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Candidate gene analysis of Tomato leaf curl New Delhi virus resistance in *Cucumis melo*

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ABSTRACT

Tomato leaf curl New Delhi virus (ToLCNDV) is a whitefly-transmited virus that causes serious damage to different cultivated plant species mainly belonging to the Solanaceae and Cucurbitaceae families. The complex epidemiological factors associated with this disease make the seeking and use of genetically resistant varieties as one of the most efficient, sustainable and frequently employed strategies to control viral infections in the fields. In the case of melon, resistance to mechanical transmission of ToLCNDV has been identified in *Cucumis melo* subsp. agrestis, in three accessions of the Momordica group and in two wild accessions. The genetic control of the resistance derived from one of the wild sources has been studied recently and a major locus in chromosome 11 has been found, along with two modifier regions located in chromosomes 2 and 12. Previous studies have reported the active role of different genes regarding ToLCNDV resistance in different species. In this work we have validated by qPCR twelve of these genes by determining the transcriptomic changes in leaves of a resistant Momodica and a susceptible Piel de Sapo genotypes along a time course experiment after infection with ToLCNDV. The transcript amount of the genes *Cm ARP4* and *Cm NAC domain protein*, was differentially higher in the inoculated susceptible genotype when compared to the inoculated resistant one. For other validated genes, the resistant accession showed higher levels of expression. The location of *Cm ARP4* in a modifier QTL of chomosome 2, suggest that the accumulation of transcripts of this gene is associated to the level of ToLCNDV accumulation and degree of symptoms development. The molecular bases of ToLCNDV resistance regarding the expression results for these candidate genes in melon are discussed as well and their potential use in TILLING platforms and breeding strategies of the species.

1. Introduction

Tomato leaf curl New Delhi virus (ToLCNDV) is a whitefly-transmitted bipartite begomovirus that represents a serious threat to different cultivated species. Although the virus was firstly reported in India infecting tomato (Padidam et al., 1995), new virus variants in this country have infected other Solanaceae crops such as potato and eggplant (Usharani et al., 2004; Pratap et al., 2011), members of other families like Cucurbitaceae as sponge gourd (Sohrab et al., 2003) or Papaveraceae as opium poppy (Srivastava et al., 2016). The virus has been also described in other Asian countries affecting cucurbits in Thailand (Ito et al., 2008), cucumber in Indonesia (Mizutani et al., 2011), chilli pepper and cotton in different provinces of Pakistan (Hussain et al., 2004; Zaidi et al., 2016) and melon in Iran (Yazdani-Khameneh et al., 2016). In the main producing areas of South-Eastern Spain, ToLCNDV has been also described severely affecting Cucurbit crops, including zucchini, melon and cucumber (Juárez et al., 2014), and Solanaceae, such as tomato (Ruiz et al., 2015). Moreover, further dispersion has been described in other countries of the Mediterranean Basin, with new reports of this virus infecting cucurbits in Tunisia (Mnari-Hattab et al., 2015), Southern Italy (Panno et al., 2016) and Morocco (Sifres et al., 2018).

Viral agents are strict intracellular parasites, therefore they cannot be controlled chemically, and prophylactic measures consist mainly in the application of practices that prevent infection (Nicaise, 2014). In many regions of the world, control strategies for begomovirus diseases focus on vector management, measures that are not always fully effective. Moreover, multiple infections of several viruses, along with the

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occurrence of viral recombination and mutation is resulting in the emergence of new strains/species of the genus Begomovirus, which may overcome resistance barriers and adapt to changing environmental conditions (Mansoor et al., 2006). Human activity has also played an important role in the emergence of serious begomovirus diseases across the globe, like the changes in cropping systems, the introduction of new crops, the movement of infected plant materials and the introduction of host susceptibility genes through the exchange of germplasm (Varma and Malathi, 2003). The complex epidemiological factors associated with these diseases, such as broad host range, accelerated rates of virus and vector evolution and the migratory behaviour of whiteflies hinder the development of effective long-term management strategies (Snehi et al., 2015). For all these reasons, the seeking and use of genetically resistant varieties is one of the most efficient, sustainable and frequently employed strategies to control viral infections in fields.

The vast amount of newly obtained genomic knowledge opened doors for the study of the underlying mechanisms that control virus resistance, knowledge of utmost importance in modern breeding. Molecular tools now allow monitoring the dynamics of genomic recombination, making possible a gene-by-gene breeding approach (Breseghello and Coelho, 2013). In this sense different strategies based on the use of resistance genes, RNA interference pathways or systemic acquired resistance mechanisms represent a vast number of plant evolutive strategies to block viruses (reviewed by Nicaise, 2014). The entry of pathogens into the host cells triggers plenty of signalling responses of defense pathways. Consequently, a huge array of host genes are altered in the infected cells. Some of these genes act providing viruses better opportunities to sustain inside the hosts, and the silencing or under expression of these genes leads to resistance. Other genes might help the host evade pathogen infection and consequently their overexpression contributes to the defense response. Among genes belonging to the first class, those that regulate the cell to cell transport of viral proteins, such as actin cytoskeleton genes (Su et al., 2010; Krenz et al., 2012) or those that interact with the virus replication protein, enhancing viral replication (Selth et al., 2005), have been reported. In the second group, many host genes involved in resistance have been also described, such as RNA dependent RNA polymerases acting by transcriptional virus gene silencing through methylation of viral genome (Butterbach et al., 2014), proteosomal subunits that inhibit viral transcription (Sahu et al., 2016), ethylene response factors that regulate PR genes transcription (Fischer and Dröge-Laser, 2004), or genes encoding membrane proteins (hexose transporter, permease like protein or lipocalin like genes), involved in virus entry and/or cell to cell trafficking (Eybishtz et al., 2010, 2009; Sade et al., 2012). Other genes, like those encoding peroxidases, are activated following virus infection as a reflection of physiological changes associated with resistance (Sahu et al., 2012).

