAA-3’</td>
</tr>
<tr>
<td>BEM COI MED</td>
<td>CY5-AGGTCGTTGATGAGAGA-CBHQ2</td>
<td>5’-GAACCTTCCATCTTGGTGGATT-3’</td>
<td>5’-AGGTCGTTGATGAGAGA-CBHQ2</td>
</tr>
<tr>
<td>TRIAL COI</td>
<td>6FAM-TGCCAAGCTGGTTCATTGTG-BHQ1</td>
<td>5’-GATGCCCTGACAGTTGATT-3’</td>
<td>5’-TTACCAAGTCTCCCAATAGAAGAAAC-3’</td>
</tr>
</tbody>
</table>

**Table 2. Probes and primers designed and used in multiplex TaqMan RT-PCR assay.**

* probes and primer designed by Tiberini *et al.* (2011).
lected belonged to *B. tabaci* MED, with an exception for an individual belonging to *T. vaporariorum*. No MEAM1 specimens were found. Fifty-eight insects showed positive to ToCV. All Sardinian samples belonged to *T. vaporariorum* and two specimens showed positive to TICV (table 3 and figure 1).

The assay was also applied to identify whitefly population at the early stages (nymphal and pupal stage) occurring on the leaves. As a consequence, twenty-six virus infected tomato leaf samples were analyzed by Triplex TaqMan RT-PCR using the specific probes for whitefly identification (figure 1). Eleven samples showed infestation of *T. vaporariorum* (all collected in Sicilian greenhouse), ten samples indicated the presence of *B. tabaci* MED infestation and five were negative (all samples collected in Sicilian greenhouse).

**Discussion**

Whiteflies and the associated viral diseases become of increasing concern worldwide. Among whitefly-transmitted virus, TICV and ToCV raise particular interest for a different vector specificity as TICV is only transmitted by *T. vaporariorum* while ToCV is transmitted by either *Trialeurodes* spp. (*T. vaporariorum* and *T. abutiloneus*) or different species of the *B. tabaci* complex. This aspect greatly stimulated researches for a better knowledge on virus-vector relationships and epidemiological studies on distribution of TICV and ToCV according to whitefly-species populations in tomato crops in many parts of the world. In the past, many protocols based on different techniques were used to provide complete data on whitefly and virus identification, resulting time consuming and requiring different specialists for pests and pathogens. Molecular diagnostics based on amplification chain reaction (PCR), associated to restriction length polymorphism (RFLP) or random amplified polymorphic DNA analysis (RAPD) or DNA sequencing, introduced the advantage of rapidly distinguishing whitefly species, without morphological identification that require a well maintained specimens (Guirao et al., 1997; Trohlich et al., 1999; De Barro et al., 2000; Bosco et al., 2006), as well as genetic polymorphisms within species when morphologically indistinguishable. More recently, fluorogenic amplification as-

**Table 3.** Results of analysis of one step triplex real time RT-PCR using the three new specific probes and primers selected for the whitefly identification and the specific probes and primers for the detection of the two viruses in insects and in tomato leaves collected in greenhouse.

<table>
<thead>
<tr>
<th>Greenhouse/locality</th>
<th>Whitefly identification</th>
<th>Virus infections in whiteflies</th>
<th>Virus infections in tomato leaves*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T. vaporariorum</em></td>
<td><em>B. tabaci</em> MED</td>
<td>ToCV</td>
</tr>
<tr>
<td>1/ Vittoria (Sicily)</td>
<td>1/6</td>
<td>0/6</td>
<td>5/6</td>
</tr>
<tr>
<td>2/ Vittoria (Sicily)</td>
<td>0/32</td>
<td>0/32</td>
<td>32/32</td>
</tr>
<tr>
<td>3/ Acate (Sicily)</td>
<td>0/26</td>
<td>0/26</td>
<td>26/26</td>
</tr>
<tr>
<td>4/ Acate (Sicily)</td>
<td>0/31</td>
<td>0/31</td>
<td>31/31</td>
</tr>
<tr>
<td>5/ Arborea (Sardinia)</td>
<td>18/18</td>
<td>0/18</td>
<td>0/18</td>
</tr>
<tr>
<td>Total</td>
<td>19/113</td>
<td>0/113</td>
<td>94/113</td>
</tr>
</tbody>
</table>

* Leaves collected and analyzed for confirmation of the crinivirus infection in greenhouse.

**Table 4.** Threshold cycles range obtained in analysis of one step triplex real time RT-PCR using the three new specific probes and primers selected for the whitefly identification and the specific probes and primers for the detection of the two viruses within insects and tomato leaves collected in greenhouse.

<table>
<thead>
<tr>
<th>Specific probes</th>
<th>Threshold cycles range in insect samples</th>
<th>Threshold cycles range in plant samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whiteflies identification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEM COI MEAM1</td>
<td>21-22</td>
<td>-</td>
</tr>
<tr>
<td>BEM COI MED</td>
<td>18-29</td>
<td>20-38</td>
</tr>
<tr>
<td>TRIAL COI</td>
<td>18-35</td>
<td>19-37</td>
</tr>
<tr>
<td>Virus identification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TICV</td>
<td>&lt;38</td>
<td>&lt;38</td>
</tr>
<tr>
<td>ToCV</td>
<td>&lt;38</td>
<td>&lt;38</td>
</tr>
<tr>
<td>Whitefly 18S</td>
<td>&gt;14</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1. Triplex real time RT-PCR assays: amplification plots of: *B. tabaci* MED (MED); *B. tabaci* MEAM1 (MEAM1); *T. vaporariorum* (TRIAL); *B. tabaci* MED nymphs/pupae in infested leaves (MED-leaves); *T. vaporariorum* nymphs/pupae in infested leaves (TRIAL-leaves); ToCV in infected whiteflies (ToCV-whitefly); TICV in infected whiteflies (TICV-whitefly); whitefly 18S internal control (Whitefly 18S). All samples (a color for each sample) were amplified by specific probes and primers.
The absence of *B. tabaci* MEAM1 in our sampling could be due to the low number of investigated greenhouses and to the scarce number of specimens found on crops due to insecticide treatments against the moth *Tuta absoluta* (Meyrick), a new serious threat of Italian tomato crops. Similar reasons apply to the absence of *B. tabaci* within samples collected in Sardinia and the single specimen of *T. vaporariorum* collected in Sicily. The occurrence and distribution of different whitefly species influenced the presence of the two criniviruses in the investigated greenhouses. Indeed, in Sardinian greenhouses, where only *T. vaporariorum* was found, plants were infected only by TICV; on the contrary, in Sicilian greenhouses, where *B. tabaci* MED was exclusively present, tomato plants were infected only by ToCV.

In conclusion, the method here developed provides researchers and technicians with a reliable and sensitive (single specimens) tool by an accurate and unequivocal identification either of whitefly (adults and nymphs) species or the two criniviruses by a single analysis saving time and cost (the same extraction process for both whitefly and virus analysis; easily processed 96 sample per day, including setup and data analysis). Scientific studies on vectors and viruses interaction, epidemiology and spatial distribution could benefit from this protocol to increase knowledge in favor of control for whiteflies and the associated viral diseases.

Acknowledgements

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