In the case of Cucurbits, information of genes regulating resistance in response to ToLCNDV infection is still lacking. In Spain, genetic resistance to ToLCNDV has been identified in melon and squashes and is being introgressed into commercial varieties (Sáez et al., 2016). In the case of melon, resistance to mechanical transmission of ToLCNDV has been identified in *Cucumis melo* subsp. *agrestis*, in three accessions of the Momordica group (exotic, highly variable, melons of Indian origin) and in two wild accessions (also from India) (López et al., 2015). The genetic control of the resistance derived from one of the wild sources has been studied recently through a QTLs approach (Sáez et al., 2017). A major *locus* in chromosome 11 has been found involved in the resistance, along with two modifier regions located in chromosomes 2 and 12. The genetic basis of the resistance in Momordica accessions has not been studied yet. Some of these accessions have been previously reported to be tolerant or resistant to other viruses (Dhillon et al., 2012) and as they are fully crossable to commercial melons, they are good sources to develop new melon varieties with tolerance to ToLCNDV. In this study we have used one of these resistant Momordica genotypes to study the molecular basis of ToLCNDV resistance in *C. melo* by analyzing the expression levels of different candidate genes previously reported to be involved in defense responses.

2. Materials and methods

2.1. Plant, whitefly, and virus materials

The Spanish strain of ToLCNDV (ES) was isolated from zucchini in greenhouses from Almería, and maintained by serial whitefly (*Bemisia tabaci*) transfer on zucchini squash plants (cv. Victoria) grown in insect-proof cages in a growth chamber at 25 °C day and 20 °C night, with a 16-h photoperiod, as described by Ruiz et al. (2017). The whitefly population used for virus inoculation was Med-Q1 cryptic species (Janssen et al., 2017). Melon accessions used in this work were the accession *Cucumis melo* subsp. *agrestis* group Momordica PI414723 (Mom-PI414Ind), kindly supplied by USDA-NPGS, and described as resistant to ToLCNDV (López et al., 2015), with symptomless plants with low viral titers after mechanical inoculation (Sáez et al., 2017), and the susceptible *Cucumis melo* subsp. *melo* group Inodorus cv. Piel de Sapo).

2.2. Virus inoculation

ToLCNDV infected whiteflies were transferred in groups of 10 adults to 16 resistant and 16 susceptible melon plants at the second true-leaf stage. The following day, the plants were inspected and whiteflies were removed by aspiration and further maintained in insect cages. The same number of resistant and susceptible plants were maintained in separate insect cages as uninfected controls. At 3 dpi, 5 dpi, 10 dpi and 15 dpi, 4 plants of each genotype, inoculated and not, were sampled. Leaf samples (1 g) were harvested and frozen in liquid nitrogen and stored at −80 °C prior to nucleic acid extraction.

2.3. Quantitative virus detection

Leaf tissue of each plant (0.5 g) was ground to a fine powder in liquid nitrogen in a pestle and mortar and placed in a sterile microcentrifugue tube. To determine the viral load in all treatments, three replications per sample were considered. Each replication was the result of an independent extraction from independent samples using pools of 4 plants as described below. Total DNA and RNA was extracted with AllPrep DNA/RNA Mini Kit from Qiagen (Madrid, Spain). The resulting pellet was resuspended in 50 µl DEPC-treated water and stored at −80 °C. DNA was quantified with a ND-2000c Spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE) and diluted to a final concentration of 50 ng/µl. Real time Taqman PCR reactions were set up in 96-well reaction plates using TaqMan PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). One microlitre aliquots of total DNA (50 ng) were used as templates in the PCR reactions of 20 µl, containing 10 µl Master Mix, 1 µl forward primer, 1 µl reverse primer, 0,5 µl probe, 1 µl total DNA and 6,5 µl DEPC-water. The primers and probe for the TaqMan assay were as described in Simon et al. (2018): specific primers were based on the KX098552 GeneBank sequence: 5′-CATTATTGCACGAATTTCCG-3′(sense) positions 921–940 and 5′-ATCGTAGCCGACTGTGTCTG-3′ (antisense) positions 1011-1030. The probe was 5′-CATGCACCTTAGACCATGGACGCT-3′ positions 967-944, labeled with 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein (JOE) and 3′end was labeled with N, *N,N′,N′*-tetramethyl-6-carboxyrhodamine (TAMRA). To normalize DNA levels in samples, we adopted the method based on the 18S ribosomal RNA gene used as a reference (Gil-Salas et al., 2009): primers CUC18S-For (5′-GGCGGATG TTGCTTTAAGGA-3′) and CUC18S-Rev (5′-GTGGTGCCCTTCCGTC AAT-3′; probe CUC18S-Ana (5′-TCCGCCAGCACCTTATGAGAAATCAA AGTC-3′) labeled with JOE (2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein) as the 5′terminal reporter dye and with BHQ1 (Black Hole Quencher One) as the 3′ quencher dye. Three technical replications